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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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Alžběta Manukjanová

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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.

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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.

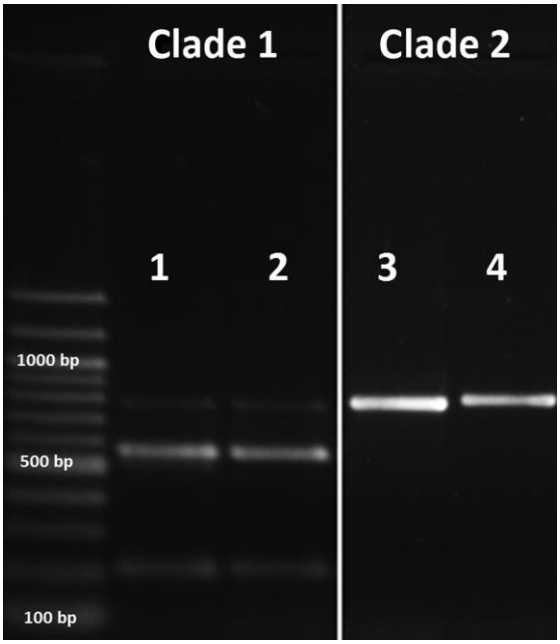

Figure 1. PCR-RFLP gel. PCR products from samples of clade 2 remain intact (702 bp), while products of clade 1 samples yield two bands of expected length (161 + 541 bp). Scale – 100 bp DNA ladder (New England Biolabs).

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.

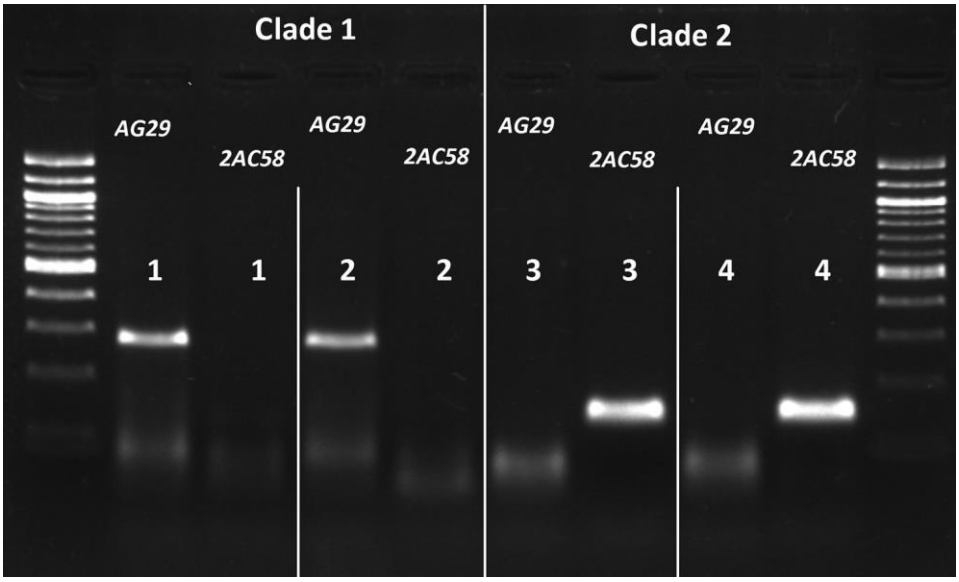

Figure 2. The agarose gel of 4 samples of *H. vernicosus*. Sample 1 and 2 belong to clade 1 and samples 3 and 4 to clade 2. Every sample was amplified with primer pairs AG29 and 2AC58, respectively. AG29 amplifies only samples of clade 1 while 2AC58 amplifies only clade 2 samples. Scale – 100 bp DNA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

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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).

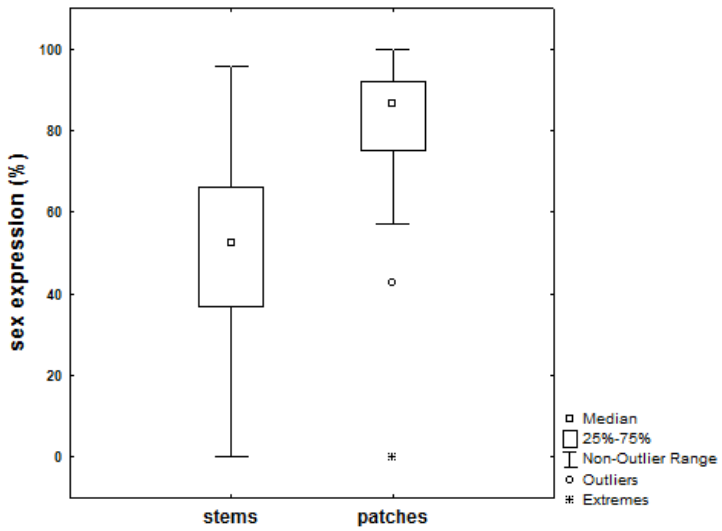

Fig. 1 The sex expression of *Hamatocaulis vernicosus* in the Czech Republic at individual localities assessed at two levels of pooling hierarchy - ("*shoots at localities"* and "*patches at localities"*).

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).

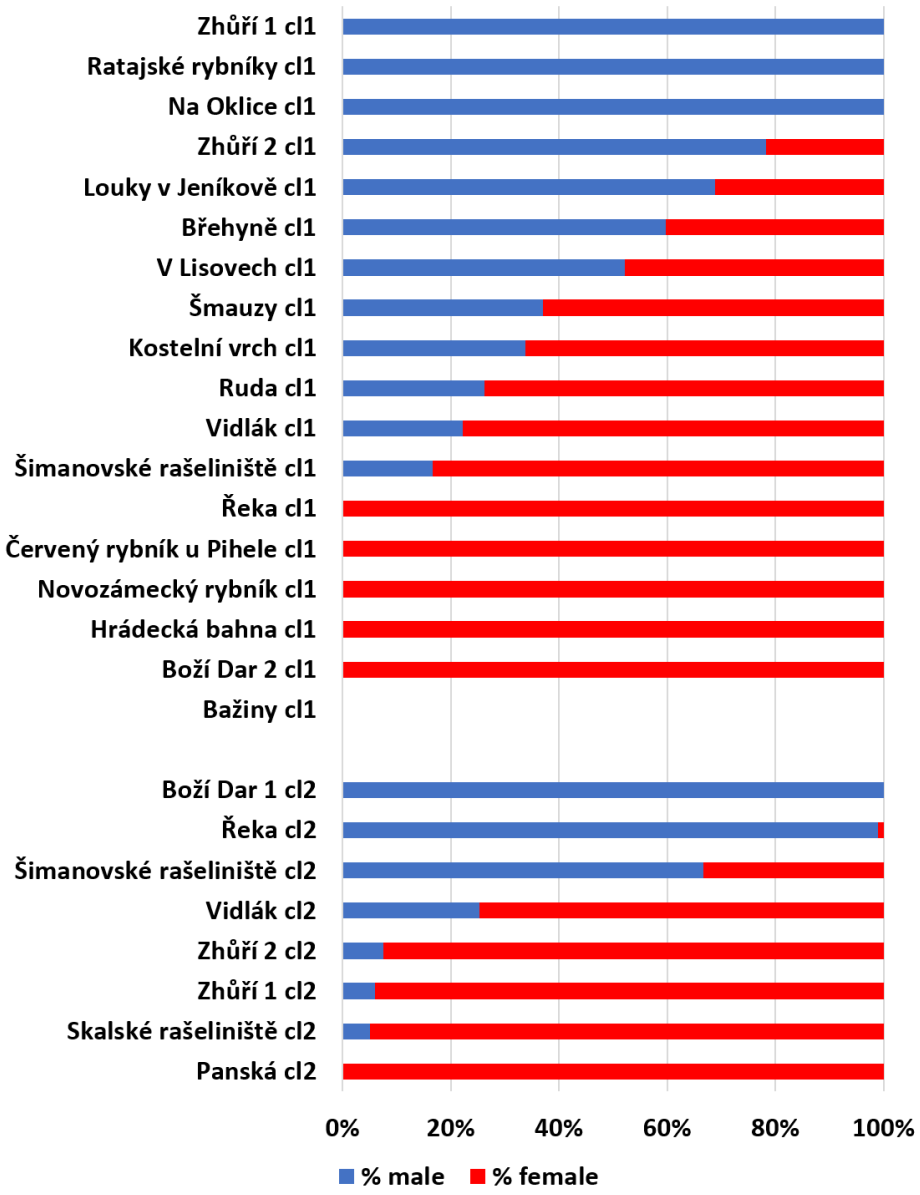

Fig. 2 The expressed sex ratio at studied localities of *H. vernicosus*. In mixed populations, only single-clade patches were used for the assessment.

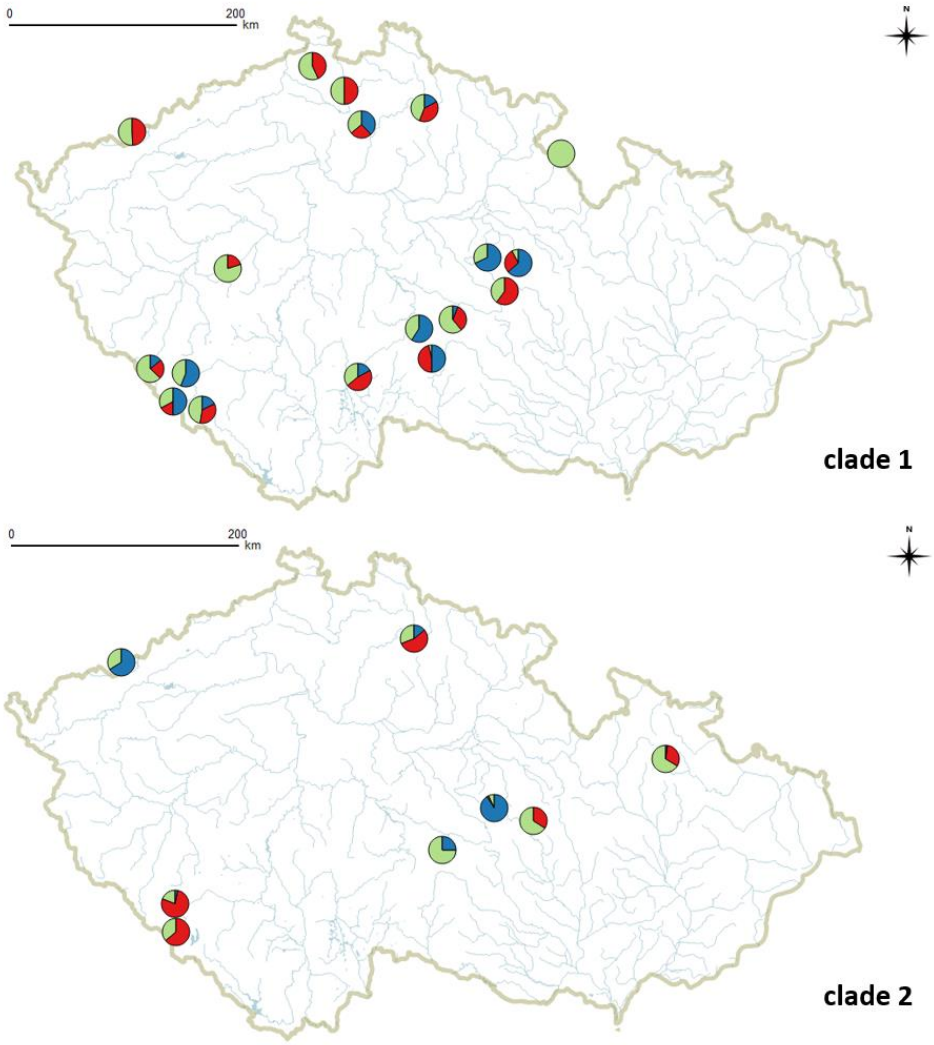

Fig 3 Rates of male (blue), female (red) and non-expressing (green) plants at studied localities of *Hamatocaulis vernicosus* clade 1 and 2.

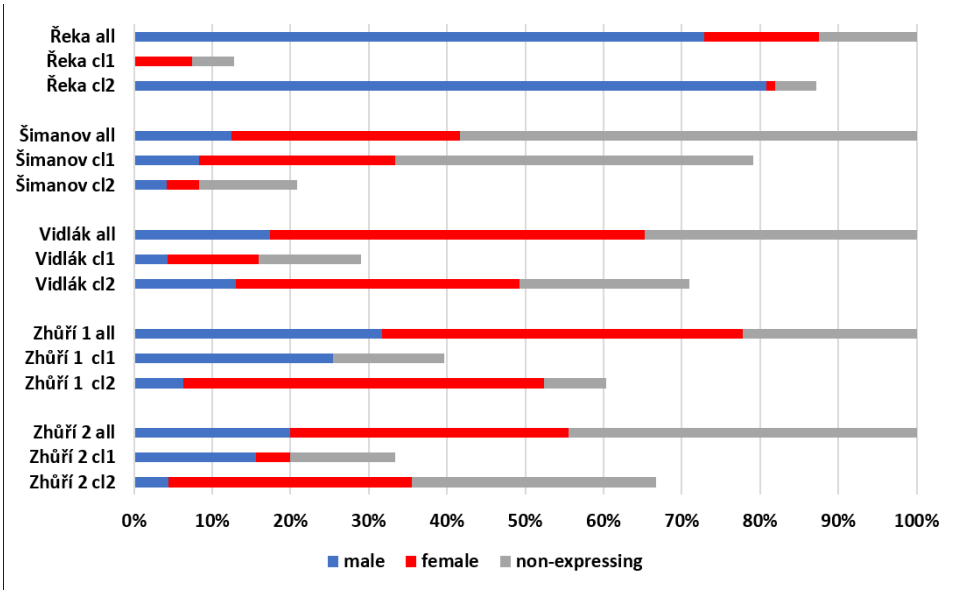

Fig. 4 Sex ratio at localities with co-occurring cryptic species. All – without distinguished clades, cl1 – clade 1, cl2 – clade 2. Only barcoded shoots were used to create this graph.

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.

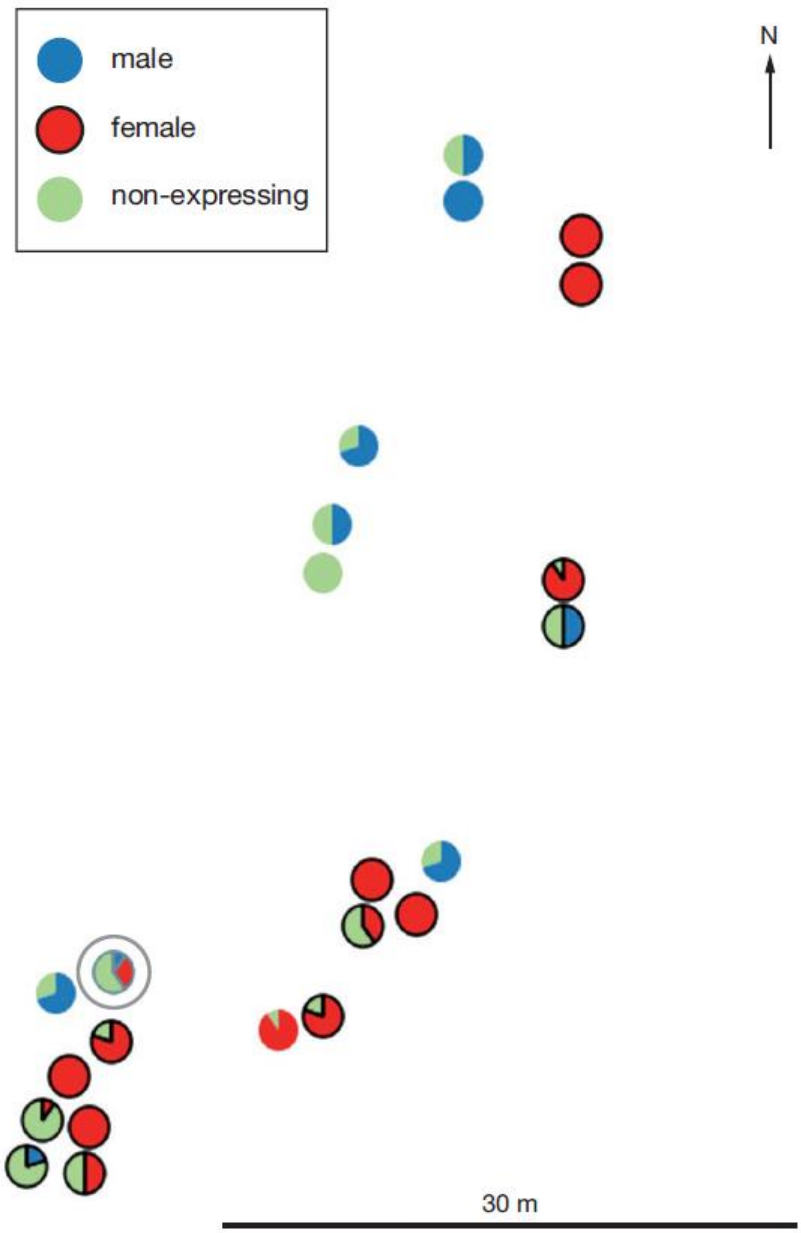

Fig. 5 The sex ratio in mixed population at locality Zhůří 1. Unbordered pie charts refer to clade 1, bordered ones represent clade 2. The patch in the larger circle contained plants of both clades, so this patch must be excluded from evaluating sex ratio in separated clades.

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**Chyba! Nenalezen zdroj odkazů.**), 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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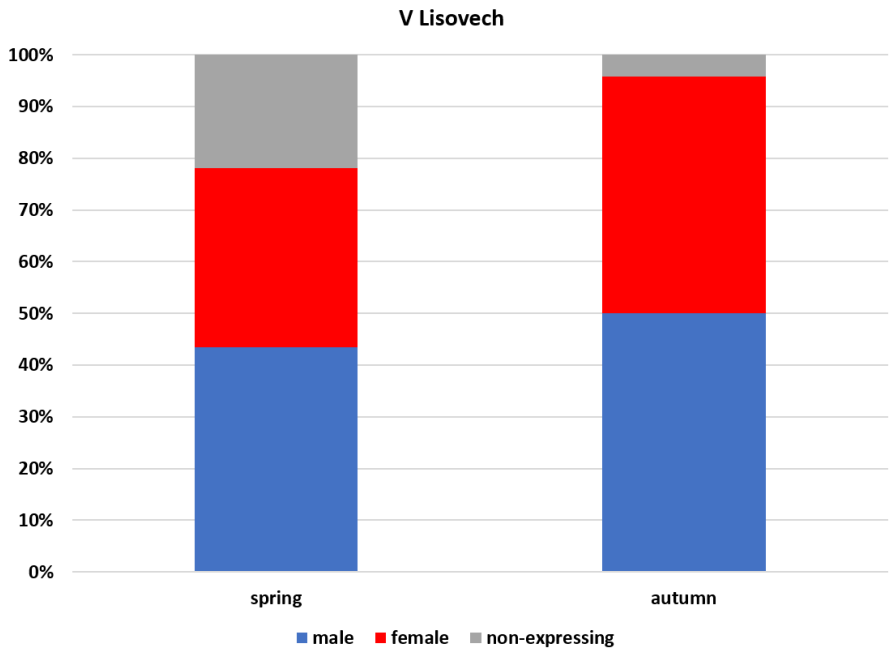
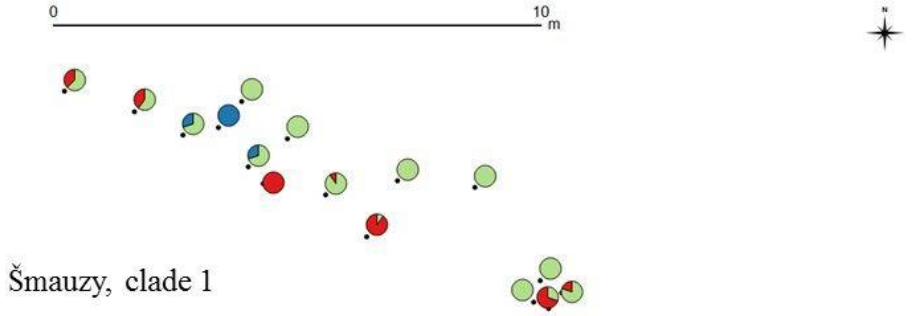
Appendices

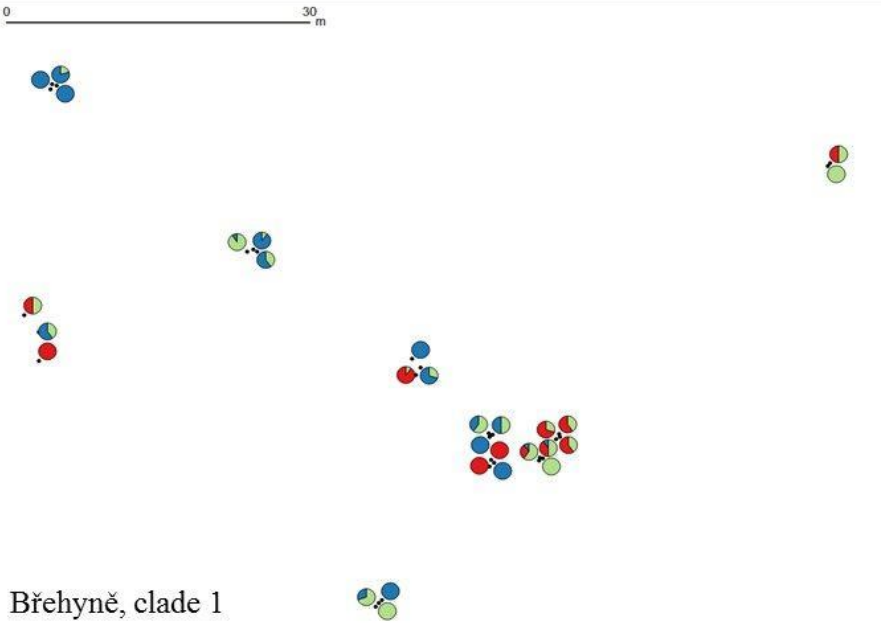
Appendix 1 Seasonal variation of the sex expression and F:M ratio at the locality V Lisovech. 24 patches were inspected on spring and 26 patches in autumn. The difference in sex ratio between the samplings counted by one-way ANOVA was statistically significant ($F(1;48) = 5.0396$; $p = 0.0294$)



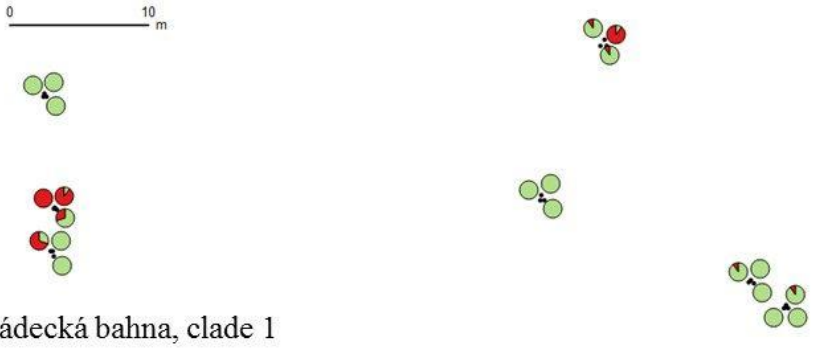
Appendix 2a-e Spatial distribution and rates of male (blue), female (red) and non-expressing (green) plants of *Hamatocaulis vernicosus* at individual localities. The small dots show the position of patches, the pie-charts observed sex ratio in the patch. Blue – male, red – female, green – sterile. All 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.



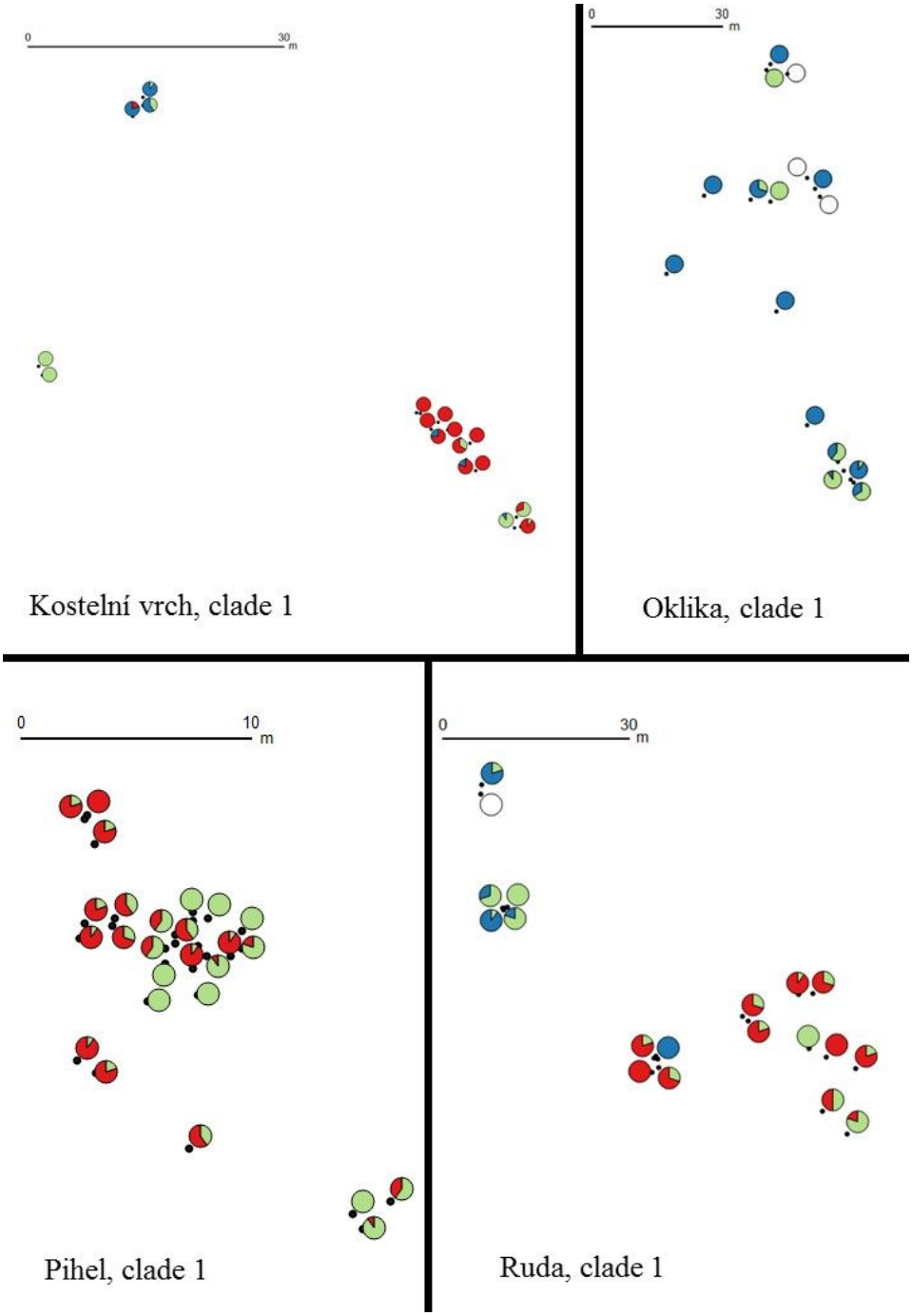
Šmauzy, clade 1

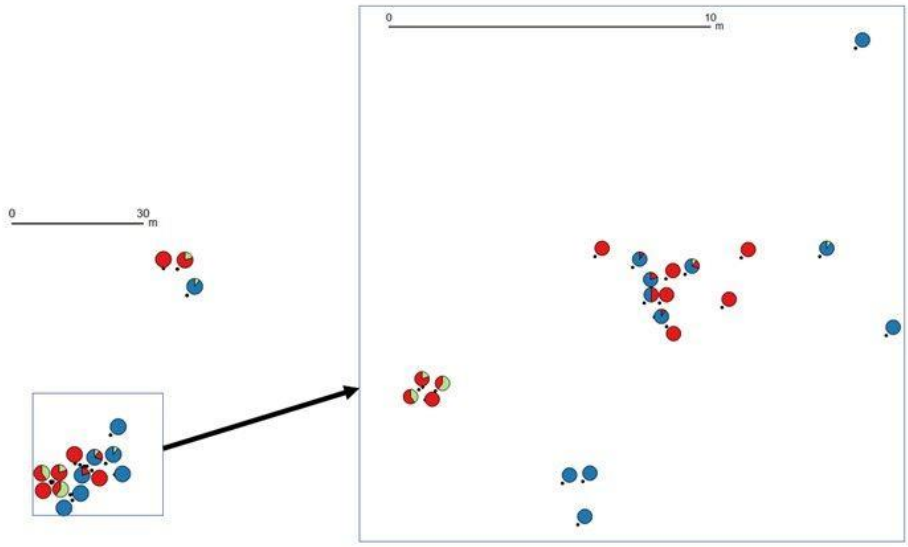


Břehyně, clade 1

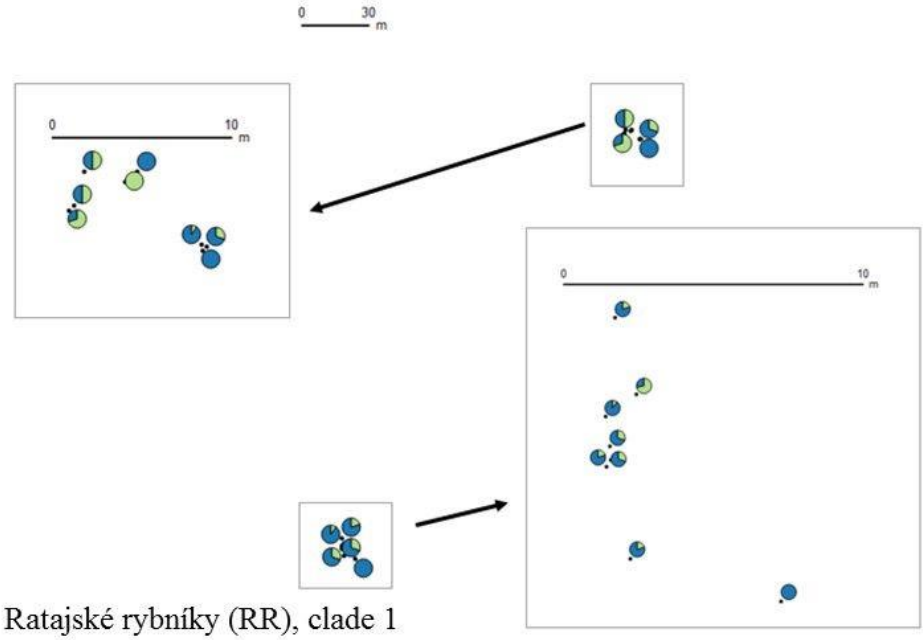


Hrádecká bahna, clade 1

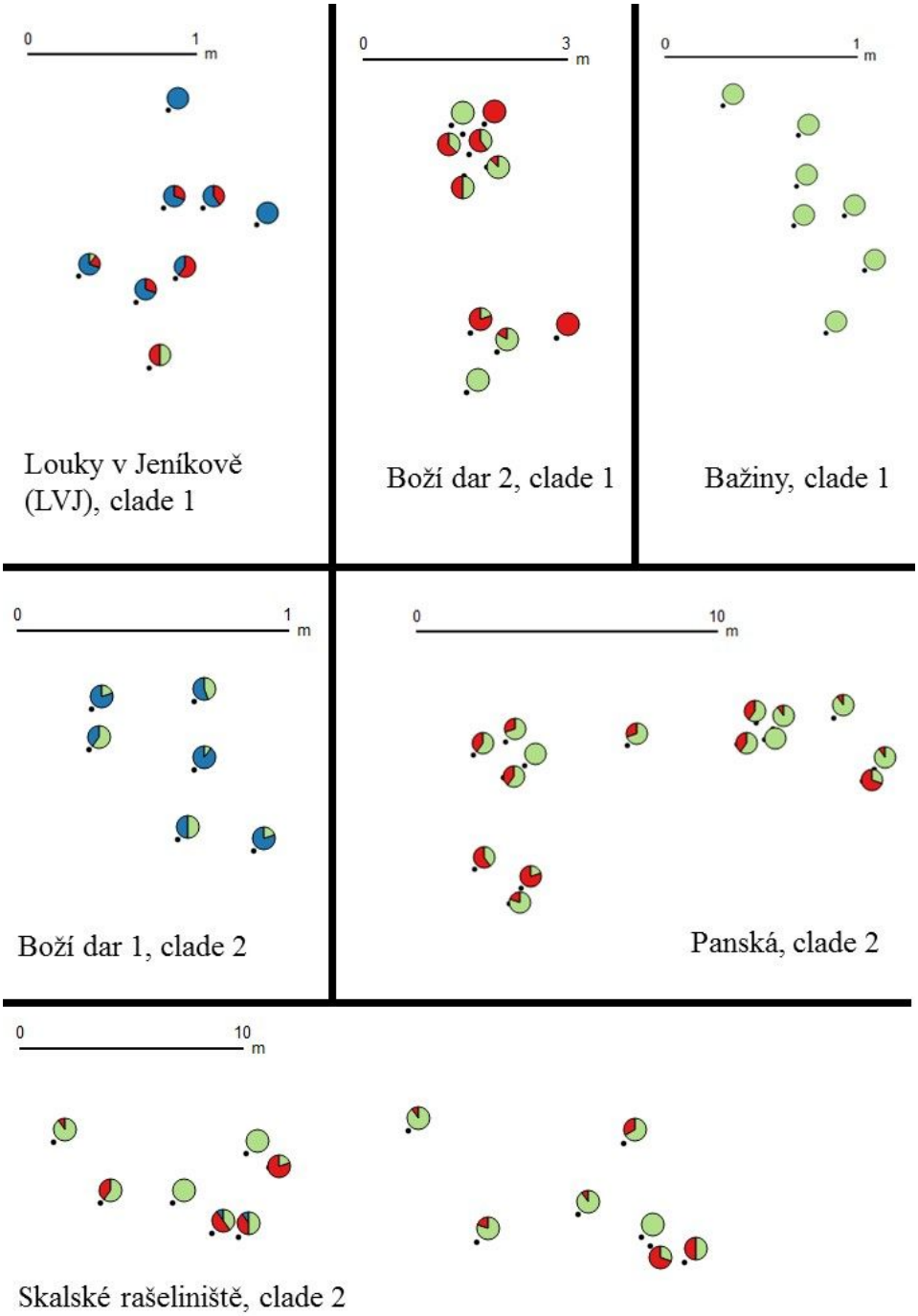

0
30
m
Kostelní vrch, clade 1
0
30
m
Oklika, clade 1
0
10
m
Pihel, clade 1
0
30
m
Ruda, clade 1

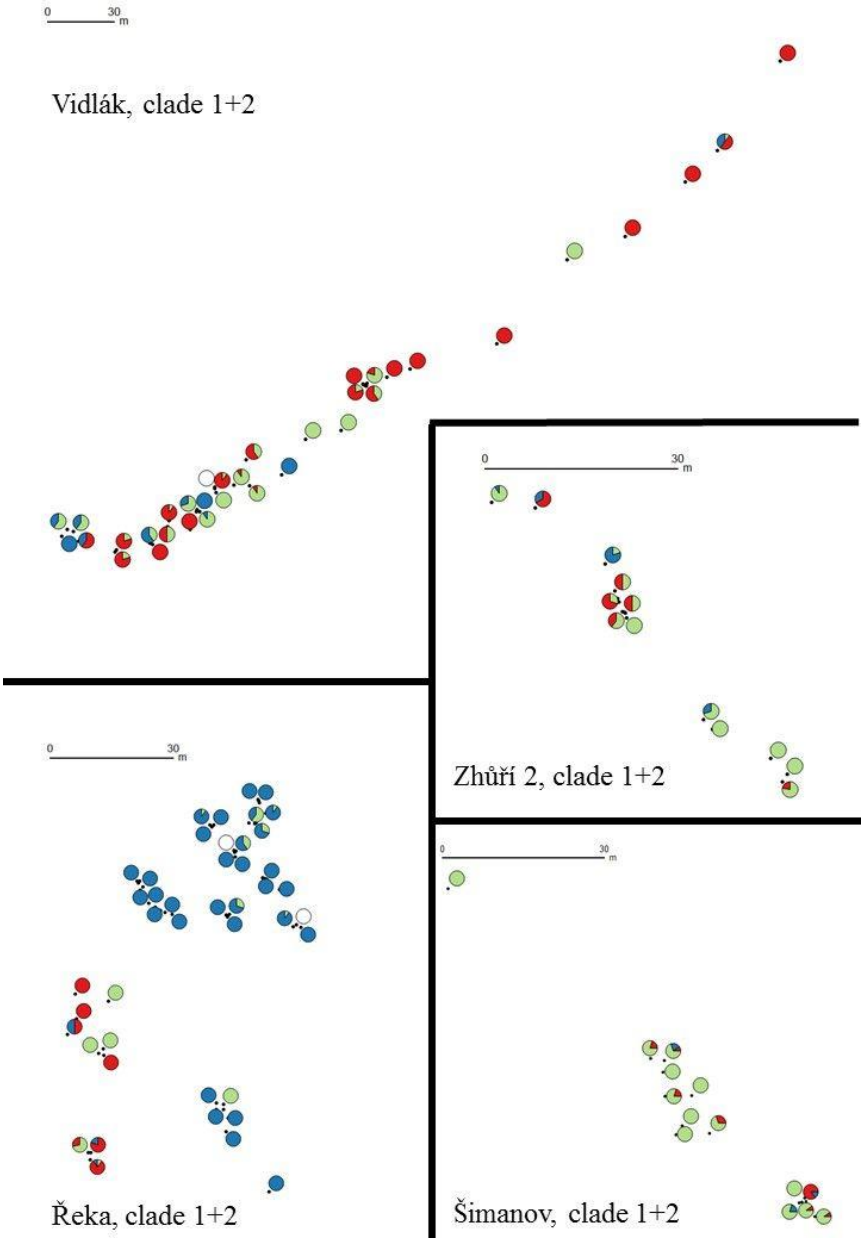


V Lisovech (autumn sampling), clade 1



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

patch	stem 1	a	stem 2	b	stem 3	c	clade in patch
	sex	clade	sex	clade	sex	clade	
45	s	1	x	x	x	x	1
46	f	1	x	x	x	x	1
47	m	2	x	x	x	x	2
48	m	2	m	2	m	2	2
49	m	2	s	2	m	2	2
50	s	2	s	2	s	2	2
51	m	2	m	2	m	2	2
52	m	2	m	2	m	2	2
53	m	2	m	2	m	2	2
54	f	1	m	2	f	1	both
55	f	1	s	1	f	1	1
56	f	1	m		f	1	1
Šimanov	**sex**	**clade**	**sex**	**clade**	**sex**	**clade**	
1	f		s		s	1	1
2	f		f	1	f		1
3	s		s		s		
4	m	1	s	1	m	1	1
5	s	1	s	1	s		1
6	f	1	s	1	s	1	1
7	f		s		f	1	1
8	s	2	s	2	s	1	both
9	s		s	2	s	1	both
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

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

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

Introduction

Hamatocaulis vernicosus is a pleurocarpous moss confined to non-calcareous rich fens. It has been listed in Annex 2 of the Habitats Directive (92/43/EEC) with respect to the considerable decrease throughout Europe during the 20th century. About 70 localities are currently known in the Czech Republic, although most of these emerged only recently as a result of targeted search.

Identity of *H. vernicosus* has been challenged after HEDENÄS & ELDENÄS (2007) discovered that this morphological species consists of two separate genetic lineages (tentatively named clade 1 and clade 2), which they considered 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 the boreal zone and was also found in the USA. The regional distribution was not studied in detail except for Sweden and Switzerland. In Sweden, northern regions were occupied exclusively by populations of the clade 2 while both clades were similarly frequent in the

southern part of the country. The existence of mixed clade 1 and 2 populations was never documented, although the sampling pattern in previous studies with mostly a single specimen per locality (HEDENÄS & ELDENÄS 2007, HEDENÄS 2018b) was not designed for revealing the co-occurrence of cryptic species. Sympatric distribution without regional segregation of clades was nevertheless observed in Switzerland and neighbouring regions of Austria. HEDENÄS & ELDENÄS (2007) were neither able to find differences in ecological niches, characterised by pH and conductivity, between the two lineages. Genetic differences between samples from northern and southern Europe have also been reported in other bryophyte species, such as *Scorpidium cossonii* (HEDENÄS 2009) but in the latter case, the reproductive isolation amongst those lineages was not found as opposed to the case of *H. vernicosus*.

Cryptic, morphologically undistinguishable 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. Cryptic species do not seem to be a rare phenomenon in bryophytes, although most of them have been detected only recently thanks to the detection power of molecular methods (SZWEYKOWSKI & KRZAKOWA 1979, BICKFORD et al. 2007, Heinrichs et al. 2010, Hedenäs et al. 2014, KYRKJEEIDE et al. 2016, PATIÑO et al. 2017, Hassel et al. 2018), and many are probably yet to be discovered. Molecularly defined lineages, morphologically undistinguishable at first, may subtly differ in morphology or ecology, although such differences may only be discovered after a scholarly executed study, as was the case of, e.g., *Conocephalum conicum* vs. *C. salebrosum* (SZWEYKOWSKI et al. 2005). New species were also recognized in *Homalothecium sericeum* s.l. (HEDENÄS et al. 2014) or *Oncophorus wahlenbergii* s.l. (HEDENÄS 2018a).

Earlier studies dealing with *H. vernicosus*, including those that have been published after the existence of cryptic lineages was discovered (e.g., ŠTECHOVÁ & KUČERA (2007), ŠTECHOVÁ et al. (2012), PÉPIN et al. (2013), MANUKJANOVÁ et al. (2014)), considered only the morphospecies, *H. vernicosus* s.l. The reason for this was the morphologically truly cryptic nature of the two lineages, which to date could only be identified using the

molecular tools, such as the sequences of nuclear (ITS) and chloroplast loci (*rpl16* and *trnL-trnF*). However, morphology of the cryptic species within *H. vernicosus* has never been analysed using the multivariate morphometric methods.

Recently, we designed cost effective methods of distinguishing *H. vernicosus* clades (MANUKJANOVÁ et al. 2018), which allow easy barcoding of larger datasets, used, *e.g.*, in population studies. We could thus revise the localities of *H. vernicosus* in the Czech Republic, in order to answer the following questions: (i) Does the distribution pattern of *H. vernicosus* cryptic lineages differ? (ii) Do the lineages co-occur at some localities? If so, is there any differentiation on the finer spatial scale? (iii) Do the lineages differ in their genetic variability? (iv) Are there any morphological characters allowing to distinguish the lineages?

Methods

Field sampling

Plant samples for molecular analyses (see below) were obtained either from herbarium specimens (herbarium CBFS) or were newly sampled *in situ*. The first screening aimed at the overall patterns of clade distribution was based on samples collected for various purposes in 1996 – 2013. Where more herbarium specimens from a site were available, several of them were sampled but always only one shoot per specimen was used. More than 100 samples from most of known *H. vernicosus* localities in the Czech Republic were screened this way.

After the first screening, fine-scale sampling at selected localities aimed at more balanced representation of clade 1 and clade 2 as well as their more regular regional representation was conducted in 2013-2016. Populations were sampled evenly over the whole extent of the species occurrence at the locality (7-49 samples per locality, depending on population size and spatial distribution), to increase the possibility of discovering both cryptic species. To decrease the probability of sampling from the same clone, samples were picked at least 20 cm apart. The position of each sampled patch was drawn into a field sketch that was later transferred into a GIS layer using the QGIS v. 2.6 software (QGIS Development Team 2015). Two of those localities – Boží Dar and Zhůří contained 2 microlocalities each (we call microlocality a situation when places of occurrence of the

target species are separated by more than 200 meters of unsuitable habitat). The localities are listed in Appendix 1.

Clade barcoding

DNA was isolated using the NaOH isolation protocol based on WERNER et al. (2002). One cm long branch from the upper green part of shoot was homogenized in 40 µl of 0.5M NaOH. The tubes were centrifuged for 1 min at 13600 rpm, the supernatant diluted 1:10 in 100mM Tris-HCl (pH 8.3) and stored at -20°C. Clade identity of plants was determined either by Sanger sequencing of the ITS region using ITS 18SF (SPAGNUOLO et al. 1996) and ITS 26SR (STECH & FRAHM 1999) primers, or one of the barcoding methods described in MANUKJANOVÁ et al. (2018).

Environmental data

The information on mean annual temperature, precipitation and number of frost days for each population was extracted from a GIS layers supplied by the Czech Hydrometeorological Institute. The differences in climatic variables between clade 1 and 2 localities were tested using one-way ANOVA in Statistica software (STATSOFT 2007).

Altitudinal distribution of the clades was compared in two central European regions. Elevation of localities in the Alps (HEDENÄS & ELDENÄS 2007) was retrieved from the herbarium database of Stockholm Museum of Natural History (http://herbarium.nrm.se/). Those two datasets were compared separately by one-way ANOVA in Statistica (STATSOFT 2007).

Ribotype network

Samples from 35 Czech localities of *H. vernicosus* were sequenced for the ITS region to compare the ribotype variability with that reported by HEDENÄS & ELDENÄS (2007) and other available ITS sequences (2 CBFS herbarium samples from Russia, unpublished). One sample per locality was used, except for mixed localities, where one sample per each clade was used. The sampling covered all known Czech clade 2 localities and 25 localities of clade 1, which were sampled to cover evenly the whole range of occurrence in the Czech Republic. Ribotype minimum spanning network (BANDELT et al. 1999) was visualised using PopArt software (LEIGH & BRYANT 2015). As this software does not allow for gaps to be coded as the fifth state, unique substitutions were added to replace gaps. In total, 104 sequences were used, 45 of the clade 1 (of which 25 were from the Czech

localities and 20 from other countries) and 59 of the clade 2 (8 from Czech localities, 53 from other countries). Genbank accession numbers of newly generated sequences are listed in Appendix 1.

Morphometry

To evaluate the morphological characters of the two cryptic species, we sampled plants from nine localities of each clade; at four of them both clades co-occurred. Three shoots per locality and clade were selected.

Selected stems were placed between object glass and cover slip and gently pressed to spread the leaves. Five randomly selected well-spread leaves from one shoot were measured and the average values used; only leaves that were not excessively deformed by pressing were measured. The measurements were realized using the QuickPHOTO MICRO 3.1 software (PROMICRA 2015), using the light microscope Olympus CX31. In total, 10 quantitative characters of leaves and 4 derived ratios were used for the analyses (Table 1, Fig. 1). Ten leaf cells were measured in the middle part of the leaf lamina including their cell walls and the average value was used. It was not possible to include sporophyte characters since the sporophytes of clade 2 plant have never been recorded in the study area, and even in clade 1 sporophytes were extremely rare.

Morphometric data were processed using MorphoTools package, a set of R functions specifically designed for plant morphometric analyses (KOUTECKÝ 2015), run in the R software (R Core Team 2018) unless specified otherwise. Descriptive statistic were computed for each population and clade (Appendix 2). As the distribution of all characters approached normal distribution, no transformation was applied for subsequent analyses. The difference between clades in individual morphological characters was tested using t-test run in Statistica software (STATSOFT 2007). To account for highly correlated characters, we produced a matrix of Pearson's correlation coefficients of the characters (Appendix 3). Based on the results, the character CoW was excluded from subsequent analyses due to high correlation with CoBW. PCA on correlation matrix (from characters standardized to zero mean and unit variance) was carried out to visualize the similarity of samples from both clades. To test the morphological differences between clades, canonical discriminant analysis

was computed; significance was tested using Monte Carlo permutation test (999 permutations). Classification discriminant analysis with cross-validation was applied to estimate the number of correctly classified individuals based on morphological data.

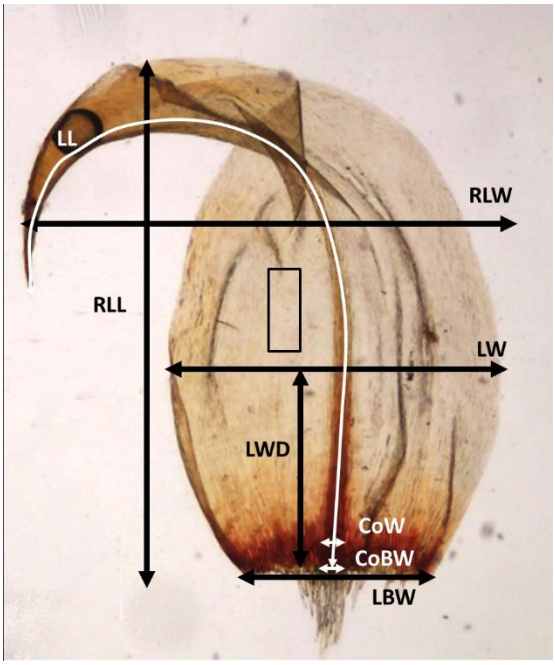


Fig. 1 Leaf dimensions measured in this study. The rectangle shows the approximate placement of the region of the cell measurement. See Table 1 for abbreviations.

Table 1 Measured leaf characteristics.

abbreviation	description
	quantitative measurements (all measurements in µm)
LL	leaf length
LW	leaf width
LBW	leaf base width
LWD	distance of the widest part of leaf from the leaf base
CoW	costa width [measured above red basal cells]
CoBW	costa base width
RLL	leaf outer rectangle length
RLW	leaf outer rectangle width
LCW	middle leaf cell width [average of 10 cells per leaf]
LCL	middle leaf cell length [average of 10 cells per leaf]
	ratios
LL_LW	leaf length / leaf width
LL_LBW	leaf length / leaf base width
LW_LBW	leaf width / leaf base width
LW_CoW	relative costa width [leaf width/ costa width]

Results

Regional clade distribution and the relation to environmental characteristics

Both clades of *Hamatocaulis vernicosus* generally avoid warm parts of the country with mean annual temperature exceeding 8°C (Fig. 2) but also avoid regions with mean annual temperature lower than 4°C, which correlates with the general distribution of suitable rich fens in the region (Štechová et al. 2012). Out of 70 investigated localities, solely clade 1 was detected at 62 localities, solely clade 2 at 3 localities, and 5 localities supported the mixed occurrence of both clades. Largest populations of both clades occurred at elevations around 600 m a.s.l. (Appendix 4). No significant differences among the clades were found for the mean annual temperature, total annual precipitation and number of frost days (Fig. 3), even though populations of clade 2 had their median and interquartile range slightly shifted to colder and wetter regions.
Comparison of elevation between localities of clade 1 and 2 showed slightly higher mean elevation of clade 2 localities (Appendix 5). However, due to the small number of samples and broad overlap in the range, the test was not significant neither for the Czech Republic nor for the Alps.

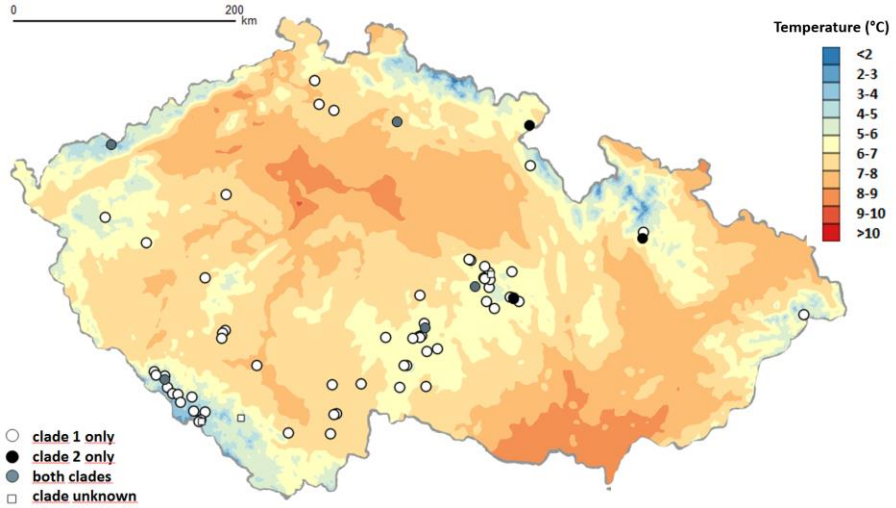


Fig. 2 Distribution of *Hamatocaulis vernicosus* clades in the Czech Republic along the gradient of mean annual temperature.

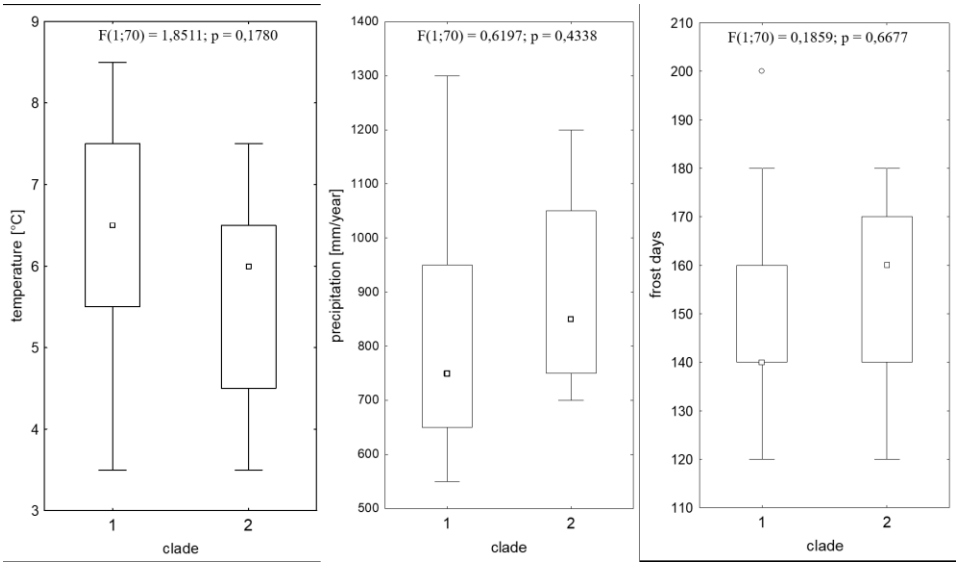


Fig 3 Mean annual temperature, precipitation and number of frost days in clade 1 and 2 localities in the Czech Republic. Point, box and whiskers denote median, interquartile range and non-outlier range, respectively.

Fine-scale clade distribution in mixed populations

Fine-scale distribution in mixed populations of both *H. vernicosus* clades often showed a pattern (Fig. 4). At the locality Řeka, the rarer plants of clade 1 occurred only in one part, co-occurring here with plants of clade 2 present throughout the whole locality. At the localities Zhůří 1 and 2, where the population grows on a gentle slope, distribution of the clades followed the micro-topography, with plants of one of the clades growing exclusively upslope and the other clade exclusively or co-occurring downslope. The upslope populations were, however, of different clades at Zhůří 1 and 2. At locality Vidlák, plants of both clades were interspersed without showing any spatial pattern. Only at one locality (Zhůří 1 and 2) both clades were similarly frequent, while at the others one of the clades markedly dominated (clade 1 at the locality Šimanov, clade 2 at the localities Vidlák and Řeka).

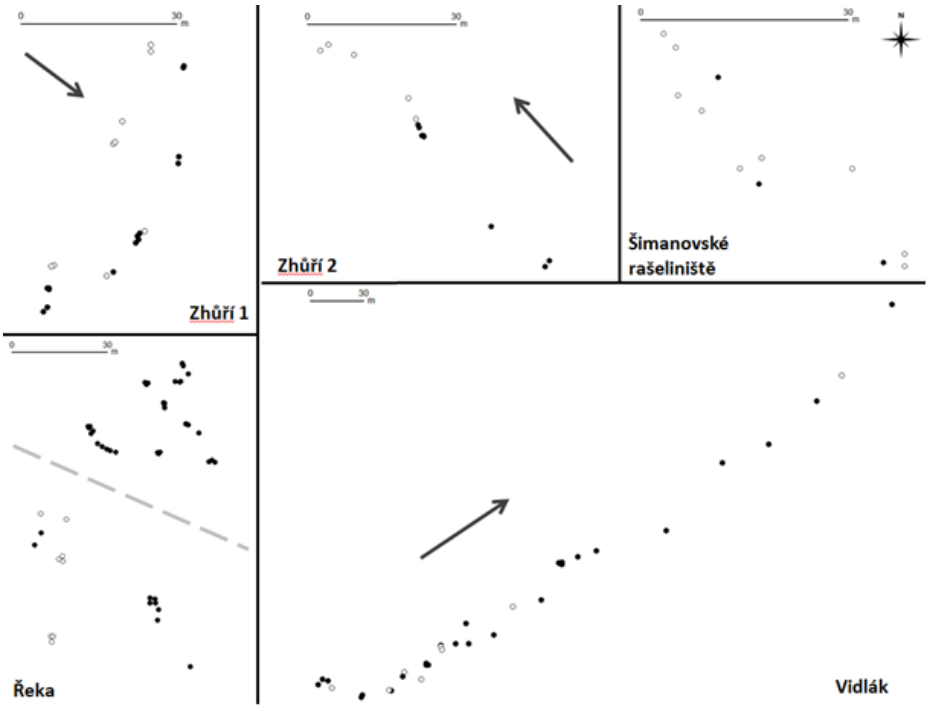


Fig. 4 Fine-scale distribution of *H. vernicosus* at localities with mixed populations of both clades. White circles mark clade 1 individuals, black circles clade 2, arrows show water movement along the slope and grey interrupted line shows a shallow ditch with a shrub line dividing the locality Řeka.

Ribotype network

Plants from the Czech Republic belonged to 3 different ITS ribotypes of clade 1, while there was no variability in clade 2 (Fig. 5). Most of the sequences from the Czech Republic were identical to those published in the study of HEDENÄS & ELDENÄS (2007). The commonest European ribotype of clade 1 was the commonest in the Czech Republic as well (65% of samples) and 13 % of the Czech samples belonged to the second commonest European clade 1 ribotype. One earlier unknown ribotype was documented at Vidlák locality once, and one new ribotype of the clade 1 was discovered among our herbarium samples from Russia. Our samples of clade 2 belonged to the commonest clade 2 ribotype worldwide.

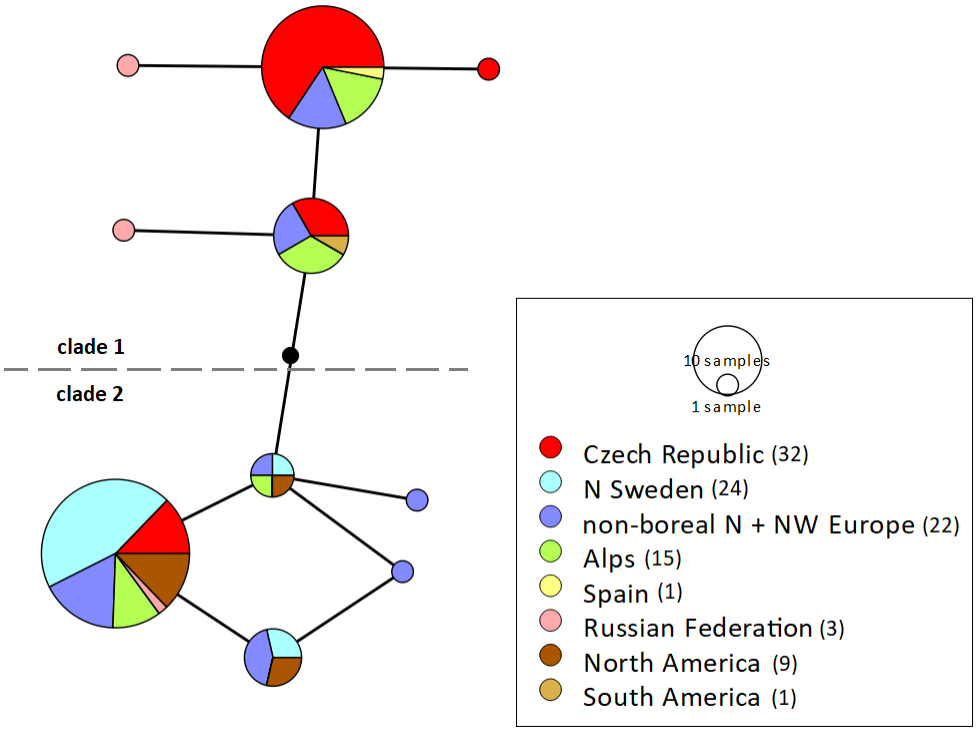


Fig. 5 Ribotype minimum spanning network of *Hamatocaulis vernicosus* based on ITS sequences with our samples added to the dataset published by Hedenäs & Eldenäs (2007). Each circle represents one unique ribotype.

Morphological differentiation of clades

Measured morphological characteristics did not cluster plants of individual clades together (Fig. 6); the variation of the characters between individual plants is generally bigger than among localities and clades (Fig. 7). No statistically significant differences of measured morphological characters values between the clades was found using t-test (Appendix 2). LDA has not revealed any statistically significant difference ($F(14)= 0.862$; $p=0.597$) that would allow for successful morphology-based classification of the plants based on the measured characters. The classification discriminant analysis of *H. vernicosus* therefore failed nearly completely at discriminating between the clades based on the measured characters (successful identification in less than 52% of cases).

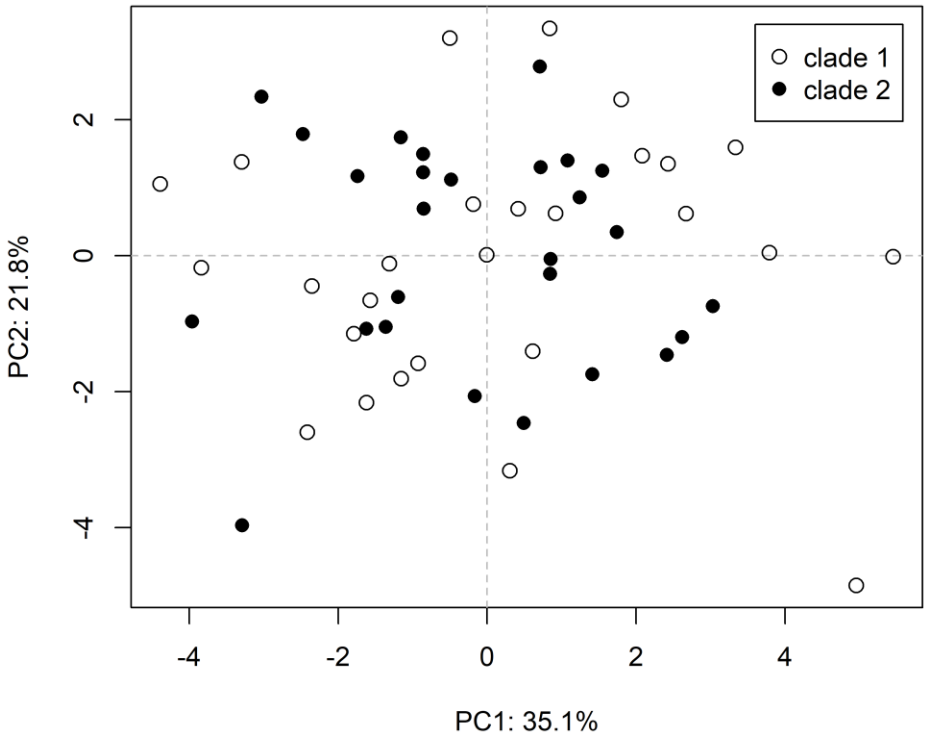


Fig. 6 Principal Component Analysis of *H. vernicosus* morphological leaf characters of clade 1 and 2. The first two axes explained 61.5% (35.1 + 21.8) of variability.

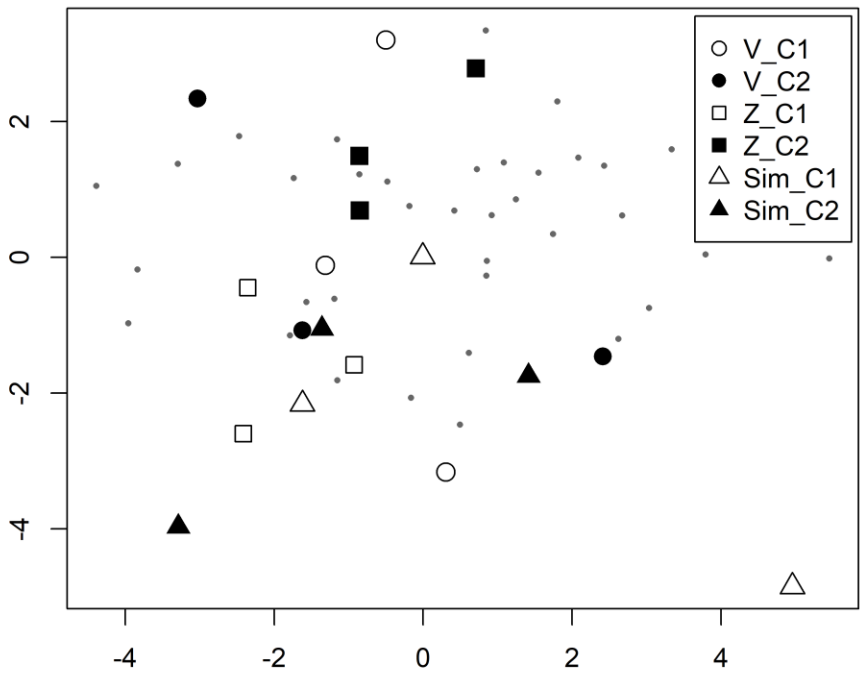


Fig. 7 PCA of *H. vernicosus* morphological leaf characters of clade 1 and 2 from localities with co-occurrence of both clades. Symbols represent individual localies (V – Vidlák, Z – Zhůří 1, Sim – Šimanov), white symbols denote clade 1 and black denote clade 2 plants. Other samples are shown as small grey dots to provide the context.

Discussion

Clade distribution and relation to environmental characteristics

The markedly commoner occurrence of clade 1 localities has not yet been reported from any region, where the two cryptic species are co-occurring, although published data only exist for Sweden, Austria and Switzerland (HEDENÄS & ELDENÄS 2007). There are almost ten times as many clade 1 localities in the Czech Republic as compared to localities of clade 2. Different frequency of occurrence is perhaps not surprising in comparison to Sweden, where *Hamatocaulis vernicosus* clade 2 is more common, being the only present cryptic species in Northern Scandinavia, but a markedly different rate between clades in the alpine countries with roughly equivalent representation of clade 1 and 2 is quite surprising. Although sampling in the latter two countries was notably less dense than in the

Czech Republic (15 vs. 70 samples), it is rather unlikely that the different ratio between clades was only caused by unrepresentative selection of samples.

Regional distribution of the cryptic species in the Czech Republic does not show different patterns for individual clades. Although the elevation of clade 2 localities is slightly higher, the difference in this characteristic between clades was not statistically significant, similarly to the situation in Austria and Switzerland (HEDENÄS & ELDENÄS 2007), if compared in the same way. The shift towards higher elevations for clade 2 in Central Europe would present a logical reflection of its more northern distribution in Europe, but the lack of statistical support for the elevational shift between clades in Central Europe makes any serious discussion of this pattern irrelevant. However, similar latitudinal and altitudinal distribution was observed in two closely related *Palustriella* species (*P. falcata* and *P. commutata*), where it was hypothesized, that *P. falcata* might have lower competition abilities in warmer regions.

Clade 2 was found in mixed populations with clade 1 in most cases, with both clades often growing in close proximity at mixed localities. Documentation of fine-scale distribution at our localities nevertheless mostly revealed the patches of both clades growing in individual clusters. Clustered distribution of clades at mixed localities indicates high ratio of vegetative reproduction, which is assumed to be the main way of "brown mosses" propagation in fens (POSCHLOD & SCHRAG 1990). On the other hand, it is not possible to state that patches consisting of one clade represent a single clone. Their genetic diversity needs to be assessed using more variable markers. Spatial patterns of the clades at localities similar to those presented for localities Zhůří 1 and 2 (Fig.4) probably generally reflect the colonization events, followed by the vegetative propagation. However, both localities at Zhůří, which are cow pastures have different management than the rest of the mixed localities, which are mowed. It seems that the mowed localities has clades more intermixed than the other localities. It is probable, that mowing disperses stem fragments at the locality with higher efficiency than natural dispersal vectors like animals, water and wind. On the other hand, BOISSELIER-DUBAYLE et al. (1995) or BIJLSMA et al. (2000) advocated rather the segregation triggered by different microhabitat

conditions. Due to the unusually dry weather in past few years, which made pH measurement and water sampling at most localities impossible or unreliable, we were unable to test the differences in site conditions directly.

Ribotype diversity

Two of three ribotypes in clade 1 and the only revealed ribotype of clade 2 belong to the commonest ones documented world-wide. The newly discovered single-point mutation in the commonest clade 1 ribotype at one Czech locality probably evolved as a somatic mutation, which is a rather common phenomenon in bryophytes (NEWTON & MISCHLER 1994). While this ribotype represents probably a rare genotype, the accidentally found ribotype in a south-Siberian population might either represent a similar stochastic event or might belong to a yet unrecorded but more common ribotype in the region which has not yet been adequately surveyed. The complete invariability of clade 2 sequences in the Czech Republic contrasts with the variability both in Sweden and in the Alps (Fig. 5), although lower variability could be anticipated in view of the low frequency of clade 2 occurrence in our region. Moreover, smaller variation in clade 2 ribotypes in Central Europe can be explained by the later migration to central Europe from glacial refugia in northern Europe (HEDENÄS & ELDENÄS 2007, KYRKJEEIDE et al. 2014). The later establishment of clade 2 in localities already occupied by clade 1 would also explain the lower number of its localities since colonization might have been impaired by competition with the other cryptic species.

Morphological differentiation

Neither our increased effort at discovering statistically significant morphological differences to distinguish the clades apart changed the morphologically cryptic nature of lineages revealed by HEDENÄS & ELDENÄS (2007), at least in the Czech Republic. Although we particularly focused on measurements of plants from mixed localities to account for site-specific conditions possibly affecting the phenotypic expression, no clustering of samples from same clade at locality was observed (Fig. 7). The cases when morphological differences between originally not recognized species regarded to be cryptic are not revealed are rather rare; much commoner are the opposite cases (HEDENÄS et al. 2014, YOUSEFI et al. 2018). It is also possible that unmeasured characters of the rarely

produced sporophyte might allow for distinguishing the two clades, as it was recently discovered for species of *Homalothecium sericeum* complex, in which gametophyte characters overlap but sporophytes are characteristic (HEDENÄS et al. 2014).

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Appendices

Appendix 1 - Table of localities, GPS coordinate (WGS 84), clade present and Genbank accession numbers of ITS sequences

Locality	N	E	clade	elevation	Genbank accession
Bažiny	50.296389	16.299722	1	620	MK313848
Borová u Poličky	49.742889	16.152944	1	630	
Branišov u Jihlavy	49.473639	15.438667	1	640	
Brouskův mlýn	48.883222	14.682417	1	450	
Břehyně - Pecopala	50.584539	14.706881	1	280	MK313848
Cikháj	49.659278	15.970806	1	680	
Červený ryb. u Pihele	50.735278	14.552897	1	300	MK313846
Dolejší ryb.	49.432747	13.821433	1	450	MK313848
Herálec, Kuchyně u ryb. Šantrůček	49.701083	15.975164	1	640	
Hrádecká bahna	49.713233	13.658972	1	400	
Hůrky	49.896103	13.18366	1	550	MK313848
Chaloupky	48.95046	13.60894	1	965	
Chalupská slať	49.002364	13.6622	1	690	
Chvojnov	49.407222	15.419167	1	605	
Jezdovická raš.	49.323611	15.461667	1	575	
Jezerní pot. u Cetlovy Hůrky	49.132917	13.356528	1	870	
Knížecí pláně	48.959295	13.627445	1	985	
Kostelní vrch	49.05564	13.46031	1	970	
Kvilda	49.008631	13.565169	1	1070	
Louky u Černého lesa	49.586108	15.942917	1	570	
Louky v Jeníkově	49.738542	15.964531	1	630	
Matenský rybník	49.151139	14.931031	1	525	
Městišťské rokle	49.218301	13.250007	1	1060	
Na Klátově	49.137708	15.452425	1	485	MK313848
Na Oklice	49.404217	15.394517	1	660	
Nad Svitákem	49.396667	15.404722	1	630	MK313848
Návesník	49.711972	15.927889	1	620	
Novozámecký ryb.	50.6125	14.5853	1	255	MK313846
Nový ryb. u Rohozné	49.803662	15.819792	1	560	MK313848
Obidová, PP	49.517849	18.523277	1	720	
Odměny u ryb. Svět	48.992033	14.725981	1	435	MK313848
Odranec	49.608572	16.141557	1	740	

Locality	N	E	clade	elevation	Genbank accession
Poutnov	50.02658	12.85082	1	675	
Prameny Klíčavy	50.145861	13.828825	1	420	
Pstruží potok	49.950278	17.220556	1	680	MK313848
Rašeliniště Kaliště	49.250278	15.296389	1	655	MK313848
Rašeliniště u Suchdola	49.132028	15.238453	1	625	
Rašelinná louka u Proseče - Obořiště	49.396915	15.127919	1	605	
Ratajské ryb.	49.769364	15.933906	1	590	
Roženecké paseky	49.606917	16.165083	1	650	MK313848
Ruda	49.145317	14.690775	1	415	
Řežabinec a Řežabinecké tůně	49.251292	14.082886	1	370	MK313848
Slunečná u Prášil	49.100653	13.400856	1	910	
Smyslov	49.419489	13.802408	1	470	
Staré jezero	48.891618	14.34017	1	440	
Strádovka	49.809397	15.803394	1	580	MK313848
Šafranice	49.548	16.013558	1	630	MK313848
Šmauzy	49.19702	13.26218	1	1030	
V Lísovech	49.246993	15.27881	1	650	
V Rájích	48.986375	14.708678	1	445	MK313846
Váhy	49.6181839	15.4019394	1	490	
Ve Sklenářích	49.391753	15.349332	1	660	MK313848
Velká Kuš	49.3943	13.794869	1	482	MK313848
Velké Janovice	49.586729	16.211739	1	620	
Velký Bor u Prášil	49.097064	13.43945	1	850	
Vílanecké rašeliniště	49.33932	15.54844	1	570	
Zhůří u Horské Kvildy	49.082111	13.554589	1	1120	
Zhůřská pláň	49.192797	13.333889	1	990	
Zlámanec	49.705311	15.932294	1	620	MK313848
Panská	49.601944	16.168822	2	720	MK313850
Řeřišný	50.504589	16.291511	2	510	MK313850
Skalské rašeliniště	49.918193	17.211387	2	680	MK313850
Božídarské rašeliniště	50.406972	12.900611	1+2	1000	MK313848, MK313850
Řeka	50.524444	15.217414	1+2	280	MK313849, MK313850
Šimanovské rašeliniště	49.172531	13.331747	1+2	960	MK313850
Vidlák - Podtrosecké údolí	49.666597	15.852992	1+2	555	MK313846, MK313850

Locality	N	E	clade	elevation	Genbank accession
Zhůří - Rašeliniště u Křemelné	49.450397	15.446708	1+2	605	MK313848, MK313850
Volákův kopec	49.731667	15.979167	NA	640	
Červený potok	48.94979	13.63091	NA	975	
Křišťanovický rybník	48.969369	13.951109	NA	795	
Russia, Burjatija, Svjatoj Nos	53.562222	108.941667	2	450	MK313850
Russia, Yakutiya, River Shandrin	NA	NA	1	NA	MK313847

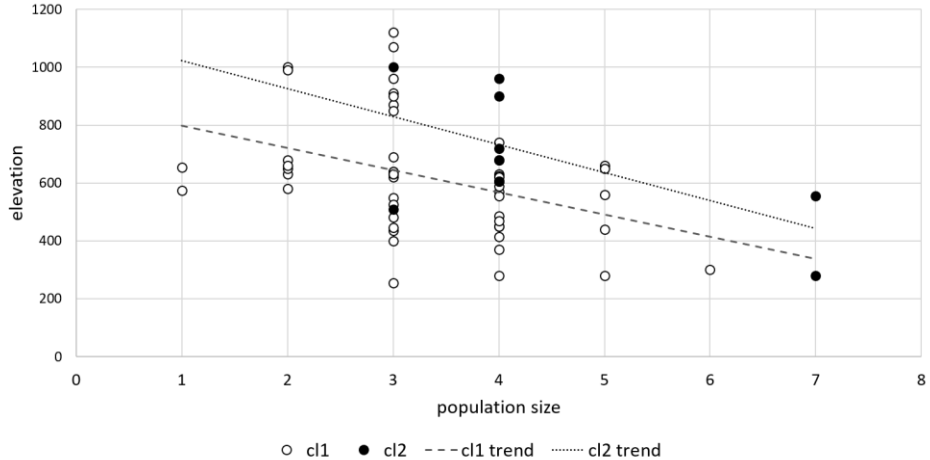
Appendix 2 Descriptive statistics of morphological characters showing standard deviation and percentiles for each morphological character. The last column shows results of T-test of difference between clade 1 and 2 for every morphological character separately.

Character	Taxon	N	Mean	SD	Min	0.05	0.25	Median	0.75	0.95	Max	T-test
LL	C1	27	2635.97	392.74	1888.2	2062.22	2465.8	2600.8	2767.7	3335.68	3762.2	t(53)=-0.71; p=0.481
LL	C2	27	2563.3	293.1	1998.8	2194.74	2335.3	2504.2	2786.2	3040.56	3086	
LW	C1	27	900.63	112.2	721	735.88	817.3	866	984.7	1075.78	1164.2	t(53)=-0.009; p=0.993
LW	C2	27	895.55	90.98	720.2	737.84	843	918.8	952.8	1036.12	1086.8	
LBW	C1	27	582.35	72.01	459	475.74	538.7	587.6	621	692.52	730.8	t(53)=0.462; p=0.646
LBW	C2	27	587.48	59.62	430.4	485.76	561.1	598.2	628.4	664.4	670.6	
LWD	C1	27	559.21	91.83	418	439.38	503.9	552.2	590.7	754.94	798.6	t(53)=0.795; p=0.43
LWD	C2	27	573.15	78.92	387.6	452.66	525.9	589	629.2	676.34	701	
CoBW	C1	27	77.09	15.79	44	57.96	64.4	75.6	88.35	102.08	105.6	t(53)=-0.479; p=0.634
CoBW	C2	27	74.88	11.49	53.2	57.32	67.7	73	81.5	92.76	94	
CoW	C1	27	56.89	11.2	38.8	40.18	47.7	57.6	66	73.59	76.8	t(53)=-0.89; p=0.377
CoW	C2	27	54.03	7.58	38	40.06	50.2	54	59.1	63	72	
RLL	C1	27	1589.76	207.26	1245.2	1311.68	1456.1	1541	1711	1832.06	2224	t(53)=-0.014; p=0.989
RLL	C2	27	1579.46	192.18	1256.6	1303.08	1442.1	1593.2	1670.4	1908.3	2062	
RLW	C1	27	1567.47	221.39	1307	1320.84	1428	1494.8	1644.4	1921.6	2271.8	t(53)=-0.387; p=0.701
RLW	C2	27	1542.57	210.08	1239.4	1277.14	1398.1	1465.8	1645.6	1891.38	2093.8	
LL_LBW	C1	27	4.58	0.62	3.56	3.65	4.22	4.51	4.93	5.49	5.99	t(53)=-1.24; p=0.22
LL_LBW	C2	27	4.4	0.52	3.42	3.69	4.03	4.24	4.89	5.18	5.35	
LL_LW	C1	27	2.95	0.34	2.4	2.5	2.63	2.95	3.17	3.38	3.8	

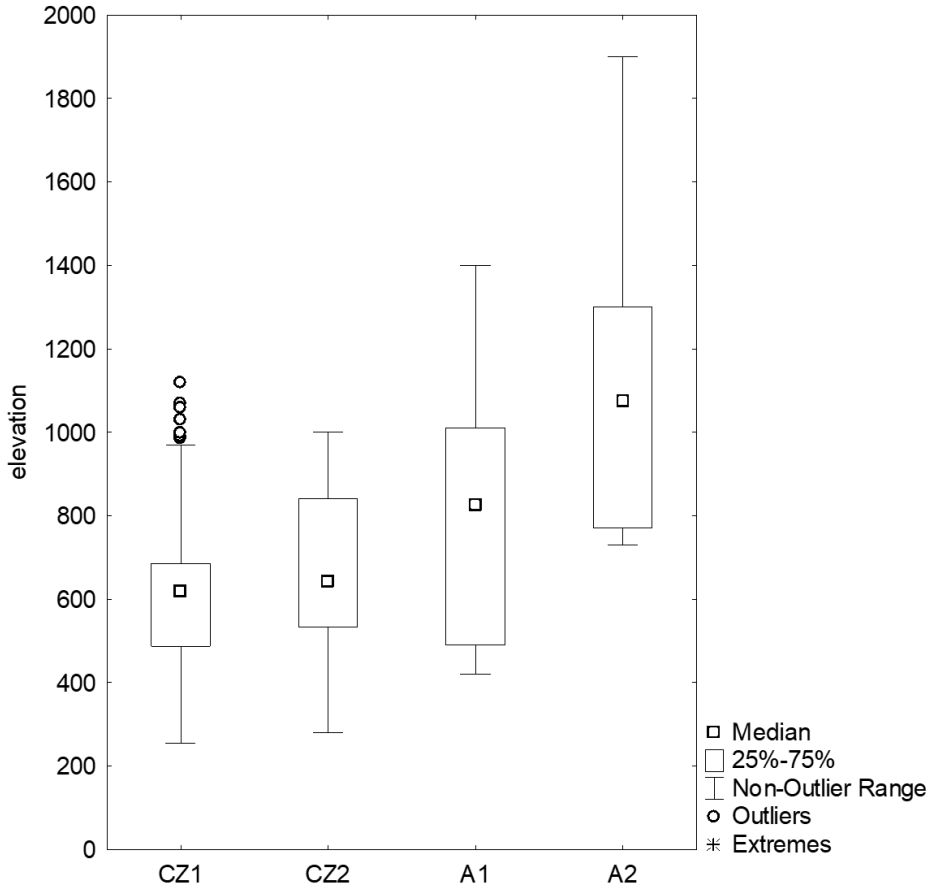
Character	Taxon	N	Mean	SD	Min	0.05	0.25	Median	0.75	0.95	Max	T-test
LL_LW	C2	27	2.89	0.32	2.42	2.45	2.64	2.83	3.03	3.46	3.57	t(53)=-0.829; p=0.411
LW_LBW	C1	27	1.56	0.15	1.33	1.35	1.46	1.56	1.7	1.77	1.89	t(53)=-0.888; p=0.379
LW_LBW	C2	27	1.53	0.11	1.32	1.38	1.46	1.53	1.59	1.71	1.74	
LW_CoW	C1	27	16.44	2.53	11.97	12.73	14.79	15.94	18.29	19.87	21.65	t(53)=0.843; p=0.403
LW_CoW	C2	27	17.1	2.37	11.97	13.73	15.27	16.97	18.42	20.71	21.91	
CW	C1	27	4.82	0.44	4.11	4.36	4.47	4.75	5.05	5.7	5.93	t(53)=1.239; p=0.221
CW	C2	27	4.97	0.56	4.08	4.29	4.57	4.9	5.17	5.98	6.55	
CL	C1	27	70.58	7.8	58.38	59.19	65.71	70.36	75.83	84.65	89.68	t(53)=0.296; p=0.768
CL	C2	27	70.83	9.53	57.17	58.31	64.84	70.02	74.94	88.7	95.9	

Appendix 3 Pearson's correlation coefficient of morphological characters

Pearson	LL	LW	LBW	LWD	CoBW	CoW	RLL	RLW	LL_LBW	LL_LW	LW_LBW	LW_CoW	CW	CL
LL	1	0,625072	0,467125	0,648099	0,511421	0,513982	0,775679	0,789846	0,510397	0,484441			0,006765	0,347035
LW	0,625072	1	0,830501	0,757797	0,692046	0,671475	0,788917	0,522278	-0,19405	0,373291	0,182331	-0,08526	0,109563	0,038911
LBW	0,467125	0,830501	1	0,672653	0,687513		0,606501	0,426875	-0,50992	0,367161	-0,38737	-0,04891	0,163083	0,017543
LWD	0,648099	0,757797	0,672653	1	0,590685	0,602409	0,626494	0,438394	-0,0444	0,064674	0,017964	-0,18874	0,205453	0,101538
CoBW	0,511421	0,692046	0,687513	0,590685	1	0,893176	0,593493	0,371123	-0,17523	0,173717	-0,04568	-0,60694	0,148169	0,179272
CoW	0,513982	0,671475		0,602409	0,893176	1	0,575927	0,346232	-0,05551	0,147232	0,134727	-0,77631	0,158931	0,230441
RLL	0,775679	0,788917	0,606501	0,626494	0,593493	0,575927	1	0,614637	0,167925	0,042373	0,228787	-0,12351	-0,03941	0,084531
RLW	0,789846	0,522278	0,426875	0,438394	0,371123	0,346232	0,614637	1	0,371088	0,337955	0,123206	-0,07622	0,080067	0,092722
LL_LBW	0,510397	-0,19405	-0,50992	-0,0444	-0,17523	-0,05551	0,167925	0,371088	1	0,823428	0,557713	-0,11914	0,148468	0,360158
LL_LW	0,484441	0,373291	0,367161	0,064674	0,173717	0,147232	0,042373	0,337955	0,823428	1	-0,0038	-0,10451	0,112067	0,480964
LW_LBW	0,172381	0,182331	-0,38737	0,017964	-0,04568	0,134727	0,228787	0,123206	0,557713	-0,0038	1	-0,09451	0,105493	0,081473
LW_CoW	-0,18191	-0,08526	-0,04891	-0,18874	-0,60694	-0,77631	-0,12351	-0,07622	-0,11914	-0,10451	-0,09451	1	0,112283	0,279265
CW	0,006765	0,109563	0,163083	0,205453	0,148169	0,158931	-0,03941	0,080067	0,148468	0,112067	0,105493	0,112283	1	0,512151
CL	0,347035	0,038911	0,017543	0,101538	0,179272	0,230441	0,084531	0,092722	0,360158	0,480964	0,081473	0,279265	0,512151	1



Appendix 4 The distribution of population size along elevation gradient. Population size was classified into 7 categories: 1: max. 100 shoots; 2: to 500 cm^2; 3: 500 cm^2- 1 m^2; 4: 1 - 5 m^2; 5: 5 - 10 m^2; 6: 10 - 20 m^2; 7: above 20 m^2



Appendix 5 Elevation of Central-European Hamatocaulis vernicosus localities of clade 1 and clade 2 in the Czech Republic (CZ) and Alps (A)
CZ: $F(1;70) = 0{,}0969$; $p = 0{,}7565$; A: $F(1;12) = 2{,}6469$; $p = 0{,}1297$

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

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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

Introduction

Hamatocaulis vernicosus is a dioicous pleurocarpous moss species, classified currently in the family Scorpidiaceae (Ignatov & Ignatova 2004). It is confined to a threatened habitat of non-calcareous rich fens (Janssen et al. 2016), considered to be in continuous decline in central Europe (Štechová & Kučera 2007, Davidson 2014). European population decrease of *Hamatocaulis vernicosus* resulted in enlisting the species in Annex 2 of the Habitats Directive (92/43/EEC), which increased the efforts in surveillance of its existing and potential localities, and also in the interest in better understanding of its habitat requirements and other aspects of its biology (Štechová & Kučera 2007, Štechová et al. 2008, 2012a, 2012b, Pépin et al. 2013, Manukjanová et al. 2014). A challenging complication of the biological research of *Hamatocaulis vernicosus* appeared after Hedenäs & Eldenäs (2007) discovered that the species consists of two separate lineages, which can be considered as cryptic species with respect to the impossibility of morphological separation. The cryptic species were called clade 1 and clade 2, or southern/northern cryptic species (Hedenäs 2018). Both lineages have been reported to display partly overlapping distribution areas, with one of them (southern, clade 1) occurring south of the boreal zone in Europe, in Peru and Russia, while the northern clade 2 was found widespread in Europe occurring most frequently in the boreal zone and was also sampled in the USA (Hedenäs & Eldenäs 2007). The genetic diversity of *Hamatocaulis vernicosus* has not yet been studied in closer detail. So far we know, based on the analysis of DNA sequence data from one nuclear and two chloroplast loci on a limited set of samples (Hedenäs & Eldenäs 2007, Hedenäs 2018), that there is some haplotype variability in both lineages, but no hint of the genetic flow among lineages has been traced. This seems to confirm that the two clades function as a distinct biological species, whose sympatric occurrence probably results from a slight niche differentiation or allopatric origin, each of them has its own evolution history.

In previous studies, we assessed the distribution of the *H. vernicosus* cryptic species at all known localities of in the Czech Republic including their detailed spatial distribution at localities with sympatric occurrence

(Manukjanová et al. in press). To provide additional insights into the biological characteristics and comparison between the lineages, particularly the possible mate limitation, we inspected the sex ratio at 21 localities in the Czech Republic (Manukjanová et al. 2019). That study showed that more than one third of the populations consisted only of plants of one sex. The clades did not significantly differ in their sex expression or sex ratio.

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

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

Having developed the SSR markers for both cryptic species of the moss *Hamatocaulis vernicosus*, we were able to compare the genetic diversity

among populations of both clades, aiming at assessment of the genetic diversity within the Czech populations and an attempt at comparison of genetic variability patterns between the two cryptic species. Revealing of these patterns can result in learning the evolutionary history of the two cryptic species in Central Europe.

Material and methods

Sampling

Sampling was performed at 22 localities of *Hamatocaulis vernicosus* between 2013 and 2017 (Table 1). The sampling sites represent almost one third of recently known localities in the Czech Republic. 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 which was the case of the macro-localities Zhůří (localities Zhůří a, Zhůří b) Boží Dar (localities Boží Dar a, Boží Dar b), and Břehyně (Břehyně a, Břehyně b). Populations were sampled evenly over the whole locality depending on population size and spatial distribution (Table 1). To decrease the probability of sampling from the same clone, patches were sampled at a distance of at least 20 cm apart. The position of each patch was drawn into a field sketch that was later transferred into a GIS layer (QGIS v. 2.6 software, QGIS Development Team 2015). The information on expressed sex ratio at localities and sex of individual shoots was obtained during our previous study utilizing the same set of sampled data (Manukjanová et al. 2019).

From each patch, one stem was chosen for microsatellite analysis to account for the within-population genetic diversity and allele frequencies. Additionally, a second stem from each patch was collected for additional analyses which addressed the clonality and genotype diversity on the scale of possible fertilization distances. These analyses were only performed in populations which, based on the analysis of one stem from each patch, did not consist of a single clone, as the probability of obtaining additional information from analysis of the second stem in uniform populations was believed to be negligible. The stems in patch were taken from plants of different sex whenever possible, to increase the number of detected genotypes. The stems were barcoded into their respective clades using one

of methods described in Manukjanová et al. (2018). With respect to the rarity of clade 2 at Czech localities, it was not possible to have an equal representation of populations of both clades; plants of *Hamatocaulis vernicosus* clade 1 occurred at 20 of the selected localities, plants of clade 2 occurred at nine, of which five supported the occurrence of both cryptic species.

SSR analysis

The set of 12 loci for clade 1 and 11 loci for clade 2 was used for the analysis (Manukjanová et al. 2018, Appendix 1). The DNA isolation, PCR protocols and fragment analysis followed the methods described in Manukjanová et al. (2018). Microsatellite alleles were coded as the number of SSR motif repeats and scored using GeneMarker v1.80 (SoftGenetics LLC, State College, USA). Samples with more than 3 missing loci were discarded from the dataset. As some of the methods cannot proceed analysis with any missing data and various programs treat missing data differently, we substituted missing allele data with existing alleles from the genetically and spatially closest plant of the same sex at the same locality, even though this approach may lead to a minor underestimation of the actual diversity.

For multilocus genotypes (MLG), their number (Ng), number of resampled MLGs (Nr) for the defined number of samples (we used Nr for 7 samples in this study) and Shannon index (H) were calculated using the GenClone 2.0 software (Haond & Belkhir 2007). The difference in Shannon index between localities with both expressed sexes and those, where both sexes were not observed was tested using Analysis of variance (ANOVA) and the relationship between population size represented by the number of samples (N) and the number of genotypes (Ng) was tested using the linear regression. Both ANOVA and linear regression were calculated in Statistica software (Statsoft).

In order to compare the genetic diversity in clade 1 and clade 2 populations at five mixed localities, we used a reduced dataset consisting only 5 loci that were variable in both clades. To maximize the number of samples, both tested stems from patch were used for analyses at mixed localities, resulting in 66 individuals of clade 1 and 126 individuals of clade 2, respectively. The genetic distances among samples were visualized using Principal

Coordinate Analysis (PCoA, Appendix 2) run in GenAlEx 6.5 add-in to Microsoft Excel software (Peakall & Smouse 2012).

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

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

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

Table 1. Population characteristics. N = number of samples, Ng = number of multilocus genotypes, Largest MLG = number of samples of the most abundant MLG, Pol = % of polymorphic loci, Nr = number of genotypes resampled for 7 samples, H = Shannon diversity index, r_d = index for multilocus linkage disequilibrium (significance of r_d values is marked as ** $p < 0.001$; * $p < 0.01$). The expressed sex at locality is coded as NA = not available, F = female, M = male.

clade	locality	sex	N (°)	E (°)	elevation (m a.s.l.)	N	Ng	largest MLG	NG/N	Pol	Nr	r_d	H
1	Bažiny	NA	50.2964	16.2997	620	7	**1**	7	0.14	0	1	-	0
1	Boží dar b	F	50.4057	12.8985	1010	10	2	8	0.2	8.33	1.94	-	0.5
1	Břehyně a	M+F	50.581	14.7189	280	29	15	11	0.52	100	5.07	0.441**	2.27
1	Břehyně b	NA	50,5845	14,7069	280	9	5	3	0.56	83.33	4.34	0.603**	1.46
1	Hrádecká bahna	F	49.7132	13.659	400	20	7	6	0.35	66.67	4.48	0.77**	1.74
1	Kostelní vrch	M+F	49.0556	13.4603	970	19	14	4	0.74	100	6.14	0.322**	2.51
1	Louky v Jeníkově	M+F	49.7385	15.9645	630	8	2	5	0.25	66.67	2	1	0.66
1	Novozámecký rybník	F	50.6125	14.5853	255	7	3	4	0.43	33.33	3	0.541*	0.96
1	Oklika	M	49.4042	15.3945	660	13	11	2	0.85	83.33	6.46	0.273**	2.35
1	Pihel	F	50.7353	14.5529	300	24	3	22	0.13	16.67	1.54	-0.043	0.33
1	Ratajské rybníky	M	49.7694	15.9339	590	16	10	4	0.63	25	5.61	0.028	2.13
1	Ruda	M+F	49.1453	14.6908	415	19	8	6	0.42	91.67	4.47	0.592**	1.79
1	Řeka	M+F	49.6666	15.853	555	9	3	7	0.33	16.67	2.52	-0.125	0.68
1	Staré jezero	M+F	48.9792	14.8973	445	40	19	11	0.48	100	5.49	0.269**	2.58
1	Šimanov	M+F	49.4504	15.4467	605	9	6	2	0.67	50	5.22	0.439**	1.74
1	Šmauzy	M+F	49.197	13.2622	1030	16	7	5	0.44	91.67	4.8	0.328**	1.77
1	V Lisovech	M+F	49.247	15.2788	650	26	18	6	0.69	100	6.03	0.325**	2.66
1	Vidlák	M+F	50.5244	15.2174	280	13	11	2	0.85	91.67	6.41	0.422**	2.35
1	Zhůří a	M	49.1725	13.3317	900	9	8	2	0.89	83.33	6.41	0.447**	2.04
1	Zhůří b	M+F	49.1707	13.3326	960	5	2	4	0.4	33.33	-	1*	0.5

clade	locality	sex	N (°)	E (°)	elevation (m a.s.l.)	N	Ng	largest MLG	NG/N	Pol	Nr	r_d	H
2	Boží dar a	M	50.407	12.9006	1000	6	**1**	6	0.17	0	1	-	0
2	Panská	F	49.6019	16.1688	720	15	**1**	15	0.07	0	1	-	0
2	Řeka	M	49.6666	15.853	555	40	3	35	0.08	45.45	1.74	0.674**	0.44
2	Řeřišný	M	50.5046	16.2915	495	13	4	5	0.31	18.18	3.63	-0.028	1.33
2	Skalské rašeliniště	M+F	49.9182	17.2114	680	14	4	11	0.29	54.55	2.61	0.679**	0.75
2	Šimanov	M+F	49.4504	15.4467	605	3	2	2	0.67	9.09	-	-	0.56
2	Vidlák	M+F	50.5244	15.2174	280	25	6	14	0.24	63.64	3.28	0.381**	1.26
2	Zhůří a	M+F	49.1725	13.3317	900	16	5	9	0.31	72.73	3.45	0.329**	1.24
2	Zhůří b	M+F	49.1707	13.3326	960	12	4	5	0.33	63.64	3.44	0.641**	1.24

Results

Genetic variability and its structure in the two cryptic species

The final dataset with one analysed sample per patch contained 452 successfully genotyped stems (Table 2). The genetic variability in clade 2 was generally lower, even when resampled for the same number of samples from locality. Both localities with the highest discovered number of genotypes were large and contained plants of both sexes. The population size is positively correlated with the number of MLGs, but the regression was statistically significant only for clade 1 (Fig. 1) and when MLGs are not resampled for equal number of samples. The percentage of clones expressed by ratio between number of MLG and number of samples at locality was significantly lower in clade 2 ($F(1;27) = 8.6586$; $p = 0.0066$). No MLG was shared between localities of clade 1 except for sublocalities Břehyně a and b which shared one MLG, while in clade 2, one MLG was shared between localities Vidlák and Šimanov over the distance of 120 km.

Table 2. Population characteristics for both *Hamatocaulis vernicosus* clades (analysis of 1 stem per patch). N = number of samples, Ng = total number of multilocus genotypes, Ng max = the highest number of multilocus genotypes per locality, mean Ng = mean number of genotypes per locality, mean Nr = mean number of genotypes resampled for 7 samples per locality. For mean values, standard deviation is shown in brackets).

	clade 1	**clade 2**
N	308	144
Ng	152	29
mean Ng	7.75 (5.35)	3.33 (1.63)
mean Ng/N	0.52 (0.22)	0,32 (0.18)
mean Nr	4.37 (1.81)	2.51(1.12)
Ng max	19	6
number of alleles per locus	4-22	2-11
mean Shannon index	1.55 (0.83)	0.76 (0.51)

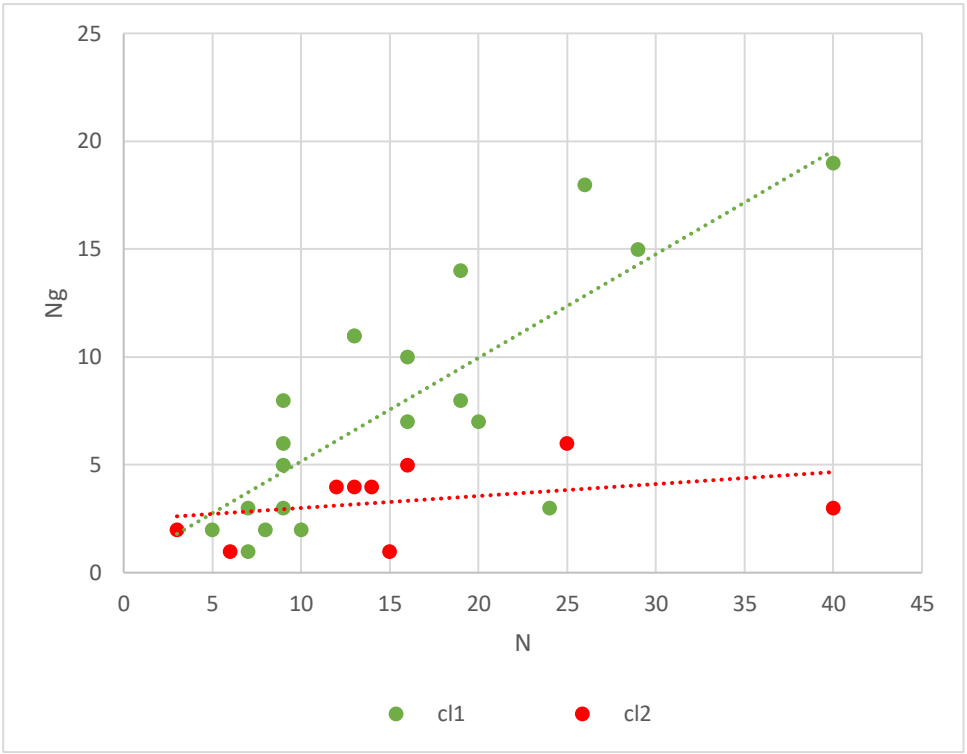

Fig. 1. – Relationship between the number of genotypes (Ng) and the population size. The correlation (r = 0.7848) was only significant (p = 0.00004) for clade 1 (cl1), not for clade 2 (cl2, r = 0.3499; p = 0.3560)

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

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

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

At each locality, we could observe plants with identical multilocus genotypes, which were considered clones (Table 1) but only one locality of clade 1 (Bažiny) and two localities of clade 2 (Boží dar a and Panská) were consisting of a single clone. The clones were variable in size, the largest clone of clade 1 at the locality Pihel accounted for 22 samples, and the largest clone among populations of clade 2 at the locality Řeka accounted for 35 samples. In Boží dar b, all samples except one and in Pihel, all samples except two belonged to came clone, but the differences applied to a single locus, where one allele was one repetition longer.

The localities where both male and female plants were expressing gametangia had higher values of Shannon index than localities with only one expressed sex present (Fig. 2), however, the results were not statistically significant (clade 1: $F(1;17) = 3.575$; $p = 0.0758$; clade 2: $F(1;7) = 2.2551$; $p = 0.1769$). The locality Bažiny where no sex expression was observed consisted of a single MLG. The putative clones at localities usually belonged to same sex (Fig. 3), however, not all stems followed that rule. The ongoing gene-flow among plants of the same clade detected by high value of linkage disequilibrium which indicate sexual reproduction involving recombination was observed only at a minimum number of localities. The high values of linkage disequilibrium (Table 1) show the prevalence of asexual reproduction, which agrees with the high level of clonality. The linkage disequilibrium values, interestingly, do not differ significantly between the clades, despite the larger genetic diversity in clade 1 (r_d: $F(1;22) = 0.0239$; $p = 0.8785$).

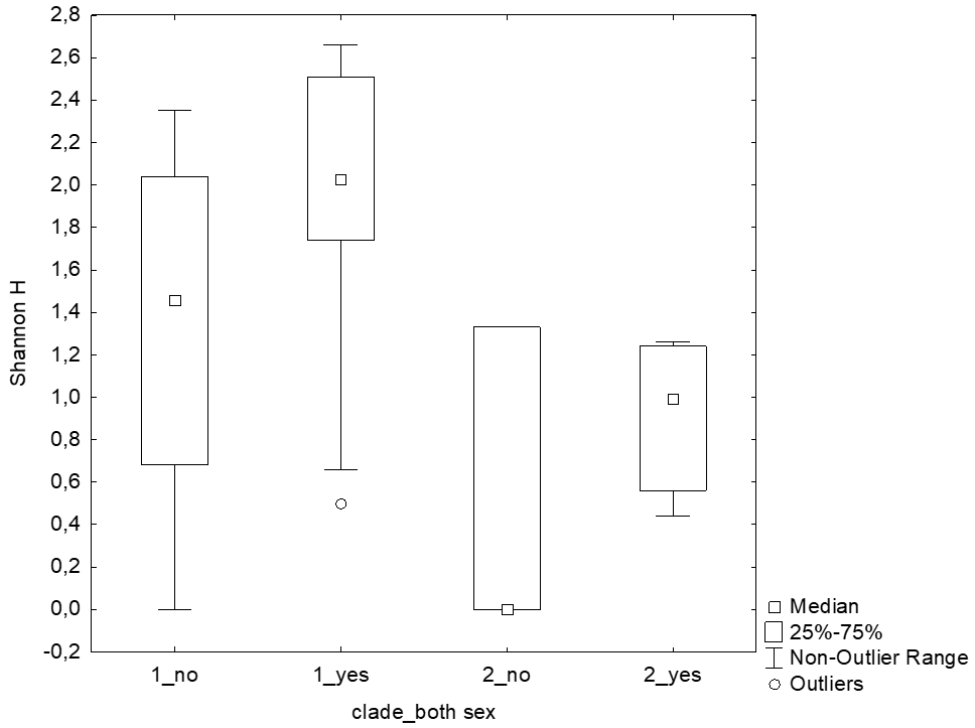

Fig. 2. – The comparison of Shannon index in populations with both male and female plants expressing gametangia and unisexual/non-expressing population in clade 1 and 2 of *H. vernicosus*.

At localities where the second stem from patch was analysed, the additionally analysed samples (139 in clade 1 and 42 in clade 2) added 30 MLGs in clade 1 and 10 MLGs in clade 2 to the dataset. This indicates that the initial sampling designed at avoiding sampling from clones was not sufficient to account for the genotype diversity and that even the spatially close patches add to the diversity of MLGs in a not negligible way, although most of samples from the same patch (89%) were identical.

Spatial genetic structure and distribution of genotypes

Genetic distances among plants of both clades were proven to be correlated with geographical distance by Mantel's test (clade 1 r=0.133, p=0.01; clade 2 r=0.288, p=0.01). The gradual decrease of similarity among MLGs of both clades is also visible from the spatial autocorrelation analysis (Fig. 4). In all distance classes, the values of Fij are positive in plants of both clades, although in plants of clade 1, the values decrease with the distance gradually

while in clade 2, the values remain exceptionally high until the distance of 50 metres.

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

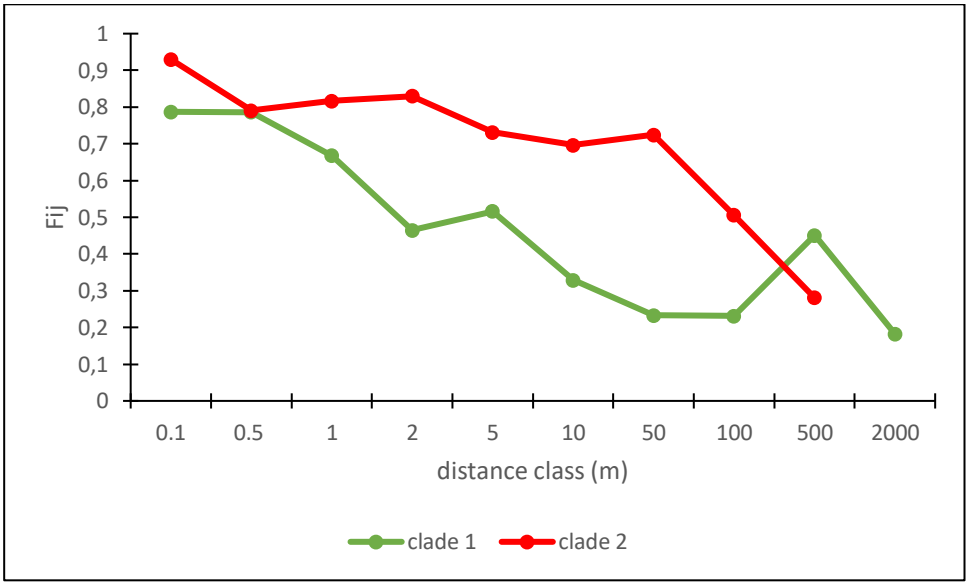

Fig. 4. – Spatial autocorrelation analysis based on microsatellite data in populations of *Hamatocaulis vernicosus* of clade 1 and 2. The Nason's kinship coefficients (Fij) are positioned along the X-axis at the mean pairwise distance within each distance class. All the values were statistically significant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

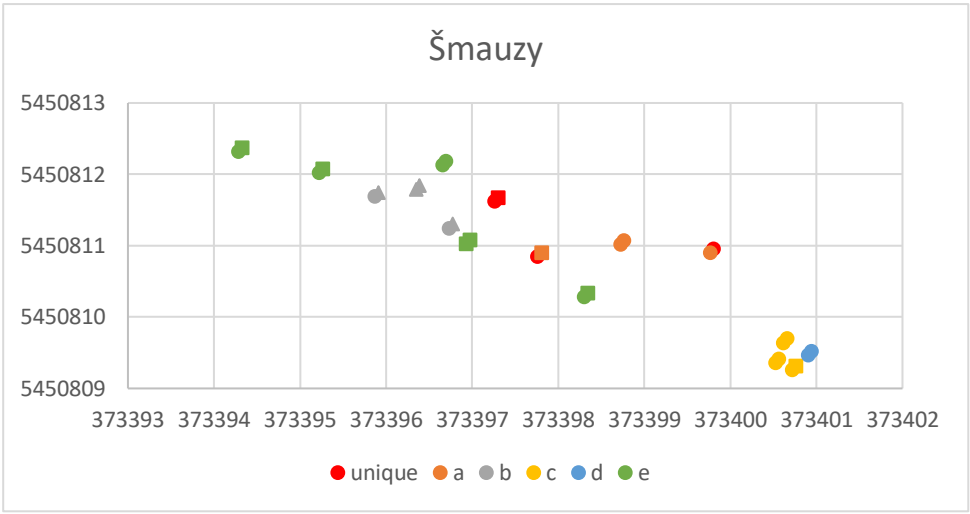

Fig. 3. – Spatial genetic structure at locality Šmauzy (clade 1). Each clone recorded more than once is depicted using a letter, unique genotypes are multiplexed into the category unique. Symbol shapes represent the sex – triangles are males, squares are females, and circles are sterile plants. The position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

The genetic distance between localities was higher in clade 1 (0.987) than in clade 2 (0.703), despite the higher average pairwise fixation index Rst values in clade 2(0.54 in clade 1 and 0.78 in clade 2, Appendix 4). The correlation between Rst and geographic distance was positive in clade 1, but non-significant in clade 2 (cl1: r = 0.1707; p = 0.0186; cl2: r = 0.0286; p = 0.8683).

Mixed localities

Genetic relationships among populations in clade 1 and 2 are illustrated using the PCoA analysis of samples of both clades from mixed localities, performed at the limited set of 5 loci which are variable in both clades (Fig. 5). While the samples from different localities were often intermixed *in situ*, the clades formed distinct clusters in ordination space. The samples from same localities were not closer to each other between clades, indicating no gene-flow between the lineages. Even at such limited mixed-localities sample set, the number of MLGs in clade 1 was higher (27 MLGs in clade 1, 22 MLGs in clade 2) despite lower number of samples (66 individuals of clade 1 and 126 individuals of clade 2).

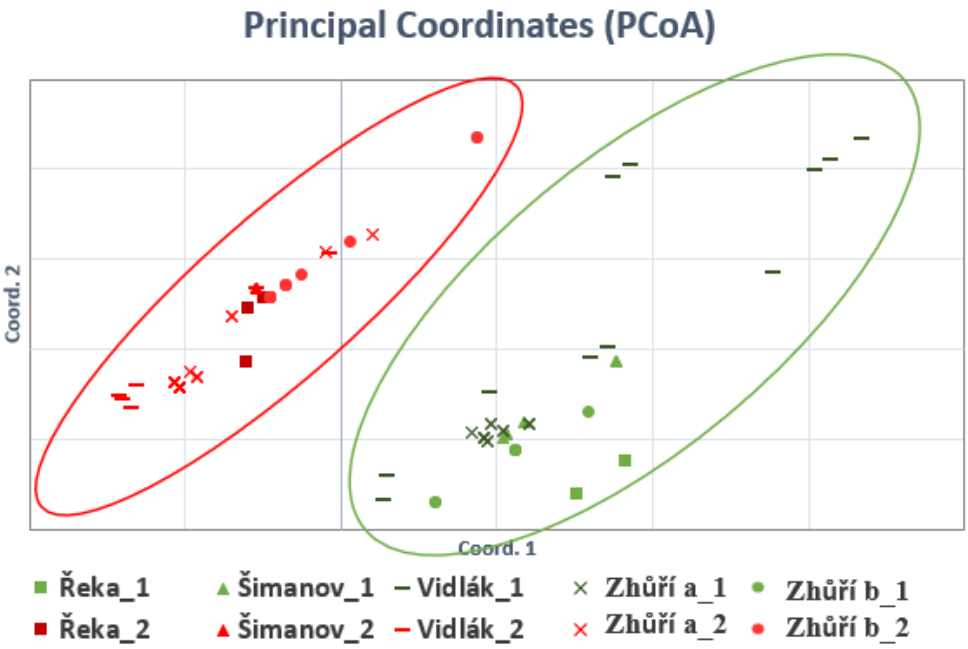

Fig. 5. – The genetic difference of clade 1 and 2 at mixed localities based on 5 variable loci visualized by principle coordinate analysis. The plants from same locality are represented with the same symbol. Clade 1 is in green color while clade 2 is red. The ellipse surrounds the samples from the same clade. The first two axes explain 53.61% and 38.11% of variability.

Discussion

Comparison of genetic variability between the two cryptic species of *Hamatocaulis vernicosus* is complicated by the surprisingly strong divergence in their microsatellite loci. While in the other similar studies, cross-amplification of SSR loci in the closely related species was generally successful both in pleurocarpous *Scorpidium* species (Kophimai et al. 2014), and in *Sphagnum* (Shaw et al. 2008a, 2008b; Johnson & Shaw 2015; Mikulášková et al. 2017), we were able to discover only five cross-amplifying variable SSR loci in the two cryptic species of *H. vernicosus*, and their variability patterns at mixed localities were non-overlapping (Fig. 5). This confirms that the mechanisms maintaining reproductive isolation between the two cryptic species of *H. vernicosus* are strong, and they indeed represent distinct biological species, despite the absence of morphological

and ecological differentiation (Hedenäs & Eldenäs 2007, Manukjanová et al. 2018).

The genetic variability of the two clades in the Czech Republic is generally correlated with the population size. Plants of clade 1 have almost ten times more localities. The larger genetic variability in clade 1 agrees with the variability of ITS ribotypes published in Manukjanová et al. (in press). Genetic variability within the clades in the Czech Republic is thus probably strongly different from the pattern in Scandinavia, where clade 2 is more diverse in the number of published ribotypes than clade 1 (Hedenäs & Eldenäs 2007). On the other hand, it is not certain, whether the pattern of genetic diversity between clades holds generally for Central Europe, as the number of ribotypes in individual clades discovered at localities in Switzerland and western Austria was similar, and hence the small genetic diversity in clade 2 might be specific to Central-Eastern European region and not to the more western part of its area of distribution. Interestingly, clade 2, while being represented by a single ITS ribotype 2 in the Czech Republic (Manukjanová et al. in press), it seems to have bigger populations at localities where both clades are present, which would suggest either its competitive advantage in the faster growth, or a broader ecological niche. Different levels of genetic diversity between the clades are probably related to different migration patterns. Clade 2 has probably migrated to the region of the Czech Republic later and following a single migration route from north-east of Europe. Such scenario is not contradicting the hypothesis discussed by Kyrkjeeide et al. (2014), suggesting the existence of glacial refugia in Scandinavia for bryophytes. Clade 2 could have survived the last glaciation in refugia in north Europe (Hedenäs & Eldenäs 2007) similarly to *Drepanocladus aduncus* (Hedenäs 2008) or *Rhytidium rugosum* (Hedenäs 2015).

The cryptic species of *H. vernicosus* also differed in their structure of genetic variability. While in clade 1, variability within and among population was almost equal, populations were less variable in clade 2 and most of the variability (84%) was observed among populations (Table 3). The populations of clade 2 in the Czech Republic are thus genetically more isolated, which also agrees with the average geographical distance between their populations. On the other hand, the mean pairwise Nei's genetic

distance between localities was higher in clade 1, despite its lower average pairwise fixation index (Rst values). Pairwise Rst values between localities are higher (for both clades) than those reported in other similar studies (Holá et al. 2015, van der Velde et al. 2001, Szövényi et al. 2008), suggesting only a limited gene flow between localities. Particularly surprising was the great genetic differentiation between sublocalities Zhůří a and b (several hundred metres apart) in clade 1, which was higher (Rst=0.947) than most other pairwise distances (0.54). The limited gene flow among localities certainly generally results from the habitat fragmentation, which has adverse effects on the genetic diversity (Pandey et al. 2016, Wilson & Provan 2003; Leonardia 2012, Kophimai et al. 2014). Rich fens faced serious destruction, degradation and fragmentation in the last centuries, which particularly affected species relying on vegetative reproduction.

Despite higher number of studied localities with clade 1, no shared MLG was found at different localities except for two cases. One genotype of *H. vernicosus* clade 1 was shared between microlocalities Břehyně a and Břehyně b, which are situated about 1 km apart, which share a similar history and might be a residuum of a single larger population, in addition to the possibility of diaspore dispersal via water or animals. In clade 2, one MLG was shared between two localities over more than 100 km, which was probably rather caused by the accidental homoplasy in microsatellite loci used (Estoup et al. 2002). The general absence of shared MLGs matches the previously published pattern in the rare epixylic hepatic *Crossocalyx hellerianus* (Holá et al. 2015). Even that hepatic grows in habitats which face significant decline and fragmentation in central Europe.

Both clades of *H. vernicosus* in The Czech Republic shared a generally low genetic diversity in populations, with the mean number of resampled MLGs per locality 4.36 in clade 1 and 2.52 in clade 2. The diversity in the more diverse clade 1 (mean Ng=7.75) was significantly lower than the diversity reported for the related fen species, *Scorpidium cossonii* (mean Ng=13.6, Kophimai et al. 2014), while the MLG diversity in another *Scorpidium* species, *S. revolvens* (mean Ng=3.5), was in close to clade 2 (mean Ng=3.33). However, the studies differed in their sampling pattern (7 samples per each of the five circular plots with 2 m radius within an area 1 ha), as did the number and variability of utilized SSR loci (14 loci for S. *cossonii* and

13 for *S. revolvens*). The smaller number of localities od both *Scorpidium* species (5 and 4) allowed to sample only the larger populations, which tend to have greater diversity (Fig. 1). In both *Scorpidium* and *Hamatocaulis* species, smaller genetic diversity was proven for the locally rarer species. Generally, rare alleles may be lost accidentally while common alleles may become fixed, resulting in low genetic diversity and high differentiation between populations (Frankham et al. 2004). Lower genetic diversity in rare species has also been reported using other genetic markers in the moss genus *Plagiomnium* (Wyatt 1992).

Availability of sexual mates did not contribute statistically significantly to higher genetic diversity, although the localities where both sexes were present displayed a slightly higher values of diversity. Most localities including the genetically more variable ones also showed high values of linkage disequilibrium, suggesting that most of the observed genetic variability does not result from successful establishment of new recombinant genotypes originated via sexual reproduction (Shaw et al. 2008, Ramaiya et al. 2010), but rather from the diversification of clones Despite this general pattern, localities with low values of linkage disequilibrium occurred (Table 1), suggesting the presence of sexual reproduction. Despite the low levels of genetic diversity, only two localities where plants of only one sex were observed were completely clonal. This shows that the absence or non-detectability of sexual mates does not necessarily imply the genetic uniformity of the whole population. Besides the possibility of overlooking the plants expressing the other sex, their disappearance could also have occurred recently and have left the genetic imprint of previously bigger and functional population. The other MLGs in unisexual populations could also have originated from external sources.

The populations of clade 2 in the Czech Republic showed higher clonality, as indicated by the significantly lower numbers of Ng/N. The micromaps of MLGs at individual localities showed that in both clades, the clones were usually grouped in clusters and the clusters of identical MLGs were of the same sex. The extreme case was seen at the locality Louky v Jeníkově, where all male plants belonged to one MLG and all female plants to another, indicating the absence of sexual reproduction. The extremely small size of this population, scattered at the area of ca. one square meter, could, however,

also indicate a recent bottleneck event, as the population was known to be bigger in past years (Štechová, personal records) and the species is occasionally known to have a rapid dynamics at localities (Štechová et al. 2015). Generally, the aggregation and expansion of clones implies the vegetative spreading as the most important means of vegetative reproduction in *H. vernicosus* (observed also earlier by During & van Tooren 1987). The expansion of clones was observed in several other molecular studies (Pfeiffer et al 2006, Bryzski et al 2018). On the other hand, the clones of both clades were generally not large and mostly differed by more than one repetition in a single locus (as exemplified by the localities Zhůří a and b. Such diversification in patches of both clades cannot easily result from a somatic mutation in a few years but rather it seems that there is a slight difference in ecological optimum of the cryptic species which in which one of them has a slight competitive advantage in the particular microhabitat (Mikulášková et al. 2015).

The one-repetition differences in samples at otherwise clonal localities Pihel and Boží Dar b suggest the occurrence of somatic mutations. This variation in microsatellite data often results from strand slippage during DNA replication (Levinson and Gutman, 1987), where a new match is allowed by excision or addition of repeats (Schlotterer and Tautz, 1992). Somatic mutations have also been observed in *Scorpidium cossonii* (Kophimai et al. 2014), and their importance to generating the genetic diversity, especially in asexually reproducing bryophytes, has been discussed in many studies (Pohjamo et al. 2008, Bączkiewicz A 2012, Newton AE, Mishler BD 1994, Skotnicki *et al*., 2005, Karlin et al. 2011). Somatic mutations probably also occur at localities supporting greater genetic diversity, which obscures their easy detection, as indicated by the unusually high values of linkage disequilibrium (Table 1).

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Appendix

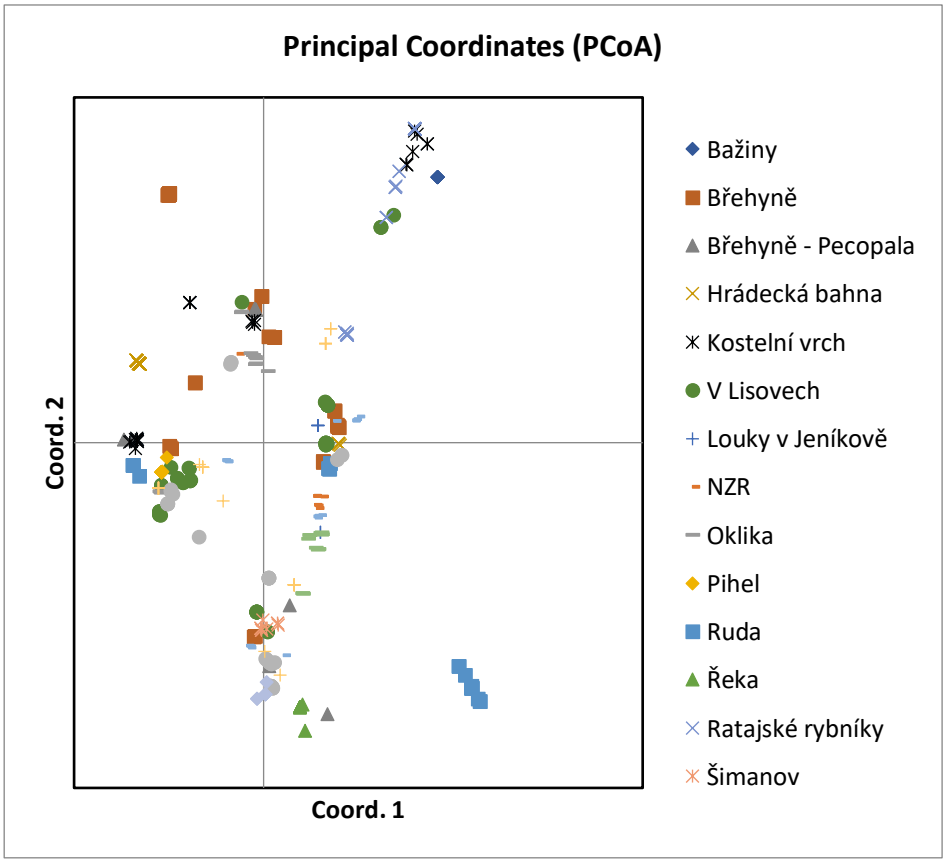
Appendix 1

The number of alleles for each loci. The loci written in bold are common for both clades

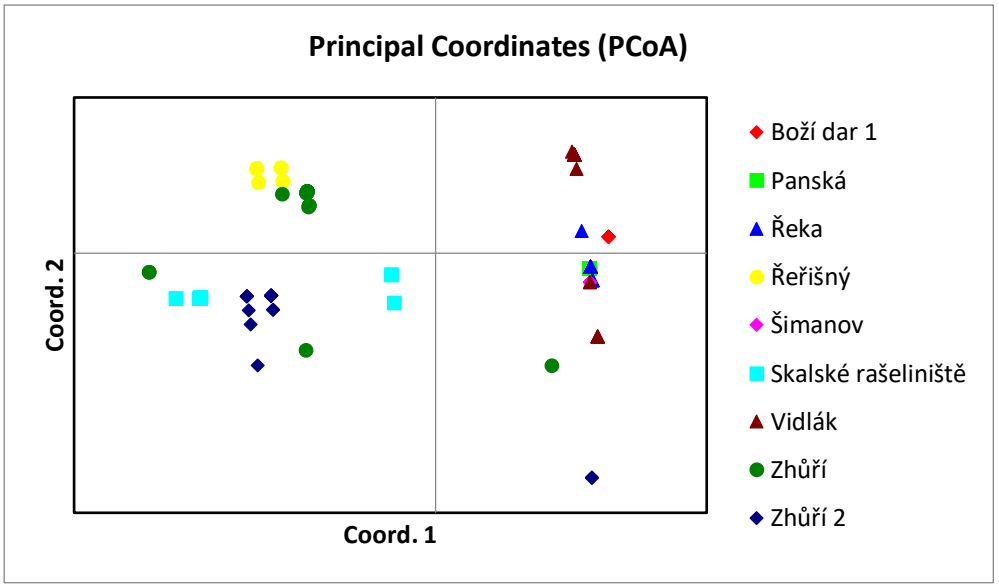
locus	AC4	2-AC45	AC209	AC30	AG29	AG39	AC115	**TC14**	**2-AC107**	**AC40**	**AC62**	**CAA111**
clade 1	4	10	10	9	22	6	4	4	21	10	20	22

locus	**TC14**	**2-AC107**	**AC40**	**AC62**	**CAA111**	2-AC58	2-AC141	2-AC134	2-AC90	2-AC74	2-AC53
clade 2	3	7	4	4	11	2	3	4	10	2	3

Appendix 2



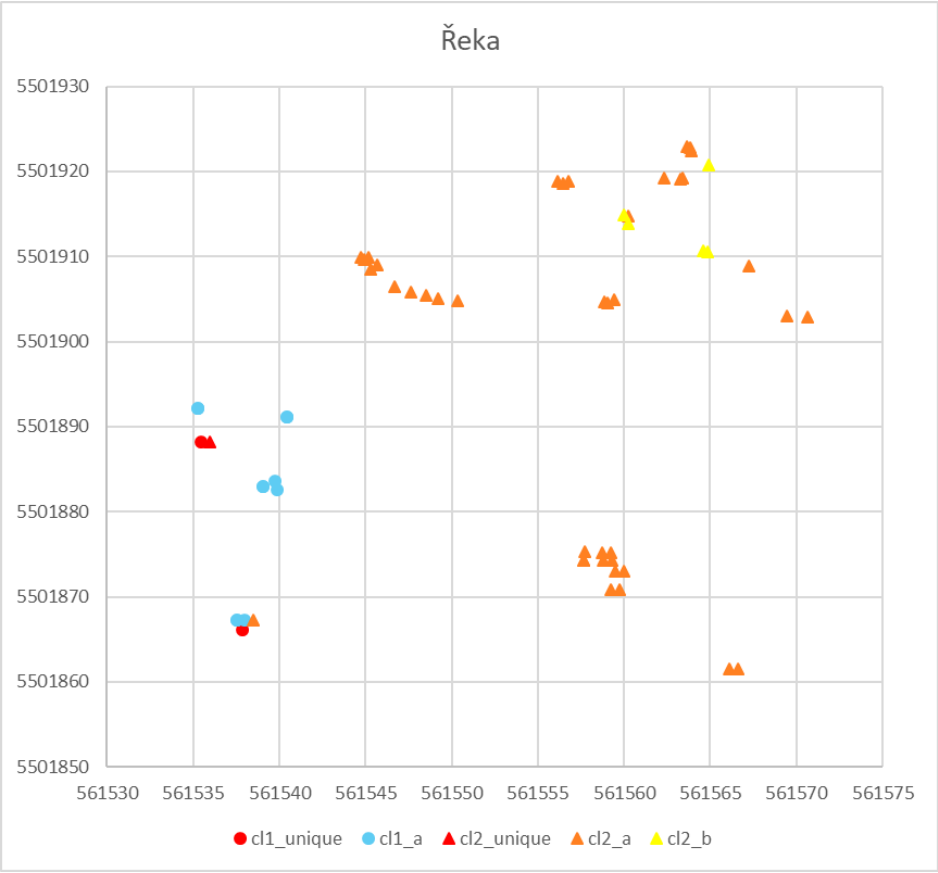
PCoA of clade 1. The first two axes explain 41.67% and 22.58% of variability.

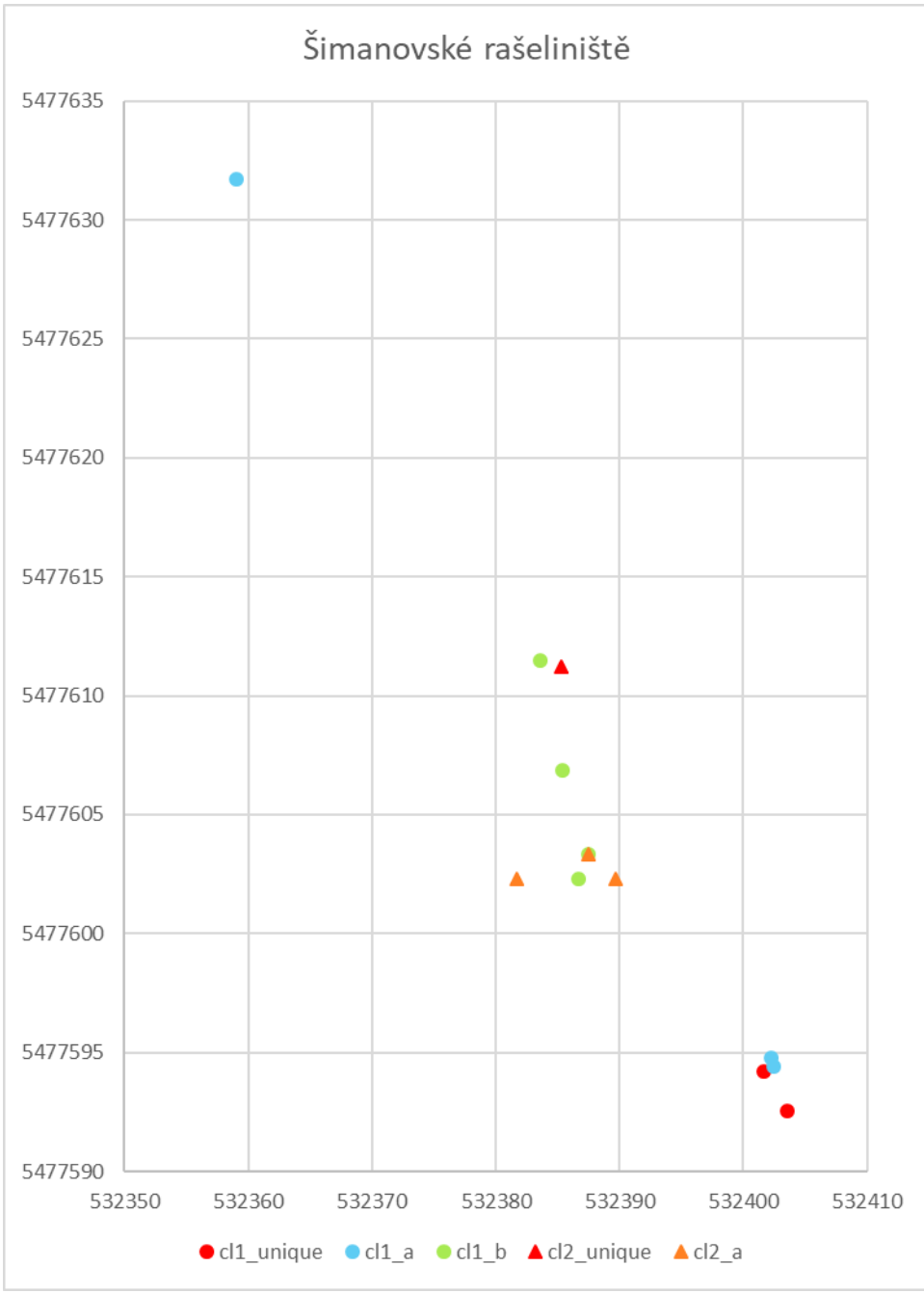


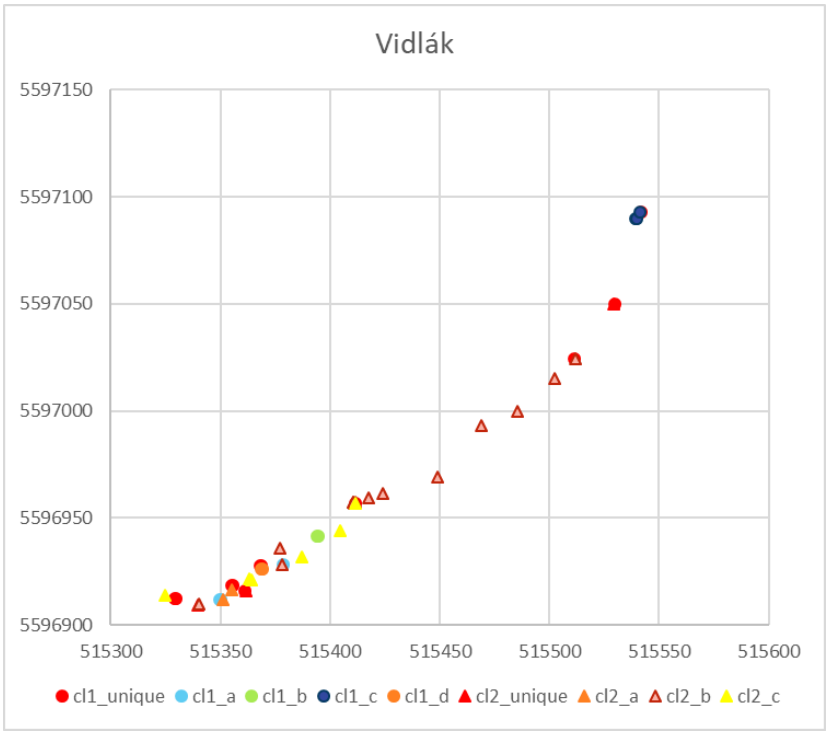
PCoA of clade 2. The first two axes explain 69.56% and 25.28% of variability.

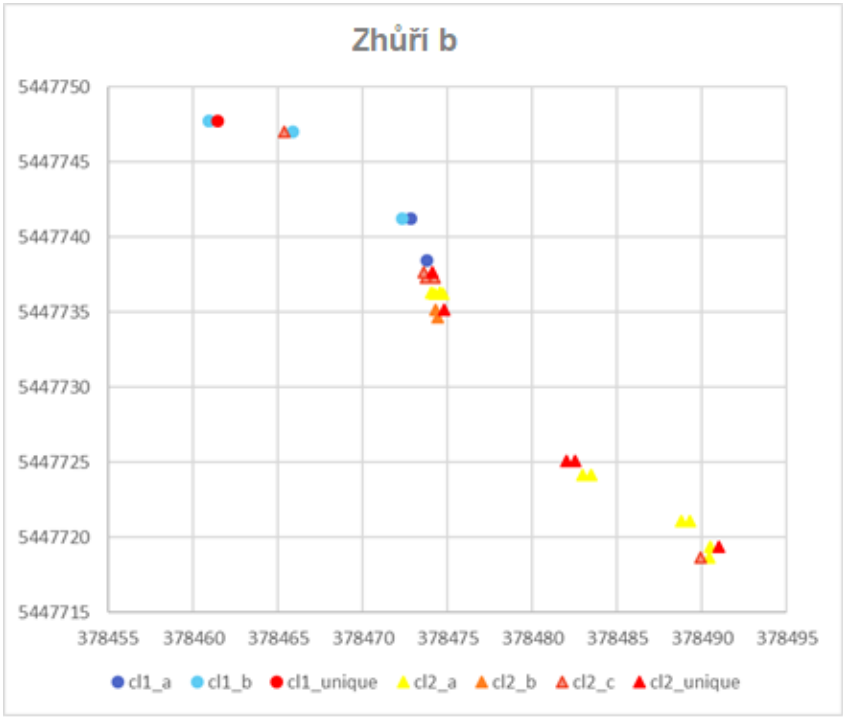
Appendix 3

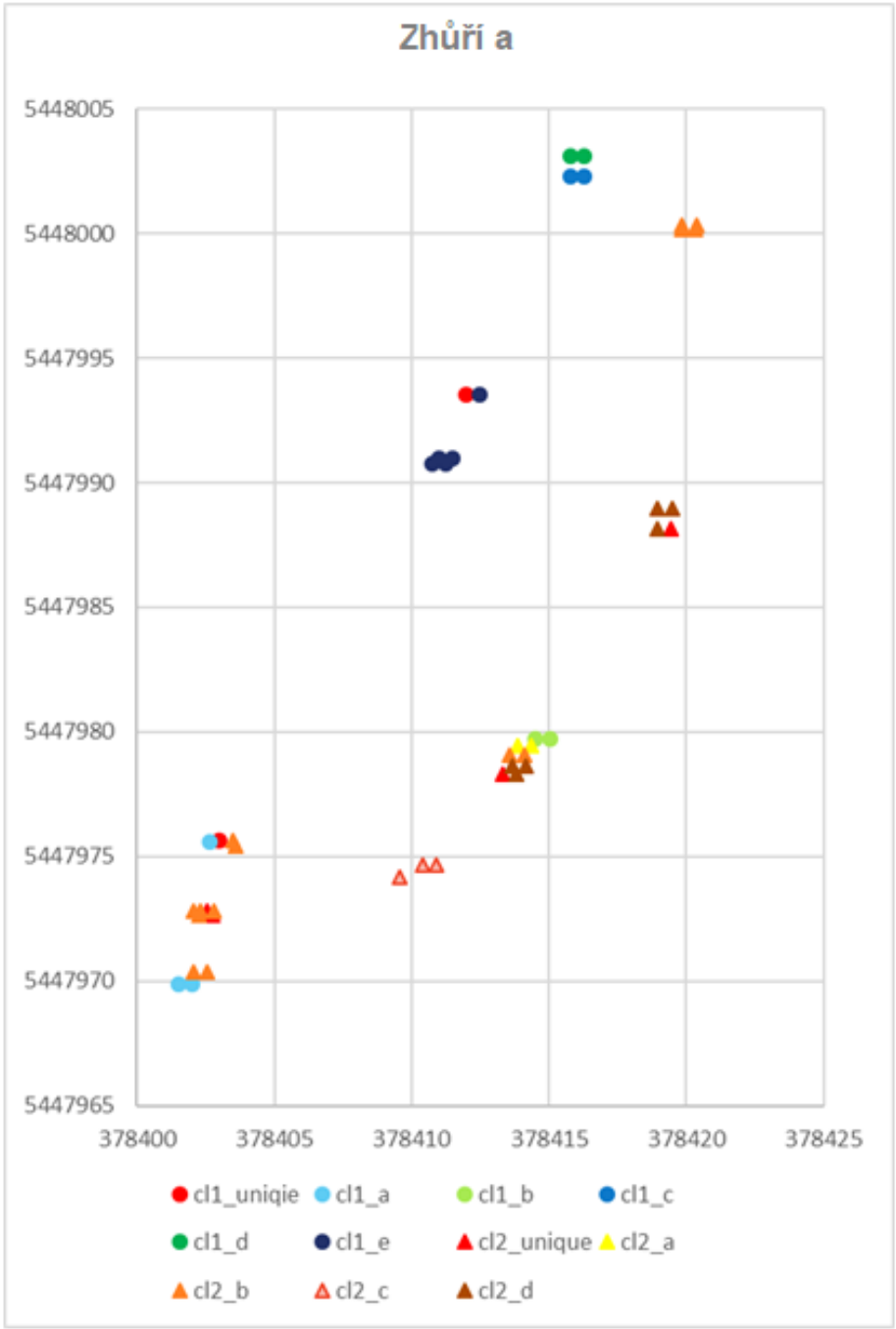
The distribution of genotypes at mixed localities. Clade 1 is represented by circles, clade 2 by triangles. The position of patches are defined by GPS coordinates in UTM format. The locations with 2 analysed individuals from one patch were manually separated by shifting their position at x-axis by 0.5m, so the marks are distinguishable in graph.




Šimanovské rašeliniště
5477635
5477630
5477625
5477620
5477615
5477610
5477605
5477600
5477595
5477590
532350
532360
532370
532380
532390
532400
532410
cl1_unique
cl1_a
cl1_b
cl2_unique
cl2_a


Vidlák
5597150
5597100
5597050
5597000
5596950
5596900
515300
515350
515400
515450
515500
515550
515600
cl1_unique
cl1_a
cl1_b
cl1_c
cl1_d
cl2_unique
cl2_a
cl2_b
cl2_c


Zhůří b
5447750
5447745
5447740
5447735
5447730
5447725
5447720
5447715
378455
378460
378465
378470
378475
378480
378485
378490
378495
cl1_a
cl1_b
cl1_unique
cl2_a
cl2_b
cl2_c
cl2_unique


Zhůří a
5448005
5448000
5447995
5447990
5447985
5447980
5447975
5447970
5447965
378400
378405
378410
378415
378420
378425
cl1_uniqie
cl1_a
cl1_b
cl1_c
cl1_d
cl1_e
cl2_unique
cl2_a
cl2_b
cl2_c
cl2_d

Appendix 4

The pairwise fixation index (Rst) between localities of clade 2

Boží dar a	Panská	Řeka	Řeřišný	Šimanov	Skalské rašeliniště	Vidlák	Zhůří a	Zhůří b	
									Boží dar a
1									Panská
0.688	0.632								Řeka
0.997	0.998	0.979							Řeřišný
0.993	0.987	0.756	0.996						Šimanov
0.953	0.964	0.961	0.894	0.937					Skalské rašeliniště
0.077	0.304	0.378	0.78	0.278	0.824				Vidlák
0.947	0.963	0.956	0.547	0.943	0.793	0.735			Zhůří a
0.83	0.857	0.904	0.774	0.749	0.306	0.745	0.681		Zhůří b

The pairwise fixation index (Rst) between localities of clade 1

Bažiny	Břehyně b	Břehyně a	Hrádecká bahna	Kostelní vrch	V Lisovech	Louky v Jeníkově	NZR	Oklika	Pihel	Ruda	Řeka	Ratajské rybníky	Šimanov	Staré jezero	Šmauzy	Vidlák	Zhůří a	Zhůří b	Boží dar b	
																				Bažiny
0.756																				Břehyně b
0.676	0																			Břehyně a
0.905	0.298	0.313																		Hrádecká bahna
0.584	0.061	0.101	0.247																	Kostelní vrch
0.67	0.133	0.136	0.453	0.158																V Lisovech
0.845	0.398	0.34	0.747	0.346	0.158															Louky v Jeníkově
0.945	0.422	0.423	0.75	0.266	0.226	0.624														NZR
0.783	0.176	0.195	0.338	0	0.205	0.535	0.473													Oklika
1	0.705	0.508	0.842	0.594	0.515	0.914	0.972	0.777												Pihel
0.517	0.388	0.434	0.632	0.387	0.26	0.155	0.279	0.418	0.69											Ruda
0.99	0.666	0.621	0.838	0.574	0.432	0.745	0.861	0.728	0.992	0.347										Řeka
0.771	0.684	0.593	0.866	0.525	0.585	0.767	0.837	0.74	0.975	0.551	0.934									Ratajské rybníky
0.963	0.504	0.461	0.78	0.485	0.302	0.555	0.816	0.646	0.947	0.21	0.825	0.918								Šimanov
0.815	0.366	0.385	0.564	0.389	0.158	0.338	0.405	0.421	0.644	0.315	0.365	0.765	0.202							Staré jezero
0.748	0.073	0.129	0.377	0.144	0.039	0.262	0.317	0.194	0.624	0.308	0.481	0.686	0.313	0.095						Šmauzy
0.637	0.313	0.311	0.561	0.273	0.191	0.213	0.486	0.3	0.671	0.161	0.522	0.667	0.312	0.329	0.25					Vidlák
0.98	0.554	0.446	0.797	0.375	0.216	0.46	0.778	0.562	0.988	0.137	0.9	0.895	0.793	0.267	0.282	0.306				Zhůří a
0.995	0.576	0.597	0.806	0.469	0.411	0.785	0.812	0.618	0.995	0.331	0.917	0.94	0.832	0.413	0.474	0.47	0.947			Zhůří b
1	0.333	0.175	0.696	0.132	0.279	0.805	0.882	0.366	0.999	0.476	0.983	0.896	0.931	0.573	0.341	0.503	0.961	0.991		Boží dar b

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
e-mail: a.manukjanova@gmail.com

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.

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