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Molecular ecology of cryptic species of the fen moss *Hamatocaulis vernicosus*

Ph.D. Thesis

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Annotation
This dissertation thesis aims at cryptic species of a rare fen moss *Hamatocaulis vernicosus*. It covers their distribution in the Czech Republic, potential morphological differences, sex ratio in populations and their genetic diversity.

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Cover photo
Hamatocaulis vernicosus at the locality Prameny Klíčavy. Photo by Táňa Štechová

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně, pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

Manukjanová A., Košnar J. & Kučera J. 2018. Microsatellite primers for the cryptic species of the moss *Hamatocaulis vernicosus* and methods for their quick barcoding. – Journal of Bryology 40: 302–305. (IF=1.08)

AM performed most of the sampling, lab work, data evaluation and writing the manuscript. JKo assisted with the primers design. JK assisted with the data evaluation and writing the manuscript.

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AM performed most of the sampling, lab work, data evaluation and writing the manuscript. JKo assisted with laboratory work and writing the manuscript. JK assisted with the experiment design, data analysis and writing the manuscript.

Contents:

Chapter 1: General introduction

Scope of the thesis

The thesis aims at revealing the possible differences between the two currently recognized cryptic species of the rare fen moss *Hamatocaulis vernicosus* s.l. It thoroughly documents their regional distribution and at localities where their co-occurrence was recorded, detailed spatial patterns were investigated. Further studies were aimed at differences in the expressed sex ratio, the genetic diversity and its structure in populations inhabiting the territory of the Czech Republic. Those three aspects are strongly connected. The distribution and population diversity are dependent on reproductive mode, dispersal events and mate limitations at localities.

Since cryptic species often represent lineages with different life history and sometimes even unidentical ecological preferences as any other closely related, morphologically defined moss species, neglecting them can cause inconsistent results in experiments. However, as unequivocal barcoding of cryptic species which is only possible using the molecular methods incurs relatively high financial costs, not many studies treat their biology in detail.

Hamatocaulis vernicosus

Hamatocaulis vernicosus s. l. is a pleurocarpous moss of the family Scorpidiaceae (Ignatov & Ignatova 2004). It belongs to the ecological group of so-called "brown mosses" – pleurocarpous mosses growing in fens that have usually brownish-green color and share many ecological characteristics (Zoltai & Vitt 1995, Mälson 2008). *H. vernicosus* is confined to a threatened habitat of non-calcareous rich fens (habitat D4.1a; Janssen et al. 2016), which is considered to be in continuous decline in Central Europe (Davidson 2014, Štechová & Kučera 2007). During the last century, a considerable number of rich fens was damaged or destroyed, often changed in arable land by draining, or damaged by eutrophication, acidification or cessation of traditional management (Rybníček & Rybníčková 1974, Bergamini et al. 2001). As a result of considerable decrease of its localities throughout Europe during the 20th century (Glime 1982, Heras & Infante 2000, Church et al. 2001, Hedenäs et al. 2003,

Štechová & Kučera 2007, Štechová et al. 2008), *H. vernicosus* has been enlisted in Annex 2 of the Habitats Directive (92/43/EEC). In the Czech Republic, the species is considered vulnerable (VU) according the IUCN criteria (IUCN 1994, Kučera & Váňa 2012). The exceptionally thorough long-term monitoring of *H. vernicosus* in the Czech Republic supported by the Czech Agency for landscape protection (AOPK ČR) has been done mostly by T. Štechová since 2005. During those years, up to 70 localities (the new ones being added every year; Štechová et al. 2012) were monitored (manuscripts deposited at AOPK ČR). The monitoring includes records of recent localities vegetation, pH, conductivity, water level and population characteristics such as its size, vitality and size change.

Reproduction biology of *H. vernicosus* s. l. has been studied before, however, none of the studies addressed the differences between its cryptic species. Pépin *et al.* (2013) examined the causes of its sporophyte absence in the French Central Massif, which is likely resulted from the generally unfavourable site conditions and limited mate availability. 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 family Calliergonaceae and Amblystegiaceae, while its sporophyte production was average. Sporophytes are rarely produced in the Czech Republic (Štechová et al. 2008) as well as in other countries (Smith 1978, Hedenäs et al. 2003, Pépin et al. 2013), and local population maintenance relies presumably mainly on the clonal growth.

Hedenäs & Eldenäs (2007) discovered that *H. vernicosus* s. l. consists in fact of two separate lineages (tentatively named clade 1 and clade 2), which they consider cryptic species. Later, they were referred as southern and northern cryptic species, respectively (Hedenäs 2018b). Both lineages were reported to have partly overlapping distribution areas (Hedenäs & Eldenäs 2007). Clade 1 (southern) was sampled in temperate zone of Europe, in Peru and Russia, while clade 2 (northern) was found widespread in Europe including its the boreal zone and was also found in the USA. The information about detailed distribution of those lineages except for Sweden and Switzerland is scarce. In Sweden, northern regions were occupied exclusively by populations of the clade 2 while both clades were similarly frequent in southern part of the country. Contrary to northernmost Europe,

the central Europe hosts both those lineages, their proportion in this region outside of Switzerland remained unknown. The mixed clade 1 and 2 populations were never documented, probably because of the sampling pattern in previous studies with mostly a single specimen per locality (Hedenäs & Eldenäs 2007, Hedenäs 2018b). Hedenäs & Eldenäs (2007) did not reported any differences in ecological niches, characterised by pH and conductivity, between the two lineages.

Cryptic species

Cryptic species represent a gap in the knowledge of biodiversity; their detection and investigation are important for the understanding of the evolution and speciation, and for the protection of rare species, as well as for other fields of science and practice (Shneyer & Kotseruba 2014). Although the cryptic species are morphologically undistinguishable, they function as biologically separate species and their mutual hybridization is not more frequent than between any other closely related morphologically defined species. It is not uncommon for cryptic species to have genetic distances equal to distances among non-cryptic species (Wachowiak et al. 2007). The species differing only in minute morphological or ecological characters, whose distinguishing characters have been elaborated only after the lineages have been delimited molecularly, are often called semi-cryptic. Examples of such species include, e.g., *Homalothecium sericeum* s. str., *H. mandonii* (Mitt.) Geh. and *H. meridionale* (M.Fleisch. & Warnst.) Hedenäs which differ in their previously neglected sporophytic traits while their gametophytic characters are overlapping (Hedenäs et al. 2014). The relatively common occurrence of cryptic species in bryophytes is enhanced by the small size and relative simplicity of moss plants and the influence of the environment in the evolution of those characters (Pandey et al. 2016). This fact makes mosses difficult organisms to determine at all (Wyatt et al. 1989) but on the other hand, it makes them an ideal study group for investigating this phenomenon with respect to the broad geographic distributions (Carter 2012). The existence of cryptic species might in many cases offer the explanation for the striking biogeographic feature of bryophytes, their seemingly extremely low rate of endemism (Hutsemékers et al. 2012).

Following the discovery of cryptic species in *Conocephalum conicum* (Szweykowski & Bobowicz 1979), sibling species have been discovered in many bryophyte species during the last few decades. It is often the consequence of application of molecular methods (Szweykowski & Krzakowa 1979, Bickford et al. 2007, Heinrichs et al. 2010, Hedenäs et al. 2014), and many more cryptic species are probably yet to be discovered (Shaw 2000a). Even though in many cases distinguishing morphological or ecological characters are minute and overlapping, the truly cryptic species, which seems to be the case of *H. vernicosus* (Hedenäs & Eldenäs 2007, Manukjanová et al 2019b), remain a rare phenomenon.

Bryophyte reproduction

Reproduction has a key role in life cycle of all organisms. In bryophytes, two main reproduction modes coexist in most species. The sexual, generative reproduction serves as a main source of genetic variability and plays a major role in the long-distance dispersal. The vegetative reproduction, even though it produces mere clones of the original plant, has an irreplaceable role in short distance dispersal and maintaining populations.

Sexual reproduction

The sexual reproduction has a similar course in all mosses, even though some details may differ. The spores that develop on sporophyte after meiosis, give life to new gametophytes. Due to the chromosome segregation and crossing-over during meiosis, sexual reproduction is the biggest source of genetic variability that drives evolution. However, in case of self-fertilization, new plants still function as clones of maternal plants, which is why some monoicous bryophytes have mechanisms to prevent it (Eppley et al. 2007, Glime & Bisang 2017). Monoicous species usually have more abundant sporophytes than dioicous ones, because of the short proximity of opposite sex gametangia, but the self-fertilization may significantly lower the genetic diversity of such species (Kophimai et al. 2014). Mating between gametes from different haploid individuals produced from the same diploid parent (intergametophytic selfing) results in a 50% reduction in homozygosity, which is equivalent to selfing in animals and seed plants. In contrast, mating between gametes produced

from the same haploid individual (intragametophytic selfing) results in complete homozygosity in a single generation (Hedrick 1987). The forming of clones via "asexual spores" after self-fertilization is sometimes also considered asexual reproduction, even though they were formed via sexual process (Newton & Mishler 1994).

More than half of the moss species (including the target species, *H. vernicosus*) are dioicous (Wyatt 1982, 1985, Tan & Pócs 2000, Frahm 2007, Frey & Kürschner 2011). Those species tend to produce sporophytes with lower frequency (Gemmell 1950, Smith 1978, Longton 1992), being caused both by lower sex expression and unbalanced sex ratio and sex distribution in populations, because the effective fertilization distance is only a few centimeters (Shaw & Goffinet 2000). 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).

While the spore germination can exceed 90% when conditions are ideal, there are many factors that decrease the germination rate (Dalen et Söderström 1999). The real in situ germination and subsequent establishment is estimated to be below 10^{-4} (Hassel & Söderström 1999), being crucially affected by the competition and habitat conditions. The spores often germinate better in places further from mother plant, which increase their value in colonizing new localities (Shaw & Goffinet 2000). The disadvantage of sexual reproduction can also result from the long process of protonematal growth, which is a rather vulnerable stage.

Sporophyte production occurs in most of the unisexual species at least occasionally. Fertilization in bryophytes depends on the presence of liquid water, which is needed for delivery of motile spermatozoids to the egg cell (Glime & Bisang 2017). This requires the close proximity of male and female gametangia (Longton, 1976). However, excessive dominance of vegetative reproduction may cause clones to form patches of considerable size. This may entail spatial segregation of the sexes, similarly to cases of spore establishment from long-distance dispersal. The short distance required for fertilization is further complicated by unequal distribution of male and female plants at localities (Teleganova & Ignatov 2007). Another complication might be the low sex expression or markedly biased sex ratio

(Bisang & Hedenäs 2005). At some localities, only populations of one sex survived (Gemmell 1950, Longton & Schuster 1983, Fritz 2009). The sex ratio can furthermore change at geographical and scale. In some species, sporophytes have been observed only in the center of distribution, while outside it the populations are sterile (Longton 2006).

Sex ratio has major effect on bryophyte reproduction success. 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 their combination (reviewed by Glime & Bisang 2017). Stark et al. (2000) and Haig (2016) suggested that female-biased sex ratio is a result of higher investments into antheridia production in prezygotic phase, than into archegonia. Female-biased sex ratio probably developed because of the high importance of female plants as sporophyte bearers. While many spermatozoids mature in one antheridium, there is only a single egg cell in each archegonium.

Sex ratios at the level of spore development have only been studied in a few mosses and confirmed mostly the expected balanced ratio resulting from the 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 ratios (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).

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. believed to represent cryptic species, differed in their expressed sex ratio and sex expression levels.

Vegetative reproduction

Vegetative reproduction in bryophytes is considerably diverse (Longton & Schuster 1983, Frey & Kürschner 2011). The two main types are forming of specialised particles and spreading by unspecialized parts of stems. The former type is usually accomplished by forming gemmae or special shoots or leaves that break off easily and form new individual. Global comparative phylogenetic analyses suggest a weak or no correlation between the presence of asexual propagules and dioicy in mosses and liverworts (Laenen et al. 2016). The unspecialized way takes form of simple patch growth caused by stem branching, which can reach considerable distances during decades, or by stem fragmentation. For fen pleurocarpous mosses like *H. vernicosus,* vegetative reproduction by unspecialized gametophyte fragments is a usual mean of reproduction (Pfeiffer et al. 2006, Fritz 2009, Lieske 2010) enhanced by indefinite apical growth of shoots. The apical part has the highest potential of regeneration, even though the lateral branches and central part of stem possess considerable regeneration abilities as well (Westerdijk 1907, Poschlod & Schrag 1990).

Another phenomenon at the border between sexual and asexual reproduction are apogamy and apospory (Shaw et Goffinet 2000, Bryan 2001). Apospory is a regeneration of part of the sporophyte forming a diploid gametophyte (Bryan 2001). Apogamy is sporophyte growth from gametophyte tissue without gametangia and fertilization. However, the real influence of those rare meanings of reproduction on actual populations outside laboratory experiments is not yet known.

Vegetative reproduction, which main purpose is short-distance dispersal and preventing of genotype disappearance at localities (During & van Tooren 1987, Økland 1995, Longton 2006), has several advantages. No need for sexual partner in close proximity is advantageous especially for rare dioicous species (Bisang et al. 2004, Frahm 2007). Similarly, populations may be sterile in unfavorable ecological conditions, due to the high energetic cost of producing gametangia (Pohjamo & Laaka-Lindberg 2003). Some species are known only sterile, it is even possible that

generative reproduction disappeared completely in them (Frahm 2007, Frey & Kürschner 2011). In such situations, vegetative reproduction sustains populations or is responsible for biomass increase during colonization of new localities (Laaka-Lindberg 1999). It is obvious, that vegetative reproduction is indispensable for fen mosses (Miller & Ambrose 1976, Poschlod & Schrag 1990).

Another major advantage of vegetative reproduction is that it can take place whenever an opportunity occurs, not being limited to a certain part of the year. Temporary absence of conditions suitable for sexual reproduction may be particularly problematic for bryophytes which produce sporophyte only once a year. In general, mortality of diaspores and germinating shoots seems to be much lower in asexual reproduction (Söderström 1994, Mälson & Rydin 2007). On the other hand, the absence of genetic variability in clones may lead to limited potential of adaptation to changing environment (Longton & Hedderson 2000). Nevertheless, an unexpectedly high genetic variation was found in bryophyte species with rare sexual reproduction (Pohjamo et al. 2008; Bączkiewicz 2012), which may imply other sources of genetic diversity than recombination events, such as somatic mutations (Newton & Mischler 1994). This effect can be studied especially in isolated populations. The species *Sphagnum palustre* in Hawaiian Islands was probably introduced by a single dispersal event about 50 thousand years ago. Since all the local plants of this dioicous species are sterile, it is presumed, that it spread across the region solely by means of vegetative reproduction. That should lead to genetically uniform population, however, after the genetic diversity was assessed by means of microsatellites, it was discovered to be comparable to the diversity of the species at the continent (Karlin et al 2012).

A major problem of vegetative reproduction is also rather limited range of diaspore distribution. This particularly applies to large diaspores such as the stem fragments (Glime & Bisang 2017). In fragmented, island-like habitat such as the fens in Central Europe, limited spreading of large diaspores might seriously impair not only the colonization of new localities, but also the gene flow among populations (Gunnarsson & Söderström 2007). On the other hand, some bryophyte species have vegetative diaspores of roughly the same size as spores, such as the

gemmae in *Crossocalyx hellerianus*, which effectively contribute to gene flow among populations (Pohjamo et al. 2006, Holá et al. 2015).

Molecular methods in population biology of bryophytes

The use of molecular methods in bryology advanced immensely during last decades, having become an integral part of studies in taxonomy, population biology, phylogeny and other scientific fields. Knowledge of genetic structure of populations of rare species and processes that create and keep the genetic diversity has practical consequences in species protection (Wyatt et al. 1992, Gunnarsson et al. 2005). Before molecular methods were used, it was assumed that bryophytes display low level of genetic variation due to natural selection acting on haploid gametophytes. High levels of genetic diversity in bryophytes have been explained by multiple-niche selections (Wyatt et al.1989), inter-locus interactions, such as the epistasis (Shaw & Beer 1999), and the common occurrence of somatic mutations (Skotnicki et al. 2005).

The early molecular studies of bryophytes which used isozyme markers started nearly 50 years ago (Meyer et al. 1974). They continued with more advanced methods, based on visualization of DNA fragments (Boisselier-Dubayle et al., 1995) but the real surge of molecular data started first with the general availability of Sanger sequencing in early 2000s. However, Sanger sequencing has many disadvantages for usage in population studies. The relatively high price for sample usually do not allow for sufficient number of samples from many populations and most of the loci are not variable enough to distinguish clones. For population studies, hypervariable markers capable of distinguishing clones with high probability are being used (Shaw et al. 2008). AFLP (Snäll et al. 2004, Pfeiffer et al. 2006, Fritz 2009, Lieske 2010), ISSR (Hassel & Gunnarsson 2003, Vanderpoorten et al. 2003, Spagnuolo et al. 2009) and microsatellites (Wilson & Provan 2003, Hutsemékers et al. 2008, 2012) were used in many studies. Nowadays, methods based on next-generation sequencing (NGS) are being used in bryology on regular basis (Rosengren et al 2015, Yousefi et al. 2017). The usage of particular method depends on many factors. Even though nowadays those methods are slowly replaced by NGS methods, older fingerprinting methods such as the microsatellites still have their

place in studying population diversity in bryophytes for many reasons (Korpelainen et al. 2007, Shaw et al. 2008b, Hutsemékers et al. 2009).

Microsatellites (SSRs) present selectively neutral markers with high levels of polymorphism. Their codominant nature is particularly important in studies including sporophytes. Their variability is sufficiently high and unlike AFLP, their reproducibility is higher (Pardo et al. 2014). Thanks to their high specificity, contaminations are less frequent than in similarly variable methods. However, being mostly species-specific, microsatellite primers often need to be developed for each species individually, even though cross-species amplification is successful in many cases. Many studies benefitted from usability of *Sphagnum* primers to more species of the genus (Provan & Wilson 2007, Shaw et al. 2008a, b, Johnson & Shaw 2015, Mikulášková et al. 2017). Another benefit of using SSRs is their relatively low cost once the primers are developed and the possibility of continuously adding the samples and populations to the dataset.

Although highly variable markers have a considerable risk of homoplasy (Estoup et al. 2002), microsatellites are considered to be one of the most reliable molecular tools in population genetic studies (Rodrigues et al 2017). With higher number of polymorphic loci, the method is reliable enough to study reproduction system or genetic diversity at various scales (Hutsemékers et al. 2008). Microsatellite markers further allow for the assessment of gene flow levels among populations and rates between sexual and asexual reproduction. As Pandey at al. reviewed (2016), microsatellite analyses of several bryophyte species repeatedly confirmed that bryophytes exhibit high level of genetic diversity (Wilson & Provan, 2003; Shaw et al., 2008; Hutsemékers et al. 2010).

Microsatellites have been frequently used in bryological studies aimed at clonality, genetic diversity and dispersal limitation. Examples include Van der Velde et al. (2001a, 2001b) and Wilson & Provan (2003), who studied species of the genus *Polytrichum*. Authors of the latter study investigated the impact of habitat fragmentation caused by peat mining in *Polytrichum commune*. Affected localities had smaller genetic diversity, probably caused by the bottleneck effect. The high inter-population variability indicated reproductive isolation of individual populations. Since *P. commune* is a species that produces sporophytes regularly, the effect of

dispersal limitation on pleurocarpous fen species which reproduce almost exclusively vegetatively should be even greater.

Studies on pleurocarpous mosses have not been plentiful, although their number is increasing with the availability of molecular methods. Hutsemékers et al. (2009) studied the genetic diversity in populations of the cosmopolitan autoicous aquatic moss, *Platyhypnidium riparioides* in southern Belgium (Wallonia). The high proportion of variability among populations, high inbreeding coefficient and a great number of clones in populations indicate high proportion of vegetative reproduction or self-fertilization. The correlation of genetic and geographical distance showed a considerable dispersal limitation, with different histories and possible reproductive isolation between northern and southern populations. These results contrast with the later study by Hutsemékers et al. (2011), who assessed the ability of this species to colonize islands. The expected low genetic diversity of island populations due to the bottleneck effect was not confirmed and rather than the expected fate of island populations representing a "population sink", they function as a dynamic source of diaspores that played major role as a glacial refugia. In another study on *Rhynchostegium riparioides s.l.*, the Eastern North American populations which differed significantly in their microsatellite pattern were recognized as semi-cryptic species, hardly distinguishable in morphology (Hutsemékers et al. 2012). Microsatellites were used for the species delimitation in several bryological studies, especially where the challenging (semi)cryptic species were concerned. In *Frullania asagrayana* s.l., two cryptic species were clearly resolved by microsatellites, even though the lineages were sequence-invariant at the two plastid loci and ITS2 (Ramaiya et al. 2010).

Microsatellite primers developed for the fen species *Scorpidium cossonii* (Kophimai et al. 2011) cross-amplified successfully in the congeneric species *S. scorpioides* and *S. revolvens*, which enabled a population genetic analyses of the latter two species, which differ in their ploidy level and sexual system (Kophimai et al. 2014). The haploid and dioicous *S. cossonii* was proved to be genetically more diverse than the (allo)diploid and monoicous *S. revolvens*.

Aims of the thesis

The thesis is focused on various aspects of biology of the cryptic species of the moss *H. vernicosus* s. l.

Paper I was aimed at the assessment of distribution of cryptic species of *H. vernicosus* in the Czech Republic. The attempt to distinguish those lineages by morphological characters has been made as well.

Paper II assessed the sex expression and expressed sex ratio of cryptic species of *H. vernicosus* in the Czech Republic.

Paper III describes the barcoding options for distinguishing of the two *H. vernicosus* cryptic species by means of PCR-RFLP and the design of suitable microsatellite primers for both cryptic species.

In Paper IV, patterns of genetic variation and spatial genetic structure of the two *H. vernicosus* cryptic species in the Czech Republic were investigated using the newly designed microsatellite markers.

References

Bączkiewicz A. (2012) Genetic diversity of leafy liverwort species (Jungermanniidae, Marchantiophyta) in Poland: Diversity of leafy liverwort species with various reproductive modes. – Biodiversity: Research and Conservation 27: 3–54.

Bergamini A., Pauli D., Peintinger M. & Schmid B. (2001) Relationships between productivity, number of shoots and number of species in bryophytes and vascular plants. – Journal of Ecology 89: 920–929.

Bickford D., Lohman D. J., Sohdi N. S., Ng P. K. L., Meier R., Winker K., Ingram K. K. & Das I. (2007) Cryptic species as a window on diversity and conservation. – Trends in Ecology and Evolution 22: 148–155.

Bisang I. & Hedenäs L. (2005) Sex ratio patterns in dioicous bryophytes re-visited. – Journal of Bryology 27: 207-219.

Bisang I. & Hedenäs L. (2013) Males are not shy in the wetland moss *Drepanocladus lycopodioides*. – International Journal of Plant Science 174: 733–9.

Bisang I., Ehrlen J., Persson C. & Hedenäs L. (2014) Family affiliation, sex ratio and sporophyte frequency in unisexual mosses. – Botanical Journal of the Linnean Society 174: 163–72.

Bisang I, Ehrlen J. & Hedenäs L. (2004) Mate limited reproductive success in two dioicous mosses. – Oikos 104: 291–298.

Bisang I., Hedenäs L. & Cronberg N., 2017 Can the meiotic sex ratio explain the sex ratio bias in adult populations in the dioicous moss *Drepanocladus lycopodioides*. – Journal of Bryology 39: 115–20.

Boisselier-Dubayle M. C., Jubier M. F., Lejeune B. & Bischler H. (1995) Genetic variability in three subspecies of *Marchantia polymorpha* (Hepaticae): Isozymes, RFLP and RAPD markers. – Taxon, 44: 363–376.

Bowker M. A., Stark L. R., Mcletchie D.N. & Mishler. B.D. (2000) Sex expression, skewed sex ratios, and microhabitat distribution in the dioecious desert moss *Syntrichia caninervis* (Pottiaceae). – American Journal of Botany 87: 517–526.

Bryan V. S. (2001) Apospory in Mosses Discovered by Nathanael Pringsheim in a Brilliant Epoch of Botany. – The Bryologist 104: 40–46.

Buczkowska K, Adamczak M. & Bączkiewicz A., (2006) Morphological and anatomical differentiation within the *Aneura pinguis* complex (Metzgeriales, Hepaticae). – Biology Letters 43: 51–68.

Carter B. E. 2012 *Scleropodium occidentale* (Brachytheciaceae), a new moss species from western North America. – The Bryologist 115: 222–230.

Church J. M., Hodgetts N. G., Preston C. D. & Stewart N. F. (2001) British Red Data Books Mosses and Liverworts. Joint Nature Conservation Committee, Peterborough.

Dalen L. & Söderstroöm L. (1999) Survival ability of moss diaspores in water – an experimental study. – Lindbergia 24: 49–58.

Davidson N. C. (2014) How much wetland has the world lost? Long-term and recent trends in global wetland area. – Marine and Freshwater Research 65: 934–941.

During H. J. & van Tooren B. F. (1987) Recent developments in bryophyte population ecology. – Trends in Ecology and Evolution 2: 89–93.

Eppley S. M., Taylor P. J. & Jesson L. K. (2006) Self-fertilization in mosses: a comparison of heterozygote deficiency between species with combined versus separate sexes. Heredity, 98, 38–44.

Estoup A., Jarne P & Cornuet J. M. (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. – Molecular Ecology 344: 1591–1604.

Frahm J. P. (2007) Diversity, dispersal and biogeography of bryophytes. – Biodiversity and Conservation 17: 277–284.

Frey W. & Kurschner H. (2011) Asexual reproduction, habitat colonization and habitat maintenance in bryophytes. – Flora - Morphology, Distribution. – Functional Ecology of Plants 206: 173–184

Fritz S. (2010) Vegetative reproduction and clonal diversity in three pleurocarpous mosses (*Pseudoscleropodium purum, Pleurozium*

schreberi, *Rhytidiadelphus squarrosus*; Bryophytina) of mesic habitats.Diss. Freie Universität Berlin.
Gemmell A. R. (1950) Studies in the Bryophyta. I. The influence of sexual mechanism on varietal production and distribution of British mosses. – New Phytologist 49: 64–71.
Glime J. M. (1982) Response of *Fontinalis hypnoides* to seasonal temperature variations. – Journal of the Hattori Botanical Laboratory 53: 181–193.
Glime J. M. & Bisang I. (2017) Sexuality: Sex Ratio and Sex Expression. Chapt. 3-2. *In:* Glime, J.M. *Bryophyte Ecology.* 3-2-1 Volume 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.
Gunnarsson U. & Söderström L. (2007) Can artificial introductions of diaspore fragments work as a conservation tool for maintaining populations of the rare peatmoss *Sphagnum angermanicum*? – Biological Conservation 135: 450–458.
Gunnarsson U., Hassel K. & Söderström L. (2005) Genetic structure of the endangered peat moss *Sphagnum angermanicum* in Sweden: A result of historic or contemporary processes? – Bryologist 108: 194–203.
Haig D. (2016) Living together and living apart: the sexual lives of bryophytes. In: Contribution to a themed issue 'Weird sex: the underappreciated diversity of sexual reproduction'. – Philosophical Transactions of the Royal Society of London, Series B 371: 20150535 1–9.
Hassel K., & Gunnarsson U. (2003) The Use of Inter Simple Sequence Repeats (ISSR) in Bryophyte Population Studies. – Lindbergia 28: 152–157.
Hassel K. & Söderström L. (1999) Spore germination in the laboratory and spore establishment in the field in *Pogonatum dentatum* (Brid.) Brid. – Lindbergia 24: 3–10.
Hedenäs L. & Eldenäs P. (2007) Cryptic speciation, habitat differentiation, and geography in *Hamatocaulis vernicosus*

(Calliergonaceae, Bryophyta). – Plant Systematics and Evolution 268: 131–145.
Hedenäs L. (2018a) Conservation status of the two cryptic species of *Hamatocaulis vernicosus* (Bryophyta) in Sweden. – Journal of Bryology 40: 307 –315.
Hedenäs L. (2018b) Oncophorus demetrii, a fifth scandinavian species of *Oncophorus* (musci) possible to recognize by morphology. – Lindbergia 41: 1–9.
Hedenäs L., Bisang I. & Schnyder N. (2003) The distribution of bryophytes in Switzerland and Liechtenstein IV. *Hamatocaulis* and *Pseudocalliergon*. – Botanica Helvetica 113: 111–123.
Hedenäs L., Oliván G. & Eldenäs P. (2005) Phylogeny of the Calliergonaceae (Bryophyta) based on molecular and morphological data. – Plant Systematics and Evolution 252: 49–61.
Hedenäs L., Desamoré A., Laenen B., Papp B., Quandt D., Gonzáles-Macebo J. M., Patiño J., Vanderpoorten A. & Stech M. (2014) Three species for the price of one with the moss *Homalothecium sericeum* s.l. – Taxon 63: 249–257.
Heinrichs J., Hentschel J., Bombosch A., Fiebig A., Reise J., Edelmann M., Kreier H-P., Schäfer-Verwimp A., Caspari S, Schmidt A. R., Zhu R-L., von Konrat M., Shaw B., Shaw A. J. (2010) One species or at least eight? Delimitation and distribution of Frullania Dumort. s. l. (Jungermanniopsida, Porellales) inferred from nuclear and chloroplast DNA markers. – Molecular Phylogenetics and Evolution 56: 1105–1114.
Hedrick P. W. (1987). Genetic load and the mating system in homosporous ferns. Evolution 41: 1282–1289.
Heras P. & Infante M. (2000) On the presence of *Hamatocaulis vernicosus* (Mitt.) Hedenäs (Amblystegiaceae) in Spain. – Journal of Bryology 22: 297–298.
Holá E., Vrba J., Linhartová R., Novozámská E., Zmrhalová M., Plášek V. & Kučera J. (2014) Thirteen years on the hunt for *Buxbaumia viridis* in the Czech Republic: still on the tip of the iceberg? – Acta Societatis Botanicorum Poloniae 83: 137–145.

Holá E., Košnar,J. & Kučera J. (2015) Comparison of genetic structure of epixylic liverwort *Crossocalyx hellerianus* between central European and Fennoscandian populations. – PLoS ONE 10: e0133134.

Hutsemékers V., Risterucci A ., Ricca M. S. Boles S. O., Hardy J., Shaw A. J & Vanderpoorten A. (2008) Identification and characterization of nuclear microsatellite loci in the aquatic moss *Platyhypnidium riparioides* (Brachytheciaceae). – Molecular Ecology Resources 8: 1130–1132.

Hutsemékers V., Hardy O. J., Mardulyn P., Shaw A. J. & Vanderpoorten A. (2009) Macroecological patterns of genetic structure and diversity in the aquatic moss *Platyhypnidium riparioides*. – New Phytologist 185: 852–864.

Hutsemékers V., Vieira C. C., Ros R. M., Huttunen S. & Vanderpoorten A. (2012) Morphology informed by phylogeny reveals unexpected patterns of species differentiation in the aquatic moss *Rhynchostegium riparioides* s.l. – Molecular Phylogenetics and Evolution 62: 748–755.

Ignatov M. S. & Ignatova E. A. (2004) Flora mkhov srednei chasti evropeiskoi Rossii. Tom 2. Fontinalaceae–Amblystegiaceae. Moss flora of the Middle European Russia. Volume 2: Fontinalaceae–Amblystegiaceae. – Arctoa 11(Supplement 2): 609–960.

IUCN (1994) IUCN Red List Categories. – IUCN, Gland, Switzerland.

Janssen J. A. M., Rodwell J. S., García Criado M., Gubbay S., Haynes T., Nieto A., Sanders N., Landucci F., Loidi J., Ssymank A., Tahvanainen T., Valderrabano M., Acosta A., Aronsson M., Arts G., Attorre F., Bijlsma R.-J., Bioret F., Biţă-Nicolae C., Biurrun I., Calix M., Capelo J., Čarni A., Chytrý M., Dengler J., Dimopoulos P., Essl F., Gardfjell H., Gigante D., Giusso del Galdo G., Hájek M., Jansen F., Jansen J., Kapfer J., Kontula T., Mickolajczak A., Molina J. A., Molnar Z., Paternoster D., Pierik A., Poulin B., Renaux B., Schaminée J. H. J., Sumberova K., Toivonen, H., Tonteri T., Tsiripidis I., Tzonev R. & Valachovič M. (2016) Red List of European Habitats. 2. Terrestrial and freshwater habitats. European Commission, DG Environment, Brussels. 38p.

Johnson M. G. & Shaw A. J. (2015) Genetic diversity, sexual condition, and microhabitat preference determine mating patterns in *Sphagnum* (Sphagnaceae) peat-mosses. – Biological Journal of the Linnean Society 115: 96–113.

Karlin E. F., Hotchkiss S. C., Boles S. B., Stenøien H. K , Hassel K, Flatberg K. I. & Shaw A. J. (2012) High genetic diversity in a remote island population system: sans sex. – New Phytologist 193: 1088–1097.

Kophimai Y., Cornejo C., Peintinger M., Bergamini A. & Scheidegger C (2011). Characterization of nuclear microsatellite loci in the calcareous fen specialist *Scorpidium cossonii* (Calliergonaceae). – American Journal of Botany 98: e290–e292.

Kophimai Y., Peintinger M., Werth W., Cornejo C., Scheidegger Ch. & Bergamini A. (2014) Ploidy level, genetic diversity, and differentiation in two closely related mosses, *Scorpidium cossonii* and *S. revolvens* (Calliergonaceae). – Journal of Bryology 36: 33–43.

Korpelainen H., Kostamo K. & Virtanen. V. (2007) Microsatellite markers identification using genome l screening and restriction-ligation. – BioTechniques 42: 479–486.

Kučera J. & Váňa J. (2012) Seznam a červený seznam mechorostů České republiky. – Přìroda 23: 1–104.

Laaka-Lindberg S. (1999) Asexual reproduction in a population of a leafy hepatic species *Lophozia silvicola* Buch in central Norway. – Plant Ecology141: 137–144.

Laenen B., Machac A., Gradstein S. R., Shaw B., Patiño J., Désamoré A., Goffinet B., et al. (2016) Increased diversification rates follow shifts to bisexuality in liverworts. – New Phytologist 210: 1121–1129.

Lieske K. (2010): Vegetative reproduction and clonal diversity in pleurocarpous mosses (Bryophytina) of xeric habitats. Dissertation. Institute of Biology, Free UniversityBerlin

Longton R. E. & Miles C.J. (1982) Studies on the reproductive biology of mosses. – Journal of the Hattori botanical laboratory 52 : 219–239.

Longton R. E. & Schuster R. M. (1983): Reproductive biology. – New Manual of Bryology, vol. 1 (ed. R. M. Schuster), pp. 396–462. Hattori Botanical Laboratory, Nichinan.

Longton R. E. (1992) Reproduction and rarity in British mosses. – Biological Conservation 59: 89–98.

Longton R. E. & Hedderson T. A. (2000) What Are Rare Species and Why Conserve Them? – Lindbergia 25: 53–61.

Longton R. E. (1976) Reproductive biology and evolutionary potential in bryophytes. – Journal of Hattori Botanical Laboratory 41: 205–223.

Longton R. E. (2006) Reproductive ecology of bryophytes: what does it tell us about the significance of sexual reproduction?– Lindbergia 31: 16–23.

Mälson K. & Rydin H. (2007) Competitive hierarchy, but no competitive exclusions in experiments with rich fen bryophytes. – Journal of Bryology 1: 41–45.

Mälson K. (2008) Plant responses after drainage and restoration in rich fens. – Acta Universitatis Upsaliensis. Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. Uppsala 439: 33pp.

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.

McLetchie D. N. (1992) Sex ratio from germination through maturity and its reproductive consequences in the liverwort *Sphaerocarpos texanus*. – Oecologia 92: 273–278.

McLetchie D. N. (2001) Sex-specific germination response in the liverwort *Sphaerocarpos texanus* (Sphaerocarpaceae) . – The Bryologist 104: 69–71.

Meyer M. W., Greenberg J. & Tedeschi T. (1974) Enzymes of the moss Funaria hygrometrica. II. The isozymes of malate dehydrogense. – The Bryologist 77: 577–581.

Mikulášková E., Veleba A., Šmerda J., Knoll A. & Hájek M. (2017) Microsatellite variation in three calcium-tolerant species of peat

moss detected specific genotypes of *Sphagnum warnstorfii* on magnesium-rich bedrock. – Preslia 89: 101–114.
Miller N. G. & Ambrose L. J. H. (1976) Growth in Culture of Wind-Blown Bryophyte Gametophyte Fragments from Arctic Canada. – The Bryologist 79: 55–63.
Mishler B. D. & Oliver M. J. (1991) Gametophytic phenology of *Tortula ruralis*, a desiccation-tolerant moss, in the Organ Mountains of southern New Mexico. – The Bryologist 94 :143–153.
Newton A. E. & Mishler B. D. (1994) The evolutionary significance of asexual reproduction in mosses. – Journal of Hattori Botanical Laboratory 76: 127–145.
Newton A. E. & Mishler B. D. (1994) The evolutionary significance of asexual reproduction in mosses. – Journal of the Hattori Botanical Laboratory 76: 127–145.
Økland R. H. (1995) Population biology of the clonal moss *Hylocomium splendens* in Norwegian boreal spruce forests. 1. Demography. – Journal of Ecology 83: 697–712.
Pandey S., Sharma V. & Alam A. (2016) Potential of microsatellites markers for the genetic analysis of bryophytes. – Notulae Scientia Biologicae 8: 37–46.
Pardo D.C. Terracciano S., Giordano S. & Spagnuolo V. (2014) Molecular Markers Based on PCR Methods: A Guideline for Mosses. – Cryptogamie, Bryologie 35: 229–246.
Pépin F., Hugonnot V. & Celle J. (2013) Sex ratio patterns and fertility of *Hamatocaulis vernicosus* (Mitt.) Hedenäs at different spatial scales. – Journal of Bryology 35: 20–26.
Pfeiffer T., Fritz S., Stech M. & Frey W. (2006) Vegetative reproduction and clonal diversity in *Rhitidium rugosum* (Rhitidiaceae, Bryopsida) inferred by morpho-anatomical and molecular analyses. – Journal of Plant Research 119: 125–135.
Pohjamo M, Laaka-Lindberg S (2003) Reproductive modes in a leafy hepatic *Anastrophyllum hellerianum*. – Perspectives in Plant Ecology 6: 159–168.
Pohjamo M, Laaka-Lindberg S, Ovaskainen O. & Korpelainen H (2006) Dispersal potential of spores and asexual propagules in the epixylic

hepatic *Anastrophyllum hellerianum*. – Evolutionary Ecology 20: 415–430.

Pohjamo M., Korpelainen H. & Kalinauskaitė N. (2008) Restricted gene flow in the clonal hepatic *Trichocolea tomentella* in fragmented landscapes. – Biological Conservation 141: 1204–1217.

Poschlod P. U. & Schrag H. (1990) Regeneration vegetativer Teilchen von "Braunmoosen". – Telma 20: 291–301.

Provan J. & Wilson P. J. (2007) Development of microsatellites for the peat moss *Sphagnum capillifolium* using ISSR cloning. – Molecular Ecology Notes 7: 254–256.

Ramaiya M., Johnson M. G., Shaw B. , Heinrichs J. , Hentschel J. , von Konrat M. , Davison P. G. & Shaw A. J. (2010) Morphologically cryptic biological species within the liverwort *Frullania asagrayana*. –American Journal of Botany 97: 1707–1718.

Rodríguez-Romero A., Hernández M., Patiño J., Werner O. & González-Mancebo J. M. (2016) Characterization of ten polymorphic microsatellite loci for the threatened species *Grimmia curviseta* Bouman (Grimmiaceae, Musci). – Journal of Bryology 39: 16–22.

Rosengren F., Hansson B. & Cronberg N. (2015) Population structure and genetic diversity in the nannandrous moss *Homalothecium lutescens*: does the dwarf male system facilitate gene flow?– BMC Evolutionary Biology 15: 270.

Rybníček K. & Rybníčková E. (1974) The Origin and Development of Waterlogged Meadows in the Central Part of the Šumava Foothills. – Folia Geobotanica et Phytotaxonomica 9: 45–70.

Shaw A. J. & Beer S. C. (1997) Gametophyte- sporophyte variation and covariation in mosses. – Advances in Bryology 6: 35– 63.

Shaw A. J. & Goffinet B. (2000) Bryophyte biology. Cambridge University Press, Cambridge, UK

Shaw A. J., Cao, T., Wang L. S., Flatberg K. I., Flatberg B., Shaw B., Zhou P., Boles S. & Terracciano S. (2008a) Genetic variation in three Chinese peat mosses (*Sphagnum*) based on microsatellite markers, with primer information and analysis of ascertainment bias. – Bryologist 111: 271–81.

Shaw A. J., Pokorny L., Shaw B, Ricca M., Boles S. & Szövényi P. (2008b) Genetic structure and genealogy in the *Sphagnum subsecundum* complex (Sphagnaceae: Bryophyta). – Molecular Phylogenetics and Evolution 49: 304–317.

Shaw A. J. (2000a) Molecular phylogeography and cryptic speciation in the mosses, *Mielichhoferia elongate* and *M. mielichhoferiana*. (Bryaceae). – Mol. Ecol. 9: 595–608.

Shaw A. J. (2000b) Population ecology, population genetics and microevolution. In: Shaw AJ, Goffinet B (eds). Bryophyte Biology. Cambridge University Press: Cambridge. pp 369–402.

Shneyer V. S. & Kotseruba V. V. 2014 Cryptic Species in Plants and Their Detection by Genetic Differentiation between Populations. – Russian Journal of Genetics: Applied Research 5: 528–541.

Skotnicki M. L., Mackenzie A. M., Clements M. A. & Selkirk P. M. (2005) DNA sequencing and genetic diversity of the 18S–26S nuclear ribosomal internal transcribed spacers (ITS) in nine Antarctic moss species. – Antarctic Science 17: 377–384.

Smith A. J. E. (1978) The Moss Flora of Britain and Ireland. – Cambridge University Press, Cambridge.

Snäll T, Fogelqvist J, Bibeiro P.J. & Lascoux L. (2004) Spatial genetic structure in two congeneric epiphytes with different dispersal strategies analysed by three different methods. – Molecular Ecology 13: 2109–2119.

Söderström L. (1994) Scope and significance of studies on reproductive biology of Bryophytes. – Journal of the Hattori Botanical Laboratory 76: 97–103.

Spagnuolo V., Terracciano S. & Giordano S. (2009) Clonal diversity and geographic structure in *Pleurochaete squarrosa* (Pottiaceae): different sampling scale approach. – Journal of Plant research 122: 161–170.

Stark L. R., Mishler B. D. & Mcletchie D. N. (2000) The cost of realized sexual reproduction: assessing patterns of reproductive allocation and sporophyte abortion in a desert moss. – American Journal of Botany 87: 1599–1608.

Stark L. R., Mcletchie D. N. & Mishler B. D. (2005) Sex expression, plant size, and spatial segregation of the sexes across a stress gradient in the desert moss *Syntrichia caninervis*. – The Bryologist 108: 183–193.

Stark L. R., Mcletchie D. & Eppley S. (2010) Sex ratios and the shy male hypothesis in the moss *Bryum argenteum* (Bryaceae). – The Bryologist 113: 788–797.

Štechová T. & Kučera J. (2007) The requirements of the rare moss, *Hamatocaulis vernicosus* (Calliergonaceae, Musci), in the Czech Republic in relation to vegetation, water chemistry and management. – Biological Conservation 135: 443–449.

Štechová T., Hájek M., Hájková P. & Navrátilová J. (2008) Comparison of habitat requirements of the mosses *Hamatocaulis vernicosus, Scorpidium cossonii* and *Warnstorfia exannulata* in different parts of temperate Europe. – Preslia 80: 399–410.

Szweykowski J. & Krzakowa M. (1979) Variation of four enzyme systems in Polish populations of *Conocephalum conicum* (L.) Dum. (Hepaticae, Marchantiales). – Bulletin of the L'Academie Polonicae, SeÂr Sciences Biologiques, 27: 37–41.

Szweykowski J., Buczkowska K. & Odrzykoski I. J. (2005) *Conocephalum salebrosum* (Marchantiopsida, Conocephalaceae)- a new Holarctic liverwort species. – Plant Systematics and Evolution 253: 133–158

Tan B. C. & Pócs T. (2000) Biogeography and conservation of bryophytes. – In: Shaw A.J. & Goffinet B. (eds.), Bryophyte biology. Cambridge University Press, Cambridge. pp 403–448.

Teleganova V. V. & Ignatov M. S. (2007) On the reproductive biology of *Ptilium crista-castrensis*. – Arctoa 16: 87–98.

Wachowiak W, Bączkiewicz A, Chudzińska E, Buczkowska K. (2007) Cryptic speciation in liverworts – a case study in the *Aneura pingui*s complex. – Botanical Journal of the Linnean Society 155: 273–282.

Vanderpoorten A., Hedenäs L. & Jacquemart A.-L. (2003) Differentiation in DNAfingerprinting and morphology among species of the pleurocarpous moss genus *Rhytidiadelphus* (Hylocomiaceae). – Taxon 52: 229–236.

van der Velde M., Van de Zande L. & Bijlsma R. (2001a) Genetic structure of *Polytrichum formosum* in relation to the breeding system as revealed by microsatellites. – Journal of Evolutionary Biology 14: 288–295.

van der Velde M., During H. J., Van De Zande L. & Bijlsma R. (2001b)The reproductive biology of *Polytrichum formosum*: clonal structure and paternity revealed by microsatellites. – Molecular Ecology 10: 2423–2434.

Westerdijk J. (1907) Zur Regeneration der Laubmoose. – Recueil Travaux Botanique Néerlandaise 3: 1–66.

Wilson P. J. & Provan J. (2003) Effect of habitat fragmentation on levels and patterns of genetic diversity in natural populations of the peat moss *Polytrichum commune*. – Proceedings of the Royal Society B: Biological Sciences 270: 881–886.

Wyatt R. (1982): Population ecology of bryophytes. – Journal of the Hattori Botanical Laboratory 52: 179–198.

Wyatt R. (1985) Terminology for Bryophyte Sexuality: Toward a Unified System. – Taxon 34: 420–425.

Wyatt R., Odrzykoski I. J. & Stoneburner A. (1989) High levels of genetic variability in the haploid moss *Plagiomnium ciliare*. – Evolution 43: 1085–1096.

Wyatt R., Odrzykoski I. J., & Stoneburner A. (1992) Isozyme evidence of reticulate evolution in mosses: *Plagiomnium medium* is an allopolyploid of *P. ellipticum* × *P. insigne*. – Systematic Botany 17: 532–550.

Yousefi N., Hassel K., Flatberg K. I., Kemppainen P.,Trucchi E., Shaw A. J., Kyrkjeeide M. O., Szövényi P. & Stenøien H. K. (2018) Divergent evolution and niche differentiation within the common peatmoss *Sphagnum magellanicum*. – American Journal of Botany 104: 1060–1072.

Zoltai S. C. & Vitt D. H. (1995) Canadian wetlands: environmental gradients and classification. – Vegetatio 118: 131–137.

Chapter 2: Microsatellite primers for the cryptic species of the moss *Hamatocaulis vernicosus* and methods for their quick barcoding.

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Microsatellite primers for the cryptic species of the moss *Hamatocaulis vernicosus* and methods for their quick barcoding

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Introduction

Hamatocaulis vernicosus (Mitt.) Hedenäs is a pleurocarpous moss assigned to the family Scorpidiaceae (Ignatov & Ignatova, 2004). It is confined to the threatened habitat of rich fens, which has suffered a considerable decrease in its localities in Europe over the last century, and therefore has been listed in Annex 2 of the EU Habitats Directive (92/43/EEC). Its occurrence and habitats have been carefully monitored since then (Hedenäs *et al.,* 2003; Štechová & Kučera, 2007; Štechová *et al.,* 2012). The scope of this surveillance has, however, been challenged after Hedenäs & Eldenäs (2007) reported the existence of two molecularly defined lineages within the morphological concept of *Hamatocaulis vernicosus*. Despite their seemingly identical ecology and morphology, no obvious signs of gene flow were found between the two lineages, although their distribution is partly overlapping. Therefore, the two lineages were regarded as representing biological species, but were only informally named clade 1 and clade 2 in view of the absence of diagnostic morphological characters. We follow the same convention here. If plants of the two clades function as separate species, all earlier data on overall distribution, population size and threat-levels at individual localities need to be reassessed for each of the separate cryptic taxa. The same applies when investigating genetic variation within the populations of *H. vernicosus*. Both clades have been found to occur in the Czech Republic during a pilot screening, a fact that has triggered several simultaneous studies by the authors.

In order to explore the genetic structure of the two cryptic species in the Central European region, we decided to use microsatellites, an approach which offers the advantages of moderate price, high reproducibility, and laboratory protocols using small amounts of template DNA. Although the costly and time-consuming approach of microsatellite development using cloning and Sanger sequencing has been replaced in recent years by next-generation sequencing of SSR-enriched genomic libraries (Sawicki *et al.,* 2012; Holá *et al.,* 2015; Pandey *et al.,* 2016), microsatellite primers still often need to be developed individually for each species, even though successful cross-species amplification has been reported in many cases, especially within the genus *Sphagnum* L. (Shaw *et al.,* 2008; Johnson & Shaw, 2015; Mikulášková *et al.,* 2017).

SSR primers design and testing

We first tested five of the published primer pairs (Sc01, 03, 04, 09 and 20) developed by Kophimai *et al.* (2011) for *Scorpidium cossonii* (Schimp.) Hedenäs on samples from both clades. *Hamatocaulis vernicosus* is relatively closely related to *Scorpidium* (Hedenäs *et al.*, 2005), and successful cross-amplification of *Scorpidium cossonii* primers was reported for *S. scorpioides* (Hedw.) Limpr. and *S. revolvens* (Sw.) Rubers (Kophimai *et al.*, 2011). However, while most of the samples of *H. vernicosus* were successfully amplified, microsatellite motifs were absent, or the sequence contained another microsatellite, which would interfere with the interpretation of fragment analysis. Therefore, we had to design new species-specific SSR primers for *H. vernicosus*.

First, we developed primers for clade 1, which is more common in the Czech Republic, expecting that cross-amplification in clade 2 might work by analogy with the *Scorpidium* study cited above. Genomic DNA was extracted using the CTAB protocol (Doyle & Doyle, 1987). The biotin-streptavidin enrichment method was used to prepare the SSR-enriched genomic library (Nunome *et al.*, 2006). The microsatellite loci were identified using 454-pyrosequencing of the SSR-enriched library as described in Drag *et al.* (2013). Reads containing putative SSR loci were filtered in BioEdit 7.0.9.0 (Hall, 1999), and those with sufficient read coverage including reverse complement reads were selected for primer design. Specific primers were designed using Primer3 (Koressaar *et al.*, 2007; Untergasser *et al.*, 2012). The final dataset for clade 1 contained 58412 reads, from which 19 PCR primer pairs were designed and further tested as described below.

Cross amplification to clade 2 revealed frequent absence of SSR motif or poor amplification. Therefore, a new set of primers for clade 2 was designed separately using the methods described above. The dataset for clade 2 comprised of 59028 reads, and we were able to design 12 PCR primer pairs for subsequent testing.

The designed SSR primers were first tested on two individuals from both clades 1 and 2. Total genomic DNA was extracted using the NaOH method (Werner *et al.*, 2002). PCRs were performed using M13-tailed assay (Schuelke, 2000) in the reaction mixture containing 0.6 μl of genomic

DNA, 0.3 μM of reverse primer, 0.3 μM of fluorescently labelled M13 dye primer, 0.075 μM of forward-tailed primer (5'-TGTAAAACGACGGCCAGT + forward primer sequence-3'), 2.8 μl of Plain PP Master mix (Top-Bio, Prague, Czech Republic), and sterile water to make up a final volume of 5.6 μl. The PCRs were performed according to the protocol of Schuelke (2000), except that the number of cycles was set to 44. For annealing temperatures, elongation time and fluorescent dyes see Table S1. The primer pairs which amplified successfully in one or both clades were verified for the presence of SSR motif in the amplified region by Sanger sequencing of PCR products. Those sequences were used for determining the length of the flanking region (sequence length without SSR motif). Fluorescently labelled PCR products were pooled and analysed using fragment analysis with GeneScan 600 LIZ (Applied Biosystems, Foster City, USA) as the internal size standard. For both clades, the set of labelled primers was optimized to analyse the sample in a single run of fragment analysis (Table S1). Microsatellite alleles were coded as a number of the SSR motif repeats and scored using GeneMarker v1.80 (SoftGenetics LLC, State College, USA).

Primer pairs yielding products without microsatellite motifs, those which amplified poorly and those containing invariable loci in all tested samples were excluded. In total, a set of 19 variable SSR primer pairs was selected. Twelve primer pairs worked well for specimens belonging to clade 1, eleven for specimens belonging to clade 2, and only five primer pairs amplified the targeted marker in both clades. Interestingly, the primer Hv2AC45, even though designed for clade 2, proved to be variable only in clade 1.

The primers were further tested on 25 samples, involving five plants from five populations belonging to each clade. The plants were sampled at least 20 cm apart to enhance the probability of sampling various genotypes of this highly clonal moss. Repeated PCR trials were necessary to obtain successful amplifications for some of the loci (especially AC62 and CAA111). For this reason, we do not recommend multiplexing more loci into a single PCR reaction. In our experience, minimizing the duration of PCR setup improved the success of amplification. The presence of population-specific null alleles was observed in locus AC62.

Clade barcoding

It is necessary to barcode each sample before the complete microsatellite assay, to ensure that the correct primer set for the particular cryptic species is used. As morphological determination is impossible, working with *Hamatocaulis vernicosus* samples requires genetic barcoding of individual plants to assign them to either of the cryptic species. This has so far been possible only using the Sanger sequencing of published differentiating loci, the nrITS region and the chloroplast loci *trn*L–*trn*F and *rpl*16. (Hedenäs & Eldenäs, 2007). In population biology studies, identification is typically needed for hundreds of individuals. Therefore, our aim was to develop an easy and cost-effective molecular method to barcode high numbers of plants to assign them to individual clades.

We first tried to develop the PCR-RFLP assay, which would utilise the differences in sequences of the ITS region of nuclear ribosomal between the two lineages for finding a restriction enzyme targeting a restriction site unique to individuals of only one of the clades. A search in the enzyme database implemented in BioEdit 7.0.9.0 (Hall, 1999) yielded the restriction endonuclease TaqII (5'...GACCGA(N)11...3'; 3'...CTG GCT(N)9 ...5'), which targets the restriction site occurring only in the ITS region of *H. vernicosus* clade 1 and cuts it into two fragments (161 + 541 bp). The amplification of the ITS region was performed according to the protocol by Hedenäs & Eldenäs (2007). The resulting products were visualized using electrophoresis on a 1.5% agarose gel. Only products making clear and strong bands are suitable for the subsequent PCR-RFLP method. The restriction reaction has been optimized for total sample volume 7.5 µl (5.625 µl of sterile water, 0.75 µl of 10× reaction buffer, 0.25 U of TaqII, 1 µl of ITS PCR product – approx. 20–100 ng of DNA) and incubated at 65°C for 6 hours. The resulting products were visualized using electrophoresis on a 1.5% agarose gel (Figure 1). Even when the restriction time was increased, some molecules of the original PCR product remained intact, forming the third weak band on the gel. However, since the two restriction products were clearly visible, there was no need to increase the amount of enzyme.

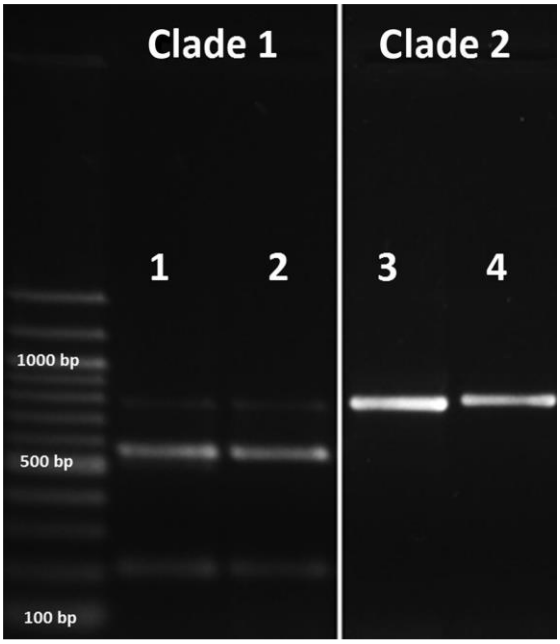

Figure 1. PCR-RFLP gel. PCR products from samples of clade 2 remain intact (702 bp), while products of clade 1 samples yield two bands of expected length (161 + 541 bp). Scale – 100 bp DNA ladder (New England Biolabs).

In addition to the above-described PCR-RFLP assay, selective amplification of some of the primers described above can also be used for clade identification. The combination of loci AG29, amplifying selectively only in clade 1, and 2AC58, which is selective for clade 2, enables barcoding of all samples (Figure 2). With respect to the reduced reliability of the SSR method (both false-positive and false-negative results are known to occur), it is nevertheless recommended that suspicious cases like bands of unusual size, multiple bands in a sample, unclear bands etc. be verified using the PCR-RFLP assay or sequencing.

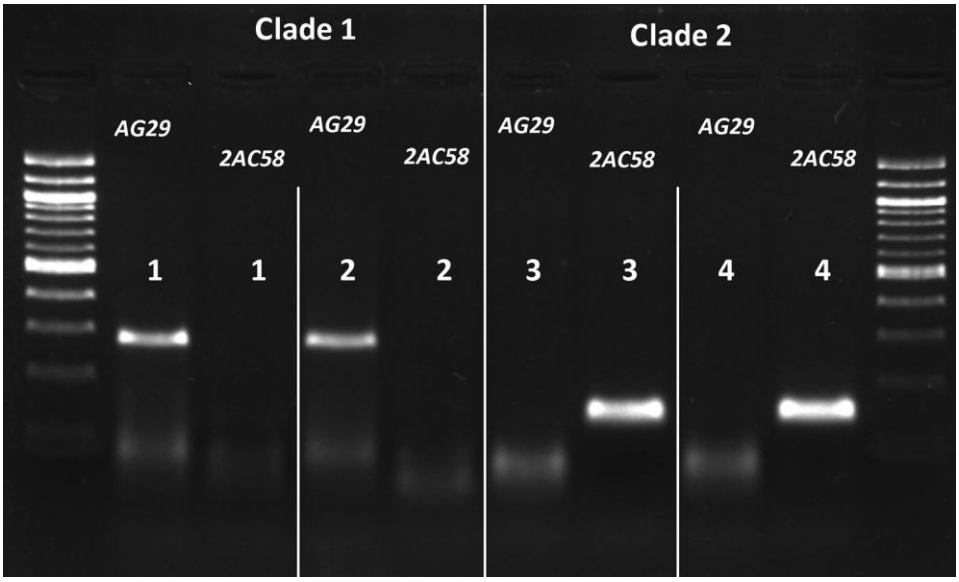

Figure 2. The agarose gel of 4 samples of *H. vernicosus*. Sample 1 and 2 belong to clade 1 and samples 3 and 4 to clade 2. Every sample was amplified with primer pairs AG29 and 2AC58, respectively. AG29 amplifies only samples of clade 1 while 2AC58 amplifies only clade 2 samples. Scale – 100 bp DNA ladder (New England Biolabs).

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References:

Doyle, J.J. & Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19: 11–15.

Drag, L., Košnar, J. & Čížek, L. 2013. Development and characterization of ten polymorphic microsatellite loci for the Great Capricorn beetle (*Cerambyx cerdo*) (Coleoptera: Cerambycidae). *Conservation Genetics Resources*, 5: 907–909.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41: 95–98.

Hedenäs, L., Bisang, I. & Schnyder, N. 2003. The distribution of bryophytes in Switzerland and Liechtenstein IV. *Hamatocaulis* and *Pseudocalliergon*. *Botanica Helvetica*, 113: 111–123.

Hedenäs, L. & Eldenäs, P. 2007. Cryptic speciation, habitat differentiation, and geography in *Hamatocaulis vernicosus* (Calliergonaceae, Bryophyta*). Plant Systematics and Evolution*, 268: 131.

Hedenäs, L., Oliván, G. & Eldenäs, P. 2005. Phylogeny of the Calliergonaceae (Bryophyta) based on molecular and morphological data. *Plant Systematics and Evolution*, 252: 49–61.

Holá, E., Košnar, J. & Kučera, J. 2015. Comparison of genetic structure of epixylic liverwort *Crossocalyx hellerianu*s between central European and Fennoscandian populations. – *PLoS ONE*, 10(7): e0133134.

Ignatov, M.S. & Ignatova, E.A. 2004. Flora mkhov srednei chasti evropeiskoi Rossii. Tom 2. Fontinalaceae—Amblystegiaceae. *Arctoa*, 11 (Supplement 2): 609– 960.

Johnson, M.G. & Shaw, A.J. 2015. Genetic diversity, sexual condition, and microhabitat preference determine mating patterns in *Sphagnum* (Sphagnaceae) peat-mosses. *Biological Journal of the Linnean Society*, 115: 96–113.

Kophimai, Y., Cornejo, C., Peintinger, M., Bergamini, A. & Scheidegger, C. 2011. Characterization of nuclear microsatellite loci in the calcareous fen specialist *Scorpidium cossonii* (Calliergonaceae). *American Journal of Botany*, 98(10): e290–2.

Koressaar, T., Jõers, K. & Remm, M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics*, 23: 1289–1291.

Mikulášková, E., Veleba, A., Šmerda, J., Knoll, A. & Hájek, M. 2017. Microsatellite variation in three calcium-tolerant species of peat moss detected specific genotypes of *Sphagnum warnstorfii* on magnesium-rich bedrock. *Preslia*, 89: 101–114.

Nunome, T., Negoro, S., Miyatake, K., Yamaguchi, H. & Fukuoka, H. 2006. A protocol for the construction of microsatellite enriched genomic library. *Plant Molecular Biology Reporter,* 24: 305–312.

Pandey, S., Sharma, V. & Alam, A. 2016. Potential of microsatellites markers for the genetic analysis of bryophytes. *Notulae Scientia Biologicae,* 8(1): 37–46.

Sawicki, J., Kwasniewski, M., Szczecinska, M., Chwialkowska, K., Milewicz, M. & Plášek, V. 2012. Isolation and characterization of Simple Sequence Repeats (SSR) Markers from the moss Genus *Orthotrichum* using a small throughput pyrosequencing machine. *International Journal of Molecular Sciences*, 13(6): 7586–7593.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology*, 18: 233–234.

Shaw, A.J., Pokorny, L., Shaw, B., Ricca, M., Boles, S. & Szövényi, P. 2008. Genetic structure and genealogy in the *Sphagnum subsecundum* complex. *Molecular Phylogenetics and Evolution*, 49: 304–317.

Štechová, T. & Kučera, J. 2007. The requirements of the rare moss, *Hamatocaulis vernicosus* (Calliergonaceae, Musci), in the Czech Republic in relation to vegetation, water chemistry and management. *Biological Conservation*, 135: 443–449.

Štechová, T., Kučera, J. & Šmilauer, P. 2012. Factors affecting population size and vitality of *Hamatocaulis vernicosus* (Mitt.) Hedenäs (Calliergonaceae, Musci). *Wetlands Ecology and Management*, 20(4): 329–339.

Untergasser, A., Cutcutache, I., Koressaar, T.Y.J., Faircloth, B.C., Remm, M. & Rozen, S.G. 2012. Primer3 – new capabilities and interfaces. *Nucleic Acids Research*, 40: e115.

Werner, O., Ros, R.M. & Guerra, J. 2002. Direct amplification and NaOH extraction: two rapid and simple methods for preparing bryophyte DNA for polymerase chain reaction (PCR). *Journal of Bryology*, 24: 127–131.

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Table S1. The microsatellite markers developed for *Hamatocaulis vernicosus*. Loci marked in bold were successfully amplified in both cryptic species. Number of alleles (Na) is based on 25 samples from 5 populations. For PCRs with M13 dye primer, forward-tailed primer (5'-TGTAAAACGACGGCCAGT + forward primer sequence -3') is required.

Locus	Motif	Clade	Fluorescent dye	Flanking region	Ta (°C)	Elongation time (s)	Size range (bp)	Na	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Hv AC4	(CA)11	1	VIC	147	56	15	167-171	3	TCAGCACATCATCAGAATCC	CGGTGCGTCAGAGTGTAT
Hv 2-AC107	(CA)21 GA (TA)6	1	VIC	158	57	15	176-214	10	ACTCACACGATGAGCAAAACT	CTGTTTCGAGCGGTTCTCTG
Hv AC40	(AC)16	1	VIC	196	59	15	216-232	5	CCTCTCCGTACTTCCTCGTC	ACTGTTTCGTCGTGCTTGG
Hv 2-AC45	(AC)12	1	PET	113	56	10	130-141	4	TTTGAAGTCGGGTTGCCTAC	CCAAATTCATTTAGGTCAAAGTT
Hv AC209	(AC)8 AG (AT)11	1	PET	181	56	15	197-207	6	TCCTTTTGGTTACATCTCTGCT	CAATCCTCACTTTCGTTTGG
Hv AC30	(CA)22	1	PET	420	54	30	444-466	4	CTGAATTGATCTCCTCTTCTTGT	CGTTTAAGGGGTATTGGAAA
Hv TC14	(TC)10	1	NED	106	57	10	126-132	3	TGTTGATGATATGGCTCTTGC	CTACCGTCCTCACCCTCA
Hv AG29	(AG)28	1	NED	189	52	15	213-247	7	GCTCTTTGGCAAATTCTA	GGTAGGGGTAGGTAGTCAG
Hv AG39	(GA)9	1	NED	282	58	20	296-306	6	GTGACCCCCAACTACCCAAG	GGGACCAAAAGTGTTCTCA
Hv AC115	(CA)14	1	6-FAM	86	56	10	128-134	3	ATGACAAGAGGGCACACA	AATTCAGATCATTTGGCATTGTA
Hv AC62	GA (CA)25	1	6-FAM	148	52	15	164-210	8	TGCAACAAATAAAACTCAAAT	GAAGAAGGAACAACCCAAAA
Hv CAA111	(CAA)19	1	6-FAM	313	57	25	364-403	7	GGGGCATTTAGGACACTTTG	ATGGGGCTTTTGTTGTTGG
Hv 2-AC58	(CA)12	2	VIC	92	54	10	116-118	2	TCTCCCCAAAATGAACACAA	TTCAGACTCACCAAAGTGTGC
Hv 2-AC134	(CA)12	2	VIC	127	57	15	151-153	2	TCTTTCAATCCGTGCAGTCA	TAGGGCAATGAGAGGGAAAA
Hv AC40	(AC)13	2	VIC	196	59	20	222-226	3	CCTCTCCGTACTTCCTCGTC	ACTGTTTCGTCGTGCTTGG
Hv 2-AC53	(CA)12	2	VIC	257	54	25	279-283	3	TTTAGAACTACATTTCAACAACAAA	TTTGCTTTCCATCATCACTCA

Hv 2-AC107	(CA)20 (TA)4	2	PET	160	57	15	194-200	5	ACTCACACGATGAGCAAAACT	CTGTTTCGAGCGGTTCTCTG
Hv TC14	(TC)11	2	NED	106	57	10	126-128	2	TGTTGATGATATGGCTCTTGC	CTACCGTCCTCACCCTCA
Hv 2-AC90	(CA)13	2	NED	147	57	15	173-209	6	TAATTTGTGGATTGGCGTTG	TTTCTAAGGTTGCAAAATAGACCTC
Hv 2-AC74	(AC)15	2	NED	230	59	20	258-260	2	ATAACTGCCCCACCACCA	TTCTGAGTGCCCGAGTGAG
Hv 2-AC141	(AC)12	2	6-FAM	128	57	15	152-154	2	TGAAGGTTGTTCACATGGTGTC	TTGAAGGCTGAATTGGGTTT
Hv AC62	(CA)11	2	6-FAM	148	52	15	170-174	2	TGCAACAAATAAAACTCAAAT	GAAGAAGGAACAACCCAAAA
Hv CAA111	(CAA)20	2	6-FAM	313	57	25	353-392	6	GGGGCATTTAGGACACTTTG	ATGGGGCTTTTGTTGTTGG

Chapter 3: Expressed sex ratio in populations of the moss *Hamatocaulis vernicosus* (Mitt.) Hedenäs (Scorpidiaceae) in the Czech Republic with consideration of its cryptic species.

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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 ration 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 spermatozoids 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 crytic 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 the 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 estimated 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).

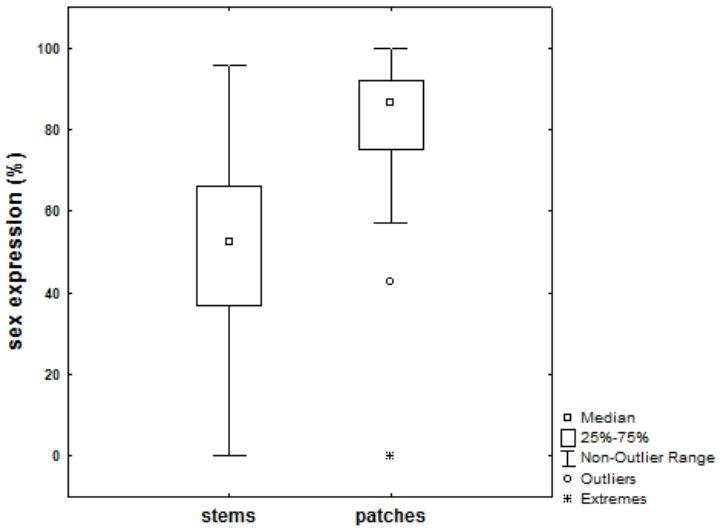

Fig. 1 The sex expression of *Hamatocaulis vernicosus* in the Czech Republic at individual localities assessed at two levels of pooling hierarchy - ("*shoots at localities"* and "*patches at localities"*).

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).

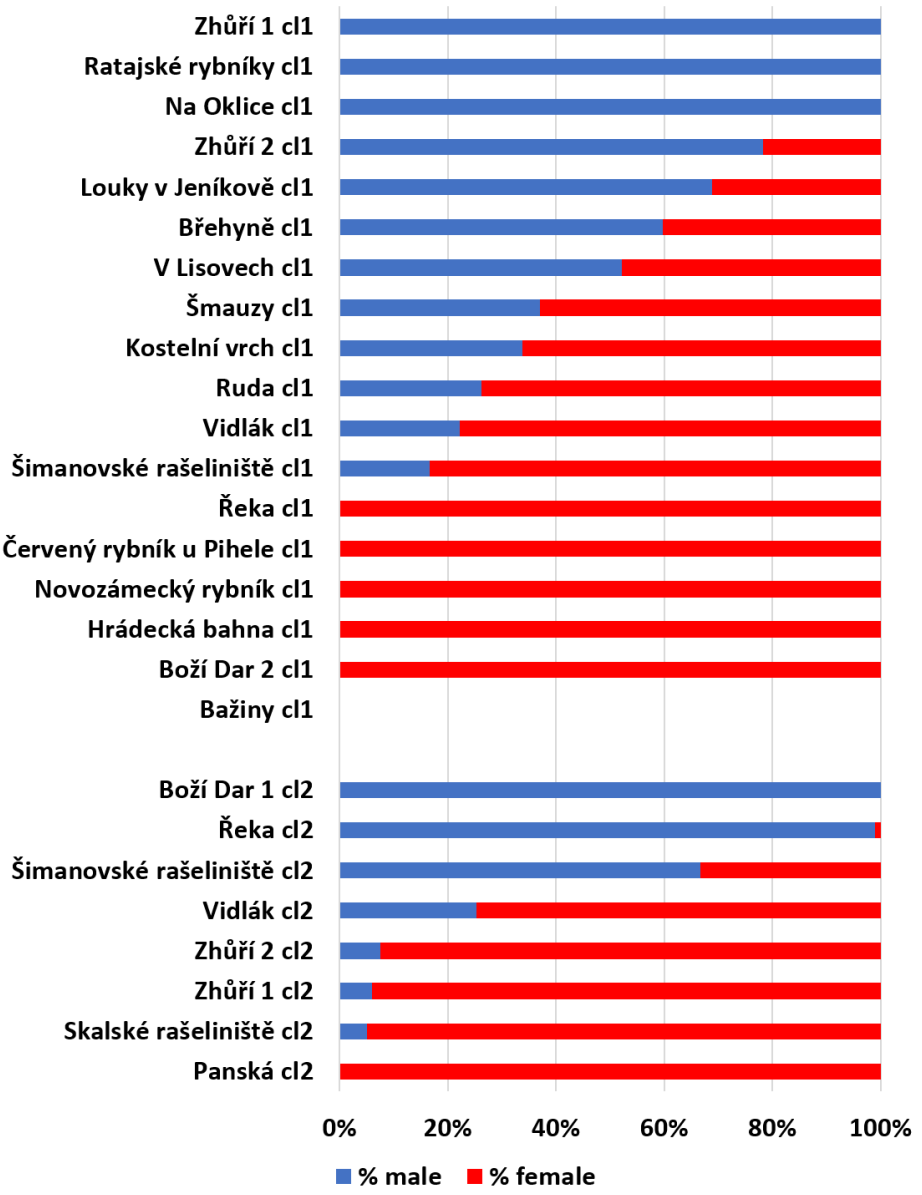

Fig. 2 The expressed sex ratio at studied localities of *H. vernicosus*. In mixed populations, only single-clade patches were used for the assessment.

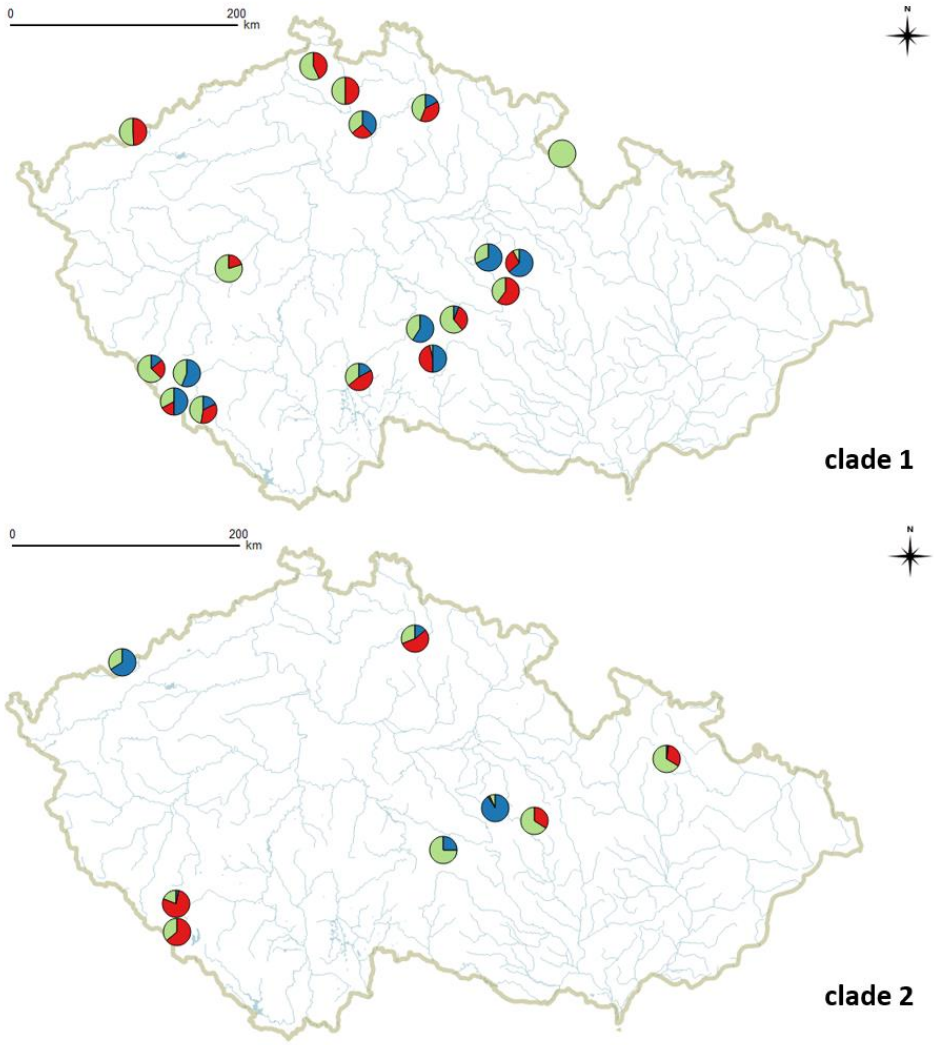

Fig 3 Rates of male (blue), female (red) and non-expressing (green) plants at studied localities of *Hamatocaulis vernicosus* clade 1 and 2.

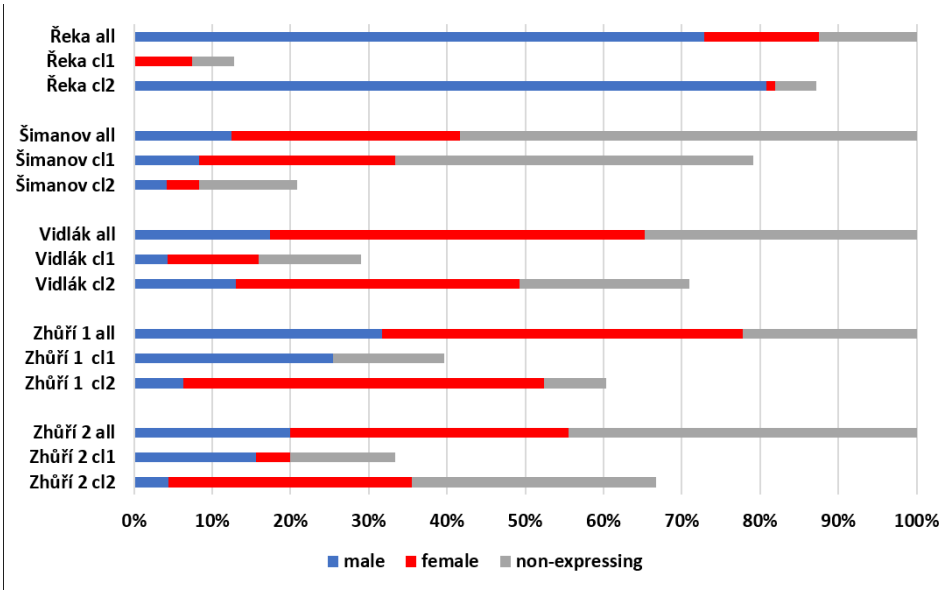

Fig. 4 Sex ratio at localities with co-occurring cryptic species. All – without distinguished clades, cl1 – clade 1, cl2 – clade 2. Only barcoded shoots were used to create this graph.

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.

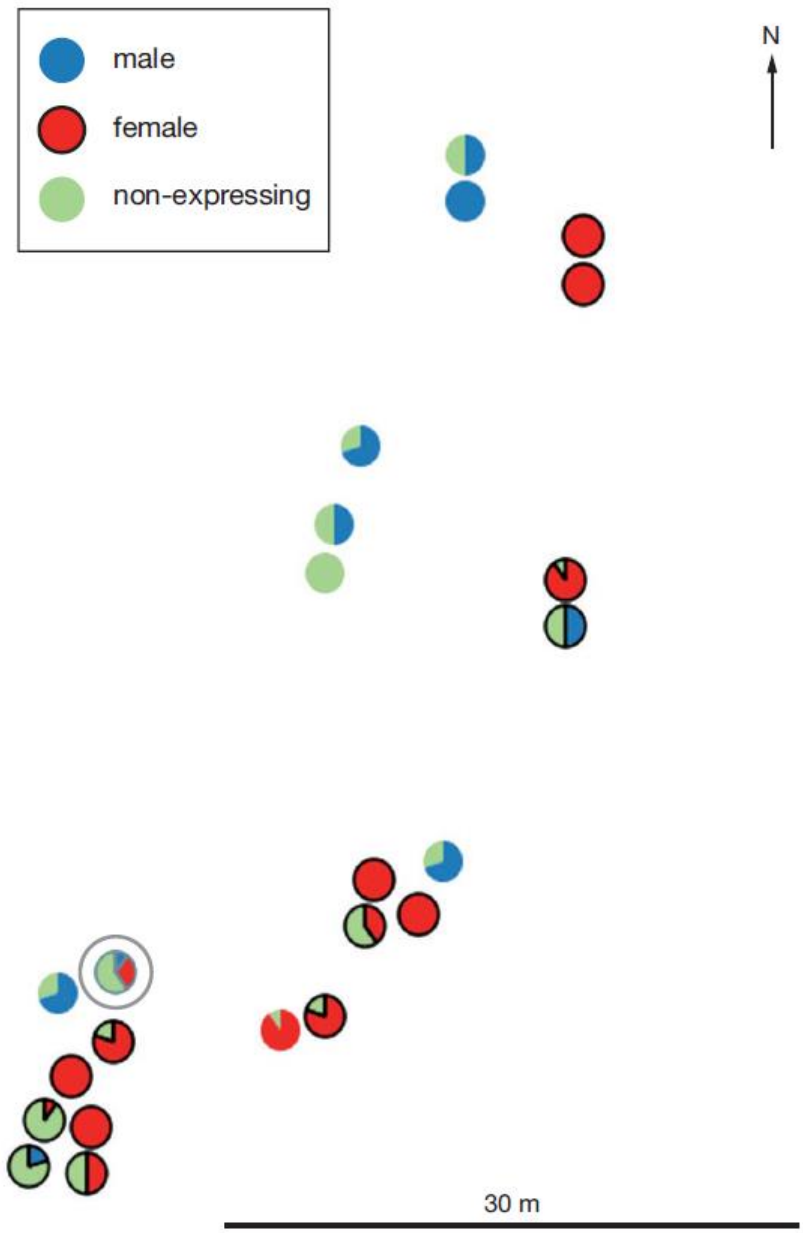

Fig. 5 The sex ratio in mixed population at locality Zhůří 1. Unbordered pie charts refer to clade 1, bordered ones represent clade 2. The patch in the larger circle contained plants of both clades, so this patch must be excluded from evaluating sex ratio in separated clades.

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 locaties"*), 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* (Buczkowska *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**Error! Reference source not found.**), 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.

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References

BISANG I. & HEDENÄS L., 2005 — Sex ratio patterns in dioicous bryophytes re-visited. *Journal of Bryology* 27: 207-219.

BISANG I. & HEDENÄS L., 2013 — Males are not shy in the wetland moss *Drepanocladus lycopodioides*. *International Journal of Plant Science* 174: 733–9.

BISANG I., EHRLEN J., PERSSON C. & HEDENÄS L., 2014 – Family affiliation, sex ratio and sporophyte frequency in unisexual mosses. *Botanical Journal of the Linnean Society* 174: 163-72.

BISANG I., HEDENÄS L. & CRONBERG N., 2017 — Can the meiotic sex ratio explain the sex ratio bias in adult populations in the dioicous moss *Drepanocladus lycopodioides*. *Journal of Bryology* 39: 115-120.

BISANG I., KORPELAINEN H. & HEDENÄS L., 2010 — Can the sex specific molecular marker of *Drepanocladus trifarius* uncover gender in related species? *Journal of Bryology* 32: 305-308.

BOWKER M. A., STARK L. R., MCLETCHIE D.N. & MISHLER. B.D., 2000 — Sex expression, skewed sex ratios, and microhabitat distribution in the dioecious desert moss *Syntrichia caninervis* (Pottiaceae). *American Journal of Botany* 87: 517-526.

BUCZKOWSKA K, ADAMCZAK M. & BĄCZKIEWICZ A., 2006 — Morphological and anatomical differentiation within the *Aneura pinguis* complex (Metzgeriales, Hepaticae). *Biology Letters* 43: 51-68.

EPPLEY S.M., ROSENSTIEL T.N., GRAVES C.B. & GARCIA E., 2011 — Limits to sexual reproduction in geothermal bryophytes. *International Journal of Plant Sciences* 172:870-978.

GLIME J. M. & BISANG I., 2017 — Sexuality: Sex Ratio and Sex Expression. Chapt. 3-2. *In:* Glime, J.M. *Bryophyte Ecology*. 3-2-1 Volume 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

HAIG D., 2016 — Living together and living apart: the sexual lives of bryophytes. In: Contribution to a themed issue ‘Weird sex: the underappreciated diversity of sexual reproduction’. *Philosophical Transactions of the Royal Society of London, Series B* 371: 20150535 1-9.

HEDENÄS L., BISANG I., KORPELAINEN H. & CRONHOLM B., 2010 — The true sex ratio in European *Pseudocalliergon trifarium* (Bryophyta: Amblystegiaceae) revealed by a novel molecular approach. *Botanical Journal of the Linnean Society* 100: 132-140.

HEDENÄS L. & ELDENÄS P., 2007 — Cryptic speciation, habitat differentiation, and geography in *Hamatocaulis vernicosus* (Calliergonaceae, Bryophyta). *Plant Syshootatics and Evolution* 268: 131-145.

HOLÁ E., VESALAINEN T., TĚŠITEL J. & LAAKA-LINDBERG S., 2014 — Sex ratio, sex-specific pattern in vegetative growth and gemma production in an aquatic liverwort, *Scapania undulata* (Marchantiophyta: Scapaniaceae). *Botanical Journal of the Linnean Society* 175(2): 229-241.

LONGTON R. E. 1976. — Reproductive biology and evolutionary potential in bryophytes. Journal ofHattori Bottanical Laboratory 41: 205-223.
LONGTON R.E. & MILES C.J., 1982 — Studies on the reproductive biology of mosses. *Journal of the Hattori Botanical Laboratory* 52: 219-239.

LONGTON R. E. & SCHUSTER R.M., 1983 — Reproductive biology. – New Manual of Bryology, vol. 1 (ed. R. M. Schuster), Hattori Botanical Laboratory, Nichinan. pp. 396-462.

MANUKJANOVÁ A., KOŠNAR J. & KUČERA J. 2018. Microsatellite primers for the cryptic species of the moss *Hamatocaulis vernicosus* and methods for their quick barcoding. *Journal of Bryology* (in press)

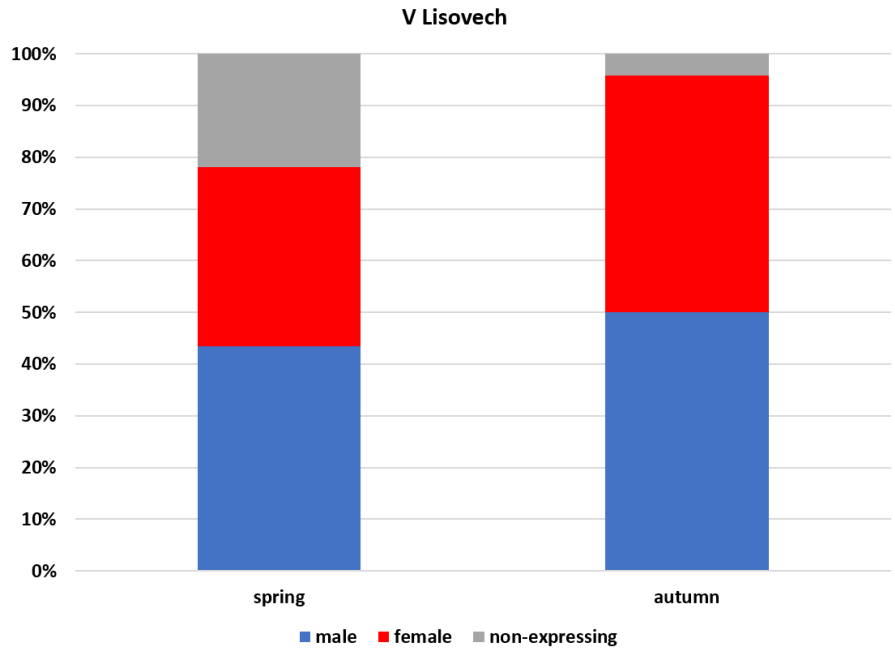
MCLETCHIE D.N., 1992 — Sex ratio from germination through maturity and its reproductive consequences in the liverwort *Sphaerocarpos texanus*. *Oecologia* 92: 273-278.

MCLETCHIE D.N., 2001 — Sex-specific germination response in the liverwort *Sphaerocarpos texanus* (Sphaerocarpaceae). *The Bryologist* 104: 69-71.

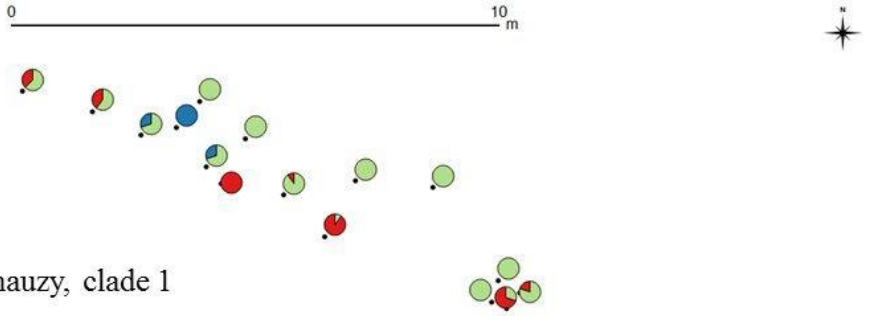
MISHLER B.D. & OLIVER M.J., 1991 — Gametophytic phenology of *Tortula ruralis*, a desiccation-tolerant moss, in the Organ Mountains of southern New Mexico. *The Bryologist* 94 :143-153.
NEWTON M.E., 1972 — Sex ratio differences in *Mnium hornum* Hedw. and *M. undulatum* Sw. in relation to spore germination and vegetative regeneration. *Annals of Botany* 36: 163-178.
PÉPIN F., HUGONNOT V. & CELLE J., 2013 — Sex ratio patterns and fertility of *Hamatocaulis vernicosus* (Mitt.) Hedenäs at different spatial scales. *Journal of bryology* 35: 20-26.
POSCHLOD P. U. & SCHRAG H., 1990 — Regeneration vegetativer Teilchen von "Braunmoosen". *Telma* 20: 291-301.
SHAW A.J., 2000 — Population ecology, population genetics and microevolution. in AJ Shaw & Goffinet B. eds. Bryophyte biology. Cambridge University Press, Cambridge. pp 369–402.
SHAW A.J., NIGUIDULA N.J. & WILSON T.M., 1992 — Reproductive biology of the rare ‘‘copper moss’’ *Mielichhoferia mielichhoferiana*. *Contributions from the University of Michigan Herbarium* 18: 131-140.
STARK L.R., MISHLER B.D. & MCLETCHIE D.N., 2000 — The cost of realized sexual reproduction: assessing patterns of reproductive allocation and sporophyte abortion in a desert moss. *American Journal of Botany* 87: 1599-1608.
STARK L.R., MCLETCHIE D.N. & MISHLER B.D., 2005 — Sex expression, plant size, and spatial segregation of the sexes across a stress gradient in the desert moss *Syntrichia caninervis*. *The Bryologist* 108: 183-193.
STARK L.R., MCLETCHIE D. & EPPLEY S., 2010 — Sex ratios and the shy male hypothesis in the moss *Bryum argenteum* (Bryaceae). *The Bryologist* 113(4): 788-797.
TELEGANOVA V.V. & IGNATOV M.S., 2007 — On the reproductive biology of *Ptilium crista-castrensis*. *Arctoa* 16: 87-98.

Appendices

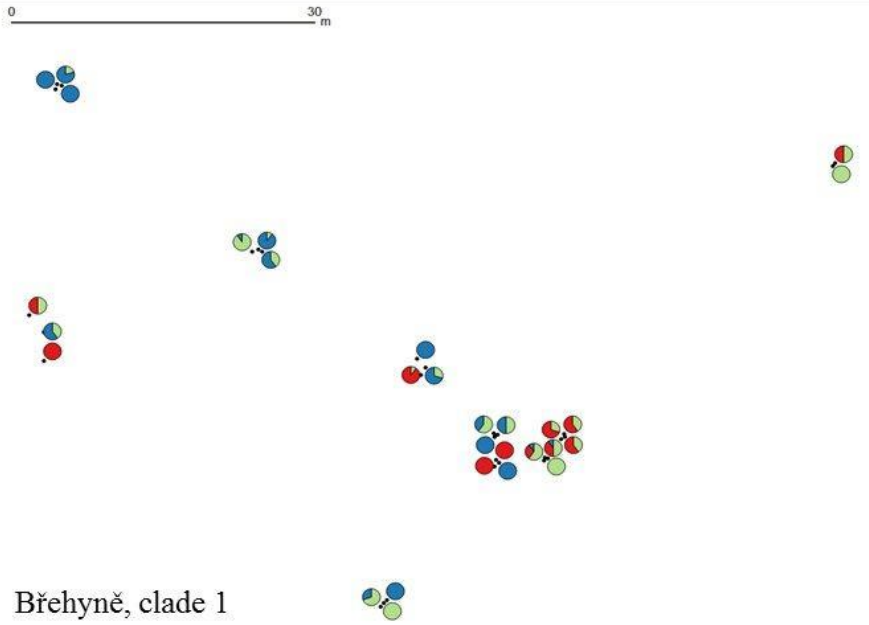
Appendix 1 Seasonal variation of the sex expression and F:M ratio at the locality V Lisovech. 24 patches were inspected on spring and 26 patches in autumn. The difference in sex ratio between the samplings counted by one-way ANOVA was statistically significant ($F(1;48) = 5.0396$; $p = 0.0294$)



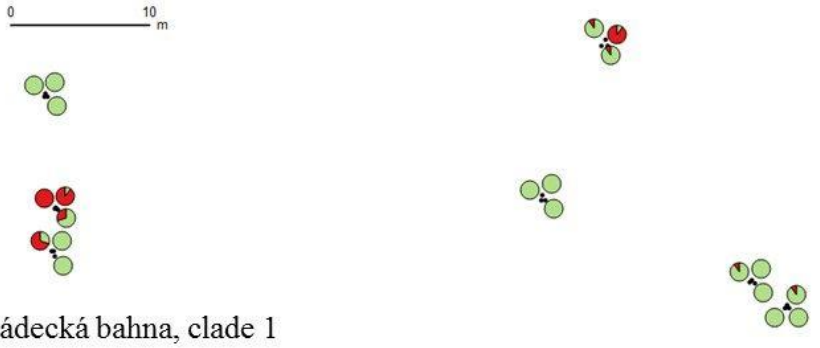
Appendix 2a-e Spatial distribution and rates of male (blue), female (red) and non-expressing (green) plants of *Hamatocaulis vernicosus* at individual localities. The small dots show the position of patches, the pie-charts observed sex ratio in the patch. Blue – male, red – female, green – sterile. All 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 in located in result section of the article, the spatial data for locality Novozámecký rybník are not available.



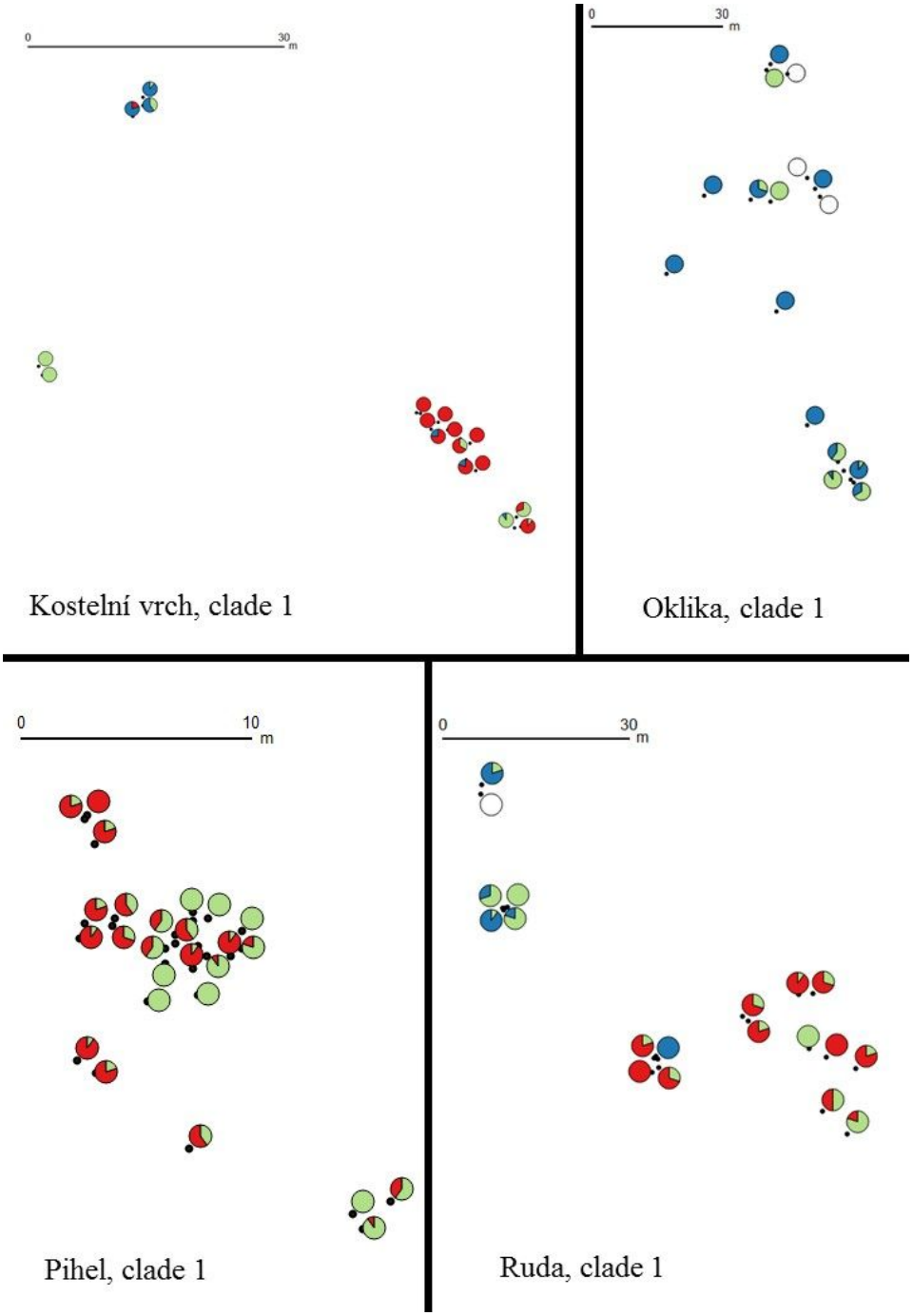
Šmauzy, clade 1

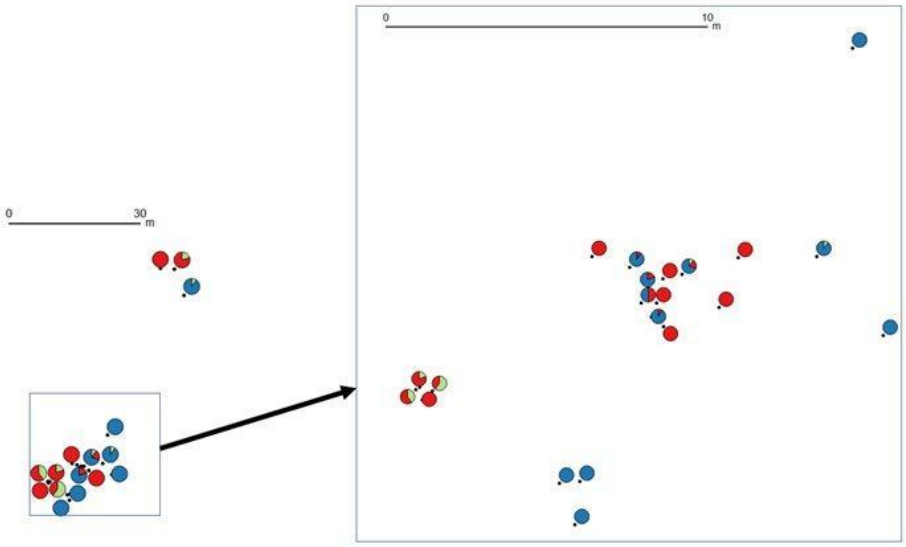


Břehyně, clade 1

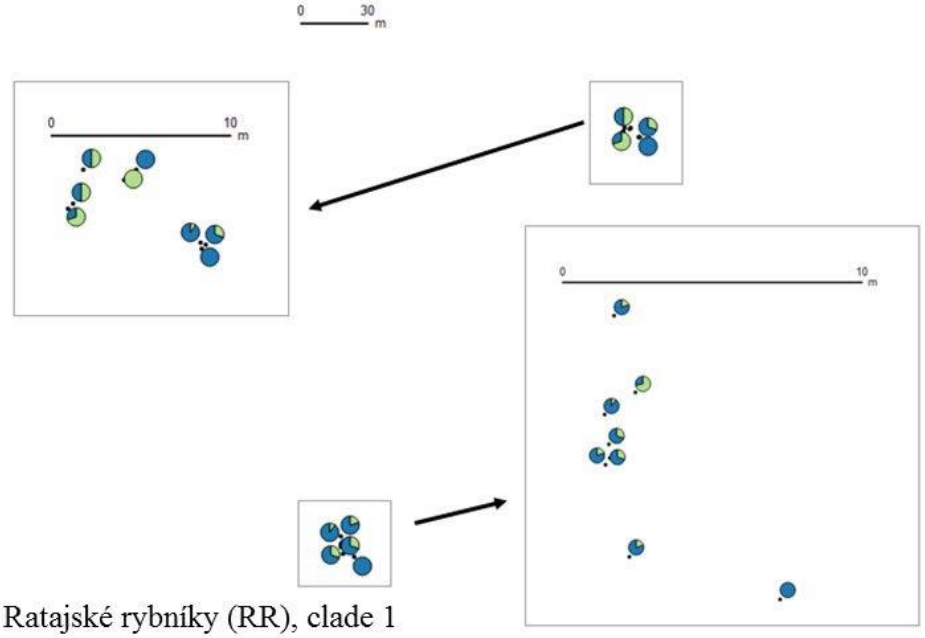


Hrádecká bahna, clade 1

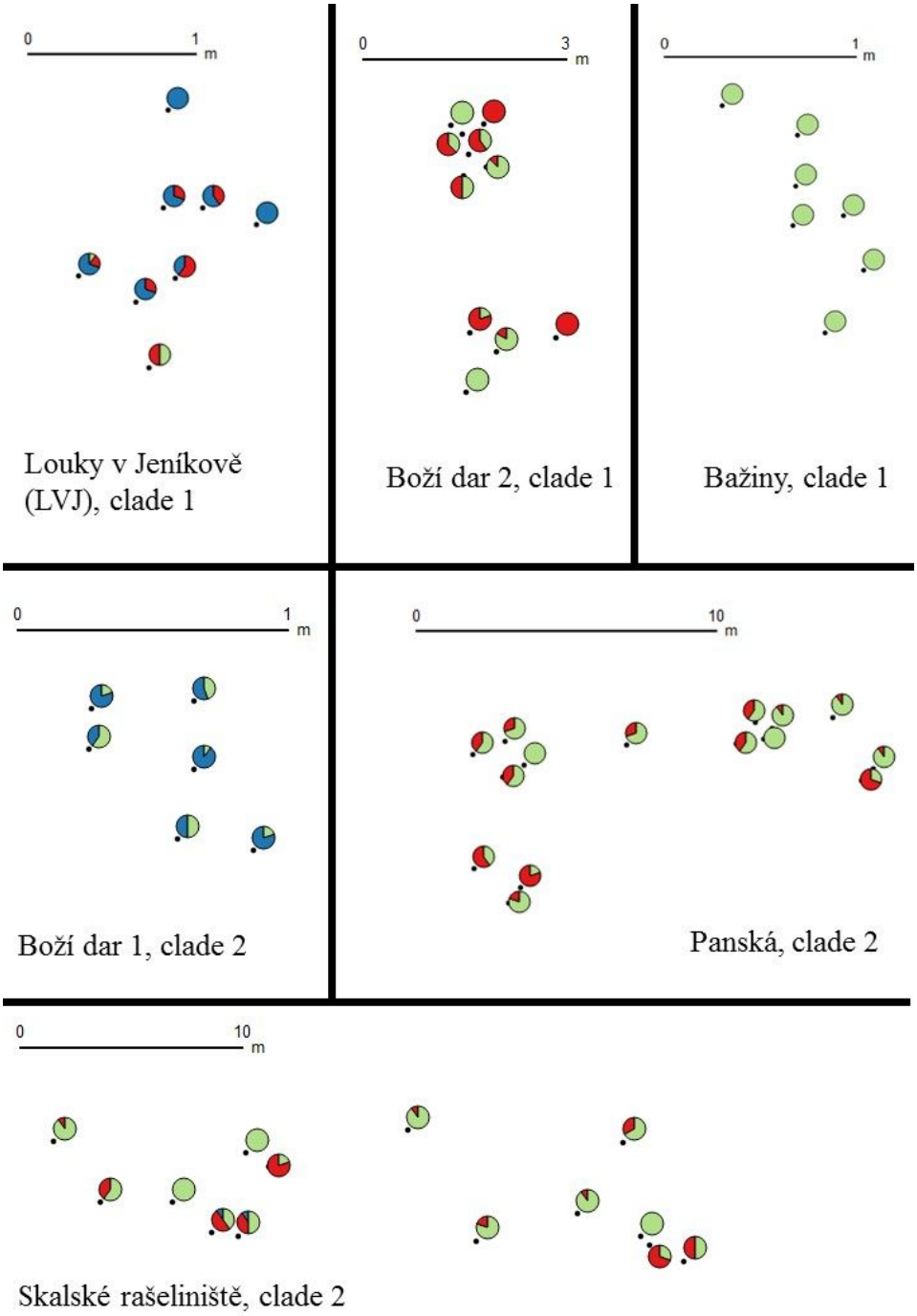

0
30
m
Kostelní vrch, clade 1
0
30
m
Oklika, clade 1
0
10
m
Pihel, clade 1
0
30
m
Ruda, clade 1

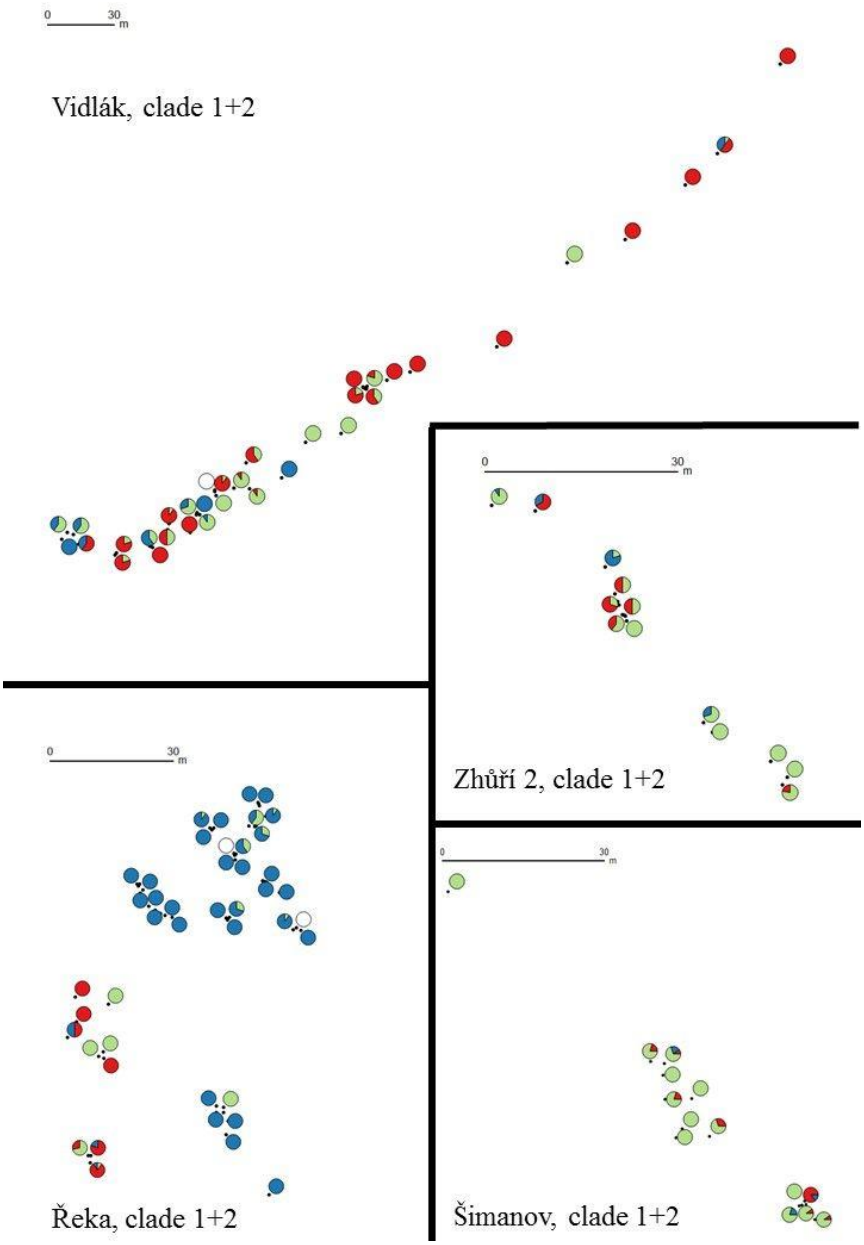


V Lisovech (autumn sampling), clade 1



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
0
10
m
Skalské rašeliniště, clade 2


0 30 m
Vidlák, clade 1+2
0 30 m
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

patch	stem 1	a	stem 2	b	stem 3	c	clade in patch
	sex	clade	sex	clade	sex	clade	
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
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

patch	stem 1	a	stem 2	b	stem 3	c	clade in patch
	sex	clade	sex	clade	sex	clade	
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
22	f	2	f	2	f	2	2
23	m	1	m	1	m	1	1
24	s	1	s	1	m	1	1
Zhůří 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

Chapter 4: Insights into the distribution patterns, habitat and morphologic differentiation of cryptic species of the moss Hamatocaulis vernicosus in the Czech Republic.

Manukjanová A., Koutecký P. Štechová T. & Kučera J. (2019). Insights into the distribution patterns, habitat and morphologic differentiation of cryptic species of the moss *Hamatocaulis vernicosus* in the Czech Republic. Herzogia 32: 183–199.

Insights into the distribution patterns, habitat and morphologic differentiation of cryptic species of the moss *Hamatocaulis vernicosus* in the Czech Republic.

Alžběta Manukjanová, Petr Koutecký, Táňa Štechová, Jan Kučera

Abstract

We studied local distribution, morphological and ecological differences between the two cryptic species of *Hamatocaulis vernicosus* in the Czech Republic. Distribution was assessed at both regional scale and within localities using several barcoding methods including direct sequencing and PCR-RFLP of ITS region of nrDNA, as well as amplification of clade-specific SSR markers. The lineage known as clade 1 occurs on more than 90% of the 70 investigated Czech localities, while clade 2 occurs at only about 10% of localities, which moreover mostly support plants of both clades. Analysis of ITS sequences from Czech samples showed considerable variability in clade 1, while plants of clade 2 were invariable. Comparison of climatic characteristics did not reveal any significant differences in mean annual temperature, precipitation and frost days between localities of both clades, although clade 2 plants tends to grow at higher elevations. Statistical evaluation of morphometric data has not revealed any character which would morphologically distinguish plants of both clades.

Zusammenfassung:
Manukjanová, A., Koutecký, P., Štechová, T. & Kučera, J. 2019. Erkenntnisse zum Verbreitungsmuster, zu den Habitatansprüchen und zur morphologischen Differenzierung der kryptischen Sippen des Laubmooses Hamatocaulis vernicosus in der Tschechischen Republik. – Herzogia 32: 183 –199.
Wir haben die lokale Verbreitung sowie die morphologischen und ökologischen Unterschiede zwischen den zwei kryptischen Sippen von Hamatocaulis vernicosus in der Tschechischen Republik untersucht. Die Verbreitung wurde sowohl regional als auch zwischen den Lokalitäten unter Verwendung verschiedener Barcoding-Methoden einschließlich direkter Sequenzierung und PCR-RFLP der ITS-Region der nrDNA, als auch durch Amplifikation von Klade-spezifischen SSR-Markern bewertet. Die Abstammungslinie Klade 1 kommt an mehr als 90% der 70 untersuchten tschechischen Lokalitäten vor, während Klade 2 nur an circa 10% der Lokalitäten festgestellt wurde, wobei an diesen Lokalitäten meistens Pflanzen beider Kladen vorkommen. Analysen der ITS-Sequenzen der tschechischen Proben zeigen eine erhebliche Variabilität von Klade 1, während Pflanzen von Klade 2 invariabel sind. Vergleiche von Klimafaktoren zeigen keine signifikanten Unterschiede in der durchschnittlichen Jahrestemperatur, dem Niederschlag und den Frosttagen zwischen den Standorten der zwei Kladen, wenngleich die Pflanzen der Klade 2 tendenziell in höheren Lagen vorkommen. Bei der statistischen Bewertung der morphometrischen Daten konnte kein Merkmal festgestellt werden, auf Grund dessen eine morphologische Unterscheidung der Pflanzen der beiden Kladen möglich wäre.

Keywords: cryptic species, bryophyte, morphometry, regional distribution, fine-scale distribution, ribotypes
Running title: Cryptic species of the moss *Hamatocaulis vernicosus* in the Czech Republic.

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The full text of this paper is a property of Herzogia journal and Bioone and is accessible at
https://bioone.org/journals/Herzogia/volume-32/issue-1/heia.32.1.2019.183/Insights-into-the-Distribution-Patterns-Habitat-and-Morphologic-Differentiation-of/10.13158/heia.32.1.2019.183.full

Chapter 5: The genetic variability in cryptic species of the rare fen moss *Hamatocaulis vernicosus*

Manukjanová A., Košnar J. & Kučera J. (in prep). The genetic variability in cryptic species of the rare fen moss *Hamatocaulis vernicosus* (manuscript submitted in Preslia)

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

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Abstract

Patterns of genetic variation in both cryptic species of the rare moss *Hamatocaulis vernicosus* have been studies in the Czech Republic, using two sets of polymorphic microsatellite loci, developed for each cryptic species separately with respect of cross-amplification failure. Reproductive isolation of the morphologically undistinguishable and commonly co-occurring species was confirmed not only by the absence of cross-compatibility in all but five of the usable primers, but also by the obvious absence of gene flow at mixed localities, where both cryptic species grow together. The genetic diversity of clade 1 (southern cryptic species), which is more common in the region, was higher than in clade 2 (northern species). At the same time, the structure of the genetic variability differed as the 84% of variability was allocated among populations in clade 2, while in clade 1 the rate of inter-population variability was only 51%. The lineages have obviously different histories in Central Europe. The high genetic isolation among populations together with high kinship coefficients insinuating low levels of sexual reproduction at most of the localities witness the detrimental effect of habitat fragmentation affecting the endangered Central European fens. Both clades have different levels of clonality, substantially higher at localities of clade 2. The effect of genetic pauperisation seems to be counteracted by the emergence of somatic mutations, observed at several localities.

Shrnutí

Genetická variabilita dvou kryptických druhů vzácného slatiništního mechu *Hamatocaulis vernicosus*

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 neúspěšných kros-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 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 leželo 84 % variability mezi populacemi, zatímco u clade 1 pouze 51 %. Je patrné, že studované linie mají na území střední Evropy odlišnou historii. Vysoká genetická izolovanost v populacích obou linií spolu s vysokým koeficientem příbuznosti poukazuje na nízkou hladinu pohlavního rozmnožování na většině lokalit a vážné důsledky fragmentace biotopu, která ovlivňuje středoevropská slatiniště. Oba druhy mají vysokou míru klonality, která je výraznější u cladu 2. Genetické ochuzení je nicméně vyváženo přítomností somatických mutací, které byly pozorovány na některých lokalitách.

Keywords: *Hamatocaulis vernicosus*, microsatellites, spatial genetic structure, cryptic species, bryophyte, dispersal limitation

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This article is under rewiew and is not yet accessible in fulltext.

Chapter 6: General conclusions

Both earlier reported cryptic species of *H. vernicosus* were discovered in the Czech Republic. The number of their localities is uneven, clade 1 occurs with approximately ten times higher frequency than clade 2, which is more frequent in Scandinavia, where most of the research has been done so far. Most of the Czech clade 2 localities also contained plants of clade 1, which is a new observation, as the co-occurrence of the two cryptic species at localities has not been recorded before. The common co-occurrence indicates that the two clades have overlapping ecological requirements. The similarity in ecology is matched by the absence of morphological distinguishing characters, which have not been found even following a scholarly morphometric analysis; the two lineages believed to be cryptic species remain thus truly cryptic, which is a rare phenomenon among plants in general.

Although morphologically undistinguishable, the two lineages likely represent two biological species, as indicated by the microsatellite data, which revealed no gene flow between them, even at the mixed localities. The extent and structure of genetic diversity in populations of clade 1 and 2, assessed using the SSR data also markedly differs at the territory of the Czech Republic. The variability in microsatellite loci largely matches the variability ITS ribotypes, which is markedly different from published data from other regions, especially northern Scandinavia. This comparison shows that the population of clade 2 in the Czech Republic represents only a small part of its variability worldwide. Unlike the likely long migration history of the clade 1, clade 2 seems to have been established at our territory following a single or very few migration events in Holocene. Investigation of detailed genotype distribution at the localities revealed the aggregation of clones and closely related genotypes, although often the clusters showed the internal genetic differentiation likely resulting from the ongoing somatic mutations.

The cryptic species differed neither in their sex expression nor in the sex ratio. However, the overall seemingly well-balanced sex ratio at mixed localities often obscured situations when severe mate limitation in one of the cryptic species occurred.

Since clade 2 is rare in the Czech Republic, protection or at least particular monitoring of its localities should be proposed above the general protection given to it as an Annex 2 species of the Habitats Directive (92/43/EEC). However, most of the populations seem to be rather stable. Future studies should probably aim at deciphering the potential ecological differences between the cryptic species, since these were not studied in greater detail in this thesis, mainly because of the small number of clade 2 localities in the Czech Republic, and the problem posed by mixed populations as well as the atypically dry summers in the last 3 years, which prevented sampling of water for analysis at many localities.

Chapter 7: Shrnutí (Summary in Czech)

Oba nedávno zaznamenané kryptické druhy *Hamatocaulis vernicosus* byly nalezeny i v České republice. Clade 1 má nicméně téměř desetkrát více lokalit, což je opačná situace než ve Skandinávii, kde byla zatím prováděna většina výzkumu na těchto kryptických liniích. Obě linie *H. vernicosus* se zatím nepodařilo odlišit žádnými morfologickými znaky ani při detailní morfologické studii a zůstávají tak zcela kryptické, což je u rostlin poměrně vzácný jev. Na většině českých lokalit cladu 2 se rovněž nachází clade 1, což ukazuje jejich značně se překrývající ekologické preference. Směsné lokality nebyly dosud zaznamenány v žádné studii.

Navzdory morfologické nerozlišitelnosti představují obě linie samostatné biologické druhy, což se ukázalo při morfologických analýzách, kdy nebyl zaznamenán genový tok mezi clady ani na směsných lokalitách. , Při srovnání genetické diverzity v populacích cladu 1 a 2 se ukázalo, že clade 2 má na území ČR extrémně nízkou variabilitu, což se projevuje jak v sekvencích ITS úseku, tak na variabilitě mikrosatelitů. Z variability ITS je zřejmé, že se na území ČR vyskytuje pouze malý výsek jeho genetické variability. Na rozdíl od pravděpodobně dlouhé migrační historie cladu 1 v tomto regionu, clade 2 osídlil území ČR patrně mnohem později v rámci jedné či několika málo holocenních migračních událostech. Studium detailní distribuce genotypů na jednotlivých lokalitách ukázalo shlukování klonů a blízce příbuzných genotypů, i když shluky občas ukazovali ojedinělé genetické rozdíly Mikrosatelitní data neindikují probíhající hybridizaci mezi cladem 1 a 2, dokonce ani na směsných lokalitách. V rámci směsných lokalit se rostliny obou cladů často vyskytují v clusterech, které jsou ale místy geneticky diverzifikované, patrně díky výskytu somatických mutací.

Mezi studovanými kryptickými druhy nebyl nalezen rozdíl v expresi gametangií ani v poměru pohlaví. Na lokalitách s výskytem obou cladů se nicméně stávalo, že pokud se kryptické druhy nerozlišovaly, zdál se poměr pohlaví vyrovnaný, zatímco když se oddělily, ukázala se jejich

výrazná disproporce, vedoucí k potenciální limitaci pohlavního rozmnožování z důvodu nedostupnosti gamet opačného pohlaví.
Jelikož je clade 2 v ČR vzácný, je třeba stav jeho populací pečlivě monitorovat, nicméně zatím se nezdá, že by jej ohrožoval náhlý ústup. Populace cladu 2 se prozatím zdají být stabilní a dočasná všeobecná ochrana jakožto druhu náležející do přílohy 2 evropské směrnice o stanovištích se zdá být dostatečná. Případné navazující studie by bylo vhodné zaměřit na hledání rozdílů mezi kryptickými druhy *H. vernicosus* v ekologických a mikrostanovištních preferencích.

Chapter 8: Curriculum vitae

Date of birth: 01.01.1986
Nationality: Czech
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Education

- 2011 – 2019: Doctoral studies, botany, University of South Bohemia, Faculty of Science, topic: **Ecology and molecular ecology of fen mosses** (supervisor J. Kučera)
- 2012 – 2014: Master studies, biology teaching, University of South Bohemia, Faculty of Science
- 2008 – 2011: Master studies, botany, University of South Bohemia, Faculty of Science, topic: **Vybrané ekologické charakteristiky mechu** *Hamatocaulis vernicosus* /The ecological characteristics of the moss *Hamatocaulis vernicosus*. (supervisor T. Štechová)
- 2005 – 2008: Bachelor studies, botany, University of South Bohemia, Faculty of Science, topic: **Kompetiční a regenerační schopnosti mechu** *Hamatocaulis vernicosus* /Competitive and regenerative abilities of the moss *Hamatocaulis vernicosus*.(supervisor T. Štechová)

Internship

- 2014: J. Shaw Laboratory, Department of Biology, Duke University, Durham, North Carolina, USA
- 2009: Summer course of peatland ecology, Uppsala University, Sweden

Conference

- 2012: 8th Conference of European Committee for Conservation of Bryophytes, Budapest, poster: Desiccation tolerance of fen bryophytes
- 2009: 2 nd European Congress of Conservation Biology, Prague, poster: Desiccation tolerance and regeneration ability of fen bryophyte species

Teaching

The teaching was mostly aimed at TA of practical part of various botanical courses:

KBO/137 Základní kurz botaniky, fykologie a mykologie/ Basic course in botany, phycology and mycology – practical part (botany)

KBO/132 Botanika vyšších rostlin – malá/ Botany of higher plants – basic – practical part, 1 lecture (bryology)

KBO/138 Botanika vyšších rostlin – velká 1/ Botany of higher plants – advanced 1 – practical part (bryology)

KBO/004 Biologická laboratorní technika / Laboratory techniques in biology – 1practical part (microscoping dyed *Sphagnum* cells)

Work experiences

- Laboratory technician in botanical molecular laboratory – University of South Bohemia
- Various bryological inventories and monitoring of rare mosses.

Publications

Publicatios with IF:

Manukjanová A., Kučera J. & Štechová, T. 2014. Drought survival test of eight fen moss species. – Cryptogamie, Bryologie 35: 397–403.

Carter B.E., Larraín J., **Manukjanová** A., Shaw B., Shaw A.J., Heinrichs J., de Lange P., Suleiman M., Thouvenot L. & von Konrat M. 2017. Species delimitation and biogeography of a southern hemisphere liverwort clade, *Frullania* subgenus *Microfrullania* (Frullaniaceae, Marchantiophyta). – Molecular Phylogenetics and Evolution 107: 16-26

Manukjanová A., Košnar J. & Kučera J. 2018. Microsatellite primers for the cryptic species of the moss *Hamatocaulis vernicosus* and methods for their quick barcoding. – Journal of Bryology 40: 302–305

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.

Manukjanová A., Koutecký P. Štechová T. & Kučera J. 2019. Cryptic species of the moss *Hamatocaulis vernicosus* in the Czech Republic. – Herzogia 32: 183–199

Kučera J. Kuznetsova O., **Manukjanová** A. & Ignatov M.I. (in press). Phylogenetic revision of the genus *Hypnum*: towards the completion. Accepted in Taxon

Publicatios without IF:

Dřevojan P., Holá E., Jandová L., Košnar J., Kubešová S., Kučera J., **Manukjanová** A., Mikulášková E., Müller F., Peterka T., Štechová T. & Štěrbová J. 2018. Zajímavé bryofloristické nálezy XXX. – Bryonora 62: 76–83

Štechová T., **Manukjanová** A. & Bradáčová J. 2018. Nálezy vzácných mechorostů na slatinných loukách ve Slavkovském lese. – Arnika 2/2017: 46–49

Kučera J., Fialová L., Kubešová S., Kyselá M., **Manukjanová** A., Mikulášková E., Skoupá Z. & Tkáčiková J. 2017. Mechorosty zaznamenané v průběhu podzimních bryologicko-lichenologických dnů v Českém ráji (Sedmihorky) v roce 2015. – Bryonora 60: 13–23

Kučera J., Dřevojan P., Bradáčová J., Fialová L., Godovičová K., Janošík L., Kubešová S., **Manukjanová** A., Mikulášková E., Skoupá Z. & Tkáčiková J. 2017. Mechorosty zaznamenané v průběhu jarního bryologicko-lichenologického setkání na Pálavě v roce 2017. – Bryonora 60: 1–12

Kučera J., Dřevojan P., Hradílek Z., Kubešová S., Laburdová J., Lysák F., **Manukjanová** A., Koval Š., Peterka T., Soldán Z., Štechová T. & Zmrhalová M. 2016. Zajímavé bryofloristické nálezy XXVI.. – Bryonora 58: 73-78. (www link)

Kučera J., Dřevojan P., Hradílek Z., Kubešová S., Laburdová J., Lysák F., **Manukjanová** A., Koval Š., Peterka T., Soldán Z., Štechová T. & Zmrhalová M. 2016. Zajímavé bryofloristické nálezy XXVI. – Bryonora 58: 73-78.

Kubešová S., Kučera J., Jandová J., **Manukjanová** A., Novotný I., Táborská M. & Tkáčiková J. 2016. Mechorosty zaznamenané během jarního Bryologicko-lichenologického setkání na Mohelenském mlýně v dubnu 2016. – Bryonora 58: 28-37.

Kučera J., Bradáčová J., Fialová L., Jandová J., **Manukjanová** A., Oliveriusová D., Plaček J., Tkáčiková J. & Vicherová E. 2016. Mechorosty zaznamenané v průběhu Bryologicko-lichenologických dnů na Semilsku v září 2016. – Bryonora 58: 18-27.

Kučera J., Dřevojan P., Ekrtová E., Holá E., Koval Š., **Manukjanová** A., Peterka T., Procházková J., Štechová T., Táborská M., Tkáčiková J., Vicherová E. & Zmrhalová M. 2016. Zajímavé bryofloristické nálezy XXV. – Bryonora 57: 83-91

Kučera J., Hradílek H., Holá E., Košnar J., Kubešová S., **Manukjanová** A., Marková I., Mikulášková E., Uhereková Šmelková D. & Vicherová E. 2015[2016]. Mechorosty zaznamenané během exkurzí Bryologicko-lichenologických dnů v Podyjí (duben 2011). – Thayensia (Znojmo) 12: 49–64.

Kučera J., Bradáčová J., Holá E., Jandová J., Košnar J., Kubešová S., Kyselá M., Laburdová J., **Manukjanová** A., Tkáčiková J. & Štechová T. 2015. Mechorosty zaznamenané v průběhu terénních bryologických kurzů Jihočeské Univerzity na Šumavě v roce 2014. – Bryonora 56: 88–104

Holá E., **Manukjanová** A., Ekrtová E. & Štechová T. 2015. Citlivost rašeliništních mechů na správné provádění managementu, Ochrana přírody 2015:24-25

Štechová T., Holá E., Ekrtová E., **Manukjanová** A. & Kučera J. 2014[2015]. Monitoring ohrožených rašeliništních mechorostů a péče o jejich lokality: metodika AOPK ČR. – Agentura ochrany přírody a krajiny České republiky, Praha [64 pp.]

Manukjanová A., Kučera J. & Štechová, T. 2014. Drought survival test of eight fen moss species. – Cryptogamie, Bryologie 35: 397–403.

Štechová T., **Manukjanová** A., Vicherová E. & Kučera J. 2014. Výskyt vzácných a ohrožených druhů rašeliníků na Třeboňsku. – Sborník Jihočeského muzea v Českých Budějovicích, Přír. Vědy, 53: 120–127.

Štechová T., **Manukjanová** A. & Ondráček O. 2014. Bryoflóra vybraných rašelinišť a pramenišť v okolí Božího Daru v Krušných horách. – Severočeskou Přírodou 44: 103–114

Kučera J., Bradáčová J., Holá E., Kubešová S., **Manukjanová** A., Mikulášková E., Štechová T., Tkáčiková J. & Vicherová E. 2013. Results of the bryofloristic courses of the Department of Botany, University of South Bohemia, in 2012 and 2013. – Časopis Slezského Zemského Muzea, Sér. A, 62: 173–184.

Kučera J., Plášek V., Kubešová S., Bradáčová J., Holá E., Košnar J., Kyselá M., **Manukjanová** A., Mikulášková E., Procházková J., Táborská M., Tkáčiková J. & Vicherová E. 2014. Mechorosty zaznamenané během podzimních 26. bryologicko-lichenologických dní (2013) v Beskydech. – Bryonora 54: 11–21.

Štechová T., **Manukjanová** A., Holá E., Kubešová S., Novotný I. & Zmrhalová M. 2010. Současný stav populací druhů *Helodium blandowii* (Thuidiaceae) a Scorpidium scorpioides (Calliergonaceae) v České Republice. – Bryonora 46: 22–32.

Štechová T., **Manukjanová** A. et Čejková A. 2011. Bryoflóra tří rašelinných luk v Orlických horách. – Bryonora 47: 52–56.

Štechová T., Holá E., **Manukjanová** A. & Mikulášková E. 2010. Distribution and habitat requirements of the moss *Hamatocaulis vernicosus* (Mitt.) Hedenäs in the Bohemian Forest. – Silva Gabreta 16: 1–11.

Manukjanová A. & Velehradská T. (2010): Paludella squarrosa. – In: Kučera J. [ed.], Zajímavé bryofloristické nálezy XVI., Bryonora 46: 74

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