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(A)sexual Life of Liverworts

Ph.D. Thesis

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Annotation

This thesis comprises of two published papers and one accepted manuscript, focused on various aspects of liverwort reproduction. Treated aspects include patterns of asexual reproduction, sex ratio and sex-specific pattern in vegetative growth, and patterns of genetic variation and spatial genetic structure of populations differing in availability of substrate on localities and the population connectivity, and consequently in size, density, and prevailing reproductive mode. These characteristics were studied on representatives of the family Scapaniaceae *s.l.*, belonging to the largest liverwort order Jungermanniales. The results showed that asexual propagules were formed and present in course of the whole growing season and can be considered as a sufficient substitution for sexual reproduction. In contrast with the female-biased sex ratio observed earlier in most dioicous bryophytes, unexpectedly high male-biased sex ratio was observed in the aquatic liverwort, which was speculated to represent a strategy to overcome sperm dilution in aquatic environment. In addition, no size differences between female and male shoots were detected, although the evidence for higher cost of sexual reproduction in females was found. The study of population genetic structure has shown that even small and predominantly asexually reproducing populations are important sources of genetic variation. However, we were able to demonstrate notably low levels of gene flow among populations where habitat fragmentation poses a significant barrier to dispersal of diaspores. The fine scale study of spatial genetic structure revealed a strong aggregation of genotypes, particularly in smaller populations, and at the same time showed that asexual reproduction is an efficient mean of maintaining the populations at not only the short distances, given the spatial extent of clones spanning dozens of meters.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 26.6.2015 ....

Eva Holá

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“Mr. Wine gave me a pencil. It was long and yeller. There was a certain way you sharpened it, so that you didn’t make the point too thin. If you made the point too thin it would break, and you would have to sharpen it again; which used up the pencil for no need at all.”

Forrest Carter, *The Education of Little Tree*

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List of papers and author’s contribution

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

I. **Holá E.**, Kučera J., Těšitel J. 2011. Comparison of gemma production among three *Lophozia* species during the growing season. Flora 206, 763–768 (IF = 1.639).

II. **Holá E.**, Vesalainen T., Těšitel J., Laaka-Lindberg S. 2014. Sex ratio, sex-specific pattern in vegetative growth and gemma production in an aquatic liverwort, *Scapania undulata* (Marchantiophyta: Scapaniaceae). Bot J Linn Soc 175, 229–241 (IF = 2.534).

III. **Holá E.**, Košnar J., Kučera J. Comparison of genetic structure of epixylic liverwort *Crossocalyx hellerianus* between Central European and Fennoscandian populations. PLoS One (accepted manuscript).

The following table shows major contributions of authors to the original papers.

	Paper I	**Paper II**	**Paper III**
Original idea	EH	SL-L	EH
Field work	EH	TV	EH, JKo
Ecological data	EH	EH	EH, JKo
Molecular data	-	-	EH, JKo
Data analyses	JT, EH	JT, EH	EH, JKo
Manuscript preparation	EH, JT, JK	EH, JT, SL-L	EH, JKo, JK

EH = Eva Holá, JK = Jan Kučera, JKo = Jiří Košnar, SL-L = Sanna Laaka-Lindberg,
JT = Jakub Těšitel, TV = Tarja Vesalainen

Contents

General introduction **1**

Paper I

Comparison of gemma production among three *Lophozia* species during the growing season **23**

Paper II

Sex ratio, sex-specific pattern in vegetative growth and gemma production in an aquatic liverwort, *Scapania undulata* (Marchantiophyta: Scapaniaceae) **39**

Paper III

Comparison of genetic structure of epixylic liverwort *Crossocalyx hellerianus* between Central European and Fennoscandian populations **65**

General conclusions **97**

Curriculum vitae **103**

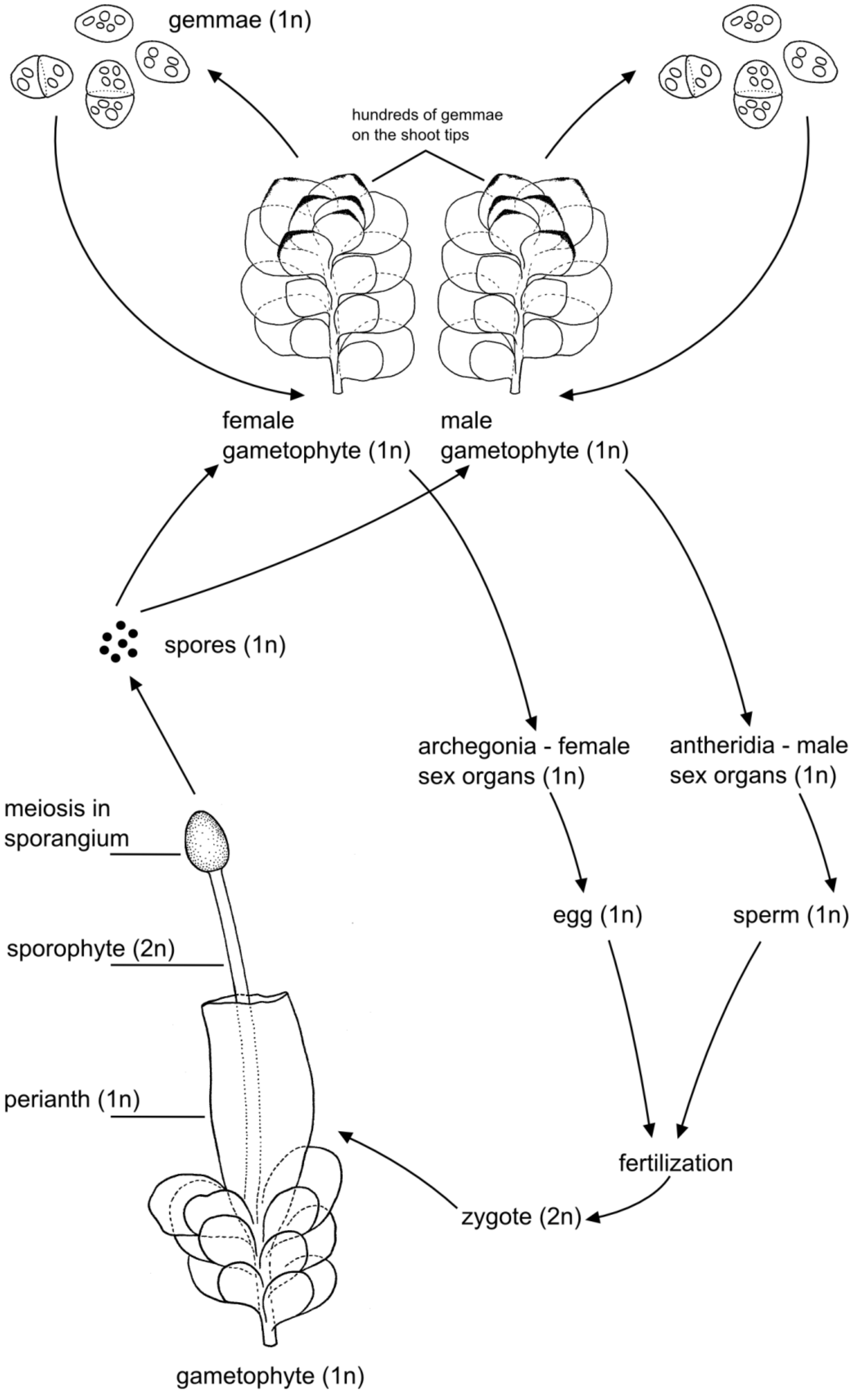
General introduction

General introduction

Bryophytes include three groups (phyla) of plants: the liverworts (Marchantiophyta), mosses (Bryophyta), and hornworts (Anthocerotophyta). They are represented by approximately 15 000–20 000 species in the world. Bryophytes are the only extant representatives of land plants with unbranched sporangia and their life cycle (Fig. 1) is unique among land plants in the dominance of elaborate autotrophic haploid gametophyte. A transition from a gametophyte-dominated to a sporophyte-dominated life cycle has marked the evolution of polysporangiate plants (Ligrone *et al.*, 2012). Some other features of bryophyte reproduction, such as the production of sexual spores and their dispersal mostly by the wind currents, and the necessity of aquatic environment for the transportation of male gametes to female ones are shared with pteridophytes (*sensu* Smith *et al.*, 2006) and affect patterns and processes in bryophyte populations and communities.

Liverworts are believed to represent the phylogenetically oldest phylum of probably paraphyletic bryophytes (Shaw *et al.*, 2011). Their principal characters include the short-lived sporophyte (persisting for at most a few weeks), stomata absent in both generations, seta hyaline and elongating after maturation of the sporangium (capsule). The capsule lacks peristome teeth and sterile tissue of the columella and spores are released mostly following the split of the sporangial wall into four valves, aided by hygroscopic elaters with spiral wall thickenings, which are produced from sporogenous tissue together with spores. Gametophytic protonema in liverworts is extremely reduced and produces only a single bud that develops into a leafy or thallose gametophore.

All studied species in this thesis belong to the largest liverwort order Jungermanniales (about 3000 species), and have been assigned to the family Scapaniaceae *s.l.* (Crandall-Stotler *et al.*, 2009). Liverworts of this order occupy a broad range of terrestrial habitats but despite this fact they have not been often used in ecological or genetic studies with respect to their minute size.


gemmae (1n)
hundreds of gemmae on the shoot tips
female gametophyte (1n)
male gametophyte (1n)
spores (1n)
archegonia - female sex organs (1n)
antheridia - male sex organs (1n)
meiosis in sporangium
egg (1n)
sperm (1n)
sporophyte (2n)
perianth (1n)
fertilization
zygote (2n)
gametophyte (1n)

← **Figure 1.** Life cycle of liverworts. Antheridia and archegonia, the male and female sex organs, respectively, are produced on haploid gametophytic plants. Spermatozoids are released from antheridia and move towards the archegonia in liquid water. Fertilized egg develops into a diploid zygote, which differentiates into the diploid sporophyte consisting mainly of the elongate seta and ellipsoid or spherical sporangium, which remains attached to and is nourished by the gametophyte. Sporogenous tissue in the capsule undergoes meiosis, resulting in the synchronous production of haploid spores, released from the sporangium upon opening. Germinating spores give rise to a new generation of haploid gametophytes. Various parts of liverwort gametophores can produce asexual propagules, most typically the specialized gemmae which germinate into new, physiologically independent plants.

Reproduction of bryophytes

The term reproduction of bryophytes is defined as the production of a new, physiologically independent plant (Mishler, 1988) and involves both sexual and asexual reproduction. The reproduction is sexual if the new plant develops from a meiotic spore that resulted from fertilization (fusion of egg and spermatozoid), whereas asexual reproduction produces offspring by means of mitotically dividing cells (Mishler, 1988).

Sexual reproduction

Spatial proximity of antheridia and archegonia is essential for successful sexual reproduction due to a restricted spermatozoid motility radius (Bisang *et al.*, 2004). Sperm range in most species spans less than half a meter and known minimum and maximum distances for sperm dispersal vary from 5–15 mm in *Splachnum ampullaceum* to 230 cm in *Dawsonia longiflora* and 2–5 m in epiphytes (Glime, 2013). Spatial proximity of male and female gametangia can be ensured by their co-occurrence on the same individual in monoicous bryophytes[1]. Nevertheless, more than half of mosses (Bryophyta), roughly two thirds of liverworts (Marchantiophyta) and more than one third of hornworts (Anthocerotophyta) occurring worldwide are dioicous – producing gametophytes bearing exclusively archegonia or antheridia (Wyatt, 1985; Villarreal & Renner, 2013).

Both dioicy and monoicy have a few drawbacks. Higher proportion of dioicous species fails to produce sporophytes (Longton & Schuster, 1983; Longton, 1992; Laaka-Lindberg *et al.*, 2000), probably as a consequence of already mentioned sperm range limitation. Moreover, in species with separate sexes, spores of only one gender may reach new sites, and such populations are not capable of sexual

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[1] The termination ‘oicous’ may be considered as misspelling, however, it refers to sexuality on bryophyte gametophytes, as opposed to the termination ‘oecious’, referring to presence of microspores or megaspores on sporophyte(s) of seed plants. Both terms are nevertheless functionally comparable.

reproduction, as reported for *Didymodon nevadensis* (Zander *et al.*, 1995) in Nevada where only females are known. Only one sex is known in the British liverworts *Telaranea murphyae* and *T. longii* (Paton, 1999). Colonization of new sites by spores of only one sex is in some cases prevented by the production of sticky spores in clusters (*Schistostega pennata*) or dispersal of meiotic tetrads (*Cryptothallus, Sphaerocarpos,* Glime, 2014a and b).

Monoicy greatly increases the fertilization chances (Longton & Schuster, 1983; Longton, 1992; Laaka-Lindberg *et al.,* 2000) but the fertilization occurs at cost of high rates of intragametophytic selfing, which results in genetic uniformity including fully homozygous sporophyte generation (Eppley *et al.*, 2007; Jesson *et al.*, 2011; Perroud *et al.*, 2011). Indeed, significantly higher inbreeding rate has been reported in monoicous species than in dioicous species (Eppley *et al.*, 2007). However, monoicous species can remove deleterious alleles after one generation of intragametophytic self-fertilization resulting in no or little inbreeding depression which would be assumed in dioicous species (Eppley *et al.*, 2007, Taylor *et al.*, 2007). Mechanisms suppressing inbreeding, such as the different maturation times of male and female organs on the same individual have not been observed in bryophytes (Glime & Bisang, 2014a).

The dominance of unisexual species in bryophytes is remarkable among higher plants, where the dioecy rate is reported to be only about 6% for angiosperms (Renner, 2014) and 36% for gymnosperms (Ming *et al.*, 2011). Similarly, most gametophytes of pteridophytes are potentially bisexual (Wyatt, 1994). Dioecy in seed plants is more widespread in the tropical zone, particularly applying to the woody dominants of tropical forests (Renner, 2014). Relatively more frequent occurrence of dioicy in the tropics has also been reported for liverworts (Wyatt & Anderson, 1984), although a very quick survey of reproductive systems in a few liverwort genera (non-tropical *vs.* tropical) did not support this statement (Table 1). Similarly, while Longton & Schuster (1983) estimated about 85% of the tropical species of Ptychantheae and Brachiolejeuneae to be dioicous, Gradstein (1987) noticed that only 30% of neotropical species of Ptychantheae and Brachiolejeuneae are strictly dioicous. It is because one sex is less frequently developed in population and therefore species may give impression of dioicous species (Gradstein, 1994).

Table 1.
A brief survey of reproductive systems in a few mainly tropical versus mainly non-tropical liverwort genera.

	species of mostly boreal or temperate regions		species of mostly tropical regions	
	Lophozia (Damsholt, 2002)	Scapania (Damsholt, 2002)	Radula (Devos *et al.*, 2011)	Prionolejeunea (Borges, 2006)
dioicous	85%	87.50%	81.70%	25%
monoicous	12.50%	6.25%	9.70%	67%
polyoicous	-	-	-	2%
unknown	2.50%	6.25%	8.60%	6%

In bryophytes, monoicy may be considered as derived character (Devos *et al.*, 2011; Glime, 2013). Polyploidy enables genes for both male and female sex organs to be combined into one gametophytic individual, depending on the pairing of chromosomes UU, VV, UV and self-fertilization may be way how to maintain the monoicous populations. Indeed, polyploidization is traditionally considered as a mechanism of transition from dioicy to monoicy among bryophytes (Crawford *et al.*, 2009), however recent study demonstrates that monoicy may originate without a genome-doubling event (Devos *et al.*, 2011). Conversely, the transition from monoicy to dioicy requires at least two mutations – a male sterility mutation forming females, and a female sterility mutation forming males (McDaniel & Perroud, 2012). Even though transition mechanisms of developing monoicy and dioicy are known, the ancestral sexual system of bryophytes is not known. Phylogenetic data indicate that both monoicy and dioicy have evolved several times in bryophytes (Devos *et al.*, 2011; McDaniel *et al.*, 2013).

All unisexual species have the potential to evolve sex chromosomes. Liverworts were the first group of plants with cytological evidence for sex chromosomes (genus *Sphaerocarpos*, Allen, 1917; Anderson, 2000) although to these days, sex chromosomes are known only in a few bryophyte species (reviewed in Ming *et al.*, 2011). Sex determination in many systems occurs at the diploid stage but in bryophytes, sexes can also be determined by genetic factors at the haploid stage. The sex chromosomes of haploid bryophytes and algae are described as U (female) and V (male) chromosomes (Fig. 2; Bachtrog *et al.*, 2011). The liverwort *Marchantia polymorpha* is a model species that has allowed researchers to better understand the sex determination in bryophytes. In this species, V-chromosome-specific gene expressed in the male organs has been identified (Okada *et al.*, 2000).

64 genes were identified on V chromosome, of which 14 were detected only in the male genome and were expressed exclusively in male reproductive organs but not in vegetative thalli, suggesting their participation in male reproductive functions (Yamato *et al.*, 2007).

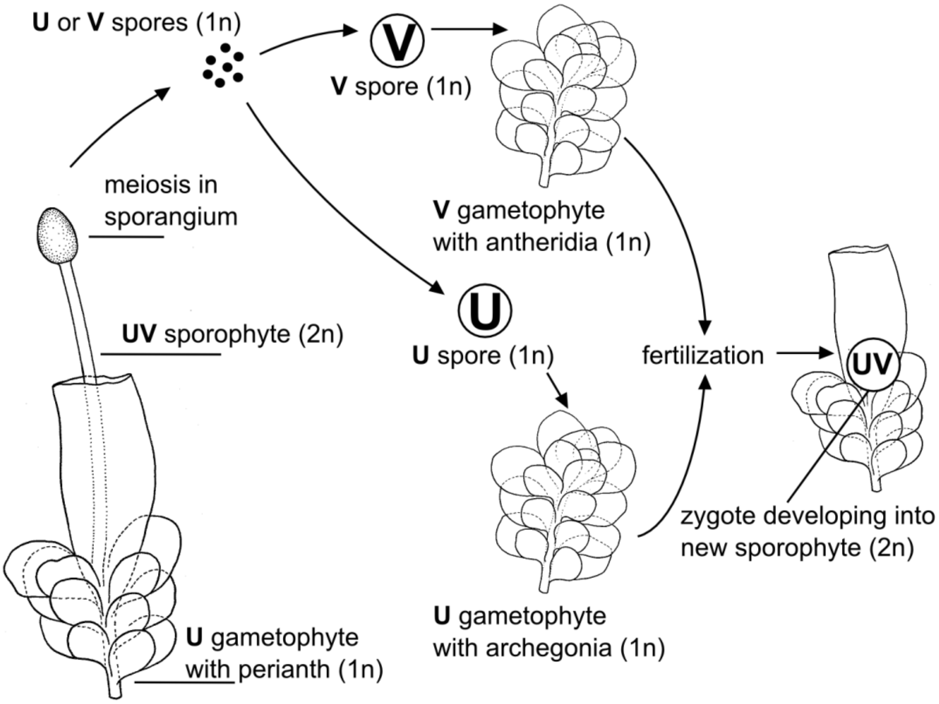

Figure 2. Haploid sex-determination (UV) systems, modified from Bachtrog *et al.* (2011). Sex is expressed in the haploid phase, with U chromosomes confined to females and V chromosomes confined to males. Male and female gametophytes mitotically produce sperm in antheridia and eggs in archegonia, respectively. Fertilization results in always heterogametic UV diploid phase. Meiotic segregation in the UV diploid sporangium results in the production of female (U) and male (V) spores, which give rise to haploid individuals.

The sex in unisexual plants (both vascular plants and bryophytes) is determined by a chromosomal mechanism (Bachtrog *et al.*, 2011). Therefore, a 1:1 sex ratio can be expected in the offspring at meiosis, although expressed balanced sex ratio is scarce. Male-biased sex ratios are most commonly seen among dioecious vascular plants (Obeso, 2002; Field *et al.*, 2013), while female-biased sex ratios prevail in bryophytes (Bisang & Hedenäs, 2005; updated by Glime & Bisang, 2014a). The latter study compiled expressed sex ratios in 138 taxa of dioicous bryophytes (86 mosses and 52 liverworts) using two different approaches: [a] rough sex assessment per cluster of individuals (herbarium specimen or patch in the field where size is undefined and may vary) and [b] precise sex assessment by examination of each individual shoot or thallus in equally sized patches. Eighty-six per cent of the taxa studied by the first method and 81% of taxa studied by the second method exhibited the female-biased sex ratio. However, expressed male-biased sex ratio in bryophytes has been reported in, e.g., *Ceratodon purpureus* (McDaniel *et al.*, 2007), *Crossomitrium patrisiae* (Alvarenga *et al.*, 2013), epiphytic *Hypnum cupressiforme* (Bisang & Hedenäs, 2005), *Mielichhoferia mielichhoferiana* (Shaw *et al.*, 1992), *Mnium hornum* (Newton, 1972), *Octoblepharum albidum* (Maciel-Silva *et al.*, 2013) and the liverworts *Lophozia silvicola* (Laaka-Lindberg, 2005) and *Scapania undulata* (Paper II).

Many explanations have been suggested to clarify the female-biased sex ratio in bryophytes although no single explanation has been widely accepted. The disparity in favour of females can be caused by differential sex-specific spore abortion, earlier germination of female spores, or faster growth of female protonemata in the early stages (Newton, 1972; McLetchie, 1992). However, the study of Stark *et al.* (2010) did not detect any skewed offspring sex ratio. Another widely hypothesized explanation is that female-biased sex ratio results from the higher prezygotic investment of sexual reproduction in males (Stark *et al.*, 2000; Horsley *et al.*, 2011). Bisang *et al.* (2006) proposed that the cost of reproduction did not explain the female-biased sex ratio, because no difference in measurements of annual male and female vegetative segments was observed. The most strongly female-biased sex ratios occur in extreme environments as a consequence of gender specific tolerance to harsh condition (e.g., desiccation or freezing tolerance, nitrogen requirement; Stark *et al.*, 2005; Stark *et al.*, 2010). Another explanation of female-biased sex ratio is the ‘shy male hypothesis’, postulating that males express sexual structures at lower rates than females, although the true sex ratio might be balanced (Stark *et al.*, 2005; Stark *et al.*, 2010). The biased ratios may also result from random processes during recruitment and colonization (Rydgren & Økland, 2002a), evolution of sex-specific life history traits (Fuselier & McLetchie, 2004), or phylogenetic history (Bisang & Hedenäs, 2005). In addition, regulation of gametangia formation by environmental factors, such as the photoperiod, pH (Chopra & Bhatla, 1983; Maciel-Silva *et al.*, 2012) or growth hormones, e.g., auxin, gibberellins and cytokinins (Bhatla & Chopra, 1981; Schumaker & Dietrich, 1998) has been demonstrated.

As mentioned above, the investigation of sex ratio in dioicous bryophytes is obscured by non-expressing gametophytes, which are common across various phylogenetic and ecological groups (see, e.g., McLetchie & Puterbaugh, 2000; Pohjamo & Laaka-Lindberg, 2003). Studies dealing with bryophyte sex ratios mostly reflect only sex-expressing shoots (i.e. those with visible gametangia), although the expressing rate may be very low (only about 13% has been reported for *Bryum apiculatum* by Mota De Oliveira & Pôrto, 2002, and 17% for *Marchantia inflexa* by McLetchie & Puterbaugh, 2000). On the other hand, a high expression rate was demonstrated in other studies – 75% for *Hylocomium splendens* (Rydgren & Økland, 2002a) and 76% for *Crossomitrium patrisiae* (Alvarenga *et al.*, 2013).

True (genetic) sex ratio, sexing of shoot includes individuals with and without gametangia, has not been often examined. Cytological approach was used by Newton (1971), with size of heterochromatin body (small *vs.* large) utilized as a sexual marker for *Plagiomnium undulatum*. In *Frullania dilatata*, flow cytometry measurements of genome size revealed that male plants have eight chromosomes with one large heterochromatic chromosome (sex-chromosome) whereas female plants have nine chromosomes with two heterochromatic chromosomes (Temsch *et al.*, 2010). Sex-specific markers have been discovered in several species: *Ceratodon purpureus* (McDaniel *et al.*, 2007), *Drepanocladus lycopodioides* (Bisang & Hedenäs, 2013), *Marchantia polymorpha* (Okada *et al.*, 2000; Yamato *et al.*, 2007), *Nyholmiella obtusifolia* (Milewicz & Sawicki, 2011), *Drepanocladus trifarius* (Korpelainen *et al.*, 2008; Bisang *et al.*, 2010) and *Sphaerocarpos texanus* (McLetchie & Collins, 2001). In studies of *D. trifarius* and *D. lycopodioides*, the expressed sex ratio and the true (genetic) sex ratio were both clearly female biased and did not differ significantly from each other (Hedenäs *et al.*, 2010; Bisang & Hedenäs, 2013). Hence, it can be expected that the true (genetic) sex ratio could be similar to the expressed sex ratio in other studied species, at least in fen bryophytes.

Labile sex expression in response to environmental, demographic or other factors occurs in many plant taxa and has most often been reported in homosporous pteridophytes (Korpelainen, 1998). Lability of the sex expression has also been speculated for some bryophytes (Korpelainen, 1998), regulated by environmental factors (Chopra & Bhatla, 1983), hormones (Bhatla & Chopra, 1981), or population factors such as the shoot density (Kimmerer, 1991). Another type of sexual plasticity is the rhizautoicy. Rhizautoicy (also rhizoautoicy) is the situation of ostensibly separate male and female shoots which are connected by rhizoids or protonema beneath the substrate surface. It has been detected in *Aloina bifrons* (Stark & Brinda, 2013), previously described as dioicous.

Asexual reproduction

Asexual reproduction and formation of asexual propagules occurring in the gametophytic phase is a widespread feature of bryophytes (Longton, 1992; Laaka-Lindberg *et al.*, 2000), unique among land plants. In polysporangiate embryophytes, vegetative reproduction of gametophyte is confined to only three families of homosporous ferns: Vittariaceae, Hymenophyllaceae and Grammitidaceae (Farrar, 1990). On the other hand, even seed plants commonly produce asexual offspring formed from vegetative tissues of sporophytes.

Asexual propagules of bryophytes (Frey & Kürschner, 2011) in strict sense are highly specialized uni- to multicellular vegetative structures that are easily detached from their mother plant. They are exemplified by specialised caducous leaves and leaf apices or shoots, gemmae, or tubers (for more details see Frey & Kürschner, 2011). Similarly, vegetative growth by fragmentation of gametophytes (or parts of gametophytes) is considered as asexual reproduction in the broad sense (Frey & Kürschner, 2011). Asexually formed spores are also assigned to asexual propagules s. str. (Newton & Mishler, 1994; Frey & Kürschner, 2011), however, they are formed by regular fertilization process (fusion of egg and spermatozoid) and result from a meiotic division of sporocytes[2]. Asexual spores, resulting from selfing on the same physical individual have the ecological role of spores, but they are genetically identical to mother plant. They may be established by asexual clone selfing (physical individuals derived from one single germination event and later separated in more individuals) or spore clone selfing (different physical individuals originated from spores of one capsule that are genetically identical because the capsule arose from selfing). Apogamy, formation of haploid sporophytes from gametophytic tissue, and apospory, formation of diploid gametophyte from sporophytic tissue, are also considered as asexual reproduction (a life cycle without sex and meiosis), however, they have only been observed *in vitro* (Goffinet *et al.*, 2009).

Specialized asexual reproduction is very common among liverworts. Almost half of the species (46%) in the British hepatic flora has been reported to produce

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[2] Two views on definitions of sexual reproduction are possible. (1) Process of spore formation is crucial for the definition of sexual reproduction. The plants invest energy to production of male and female sex organs and following sporophyte production regardless of the final genetic information carried by spores. In this case spores formed by the self-fertilization could be assigned as sexual propagules. (2) Result of the process is critical for definition. Sexual reproduction requires that sexually formed propagules are not genetically identical. In this case, spores originated as a consequence of selfing are not considered as sexual propagules.

asexual propagules at least occasionally (Laaka-Lindberg *et al.*, 2000). In contrast, only 18% of the British mosses develop specialized asexual propagules (Longton, 1992). No similar studies have been done in other countries, although Schuster (1988) suggests that both frequency and diversity of asexual propagules are smaller in tropics than in alpine and arctic areas. A little piece of information about sporophyte and/or propagule frequency is available in some regional bryofloristic studies (e.g., Kučera *et al.*, 2004; Kučera, 2009). Slightly similar rates of gemmiparous bryophytes can be seen – cf. Kučera *et al.*, 2004 (Fig. 3) and Longton (1992); Laaka-Lindberg *et al.* (2000). In addition, data about numbers of gemmiparous bryophytes from bryofloristic studies are probably much more realistic (reflecting the current situation) than the excerpt of data from national floras where numbers of gemmiparous species can be overestimated because single sporadic presence of gemmae may be recorded.

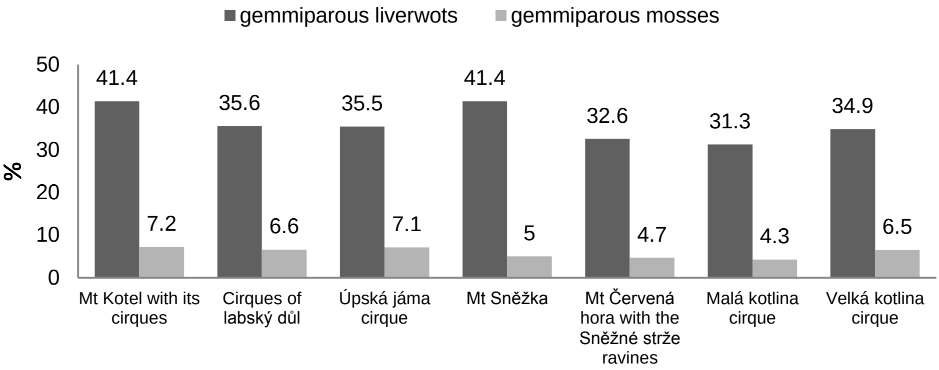

Figure 3. Percentages of gemmae-forming liverworts and mosses from survey of the glacial cirques in the High Sudetes (the data were obtained from Kučera *et al.*, 2004).

Production of asexual propagules is closely related to dioicy in mosses suggesting that asexual propagules serve as a backup option for sexual reproduction when one of the sexes is missing. (Longton & Schuster, 1983; Longton, 1992). However, the possibility of intragametophytic selfing in monoicous mosses leads to production of new propagules making production of specialized asexual propagules redundant. Moreover, production of genetically identical individuals already well-adapted to environmental conditions can be seen as advantage in some cases, such as the soil colonists (Longton & Schuster, 1983).

The production of asexual propagules by liverworts was not found to be positively correlated with dioicy and the proportion of species producing asexual propagules was almost the same in monoicous and dioicous species (Laaka-Lindberg *et al.,* 2000; Devos *et al.,* 2011; Manyanga *et al.*, 2011). Higher rates of gemmiparous liverwort as compared to mosses could be a compensation for the reduced proliferation capacity of liverwort protonema. In addition, no clear relationship between the production of asexual propagules and the rareness of liverworts has been found (Laaka-Lindberg *et al.,* 2000; Paper I).

Combination of sexual and asexual reproduction is very common among bryophytes (Longton & Schuster, 1983), enabling such species to enjoy advantages of both reproductive modes. Asexually raised shoots are less costly (in the sense of bypassing the production of gametangia and sporophytes; Laaka-Lindberg, 2001; Pohjamo & Laaka-Lindberg, 2003), may be produced under more stressful conditions (Longton & Schuster, 1983), and the reproduction may begin in the early age of shoots before reaching sexual maturity (Pohjamo & Laaka-Lindberg, 2003). In addition, gemmae germinate more quickly than spores (Pohjamo & Laaka-Lindberg, 2003). The asexual reproduction can thus help to maintain or enlarge populations, however, the genetic uniformity of asexual propagules does not enable the adaptation to changing environmental factors.

It has been traditionally postulated that asexual reproduction results in a more local dispersal of propagules (Newton & Mishler, 1994; Söderström, 1994) with respect to the generally larger size of vegetative propagules. Moreover, no specialised release mechanisms are known for asexual propagules (Shaw, 2000). Nevertheless, the contribution of small-sized asexual propagules to the long-distance dispersal has been recently evidenced by, e.g., Pohjamo *et al.* (2006).

Cost of reproduction

A basic assumption of the life history theory is that reproduction is costly due to the trade-off between current reproduction and future performance (cost of reproduction; Reznick *et al.*, 2000). In bryophytes, detection of trade-offs may be simpler than in seed plants because bryophytes lack storage organs and their sporophytes are dependent on gametophytes. On the other hand, it is difficult to handle and identify individuals non-destructively (but see Rydgren & Økland, 2002b). Correlations between the current reproduction and future performance can be positive or negative and can be studied at several levels. These include, e.g., the differences in sexual reproduction in males *vs.* females, sexual reproduction *vs.*

vegetative growth or asexual reproduction, or vegetative growth *vs.* asexual reproduction (reviewed in Stark *et al.*, 2009).

High cost of female reproduction (Laaka-Lindberg, 2001; Paper II) and sporophyte production has been demonstrated in several studies (Ehrlén *et al.*, 2000; Bisang & Ehrlén, 2002; Rydgren & Økland, 2003; Rydgren *et al.*, 2010), whereas the cost of male reproduction has rarely been addressed. In one such a study, no cost of male reproduction has been detected (Bisang *et al.*, 2006). The higher cost of reproduction in females is in conflict with the ‘cost of sex’ hypothesis, which suggests that the expensive sex is the rarer one (Stark *et al.*, 2000). This contradicts the prevailing female biased sex ratio in bryophytes (Bisang & Hedenäs, 2005).

Sexual reproduction negatively affects the vegetative growth including number of branches per shoot (Ehrlén *et al.*, 2000; Laaka-Lindberg, 2001; Bisang & Ehrlén, 2002; Rydgren & Økland, 2003; Stark *et al.*, 2009) as well as the production of generative structures (Stark *et al.*, 2009) or gemmae (Laaka-Lindberg, 2001; Manyanga *et al.*, 2011). Trade-off between production of sexual structures and gemmae can be solved by temporal separation. This can be observed in *Tetraphis pellucida*, in which the terminal gemma cup and gametangia never emerge at the same time. In addition, very few studies addressed the trade-off between asexual propagule production and vegetative growth, both of which are considered as low cost in comparison with sexual reproduction. Negative trade-off was only found among number of meristematic tips and asexual reproduction (McLetchie & Puterbaugh, 2000). The study of Pohjamo & Laaka-Lindberg (2003) and Paper II did not detect a trade-off between gemma production and survival or growth of gemmiparous shoots.

Sexually dimorphic traits between females and males may reflect the trade-offs originating from the differential cost of reproduction. The cost of reproduction hypothesis says that females may invest a higher cost of reproduction in the sense of lower vegetative growth rate (Obeso, 2002 and 2004). In contrast to seed plants, bryophyte female shoots have to nourish the sporophytes, therefore larger females are expected (Glime & Bisang, 2014b). This was confirmed in multiple studies (McLetchie & Puterbaugh, 2000; Laaka-Lindberg, 2001; Pohjamo & Laaka-Lindberg, 2004; Horsley *et al.*, 2011); male plants are sometimes reduced to so-called dwarf males (Hedenäs & Bisang, 2011).

Genetic variation connected with reproduction

It has been widely supposed that bryophytes display lower levels of genetic variability than other plants (Wyatt, 1994). Deleterious alleles can be ‘hidden’ in the diploid heterozygous organisms while newly formed deleterious mutations in haploid organisms are directly expressed and eliminated by natural selection. Moreover, it has been speculated that genetic diversity of liverworts is much lower than mosses (Wyatt, 1994). However, recent studies, using both enzymatic and DNA-markers, showed large genetic diversity in both leafy liverworts (Pohjamo *et al.*, 2008; Korpelainen *et al.*, 2011; Bączkiewicz, 2012) and mosses (Karlin *et al.*, 2012; Korpelainen *et al.*, 2013; Leonardía *et al.*, 2013). The level of their genetic diversity is comparable with vascular plants (Shaw, 2000) In thallose liverworts, level of genetic diversity was found to be lower than in leafy liverworts and mosses but this conclusion was solely based on isozymes studies (reviewed by Bączkiewicz, 2012) that are not selectively neutral and their variability can be limited (Shaw, 2000). Lower diversity in thallose liverworts was explained by their simpler morphological character (Bączkiewicz, 2012).

Certain reproductive characteristics of bryophytes may reduce their genetic variation. This applies to the above-mentioned prevailing asexual reproduction or clonal growth, rarity of sexual reproduction, high levels of self-fertilization as well as evolutionary forces (random genetic drift, low gene flow). However, the study of Bączkiewicz (2012) did not show differences in levels of genetic diversity between sexually and asexually reproducing species or monoicous and dioicous species. The allelic variation and heterozygosity rates were found to be independent of mating patterns of bryophytes in the absence of evolutionary forces (Stenøien & Såstad, 2001).

An unexpectedly high genetic variation was found in bryophyte species with rare sexual reproduction (Pohjamo *et al.*, 2008; Bączkiewicz, 2012; Paper III), which may imply other sources of genetic diversity than recombination events such as somatic mutations. Somatic mutations have been suspected to be the major source of genetic variability in the Hawaiian peatmoss *Sphagnum palustre*. Although its Hawaiian population probably originated from a single founder event and reproduced only asexually, it displays the genetic diversity comparable with other mainland regions (Karlin *et al.*, 2012). The reason for the rapid propagation of somatic mutation events might result from the growth mode of bryophytes. A single apical cell is responsible for the shoot growth, and each somatic mutation in this cell is propagated to all shoot parts, which originated from mitotic divisions following the mutation event. Similarly, any somatic mutation that occurred in leaf cells that gave rise to the gemmae, which often are only 1-2 celled, can easily be directly expressed in the progeny.

The mathematic model published by Bengtsson (2003) shows that large population with stable reproductive system can be highly variable with only small number of reproducing individuals per generation. The model was confirmed by empirical study of Fritz (2009), in which slightly higher genetic diversity of three clonal reproducing mosses with very rare sporophytes has been observed. Extraordinarily high genetic diversity in predominantly asexual or self-fertilizing bryophyte populations may be also affected by the high levels of gene flow between populations or establishment by multiple genotypes.

Aims of the thesis

The thesis is focused on particular aspects of reproduction in a little studied bryophyte group – liverworts. Although studies dealing with bryophyte reproduction in broad sense, such as the sexual and asexual cycles; propagule development, dispersal and establishment; reproductive effort and cost; trade-off among life-history traits are on the uptrend, little attention has been paid to liverworts (Fig. 4).

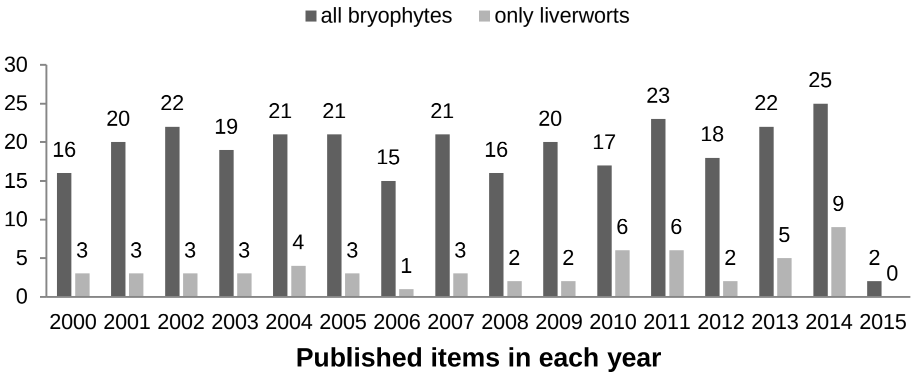

Figure 4. Studies dealing with bryophyte reproduction published from 2000 to 2015 (April) based on Web of Science database, using search topics **all bryophytes** (= 'reproduction AND bryophytes' OR 'reproduction AND mosses' OR 'reproduction AND liverworts' OR 'reproduction AND hornworts') and **only liverworts** (= 'reproduction AND liverworts').

The aim of **Paper I** was to assess the production and germinability of asexual gemmae in the rare liverwort *Lophozia ascendens* and to compare these reproductive characteristics with two common congeneric species, *L. ventricosa* and *L. longiflora*.

Paper II dealt with the reproductive system of *Scapania undulata*. Data on vegetative growth and asexual/sexual reproduction enabled us to calculate expressed sex ratio, quantify sex-specific differences in vegetative growth traits and shoot architecture and determine trade-off between sexual reproduction and asexual production.

In **Paper III**, patterns of genetic variation and spatial genetic structure were investigated in populations of *Crossocalyx hellerianus* using microsatellite markers developed specifically for this study. Studied populations were located in Fennoscandia and Central Europe, with localities differing in availability of substrate and the population connectivity, and their populations consequently different in size, density, and prevailing reproductive mode.

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Paper I

Comparison of gemma production among three *Lophozia* species during the growing season

Flora 206, 763–768

Comparison of gemma production among three *Lophozia* species during the growing season[1]

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Abstract

The extent and seasonal pattern of asexual reproduction and ability to germinate in the rare liverwort *Lophozia ascendens* and the common liverworts *L. ventricosa* and *L. longiflora* were studied in the Boubínský prales National Nature Reserve in Šumava Mts. (Bohemian Forest), South Bohemia, Czech Republic. Asexual reproduction was quantified as the number of gemmae produced per individual shoot. Numbers of gemmae per shoot among sampling months differed significantly; increase of gemma production was delayed in *L. ascendens* in comparison with gemma production of *L. ventricosa* and *L. longiflora*. We suggest that gemma production is influenced by environmental factors, mainly air humidity. Germinability of gemmae was low in early spring, highest in August and September and slightly depressed in October. This pattern suggests that rather mild winters in the Czech Republic cause the lower mortality of shoots during winter and the environmental pressure towards the production of dormant gemmae is not a prominent factor affecting the population dynamics of the species under study.

Keywords

Bryophyta, vegetative reproduction, germinability, *Lophozia ascendens, Lophozia ventricosa, Lophozia longiflora*

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Paper II

Sex ratio, sex-specific pattern in vegetative growth and gemma production in an aquatic liverwort, *Scapania undulata* (Marchantiophyta: Scapaniaceae)

Bot J Linn Soc 175, 229–241

Sex ratio, sex-specific pattern in vegetative growth and gemma production in an aquatic liverwort, *Scapania undulata* (Marchantiophyta: Scapaniaceae)[1]

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Abstract

Scapania undulata is an aquatic dioicous liverwort growing in shallow streams in boreal to subtropical zones. We studied the expressed sex ratio, sex-specific differences in shoot architecture and possible trade-off between sexual and asexual reproduction in ten populations of *S. undulata* by surveying 100 plots in ten streams in southern Finland. The expressed sex ratio was male biased, in contrast with the sex ratio in most dioicous bryophytes. It was also highly variable between the streams, but individual plots frequently comprised shoots from only one sex. The overproduction of males might be a strategy to overcome sperm dilution and ensure fertilization over longer distances in water. No size differences between females and males were detected, but they differed in branching patterns. Evidence for a higher cost of sexual reproduction in females than males can be seen from the following: the male-biased sex ratio; low number of sex-expressing female shoots in female-only plots; no co-occurrence of gemmae and female sex organs on a single branch, and no more than one sexual branch per female shoot. In contrast, high gemma production of male and female sex-expressing shoots indicates a minimal trade-off between sexual and asexual reproduction.

Keywords

Aquatic plants, asexual reproduction, sex expression, sexual reproduction

Následující pasáž o rozsahu 23 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích.
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Paper III

Comparison of genetic structure of epixylic liverwort *Crossocalyx hellerianus* between Central European and Fennoscandian populations

Accepted manuscript (PLoS One)

Comparison of genetic structure of epixylic liverwort *Crossocalyx hellerianus* between Central European and Fennoscandian populations

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Abstract

Patterns of genetic variation and spatial genetic structure (SGS) were investigated in *Crossocalyx hellerianus*, a strictly epixylic dioicous liverwort (Scapaniaceae *s.l.*, Marchantiophyta). Studied populations were located in Fennoscandia and Central Europe, with localities differing in availability of substrate and the population connectivity, and their populations consequently different in size, density, and prevailing reproductive mode. A set of nine polymorphic microsatellites was successfully developed and used. Identical individuals were only found within populations. Especially in large populations, the majority of the individuals were genetically unique. Resampled number of genotypes, mean number of observed alleles per locus after rarefaction, and Nei's gene diversity in large populations reached high values and ranged between 4.41–4.97, 3.13–4.45, and 0.94–0.99, respectively. On the contrary, the values in small populations were lower and ranged between 1.00–4.42, 1.00–2.73, and 0.00–0.95, respectively. As expected, large populations were found to be more genetically diverse than small populations but relatively big diversity of genotypes was also found in small populations. This indicated that even small populations are important sources of genetic variation in bryophytes and processes causing loss of genetic variation might be compensated by other sources of variability, of which somatic mutations might play an important role. The presence of SGS was discovered in all populations. Large populations possessed less SGS, with individuals showing a pronounced decrease in kinship over 50 cm of distance. Apparent SGS of small populations even at distances up to 16 meters suggests the aggregation of similar genotypes, caused predominantly by the deposition of asexually formed gemmae. Although no strong kinship was detectable at the distances over 16 meters in both

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small and large populations, identical genotypes were occasionally detected at longer distances (20–80 m), suggesting effective dispersal of asexual propagules.

Keywords

Crossocalyx hellerianus, dispersal, liverwort, microsatellites, propagules, spatial genetic structure

Následující pasáž o rozsahu 28 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích.
Publikace vyjde tiskem v časopise PLos One.

General conclusions

General conclusions

Results of this thesis contributed to current knowledge of liverwort reproduction. Based on obtained information we can suppose that asexual propagules are produced during the whole growing season (Paper I) and formed in large quantities (Papers I and II). It can be assumed that effective asexual reproduction can be an appropriate compensation for sexual reproduction. Moreover, cost of asexual reproduction is considered to be rather low and may already start in juvenile shoots (Paper II). Although generally considered to disperse less efficiently than spores, asexual propagules were proved to travel tens of meters (Paper III). Apparently asexually reproducing populations were further proven to serve as surprisingly important sources of genetic variation, with somatic mutations having been speculated to contribute significantly to the origin of the high genetic diversity (Paper III). Sex ratio in most dioicous bryophytes was earlier reported as female-biased. Our observation however showed an unexpectedly high male-biased sex ratio in the aquatic liverwort *Scapania undulata* (Paper II). Overproduction of spermatozoids in aquatic bryophytes might be a strategy to overcome sperm dilution in water, suggesting that sex ratio could reflect the environmental conditions rather than their systematic relationship.

Paper I

Asexual propagules are produced in course of the whole growing season but their production varies among months. The highest production of gemmae was recorded in late summer. Total gemma production of the rare species, *Lophozia ascendens*, did not differ significantly from the widely distributed species, *L. ventricosa* and *L. longiflora*, however the summer increase of gemma production in *L. ascendens* was delayed in comparison to the other species. The curve of gemma production follows the air humidity pattern and might indeed be directly influenced by this climate characteristic, yet the direct causality needs to be tested. Germinability of gemmae did not differ among studied species but it was low in early spring, while it peaked in late summer. This pattern suggests that rather mild winters in the Czech Republic cause lower mortality of shoots during winter and the environmental pressure towards the production of dormant gemmae is not a prominent factor affecting the population dynamics of the species.

Paper II

The expressed sex ratio of aquatic liverwort *Scapania undulata* was male biased, in contrast with reported sex ratio of most dioicous bryophytes. The overproduction of males (spermatozoids) and ability to produce high number of asexual propagules may pose a strategy to ensure fertilization and subsequent establishment of high number of both sexual and asexual propagules in aquatic environment. No size differences between females and males were detected, but they differed in branching patterns – female shoots have more branches which are shorter. However, higher cost of sexual reproduction in females than males has been detected, as seen from the male-biased sex ratio; low number of sex-expressing female shoots in female-only plots; no co-occurrence of gemmae and female sex organs on a single branch, and no more than one sexual branch per female shoot. In addition, minimal trade-off between sexual and asexual reproduction have been detected.

Paper III

Set of nine polymorphic microsatellite markers was successfully developed and used to investigate patterns of genetic variation and spatial genetic structure (SGS) in populations of *Crossocalyx hellerianus*. The majority of the individuals were genetically unique in the big population, moreover, identical individuals were only found within populations. The observed pattern of genetic variation is congruent with the general assumption that bigger populations tend to have bigger pool of genotypes/alleles. However, it has been shown that even small and predominantly asexually reproducing populations are important sources of genetic variation probably via somatic mutation. Low levels of gene flow among populations, where habitat fragmentation poses a significant barrier to dispersal of diaspores have been demonstrated. The fine scale study of SGS revealed a strong aggregation of genotypes, particularly in smaller populations, and at the same time showed that asexual reproduction is an efficient mean of maintaining the population at not only the short distances, given the spatial extent of clones spanning dozens of meters. On the other hand, pronounced SGS in big populations seems to be reduced by the relatively efficient dispersal of both spores and gemmae.

Future perspectives

Accomplished studies raised new interesting questions. Further experiments should test the assumption that the pattern of gemma production is directly affected by the pattern of air humidity. In studies of sex ratio, sex specific genetic markers could reveal the true sex ratio, which is obscured by non-expressing shoots, allowing more precise and additional conclusions. The hypothesis on male-biased sex ratio in aquatic bryophytes should be tested on a more representative array of aquatic species, as *S. undulata* has to date been the only aquatic species in which expressed sex ratio has been studied. Further studies could also be directed towards the role of somatic mutations in generating the genetic diversity of bryophytes with the low rate of sexual reproduction. Comparison of somatic mutation rates between gemmiparous and non- gemmiparous liverworts and the mosses would yield additional insights into the mechanisms of generating the genetic diversity, as in gemmiparous liverworts, somatic mutations that occur in leaf cells that give rise to the gemmae will be directly expressed in the progeny, while this mode of expression is different in non-gemmiparous bryophytes.

Curriculum vitae

Curriculum vitae

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Education

University of South Bohemia, České Budějovice, Czech Republic

- **Ph.D. study**, Botany, Jan Kučera's lab, since 2008
    - thesis about reproduction of liverworts
    - including one-year stay in Dr. Sanna Laaka-Lindberg's lab (University of Helsinki, Finland)
- **M.S.** (Mgr. in Czech), Botany, Jan Kučera's lab, 2006-2008
    - thesis: Ecological study of epixylic species *Lophozia ascendens* and *Anastrophyllum hellerianum* (Lophoziaceae) (in Czech, supervised by J. Kučera)
    - TOEFL, C2 level
- **B.S.** (Bc. in Czech), Biology, Jan Kučera's lab, 2002-2006
    - thesis: Bryoflora of the upper course of the Křemelná river, Šumava Mountains (in Czech, supervised by J. Kučera)

Employment

- **since 2014:** University of South Bohemia, Dept. of Botany, Hemiparasitic Orobanchaceae Research Team; responsible for using DNA-barcoding and anatomical methods to reveal host spectra of hemiparasitic plants under natural conditions
- **2008-2013:** Nature Conservation Agency of the Czech Republic; responsible for monitoring of bryophytes, lichens and fungi, and reports on the main results of the surveillance of non-vascular species under the Habitats Directive

Publications

Journals with impact factor

Holá E., Košnar J. & Kučera J. Comparison of genetic structure of epixylic liverwort *Crossocalyx hellerianus* between Central European and Fennoscandian populations – PLoS One (accepted manuscript).

Hofmeister J., Hošek J., Brabec J., Dvořák D., Beran M., Deckerová M., Burel J., Kříž M., Borovička J., Běťák J., Vašutová M., Malíček J., Palice Z., Syrovátková L., Černajová I., **Holá E.**, Novozámská E., Čížek L., Iarema V., Baltaziuk K & Svoboda T. Value of old forest attributes related to cryptogam species richness in temperate forests: A quantitative assessment. – Ecological Indicators 57: 497-504.

Hofmeister J., Hošek J., **Holá E.** & Novozámská E. 2015. Decline in bryophyte diversity in predominant types of central European managed forests. – Biodiversity and Conservation doi:10.1007/s10531-015-0863-2

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Research fellowships

- May-July 2009: project number 4701472, Ella and Georg Ehrnrooth Foundation/Lammi Biological Station (University of Helsinki); microscopic measurements of frozen *Scapania undulata*, supervised by Dr. Sanna Laaka-Lindberg
- August 2009: monitoring of long-term experiment in the laboratory at Lammi Biological Station (University of Helsinki), supervised by Dr. Sanna Huttunen
- September 2009-May 2010: CIMO (=Center for International Mobility) personal fellowship, microscopic measurement and cultivation experiment at Lammi Biological Station (University of Helsinki), supervised by Dr. Sanna Laaka-Lindberg

Conferences & meetings

- 2012: 8th Conference of European Committee for Conservation of Bryophytes, Budapest, poster: *Buxbaumia viridis* – an endangered species in the Czech Republic?
- 2011: PopBio, Oxford, poster: The comparison of gemma production among the rare hepatic *Lophozia ascendens* and the common hepatics *Lophozia ventricosa* and *Lophozia longiflora* during the growing seasons 2007 and 2008 in the Czech Republic

References

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