

School of Doctoral Studies in Biological Sciences
University of South Bohemia in České Budějovice
Faculty of Science

(A)sexual Life of Liverworts

Ph.D. Thesis

Mgr. Eva Holá

Supervisor: Mgr. Jan Kučera, Ph.D.

Department of Botany, Faculty of Science,
University of South Bohemia in České Budějovice

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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

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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 <i>Lophozia</i> species during the growing season	23
Paper II	
Sex ratio, sex-specific pattern in vegetative growth and gemma production in an aquatic liverwort, <i>Scapania undulata</i> (Marchantiophyta: Scapaniaceae)	39
Paper III	
Comparison of genetic structure of epixylic liverwort <i>Crossocalyx hellerianus</i> between Central European and Fennoscandian populations	65
General conclusions	97
Curriculum vitae	103

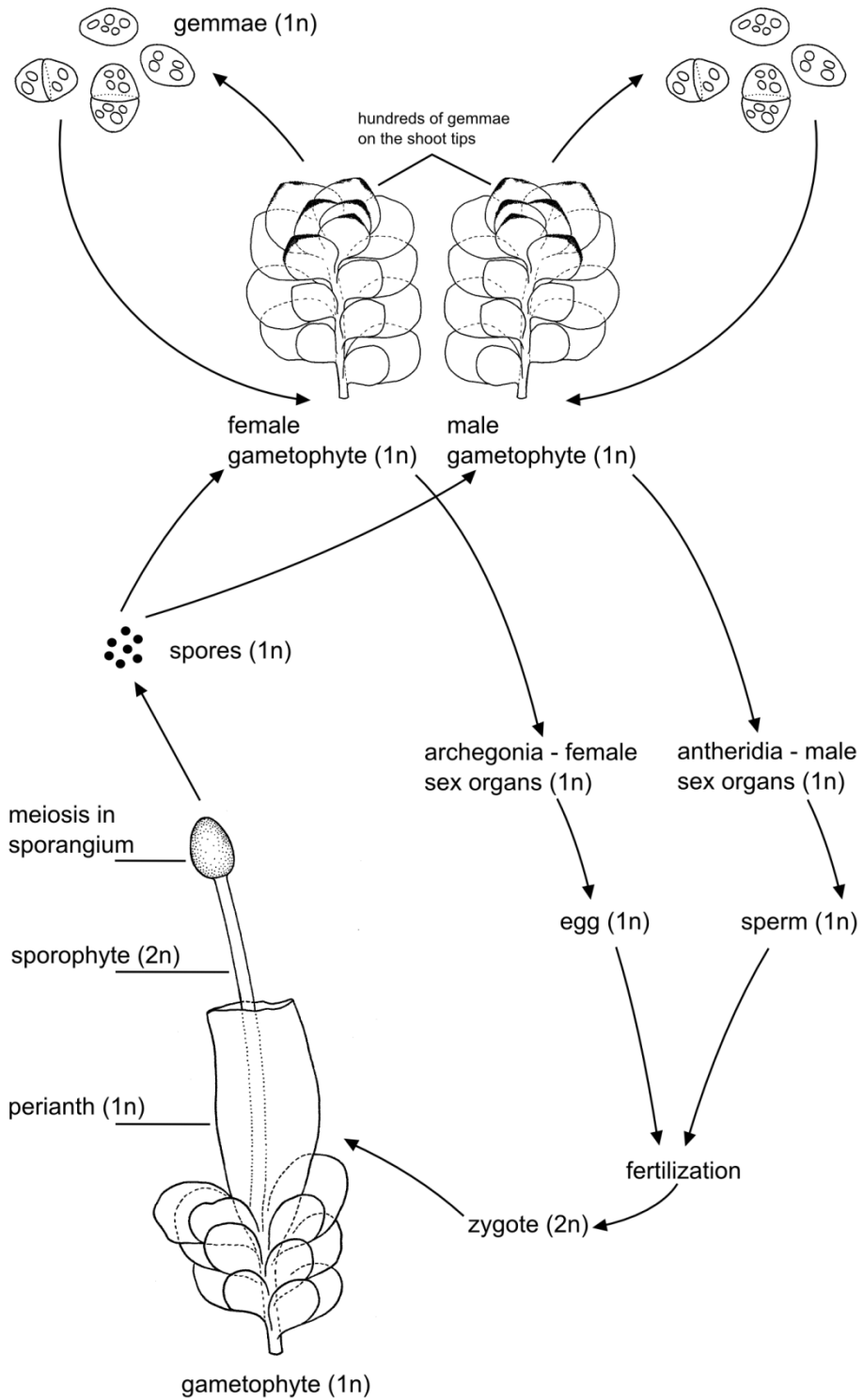
General introduction

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.



← **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 bryophytes¹. 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

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 dioecy 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 <i>et al.</i> , 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).

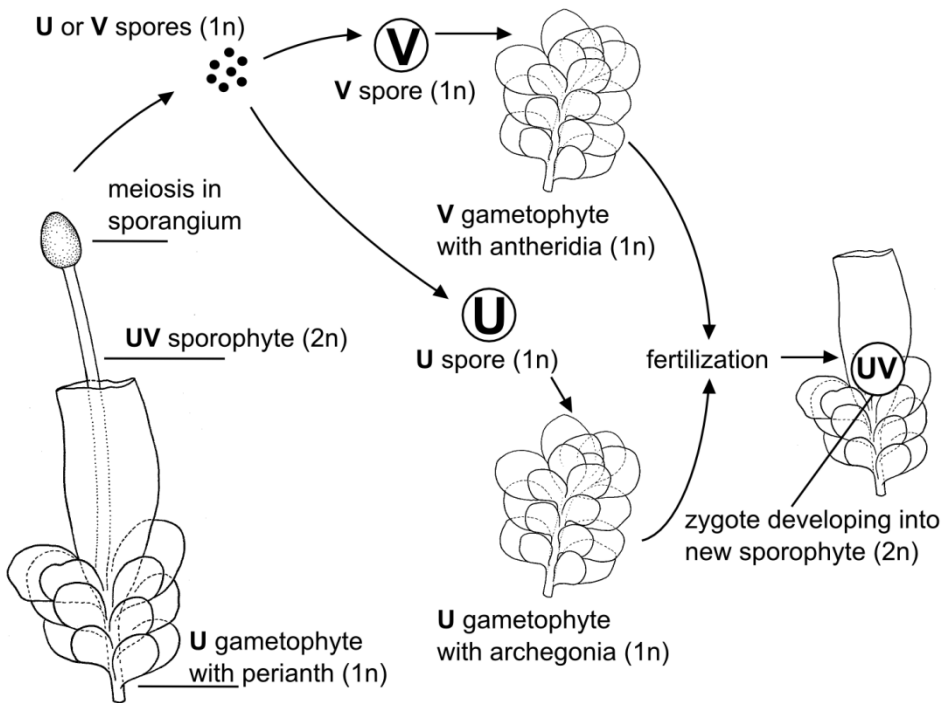


Figure 2. Haploid sex-determination (UV) systems, modified from Bachtrog *et al.* (2011). Sex is expressed in the haploid phase, with U chromosomes confined to females and V chromosomes confined to males. Male and female gametophytes mitotically produce sperm in antheridia and eggs in archegonia, respectively. 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 dioecous

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 sporocytes². 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

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.

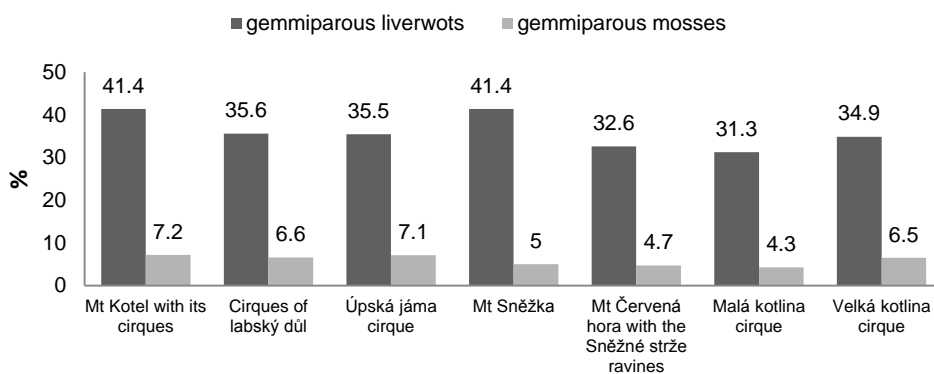


Figure 3. Percentages of gemmae-forming liverworts and mosses from survey of the glacial cirques in the High Sudetes (the data were obtained from Kučera *et al.*, 2004).

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).

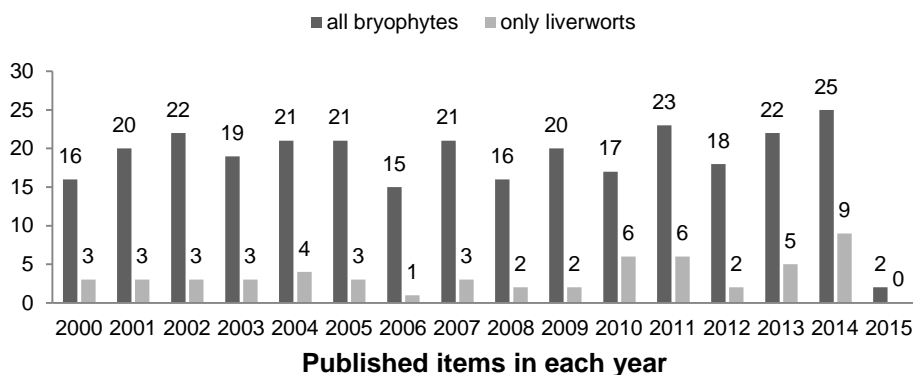


Figure 4. Studies dealing with bryophyte reproduction published from 2000 to 2015 (April) based on Web of Science database, using search topics **all bryophytes** (= ‘reproduction AND bryophytes’ OR ‘reproduction AND mosses’ OR ‘reproduction AND liverworts’ OR ‘reproduction AND hornworts’) and **only 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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Comparison of gemma production among three *Lophozia* species during the growing season

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Comparison of gemma production among three *Lophozia* species during the growing season¹

Eva Holá², Jan Kučera, Jakub Těšitel

Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic

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*

¹ Article history: received 13 October 2010; accepted 11 February 2011

² Corresponding author: Eva Holá, eva.neurazy@gmail.com

Introduction

Dead wood provides the rather high and stable level of humidity required by most of forest bryophyte species (e.g. Vellak and Paal, 1999). On the other hand, this substrate is always temporary, since the decay of wood is a dynamic process. This imposes an evolutionary pressure on epixylic bryophytes for effective dispersal and ability to establish on newly emerged favourable patches (Söderström and Herben, 1997). The proportion of epixylic bryophytes that are considered threatened has increased in Europe in recent decades (Hallingbäck and Hodgetts, 2000) as a consequence of intensive forestry management based on the cultivation of single-species even-aged plantations. Complete removal of wood during harvesting has resulted in general rareness of decaying wood, which serves as the main substrate for growth of epixylic bryophytes. Suitable environments for those species therefore became more and more fragmented, which in combination with the temporary character of these patches resulted in the massive retreat of their populations.

The term reproduction, defined as the production of a new, physiologically independent plant (Mishler, 1988) involves both sexual and asexual reproduction. The asexual mode of reproduction is widespread in liverworts. For instance, almost half of the species of the British liverwort flora produce asexual propagules (Laaka-Lindberg et al., 2000) and similar proportions can be found elsewhere in Europe (cf. Kučera, 2009). The asexual reproduction encompasses various phenomena such as branching of gametophytes with consequent separation of the branches, fragmentation of gametophytes, or small-sized propagules that are produced in relatively large numbers, such as bulbils, tubers and gemmae (Frey and Kürschner, 2011). Compared to sexual reproduction, reproducing asexually comes with several disadvantages, producing propagules that are genetically identical to the mother plant and disperse more locally than spores (Söderström, 1994). On the other hand, a recent study by Pohjamo et al. (2006) revealed that asexual propagules may also contribute to the long-distance dispersal. In addition, demands for resources are lower in asexual reproduction (Laaka-Lindberg, 2001; Pohjamo and Laaka-Lindberg, 2003) enabling persistence of bryophyte populations even in suboptimal conditions (Laaka-Lindberg, 1999).

The amount and/or seasonality of gemma production can be crucial for liverworts depending on the production of asexual propagules, but few studies (e.g. Laaka-Lindberg, 1999; Pohjamo and Laaka-Lindberg, 2003) report estimates of asexual propagule production or the seasonal pattern of asexual reproduction. Laaka-Lindberg et al. (2000) found no clear relationship between the production

of asexual propagules and the rareness of liverworts in the British liverwort flora. But no study has hitherto been conducted comparing propagule production between rare and common, yet phylogenetically closely related species of liverworts.

Dormancy can be considered a way of dispersal in time helping to escape from temporarily unfavourable environmental conditions (Evans and Cabin, 1995), competition of siblings (Hyatt and Evans, 1998), or may contribute to conserve the genetic variability within the population (During, 2001). Investigation of bryophyte diaspore banks shows that they occur in various habitats, including boreal coniferous forests (Jonsson, 1993), grasslands (Hock et al., 2004), Mediterranean scrublands and forests (During et al., 1987), or Antarctic fell-fields (Lewis Smith, 1987). Diaspore bank experiments cannot distinguish type of diaspores (sexual spores, gemmae, tubers, plant fragments) but both spore- and gemmae producing species with rare sexual reproduction are present on the species list of those experiments, including the genus *Lophozia* (Jonsson, 1993). About in vitro germination pattern of gemmae has been reported by only a few studies (Laaka-Lindberg, 1999; Laaka-Lindberg and Heino, 2001).

Low success of the establishment process is considered the main cause of bryophyte species' rareness (Wiklund and Rydin, 2004). A comparative study of gemma production pattern and germination processes in liverworts can therefore contribute to a better understanding of their population dynamics and identify parameters underpinning differential abundance of populations of individual species. The aim of this study is to assess the asexual production and germinability of gemmae in the rare liverwort *Lophozia ascendens* and to compare these reproductive characteristics to those of two common congeneric species, *L. ventricosa* and *L. longiflora*.

Materials and methods

The nomenclature of bryophytes follows Kučera and Váňa (2005), that of vascular plants follows Kubát et al. (2002) and that of plant communities follows Chytrý and Tichý (2003).

Studied species

All three studied liverworts, *Lophozia ascendens*, *L. ventricosa* and *L. longiflora* are members of the family Lophoziaceae belonging to Sect. *Lophozia* and are probably closely related (Vilnet et al., 2008). They are dioicous and reproduce both sexually by spores and asexually by 1–2 celled gemmae (Fig. 1), which are produced in considerable amounts on the top of the uppermost leaves. *Lophozia ascendens*

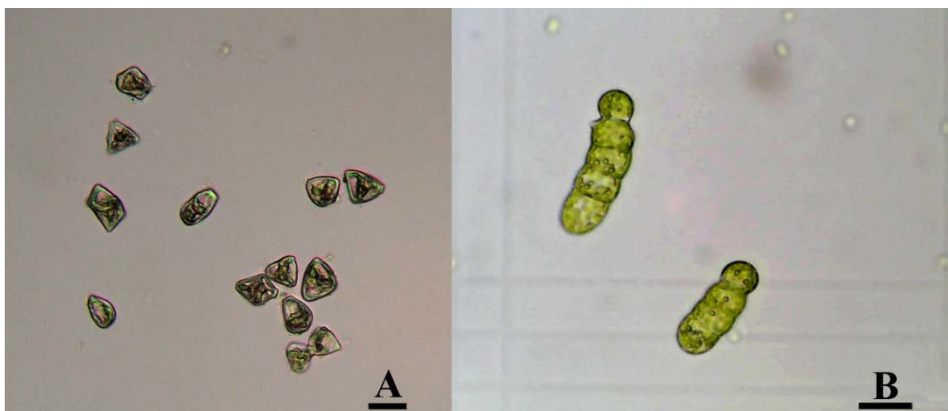


Fig. 1. (A) Gemmae of *L. ascendens* prior to germination, (B) germinated gemmae of *L. ascendens*. Scale bar: 20 μ m.

produces gemmae frequently, while sporophytes are only rarely produced in the Czech Republic (Váňa, 2005). In contrast, production of gemmae has been reported as rare in *L. longiflora* (Damsholt, 2002; Laaka-Lindberg et al., 2005). This species tends to rely on sexual reproduction (Laaka-Lindberg et al., 2005; Váňa, 2005); in some regions however the gemmae are produced copiously (Paton, 1999). The commonest of the species, *L. ventricosa*, produces gemmae frequently and sporulates occasionally (e.g. Damsholt, 2002; Paton, 1999). *L. ascendens* is considered to be a strict substrate specialist, growing exclusively on decomposing wood, while *L. ventricosa* and *L. longiflora* occasionally grow on other substrates such as mineral soil and rock (Damsholt, 2002). *L. ascendens* is considered a threatened species (EN category according to IUCN; Kučera and Váňa, 2005) with very few localities of occurrence in the Czech Republic, while *L. ventricosa* and *L. longiflora* are not endangered (LC; Kučera and Váňa, 2005).

Study area

The study area is located in the Boubínský prales National Nature Reserve (46.7 ha; 960–1040 m a.s.l.) in the Šumava Mts. (Bohemian Forest), Czech Republic. This primeval forest reserve was declared as protected in 1858. Therefore almost no human impact exists on its core zone. In addition, a fencing installed in 1967 stopped excessive deer grazing. Herb-rich forests (alliance Fagion) and, in particular, acidophilus beech forests (alliance) prevail in the vegetation, with admixtures of *Sorbus aucuparia* and *Acer pseudoplatanus* (Albrecht et al., 2003). The vegetation of the southern part of the reserve is dominated by *Picea abies*. The amount of decaying wood is rather high, corresponding to almost one third

of living trees in the reserve in the 1980s (Průša, 1985). Its quantity has recently increased, following the impact of the Kyrill windstorm (Klimánek et al., 2008) which uprooted a substantial proportion of local trees in 2007. The epixylic bryoflora of the reserve is exceptionally species-rich, hosting large populations of otherwise uncommon *Nowellia curvifolia*, *Cephalozia catenulata*, *Calypogeia suecica*, *Riccardia palmata*. In addition, several rare and threatened species have been reported to occur on the site including *Lophozia ascendens*, *Anastrophyllum hellerianum*, *Harpanthus scutatus* and *Buxbaumia viridis* (Albrecht et al., 2003; Kučera, 2006).

Sampling, recording of gemma production and germinability

For each of the species under study, three patches per log were selected. Material from each patch (0.5 cm × 0.5 cm) was collected once per month in the course of the growing season (mid-April to mid-October 2007). Gemma production and germinability of gemmae were estimated from 5 individual shoots that, following a certain pattern, were picked from each sample (one shoot in the middle of each side of the patch, and one in the centre of the sample). The gemmae of one shoot were rinsed into 0.5 ml of Knop's solution (Nehira, 1988) in a micro-tube. The tubes with gemmae were kept at room temperature (20–22°C) under diffuse light for three weeks. The numbers of gemmae (germinated and ungerminated) present on shoots were counted from each sample using a counting grid (Bürker 0.100 mm) under a microscope at 400× magnification. A drop of detergent (Triton-X 100, 0.5%) was added to the sample prior to microscoping in order to reduce surface tension. The number of gemmae per shoot was calculated as an average of three countings. Germinated gemmae were defined as having the cell walls swollen and displaying a short protonema tube (Fig. 1; cf. Laaka-Lindberg, 1999). Germinability of a sample was defined as a ratio of germinated gemmae to the total number of gemmae per shoot.

Statistical analysis

The gemma production during the growing season of the three species was analyzed by a general linear mixed-effect model. Numbers of gemmae were logarithmized prior to analysis to improve normality and homoscedasticity of residuals. Generalized linear mixed-effect model was used to analyze the germination patterns in order to account for the binomial character of the germinability variable. The effect of species identity was tested against variability between plots (i.e. mean values for plot averaged across the vegetation season) while the effects of sampling

month and the interaction was tested against the variability of individual plots across the vegetation season (i.e. mean values of samples obtained from individual plots in individual months) to account for sample interdependency and resulting complex error structure. A priori defined contrasts were used to compare the individual levels of predictors with significant overall effect on both response variables. Treatment contrasts (Crawley, 2007) were used for comparisons of *L. ascendens* to the other two species. Helmert contrasts were employed to describe the course of gemma production and germinability during the vegetation season. This type of contrasts compares the actual tested level to the mean of previous levels (e.g. July vs. mean of April, May and June) allowing capture of temporal trends in the data (Crawley, 2007).

All statistical analyses were conducted in R version 2.12 (R Development Core Team, 2010). The general linear mixed-effect model was calculated in package nlme (Pinheiro et al., 2010). The generalized linear mixed-effect model was calculated in package lme4 (Bates and Maechler, 2010).

Results

Gemma production during the growing season

Number of gemmae present on individual shoots of studied species ranged from zero to several thousands (Fig. 2) and varied significantly during the growing season (Table 1). Massive production of gemmae was particularly recorded in August and September (Fig. 2). The three species under study did not significantly differ in the total gemma production (Table 1). There was however a significant effect of the interaction between months and species due to a delayed increase in gemma production in *L. ascendens* (Fig. 2). *Lophozia longiflora* and *L. ventricosa* displayed significantly higher production of gemmae in July compared to *L. ascendens* (contrast tests: $t_{36} = 2.658$, $p = 0.012$ and $t_{36} = 3.491$, $p = 0.0013$ for each species, respectively; Fig. 2 and Table 1).

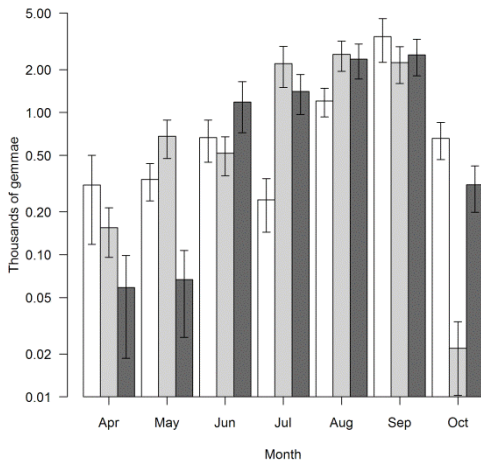


Fig. 2. Production patterns of gemmae in the three studied species; *Lophozia ascendens* – white, *L. longiflora* – light grey, *L. ventricosa* – dark grey. Bars indicate mean values, error bars represent ± 1 standard error. Tests of Helmert contrasts of the month term in the generalized linear mixed-effect model (Table 1) revealed statistically significant increase of gemma production compared to the previous period of the year in May ($t_{36} = 2.643$, $p = 0.012$), August ($t_{36} = 2.924$, $p = 0.0059$) and September ($t_{36} = 4.1843$, $p = 0.0002$). In addition, *L. longiflora* and *L. ventricosa* produced significantly more gemmae in July compared to *L. ascendens* ($t_{36} = 2.658$, $p = 0.012$ and $t_{36} = 3.491$, $p = 0.0013$, respectively) and *L. longiflora* produced significantly less gemmae in October compared to *L. ascendens* ($t_{36} = -4.262$, $p = 0.0001$). Note the logarithmic scale of the y axis.

Table 1

Summary of the general linear mixed-effect model testing the effect of species identity on production of gemmae in the course of the growing season. The first value of degrees of freedom (df) refers to the effect df while the second value refers to error df which were used in the calculations of type I error probabilities (p) from the F values of the tests of individual model terms.

	Df	F	p
Species	2.6	0.26	0.78
Month	6.36	21.61	< 0.0001
Species x Month	12.36	4.33	0.0003

Germinability of gemmae

Germinability of gemmae significantly varied in the course of the growing season (Fig. 3), the highest germinability being recorded in August and September, while in April and May gemmae of all three studied species displayed the lowest germination rates. No significant difference was detected either between individual

species or between their interaction with months (Table 2) although *L. ascendens* appeared to display slightly higher germinability in certain times of the year (in July and August, September, October) compared to the other two species (Fig. 3).

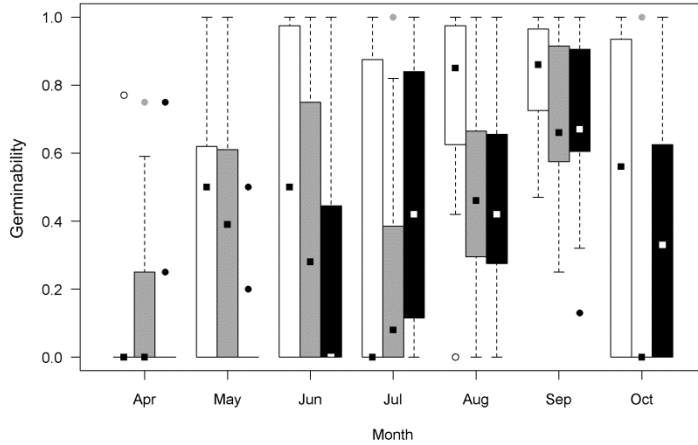


Fig. 3. Germinability of gemmae (proportion of germinated gemmae) of the three species under study, *Lophostoma ascendens* – white, *L. longiflora* – grey, *L. ventricosa* – black. Median (point), quartiles (boxes), non-outlier range (whiskers) and outliers are displayed. Tests of Helmert contrasts of the month term in the general linear mixed-effect model (Table 2) revealed statistically significant increase of germinability compared to the previous period of the year in May ($|z| = 2.525, p = 0.012$), July ($|z| = 2.322, p = 0.020$), August ($|z| = 3.672, p = 0.0002$) and September ($|z| = 5.108, p < 0.0001$).

Table 2

Summary of the generalized linear mixed-effect model testing the effect of species identity on germinability throughout the year. The df value refers to the numbers of degrees of freedom which were used in the calculations of type I error probabilities (p) from the χ^2 values of the tests of individual model terms.

	Df	χ^2	p
Species	2	4.16	0.12
Month	6	45.50	< 0.0001
Species × Month	12	12.83	0.3800

Discussion

Gemmae were present on the shoots of the endangered species *Lophozia ascendens* during the whole growing season, whereas sporophytes or perianths were not observed. Total gemma production of *L. ascendens* did not significantly differ from those of *L. ventricosa* and *L. longiflora*. This suggests that the total amount of produced gemmae alone is not the main reason why *L. ascendens* is a rare and endangered species in the Czech Republic.

Day length, temperature (Duckett and Renzaglia, 1993; Hohe et al., 2002), air humidity, and precipitation (Laaka-Lindberg, 2005) were proven to influence the initiation of sex organs or sporophyte maturation in bryophytes. Likewise, vegetative growth can be influenced by day length and temperature (Furness and Grime, 1982; Schwabe and Nachmony-Bascomb, 1963). Gemmae of *Lophozia* species are produced by mitotic cell-division on edges of young top leaves (Buch, 1911); hence both gemma production and development of gametophyte tissue are susceptible to similar environmental factors (Laaka-Lindberg, 1999; Longton, 1980). The liverwort *L. ascendens* is reported to require higher air humidity (e.g. Damsholt, 2002; Paton, 1999) than *L. ventricosa* and *L. longiflora*. Therefore, the time pattern of gemma production suggests that *L. ascendens* is more susceptible to moisture-related environmental factors compared to *L. ventricosa* and *L. longiflora*. In the Czech Republic air humidity usually peaks in late summer (Tolazs et al., 2007). This is matched by the simultaneous rise of gemmae production in *L. ascendens* after a low production during drier months (June, July). Such a late acceleration of gemma production or the low production in dry seasons can be a crucial factor restricting dispersal and establishment of the liverwort on patches that became newly available (Watson, 1981). This is also supported by Söderström and During (2005) who reported that it was rather difficult to find epixylic liverworts *Anastrophyllum hellerianum* and *L. ascendens* during dry seasons.

A comparison of gemmae numbers (Fig. 4) also shows differences among geographic areas for *L. ventricosa*. The average numbers of gemmae per shoot in Norway are more than twice as high compared to that found in the Czech Republic, which might be caused by climatic differences (Atlantic climate in Norway vs. transitional climate in the Czech Republic; Král, 2001). Norwegian liverworts benefit from the more stable air humidity and thus longer periods favourable for their growth. It may be important, however, to compare counts over several years, as illustrated by the significant interannual variance in the gemma production in the period 1997–1999 (Laaka-Lindberg and Heino, 2001), which

might be attributed to differences in pattern of the annual precipitation, particularly in the course of the growing season.

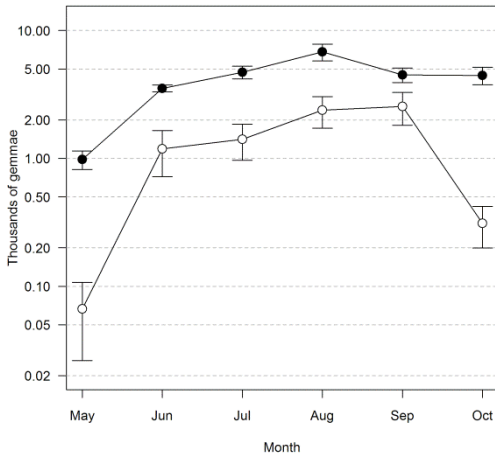


Fig. 4. Comparison of the course of production of gemmae per shoot in the *Lophozia ventricosa* population under study (open symbols) and a Norwegian population (black symbols) of the same species. Symbols and error bars represent means and ± 1 standard error, respectively. The data of the Norwegian population were obtained from Laaka-Lindberg (1999). The production of gemmae was significantly lower in the Czech population ($F_{1,72} = 52.43$, $p < 0.0001$; test of the intercept being different from zero in a linear mixed-effect model testing the difference between the Czech and Norwegian populations). Note the logarithmic scale of the y axis.

Notes about numbers of gemmae in *L. longiflora* vary in bryophyte floras (e.g. Damsholt, 2002; Paton, 1999) between “sparse” to “fairly numerous”. Studies dealing with the reproductive mode of *L. longiflora* on decaying wood in Finland indicated that only 1.5% of all colonies in the study area were gemmiparous (Laaka-Lindberg et al., 2005). Our observations for *L. longiflora* show equal numbers of gemmae in comparison with *L. ascendens* and *L. ventricosa*. The reason for this discrepancy could be the narrow concept of *L. longiflora* adopted by some authors (e.g. Saukel, 1985) or its rarity in some regions (British Isles; Paton, 1999). However, the gemma production of *L. longiflora* may also be influenced by various environmental factors related to geographical position of the populations.

Germination patterns of the three *Lophozia* species display a concave-shaped trend across the growing season with a single maximum in September (Fig. 3). Other liverwort germination studies (Laaka-Lindberg, 1999; Laaka-Lindberg and Heino, 2001) however reported a different germination pattern (highest rates at the beginning of season and lowest at the end of season) and hypothesized that

non-germinated gemmae entered dormancy at the end of the season, avoiding risk of mortality induced to non-dormant gemmae by unfavourable environmental conditions in winter. If dormancy is considered a way to escape from the unfavourable environmental conditions (Evans and Cabin, 1995) then the germination pattern observed by us leads to the suggestion that rather mild winters in the Czech Republic (with respect to temperatures and duration of snow cover, cf. Drebs et al., 2002, and Tolazs et al., 2007) might cause lower mortality of shoots during winter, and the environmental pressure towards the production of dormant gemmae could be lower compared to boreal regions. Another explanation could indicate that a high level of gemma predation (cf. Davidson et al., 1990) favours decreased dormancy of gemmae (Brown and Venable, 1991).

Gemma germinability of *L. ascendens* is generally not higher than that of the other two studied species, although *L. ascendens* appeared to display higher germinability in some cases (in July and August, September, October). This latter fact could be interpreted as a strategy that ensures a numerical advantage for species with low competitive abilities over the other two species, because the order of establishment might determine the result of competition (Watson, 1981). However, the use of liquid culture medium for bryophyte cultivation (Nehira, 1988) may artificially favour species with high demands for air humidity. This was noted by Løe and Söderström (2001), who observed *Herbertus stramineus* regenerating better in liquid culture medium than other *Herbertus* species which are not that demanding for air humidity.

Our study did not demonstrate any difference in total gemmae production between the rare and the common species of epixylic *Lophozia*. Nonetheless, the pattern of gemma production differed substantially between the species. The delayed production of gemmae in the rare *L. ascendens* in comparison to the other two species can be attributed to its higher requirement for high air humidity, which latter increases only in late summer. This constraint imposes a substantial disadvantage to this species, since gemmae of the other two species can disperse and establish on newly available patches distinctly earlier in the year, resulting in outcompeting of *L. ascendens*. This may effectively limit its ability to migrate and expand across suitable patches, which is the key parameter of the population dynamics of epixylic bryophytes.

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

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

Eva Holá*², Tarja Vesalainen[#], Jakub Těšitel*, Sanna Laaka-Lindberg[#]

* *Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic*

[#] *Finnish Natural History Museum LUOMUS, University of Helsinki, FI-00014 Helsinki, Finland*

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

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² Corresponding author: Eva Holá, eva.neurazy@gmail.com

Introduction

Hydrophily, a mode of pollen transport under or on the surface of water, occurs in only 18 aquatic genera of vascular plants (Les, 1988). In contrast with vascular plants, the reproductive system of all bryophytes is characterized by the essential presence of water in a liquid state, an environment that is required for successful sexual reproduction. Antheridia and archegonia (bryophyte male and female sex organs, respectively) are produced on haploid gametophytic plants. The spatial proximity of antheridia and archegonia is required for successful sexual reproduction because of the restricted radius of spermatozoid motility (Bisang, Ehrlén & Hedenäs, 2004). This requirement can be met by the co-occurrence of male and female branches on the same individual plant (monoecy). Nevertheless, more than one-half of mosses (Bryophyta) and approximately two-thirds of liverworts (Marchantiophyta) worldwide are dioicous, i.e. producing gametophytes bearing exclusively eggs or sperms (Wyatt, 1985; cf. dioicy with dioecy, the latter referring to the presence of sporophytes producing exclusively microspores or megaspores in seed plants; however, dioicy and dioecy are functionally comparable). Among vascular plants, a dioecious breeding system is less common, only about 6% of vascular plants being dioecious (Renner & Ricklefs, 1995).

In both dioecious plants and dioicous bryophytes, sex is determined by a chromosomal mechanism (Bachtrog et al., 2011; Janoušek, Hobza & Vyskot, 2013). Therefore, a 1 : 1 sex ratio can be expected in the offspring at meiosis. Nevertheless, the observed sex ratios in both vascular plants and bryophytes are often skewed (Obeso, 2002; Bisang & Hedenäs, 2005). Male-biased sex ratios appear to be most common among vascular plants (Lovett Doust & Laporte, 1991; Dupont & Kato, 1999; Obeso, 2002), whereas female-biased sex ratios are prevalent in bryophytes (Bisang & Hedenäs, 2005).

The most expensive sex is the rarer one. In vascular plants, females have greater reproductive allocation than males, and thus these payments often result in a higher cost of female reproduction and a male-dominated sex ratio (Silvertown & Lovett Doust, 1993; Obeso, 2002). Similarly, a substantial cost for female reproduction (Laaka-Lindberg, 2001) and sporophyte production has been demonstrated in bryophytes (Ehrlén, Bisang & Hedenäs, 2000; Bisang & Ehrlén, 2002; Rydgren & Økland, 2003; Rydgren, Halvorsen & Cronberg, 2010). Using the allocation theory as for vascular plants, there appears to be conflicting evidence between the observed female-dominated sex ratio of dioicous bryophytes and the demonstrated higher cost of reproduction in females relative to males.

The cost of male reproduction has rarely been addressed in bryophytes, but, in the few existing studies, no such cost has been detected (Bisang, Ehrlén & Hedenäs, 2006; Horsley, Stark & McLetchie, 2011). Many other explanations have been suggested to explain the female-biased sex ratio in bryophytes, including differential sex-specific spore abortion and germination rates (Newton, 1972; McLetchie, 1992), spatial segregation of sexes (Stark, McLetchie & Mishler, 2005), random processes during recruitment and colonization (Rydgren & Økland, 2002), evolution of sex-specific life history traits (Fuselier & McLetchie, 2004), habitat specialization of sexes (Stark, McLetchie & Eppley, 2010) and phylogenetic history (Bisang & Hedenäs, 2005). However, no one single explanation has been widely accepted.

Investigations of sex ratios in dioicous bryophytes are furthermore complicated by non-expressing gametophytes, which bear neither male nor female gametangia. Studies dealing with bryophyte sex ratios (see above) mostly reflect only sex-expressing shoots (i.e. male and/or female gametangia are visible on the shoots). Nevertheless, sex-specific molecular markers, which may provide the true (genetic) sex ratio, have been discovered in a few species of liverworts [*Marchantia polymorpha* L. (Okada et al., 2000; Yamato et al., 2007) and *Sphaerocarpos texanus* Aust. (McLetchie & Collins, 2001)] and mosses [*Ceratodon purpureus* (Hedw.) Brid. (McDaniel, Willis & Shaw, 2007), *Drepanocladus lycopodioides* (F.Weber & D.Mohr) Broth. ex Paris (Bisang & Hedenäs, 2013) and *Pseudocalliergon trifarium* (F.Weber & D.Mohr) Loeske (Korpelainen et al., 2008; Bisang, Korpelainen & Hedenäs, 2010; Hedenäs et al., 2010)].

Water mosses, stream bryophytes and aquatic bryophytes are difficult to define in a comprehensive way. All three terms assume that the aquatic medium is either the only or the most favoured site. More than 130 species belonging to 31 families and 62 genera have been reported as water mosses (Akiyama, 1995). Water bryophytes are commonly used as biomonitors for heavy metal pollution (e.g. Martins, Pardo & Boaventura, 2004), but little is known about their physiology (Glime & Vitt, 1984; de Carvalho, Branquinho & da Silva, 2011) or life cycle, including reproduction, spore germination, protonema formation, gametophyte and sporophyte induction and growth and spore dispersal (Glime, 1984; Glime & Vitt, 1984; Akiyama, 1995). In contrast with their terrestrial counterparts, aquatic bryophytes do not encounter constraints on fertilization connected with lack of water. However, the aquatic habitat is characterized by flowing water, which

causes difficulties with fertilization, dispersal and subsequent establishment of propagules (i.e. an excessive dilution of spermatozoids and propagules).

In this article, we deal with the reproductive system of *Scapania undulata* (L.) Dumort., an aquatic, dioicous liverwort, frequently forming large patches on stones and on the bottom of shallow streams. As a dominant species, it provides a nest and shelter for many small aquatic insects and other animals, therefore playing an important role in stream communities (e.g. Steinman & Boston, 1993). It is a central element in the south-boreal forest ecosystem of southern Finland, where this study was conducted. Through an investigation of vegetative growth and asexual and sexual reproduction in naturally occurring *S. undulata* plants at three levels of population structure (i.e. among streams, among plots within streams and within plots), we answer the following questions (see Table 1). (1) Sex ratio – What is the population structure of shoot types and expressed sex ratio in *S. undulata*? How do the incidence of shoot types and expressed sex ratio vary across the populations? (2) Differences between sexes – Are there sex-specific differences in vegetative growth traits and shoot architecture? If so, can these be explained by differential costs of sexual reproduction in males and females? (3) Reproduction trade-off – Does a trade-off occur between sexual reproduction and asexual production of gemmae? Is this trade-off sex specific?

Materials and Methods

Study species and study sites

Scapania undulata (Scapaniaceae) is a leafy aquatic liverwort which can grow up to 10 cm in length. It is a frequently fertile dioicous species with sporophytes occurring from February to July (Paton, 1999). In addition to sexual reproduction, *S. undulata* reproduces asexually by gemmae produced on the uppermost leaf edges (Fig. 1). Most frequently, it inhabits acidic to moderately base-rich rocks, boulders and stones in streams and rivers (Paton, 1999). The species grows in the boreal to subtropical zones, where it occurs rather frequently. The studied populations are located in southern Finland (Table 2) in the south-boreal vegetation zone.

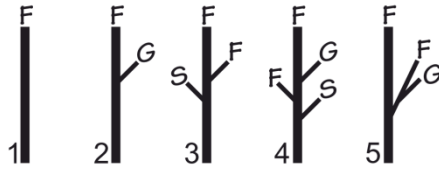
Table 1. List of the questions with details of statistical analyses.

Subject	Question	Dataset	Analysis
1. Sex ratio	<p>What is the population structure of shoot types and expressed sex ratio in <i>S. undulata</i>?</p> <p>How do the incidence of shoot types and expressed sex ratio vary across the populations?</p>	1	<p>Computation of ratios within brooks (Figure 3) and plots (Appendix S3, S4).</p> <p>Hierarchical decomposition of variance in shoot type and sex incidence (Table 3; Figure 4). Extraction of variance components from binomial generalized linear mixed models.*</p>
2. Differences between sexes	<p>Are there sex-specific differences in vegetative growth traits and shoot architecture? If so, can these be explained by differential costs of sexual reproduction in males and females?</p>	1	<p>Poisson generalized mixed model* testing difference in incidence of branched vs. unbranched shoots in relation to sex in individual plots. The model also included observation specific random contribution to deal with overdispersion.</p> <p>Linear mixed effect models* with log-transformed response variables testing differences in character values (shoot length, length of main stem, and number of branches) across the shoot types. Heteroscedasticity among individual levels of the fixed-effect predictor (shoot type) was tested and accounted for if necessary (Figure 5).</p>
3. Reproduction trade-off	<p>Does a trade-off occur between sexual reproduction and asexual production of gemmae?</p> <p>Is this trade-off sex specific?</p>	2	<p>Linear mixed-effect models* testing the effects of branch length and shoot type on the number of gemmae (log-transformed) produced on a gemmiparous branch.</p> <p>Binomial generalised linear mixed model* testing differences in proportions of gemmiparous branches among shoots of different sexes.</p>
	<p>Is this trade-off sex specific?</p>	male-only and female-only plots of 1	<p>Comparison of population structure of male-only vs. female-only plots by Poisson generalized mixed model* which included also observation specific random contribution to deal with overdispersion (Table 4).</p>

*All mixed models included stream and plot as hierarchically nested random effect terms unless stated otherwise.

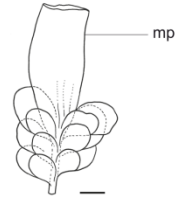
SEX-EXPRESSING SHOOTS

Female sex-expressing shoots

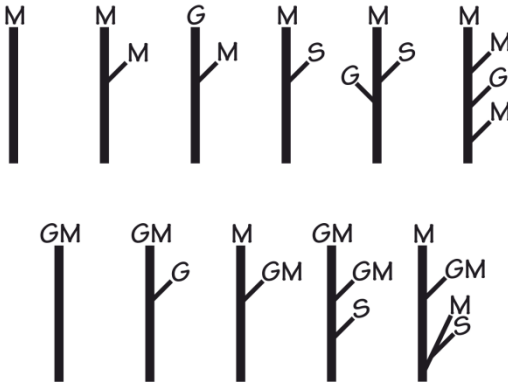


Other combinations of F, G and S branches are possible.

F - female branch



Male sex-expressing shoots

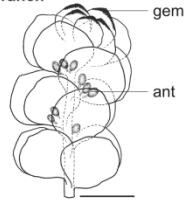


Other combinations of M, GM, G and S branches are possible.

M - male branch

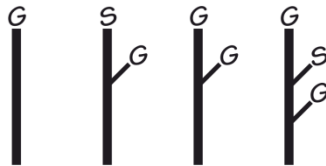


GM - gemmiparous male branch



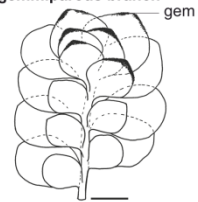
NON-EXPRESSING SHOOTS

Gemmiparous shoots

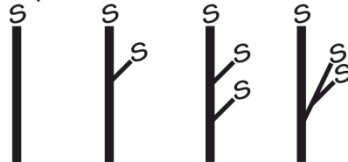


Other combinations of G and S branches are possible.

G - gemmiparous branch

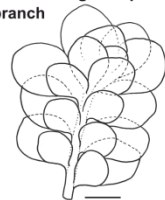


Sterile non-gemmiparous shoots



Other combinations of S branches are possible.

S - sterile non-gemmiparous branch



← **Figure 1.** Schematic diagram of *Scapania undulata* architecture. Shoots consisting of main stem (bold line) and branch/branches (thin line) are drawn. Simple unbranched (1) and branched (2, 3, 4, 5) shoots are present. Female sex-expressing shoots (F) bear at least one branch with archegonia and perichaetial leaves or perianth. Male sex-expressing shoots (M) bear at least one antheridial branch. Note that F and M shoots can and often do consist of gemmiparous and sterile non-gemmiparous branches in addition to those bearing the sex organs. Shoots without sex organs (gemmiparous and sterile non-gemmiparous shoots) are grouped together as non-expressing shoots. Gemmiparous shoots (G) bear at least one branch with gemmae. Sterile non-gemmiparous shoots (S) bear vegetative branches without any reproductive organs. Main stems and individual branches are also classified in the same way as shoots (F, M, G, S and, in addition, GM because antheridia and gemmae can be present simultaneously on one branch). ant, antheridium; gem, gemmae; mp, mature perianth. Scale bar, 1 mm.

Sampling

Ten streams in southern Finland were selected for sampling (Table 2). All are relatively intact, undisturbed habitats with as little direct effect of forest management in their immediate surroundings as possible. Although disturbance in the studied watershed areas could not be avoided completely, the stream profile, length and water quality were selected to be as similar as possible. Ten plots (c. $10 \times 10 \text{ cm}^2$) were selected in each stream (for the sampling pattern, see Fig. 2). Plots were picked randomly depending on the patchy distribution of a suitable substratum for *S. undulata* in the streams. However, a distance of $> 20 \text{ m}$ was used between plots. Plants were collected in a 1-month period (June 2008, see Table 2) to obtain comparable data. They were deep-frozen and later dissected. Each plot of shoots was evenly divided into two parts (sets of shoots). The first set (1) was used for detection of the sex ratio, and all types of shoots were sampled, including sterile non-gemmiparous and gemmiparous shoots. The second set (2) was used for the assessment of gemma production, and only gemmiparous shoots were included, regardless of sex expression.

In the first set, 18 individual shoots from each plot ($N = 1800$ in total) were sampled, following a constant sampling design (four shoots in the middle of each of the four margins of the plot, and two in the centre of the plot, see Fig. 2). The length of the main stem, branches and entire shoot, shoot and branch type, number of branches and branching pattern of each shoot (Fig. 1) were assessed to describe the shoot architecture.

The lengths of the main stems and branches were measured from the base of the apical bud to the base of the stems/branches (see fig. 1 in Laaka-Lindberg, 2001). The length of the entire shoot was calculated as the sum of the length

of the main stem and its branches. Weighing shoots was not possible because of adhering sand, gravel and other particles among the leaves. These particles might cause inaccurate measurement and their complete removal by washing is almost impossible. Moreover, shoots are evenly covered with leaves, regardless of shoot sex (F, M, G, S; see below). Therefore, we conclude that the length measurement of shoots/branches is suitable. The branching pattern (branched vs. simple unbranched shoots) and number of branches were determined simultaneously with the length measurement of the shoots. Shoots were classified into the following types: sex-expressing shoots [female (F) or male (M)] and non-expressing shoots [gemmaiparous (G) or sterile non-gemmaiparous (S)]. A detailed explanation is given in Fig. 1. In addition to shoot reproductive status, the main stems and individual branches were also classified as one of the following branch types: F, M, G, S, and male gemmaiparous branches (GM – gemmaiparous branch with antheridia in leaf axils). A few branches were damaged (by water action) which made classification into branch type (F, M, GM, G, S) impossible. Such branches were evaluated as zero branches. Sporophyte presence was observed on female shoots in all streams. As in other liverworts, the sporophytes are ephemeral in *S. undulata* (Paton, 1999). Therefore, we could not conduct any detailed analysis of their occurrence because of the temporal constraints of sampling (one stream per day was sampled) and the consequent impossibility to obtain comparable sporophyte data sampled at the same time in all ten streams.

The second set of shoots (Fig. 2) was used to estimate gemma production. Ten shoots that were gemmaiparous (i.e. at least one branch or main stem bearing gemmae) were collected in each plot ($N = 1000$ shoots). The gemmae of one gemmaiparous branch or the main stem of each shoot was rinsed off in 0.5 mL of distilled water in a micro-tube. The number of gemmae was counted using a counting grid (Bürker 0.100 mm) under a light microscope at $400 \times$ magnification. A drop of detergent (0.5% Triton X-100, Gerbu) was added to the sample prior to microscopy to reduce the surface tension. The gemma counting for each sample was repeated three times (see Holá, Kučera & Těšitel, 2011). In addition, the length of the main stems, branches and entire shoots, shoot and branch type, number of branches and the branching pattern of shoots were assessed to describe the shoot architecture of individuals of the gemmaiparous *S. undulata* shoots. Other types of asexual propagules, such as unusual propagules growing on ordinary vegetative leaves (see Glime, 1970), were looked for, but were not found.

Table 2. Study sites, their location, date of sampling and basic characteristics of streams.

Brook ID	Brook name	Coordinates [WGS 84]		Date of sampling	Length of brook channel [m]	Description of brook channel	Surrounding countryside
		lat	lon				
1	Kaitlampi	60°19'59"	24°29'06"	23.6.2008	280	sandy brook	forest mainly spruce forest
2	Luukin Myllyjärvi	60°19'36"	24°42'29"	18.6.2008	250	rocky brook	brook on the base of a rock outcrop
3	Ruuttana	61°13'36"	24°57'40"	25.6.2008	590	gravel, mainly humus-bottom	variable forest/swamp
4	Vähä Ruokojärvi	61°14'33"	25°02'06"	27.6.2008	350	rocky brook	forest brook with mires at both ends
5	Myllyoja	60°16'48"	24°39'30"	4.6.2008	250	rocky brook	rather rapidly streaming forest brook in rich herb forest
6	Valklampi	60°18'43"	24°28'07"	3.6.2008	270	rocky brook	forest, ending in a clear-cut area
7	Sudenpesänkangas	61°12'22"	25°11'28"	9.6.2008	510	rocky brook	forest brook, changing to partly ditched channel
8	Viitajärvi	61°12'24"	25°01'49"	11.6.2008	300	stony, swampy brook	variable forest/swamp
9	Haukkalampi	60°18'20"	24°30'55"	2.6.2008	325	rocky brook	variable forest
10	Vatijärvi	61°13'57"	25°05'29"	10.6.2008	330	stony, swampy brook	variable forest

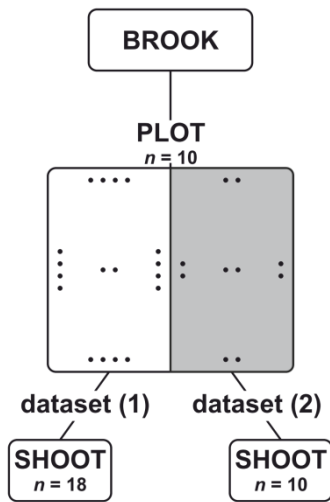


Figure 2. Schematic diagram of sampling pattern in one stream. Samples were collected from ten plots per stream and 18 (dataset 1) + ten (dataset 2) shoots were collected per plot. Black dots indicate the approximate position of sampling shoots per plot.

Data analysis

Data were analysed by (generalized) linear mixed models accounting for the sampling design and distribution of response variables. If necessary, heteroscedasticity between levels of fixed-effect factor predictors and over-dispersion were treated by adjusting the model variance structure and by the inclusion of the observation-specific random contribution, respectively. Details of individual analyses are summarized in Table 1. Model-fitted values and confidence intervals were back-transformed for the purpose of plotting the results of all of the generalized models and models involving data transformation to present the values on the original scales. All statistical analyses were conducted in R version 3.0 (R Core Team, 2013). Linear mixed models were computed with the package nlme version 3.1-109 (Pinheiro et al., 2013). Generalized linear mixed models (GLMMs) and variance components were computed with the package lme4 version 3.1-65 (Bates, Maechler & Bolker, 2011). Differences among individual levels of the predictors were tested by post-hoc Tukey’s honestly significant difference tests. Post-hoc tests were computed in the package multcomp version 1.2-19 (Hothorn, Bretz & Westfall, 2008). (R script and R workspace containing the data are given in Supporting Information, Appendices S1 and S2, respectively.)

Results

Population structure

Sterile non-gemmiparous, gemmiparous and male shoots were found in all ten streams, whereas female shoots occurred in only eight streams. The gemmiparous shoots had a substantial representation in all streams, whereas male, sterile non-gemmiparous and female shoots were found in rather smaller proportions (Fig. 3; data for individual streams are given in Supporting Information, Appendix S3). The sex ratio of sex-expressing shoots in streams varied from a complete lack of female shoots (two of ten streams), fewer female than male shoots (five streams), 1 : 1 female and male shoots (one stream) and more female than male shoots (two streams; data for each individual stream are given in Supporting Information, Appendix S4). The average sex ratio across all ten streams was 1 : 3 (female : male).

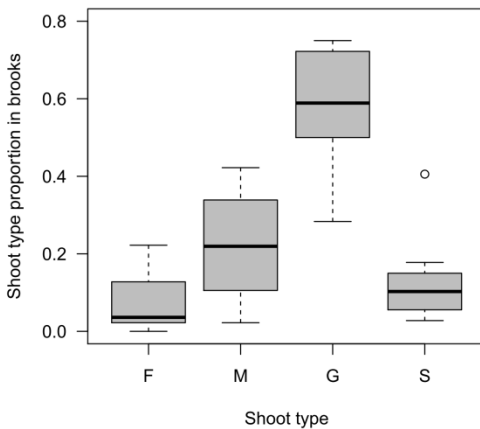


Figure 3. The proportion of female (F), male (M), gemmiparous (G) and sterile non-gemmiparous (S) shoots across the ten streams.

Hierarchical decomposition of variance (among streams, plots within streams and within plots) yielded contrasting patterns for comparisons between sex-expressing vs. non-expressing, female vs. male and gemmiparous vs. sterile non-gemmiparous (Table 3; Fig. 4). The proportion of variance in sex-expressing vs. non-expressing shoots was the lowest among the streams. The two hierarchically lower levels of variance shared approximately equal proportions of variance, accounting for almost 50% in each. In contrast, variance in female vs. male shoot proportion (i.e. sex ratio of sex-expressing shoots) was high among streams, but low within plots. This implies that many of the plots were occupied by single sex

individuals. Indeed, across the whole dataset, 16 plots consisted exclusively of females and 51 plots consisted exclusively of males, whereas only 24 plots consisted of both sexes. The proportions of variance in gemmiparous vs. sterile non-gemmiparous shoots were comparable at all three hierarchical levels.

Table 3. Likelihood-ratio test results for the variance components of shoot type and sex incidence (see Fig. 4).

Shoot type proportion	Variance among stream			Variance among stream		
	Likelihood-ratio	<i>p</i>	Df	Likelihood-ratio	<i>p</i>	Df
sex-expressing vs. non-expressing (SexE vs. NonE)	34.21	<0.0001	1	110.27	<0.0001	1
female vs. male shoots (F vs. M)	207.01	<0.0001	1	43.90	<0.0001	1
gemmiparous vs. sterile non-gemmiparous (G vs.S)	136.67	<0.0001	1	76.86	<0.0001	1

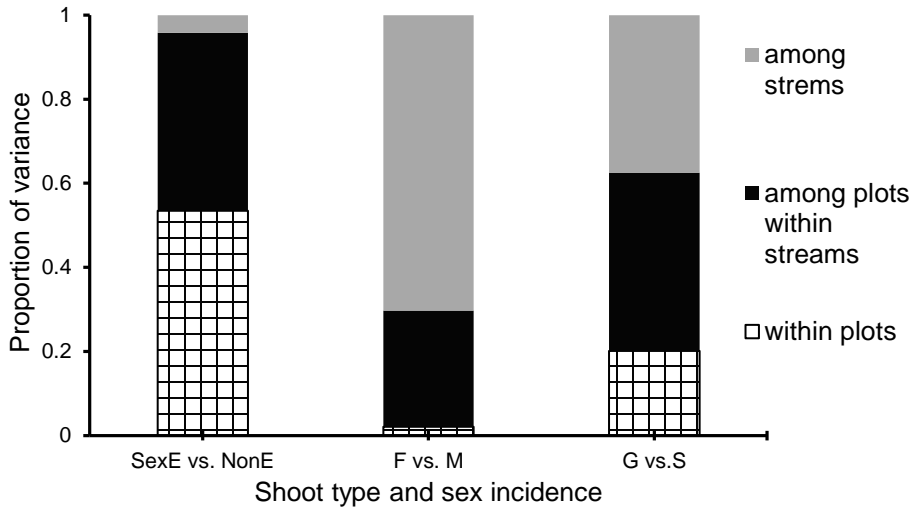


Figure 4. Hierarchical decomposition of variance in shoot type and sex incidence corresponding to the sampling levels (among streams, among plots within streams and within plots). Variance component estimates are based on a random effect extraction from a linear mixed-effect model using the restricted maximum likelihood estimation (REML). F, female; G, gemmiparous; M, male; NonE, non-expressing; S, sterile non-gemmiparous; SexE, sex-expressing. See Table 3 for significance tests of the variance components.

The proportion of unbranched shoots was significantly different among female, gemmiparous, male and sterile non-gemmiparous shoots (Poisson GLMM: likelihood ratio = 13.90; df = 3; $P = 0.003$) accounting for 10.8%, 21.3%, 19.2% and 32.3%, respectively. Female organs (archegonia and perichaetial leaves

or perianth branch) were most frequently produced on the main stems. Only 21% of female branched shoots produced female organs on a branch, and only 10% of branched shoots produced two or more branches with female organs. In contrast, antheridia (male organs) were produced approximately as frequently on the main stems (61.9% of male branched shoots) as on the branches (63.2% of male branched shoots). In addition, 36.8% of branched shoots produced two or more branches with antheridia.

Sterile non-gemmiparous shoots exhibited the shortest total shoot length and fewer branches than any other shoot type (Fig. 5). Female shoots displayed shorter main stems than the gemmiparous and male shoots (although the result between male and female main stems was statistically non-significant). They were also the most branched, resulting in their total shoot length reaching equal or slightly (statistically non-significant) higher values than the male and gemmiparous shoots (Fig. 5).

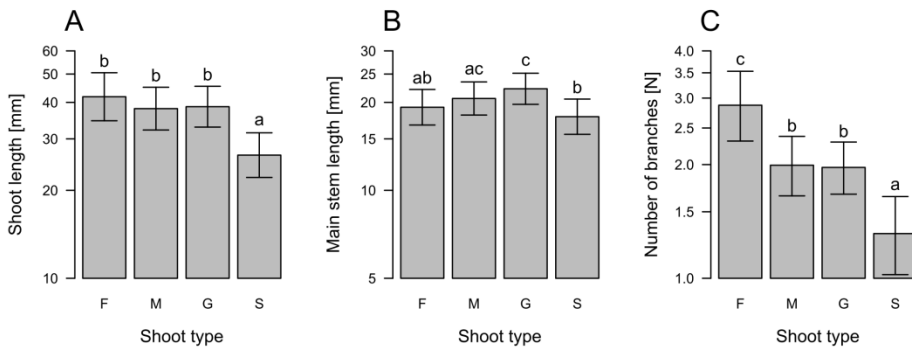


Figure 5. Shoot length (mixed-effect model: $F_{3,1697} = 25.76$; $P < 0.0001$) (A), length of the main stem (heteroscedastic mixed-effect model: $F_{3,1697} = 13.08$; $P < 0.0001$) (B) and number of branches (mixed-effect model: $F_{3,1697} = 14.35$; $P < 0.0001$) (C) in the four shoot types: F, female; $N = 130$; M, male; $N = 390$; G, gemmiparous; $N = 1047$; S, sterile non-gemmiparous; $N = 233$. Note the logarithmic scale of the y axes. Model fitted values and 95% confidence intervals are displayed. Different letters symbolize statistically significant ($P < 0.05$) differences inferred from post-hoc pairwise comparisons using Tukey's honestly significant difference tests.

Gemma production

Gemmae were only observed on the uppermost leaf edges (Fig. 1). The mean number of gemmae present on individual gemmiparous branches was 7560. There was a significant positive relationship between the number of gemmae produced and the length of the branch (linear mixed-effect model: $F_{1,895} = 8.330$; $P = 0.004$).

In addition, there was a marginally significant effect of shoot branch type ($F_{2, 895} = 3803$; $P = 0.023$), but no contrast for this factor was significant ($P > 0.1$ in all cases), making this result difficult to interpret. However, gemmiparous branches on female shoots produced slightly more gemmae.

The presence of both gemmae and archegonia with perichaetial leaves or perianth on the same branch was not recorded. In contrast, the presence of gemmae and antheridia on the same branch (the GM branch type) was seen frequently. Branches and main stems bearing both antheridia and gemmae (the GM branch type) were three times more frequent than branches and main stems with only antheridia (the M branch type). The mean number of gemmae produced by GM vs. G-only branches of male shoots was not significantly different (see paragraph above). In addition, differences in proportions of gemmiparous branches on shoots of different sex (binomial generalized mixed-effect model: likelihood ratio = 60.759; $P < 0.0001$) revealed considerably higher proportions of gemmiparous branches for gemmiparous and male shoots than female shoots.

Discussion

Population structure

The numbers of sex-expressed female and male shoots of *Scapania undulata* did not correspond to the 1 : 1 ratio or to the female-biased sex ratio, the latter of which is common in bryophytes (Bisang & Hedenäs, 2005). Instead, the expressed sex ratio in *S. undulata* populations was found to be male biased in most of the streams. However, the number of non-expressing shoots of *S. undulata* was high in all streams and thus might have biased the sex ratio estimation. In studies of *Pseudocalliergon trifarium* and *Drepanocladus 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, we expect that the true (genetic) sex ratio could be similar to the expressed sex ratio of *S. undulata*.

In bryophytes, expressed male-biased sex ratio has not often been observed. It has only been reported in a few mosses, such as *Mielichhoferia mielichhoferiana* (Funck) Loeske (Shaw, Niguidula & Wilson, 1992), *Hypnum cupressiforme* Hedw. growing on tree bark (Bisang & Hedenäs, 2005), *Crossomitrium patrisiae* (Brid.) Müll.Hal. (Alvarenga, Pôrto & Zartman, 2013) and the liverwort *Lophozia silvicola* H.Buch (Laaka-Lindberg, 2005). In contrast, male-biased sex ratios are most commonly seen among vascular plants (Lovett Doust & Laporte, 1991; Dupont &

Kato, 1999; Obeso, 2002) and only a few dioecious vascular plants are female biased (e.g. Korpelainen, 1991; Waycott, Walker & James, 1996). Balanced sex ratios are most frequently observed in algae; females are more frequent than males in only a few algal species (DeWreede & Klinger, 1988). In general, the two sexes of plants (algae, bryophytes, vascular plants) perform different functions in reproduction and may also have different resource demands. The selection pressures presented by these different resource demands could lead to the development of biased sex ratios and/or sexual dimorphism (Delph, 1990). The optimal life history strategy is that which allocates resources between growth, survival and reproduction in a manner which maximizes the total reproductive output. *Scapania undulata* is frequently fertile (see Paton, 1999). It has been demonstrated that the sex ratio may be skewed towards males at higher sporophyte frequencies (Rydgren et al., 2010). Furthermore, the regular sporophyte occurrence in this species may be a consequence of the high number of male-expressing plants, causing copious spermatozoid production, which ensures fertilization despite the effects of dilution and water flow (Glime, 1984).

The number of non-expressing shoots [gemmaiparous (58%) and sterile non-gemmaiparous (13%)] was high in all streams. The large proportion of sterile individuals within populations is commonly associated with the aquatic life form of vascular plants and as a consequence of the difficulties caused by water pollination (Lovett Doust & Laporte, 1991). Nevertheless, the high proportion of non-expressing shoots (e.g. McLetchie & Puterbaugh, 2000; Pohjamo & Laaka-Lindberg, 2003) is common across various phylogenetic and ecological groups of bryophytes.

The hierarchical decomposition of variance in sex-expressed male and female shoots is high among streams and low within plots, corresponding to the fact that nearly 70% of the plots were occupied by only one sex. Given the genetic sex determination mechanism, the sex ratio variation reflects the structure of genetic variation within the populations. The observed pattern thus corresponds closely to what one would expect to see in populations in which the local spread relies on frequent asexual reproduction by gemmae or clonal growth (Barrett, Eckert & Husband, 1993; Korpelainen, Pohjamo & Laaka-Lindberg, 2005). Sporophyte production, however, occurs less frequently (cf. the number of sex-expressed and gemmaiparous shoots) and plays its role at a greater spatial scale. In contrast with the sex ratio variation, all ten streams are comparable with respect to the proportion of sex-expressing vs. non-expressing shoots. The vast majority of variation in this proportion is on the level between and within individual plots, suggesting that

environmental factors and/or the ontogenetic stage of individuals are the underlying factors. Regulation of gametangia formation by environmental factors, e.g. photoperiod and pH, has been demonstrated in bryophytes (Chopra & Bhatla, 1983; Maciel-Silva, Valio & Rydin, 2012). In addition, the role of growth hormones is considered to be important (Schumaker & Dietrich, 1998).

Sex-specific differences in vegetative growth traits

The difference in shoot size between sterile and reproducing shoots clearly suggests a threshold size for the first sexual or gemma reproduction in *S. undulata*. Sterile shoots of *S. undulata* were significantly shorter and produced fewer branches than reproducing shoots, and therefore may be interpreted as juvenile shoots (see also Pohjamo & Laaka-Lindberg, 2004). However, the initiation of the reproductive phase of the life cycle may (e.g. Pohjamo & Laaka-Lindberg, 2004; Alvarenga et al., 2013) or may not (e.g. Ehrlén et al., 2000; Hassel, Pedersen & Söderström, 2005) be dependent on shoot size in bryophytes. In addition, shoots of < 30 mm in length produced neither gemmae nor gametangia. The gemma production cost, which may be considered to be rather low (Laaka-Lindberg, 2001; Pohjamo & Laaka-Lindberg, 2004), apparently also requires a certain minimal shoot size.

Sexually dimorphic traits may be considered as an adaptive response to the selection of different optima between males and females, or may reflect trade-offs originating from the differential cost of reproduction (Obeso, 2004; Wheelwright & Logan, 2004). A lack of or minimal differences between male and female size (Stark, McLetchie & Mishler, 2001; Bisang et al., 2006), larger males (Rydgren & Økland, 2002), larger females (McLetchie & Puterbaugh, 2000; Laaka-Lindberg, 2001; Pohjamo & Laaka-Lindberg, 2004; Horsley et al., 2011) and even extreme size differences (e.g. dwarf males; Hedenäs & Bisang, 2011) have been found in bryophytes. However, most studies have found that females are generally larger than males, which is similar to herbaceous perennials (Obeso, 2002). Nevertheless, the tendency of *S. undulata* females to produce shorter main stems and more branches and, in addition, more branched shoots than males can be interpreted as a trait providing additional opportunities for reproduction by asexual gemmae and the formation of new archegonia in the following season. This is only apparent in the context of the rare production of two or more female sexual branches on a single female shoot. Although multiple sexual branches might increase fertilization success, these would probably induce a higher cost of reproduction if fertilization is realized (McLetchie, 1992; Bisang et al., 2006). This suggests that sexual reproduction may indeed be more costly for females than

for males. Further support for a higher cost of female reproduction comes from an analysis of the population structure of putative male-only and female-only plots (i.e. plots where the presence of only one sex was revealed based on sex-expressing individuals; Table 4). Female-only plots tend to consist of a high number of gemmiparous shoots and a low number of female sex-expressing shoots. In contrast, the ratio of male to gemmiparous shoots on male-only plots was much more balanced.

Table 4. Mean model-predicted population structure of plots assumed to comprise one sex (male or female) shoots only based on the observed occurrence of sex-expressing shoots. The population structure is significantly different between plots comprising exclusively male and female sex-expressing shoots. Poisson generalized linear mixed model (GLMM) with stream and plot nested within stream as random factors; the model also includes an observation-specific random contribution to deal with over-dispersion: likelihood-ratio = 14.37; df = 2; $P = 0.001$. Test is based on comparison between the null model containing assumed population sex + shoot type and the full model which also contains the interaction between these two fixed-effect terms

	Gemmiparous	Sterile non-gemmiparous	Sexual (M/F)
Male-only ($N = 51$)	0.556	0.133	0.311
Female-only ($N = 16$)	0.713	0.051	0.236

Asexual reproduction

Asexual reproduction by gemmae (produced by a mitotic cell division) was omnipresent, except in sterile shoots. The gemmae probably represent the dominant mechanism of local spread of *S. undulata* and the only means of reproduction in streams in which only one expressed sex was detected. The lack of difference in gemma production between G and GM branches of male shoots and the slightly higher gemma production of female shoots suggest a low energetic cost for male reproduction and an absence of any strong trade-off between the production of sex organs and gemma production in *S. undulata*. Such findings contrast with the strong trade-off between sexual and asexual reproduction among female, male and gemmiparous shoots reported in *Lophozia silvicola* (Laaka-Lindberg, 2001) and a higher gemma cup production in males of *Marchantia inflexa* Nees & Mont. (McLetchie & Puterbaugh, 2000). The high production of asexual propagules and rather low cost of sexual reproduction may be a suitable strategy for the spread and colonization of new patches of the aquatic environment for water bryophytes.

Conclusion

Many bryophytes inhabit aquatic ecosystems in which large masses of water or velocity of the water stream cause difficulties with fertilization, dispersal or establishment (e.g. dilution of spermatozoids and propagules, reduced propagule establishment). However, little information is known about their life cycles. This study of *Scapania undulata* may provide a view of the life cycle of aquatic liverworts. The unexpected and high male-biased sex ratio (i.e. the overproduction of spermatozoids) might be a strategy in water liverworts to overcome sperm dilution and ensure fertilization. Sufficient production of sexual and asexual propagules enables subsequent establishment over longer distances in water.

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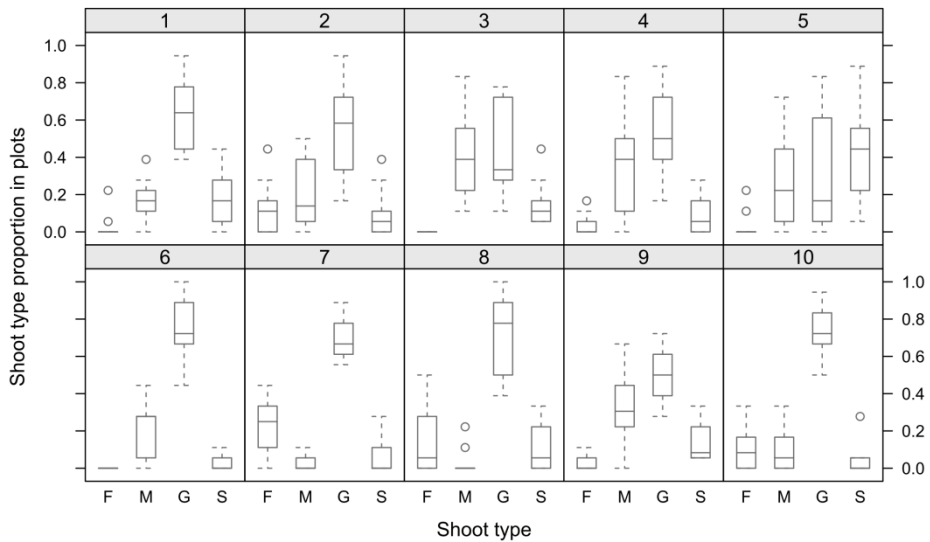
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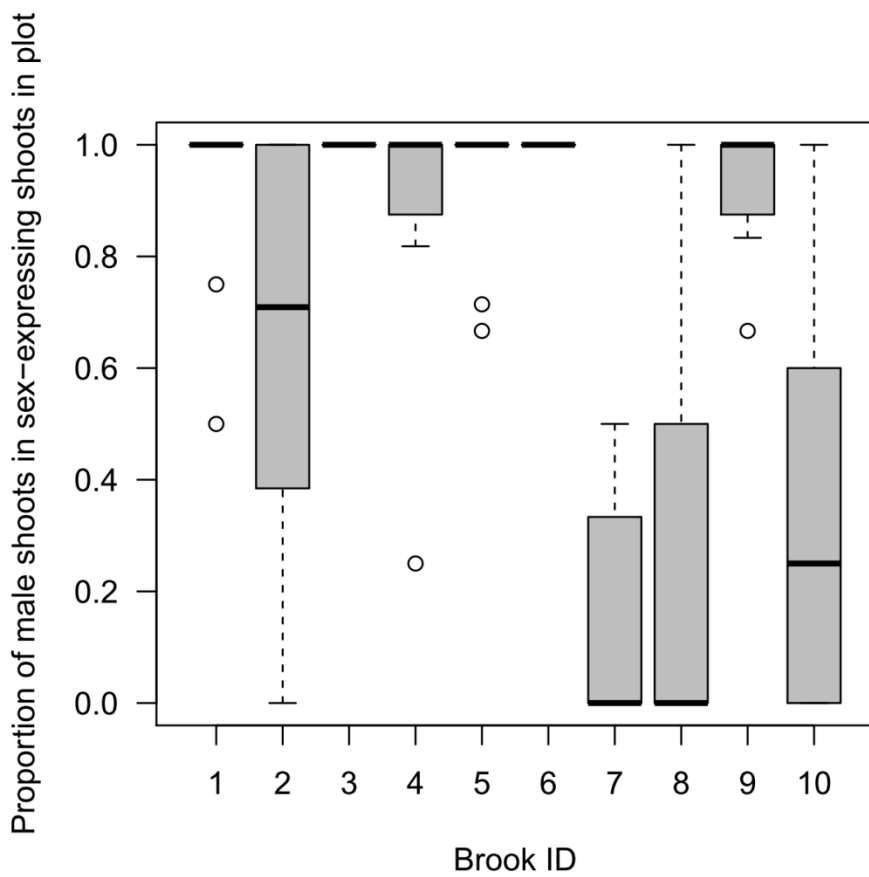
Supporting information

Appendix S1. R code used to compute statistical analyses and plot the figures. It may be found in the online version of this article at the publisher's web-site.

Appendix S2. R workspace containing the primary data. It may be found in the online version of this article at the publisher's web-site.



Appendix S3. A trellis plot showing the proportion of female (F), male (M), gemmiparous (G) and sterile non-gemmiparous (S) shoots for each stream. Numbers of individual charts correspond to Stream ID in Table 2.



Appendix S4. Proportion of male shoots in sex-expressing shoots for each stream. Stream ID corresponds to Table 2.

**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

Eva Holá¹, Jiří Košnar, Jan Kučera

Department of Botany, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

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

¹ Corresponding author: Eva Holá, eva.neurazy@gmail.com

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

Introduction

The structure of genetic diversity on fine scales within populations and on larger scales among populations may bring valuable insights into the reproductive systems of studied organisms including the assessment of reproductive effort, rates of sexual and vegetative reproduction, dispersal capacity of diaspores and levels of gene flow among populations. Bryophytes are generally considered to possess high dispersal capacity of their sexually originating spores [1, 2], which however is often impaired by the relatively low reproductive effort allocated into the production of energetically costly sporophytes. In dioicous bryophytes, which constitute a significant proportion as opposed to the situation in remaining land plants [3, 4], the sexual reproduction is further complicated by the necessity of spatial proximity of male and female gametangia, as the dispersal range for sperm is generally very short [5, 6]. On the other hand, most bryophytes also propagate by means of vegetative fragments, and a notable proportion of bryophytes produce specialized vegetative diaspores, such as the gametophytic gemmae, which were proven to possess a dispersal capacity comparable to spores and even effectively contributing to gene flow among populations [7]. Recruitment of progeny is nevertheless not only dependent on the formation and dispersal capacity of diaspores, but also on the diaspore establishment and sustainable growth conditions for mature plants. A significant proportion of bryophytes are known to be strictly specialized in particular substrates or habitats [8], and one such examples of habitat specialization are the epixylic species, i.e. species growing on decomposing wood matter. Decomposing wood supports a rich community of plants, fungi and animals [9]. Decaying logs are a very dynamic substratum with a non-random patchy distribution, restricted duration and time-variable quality [8, 10] where composition of bryophyte communities changes following the decay stage of logs [11]. Moreover, a sufficient amount of decomposing wood is missing from most human-managed forests and is only present in natural and old-growth forests. These unfortunately belong to prime examples of habitats under globally strong anthropogenic pressure [12]. Epixylic species are thus handicapped on two scales. The suitable substrate is not continuously available, as exemplified in a study of the epixylic liverwort *Ptilidium pulcherrimum*, which showed that less than 1% of produced spores were deposited on substrate suitable for establishment [13]. On the landscape scale, extensive forestry has resulted in considerable decrease and fragmentation of forest habitats, in which the specific substrate occurs. To date, no strictly epixylic bryophyte has been studied, although the genetic diversity and structure of epiphytic forest bryophytes has been addressed in several studies

[14–16]. Genetic variation in wood living fungi and beetles, to our knowledge the only studied epixylic organisms, showed low gene flow and low genetic variation among isolated and fragmented populations similarly as it was the case in other forest dwelling species [16–18].

Recent studies of population genetic variability and spatial genetic structure using DNA fingerprinting methods have shown a remarkable variability of results, showing the uniqueness of parameters of individual reproduction systems in different taxa. One of the most interesting findings is that the level of genetic differentiation among bryophytes reproducing mostly or exclusively vegetatively was in several cases surprisingly high [19–21]. The genetic variability in mostly non-sexual populations can be maintained by migration from neighboring populations, occasional sporophyte production, or by the accumulation of somatic mutations [19, 20, 22]. Studies of spatial genetic structure (SGS) in bryophyte populations are also relatively rare [14, 23–26]. Only one study [23] focused on small-scale pattern of SGS in the liverwort species *Barbilophozia attenuata* Mart. (Loeske), which is a species closely related to our object of study, possessing a similar reproduction mode.

We have studied *Crossocalyx hellerianus* (Nees ex Lindenb.) Meyl., a minute, circumboreally distributed dioicous epixylic liverwort (Fig. 1) of the family Scapaniaceae *s.l.* (Anastrophyllaceae [27, 28]). Both sexually formed spores and asexual gemmae are produced, with both being approximately 10–12 µm in diameter. Sexual reproduction is described as occasional in Nordic countries (sporophyte formation was observed in 2.5–12% of the colonies [29]), whereas in other parts of European distribution area it might be much rarer, e.g. they were never reported from Ireland and Britain [30, 31]. On the contrary, gemmae are always present and generally abundant. It is considered to be a colonist species with the potential life span of only a few years [32], inhabiting decaying logs (mostly of spruce) of intermediate decay stages [33]. With respect to its habitat preference, it usually occurs in old-growth spruce forests with high amounts of coarse woody debris [33] and therefore it is relatively rare in all parts of its distribution area. In the countries of this study, it has been classified as Near Threatened (NT) in Finland [34], and Endangered (EN) in the Czech Republic [35] according to IUCN criteria. In the latter country, only 8 populations are recently known, with only one population classified as large (see below for definitions).

The study populations, located in Scandinavia and Central Europe, differ in size, density, prevailing reproductive mode, and population connectivity. Thus, these populations represent a suitable study system for investigations on patterns

of genetic variation with regard to the above mentioned population characteristics. The studied liverwort moreover produces sexual and vegetative diaspores of potentially very similar dispersal capacities with respect to their size, which facilitates the inference on dispersal efficiency. We hypothesized that the population size or density and prevailing reproductive mode would be mirrored in the population genetic diversity and fine-scale spatial genetic structure. Microsatellite markers, which have been developed for his study, further allowed for the assessment of gene flow levels among populations and rates between sexual and asexual reproduction.

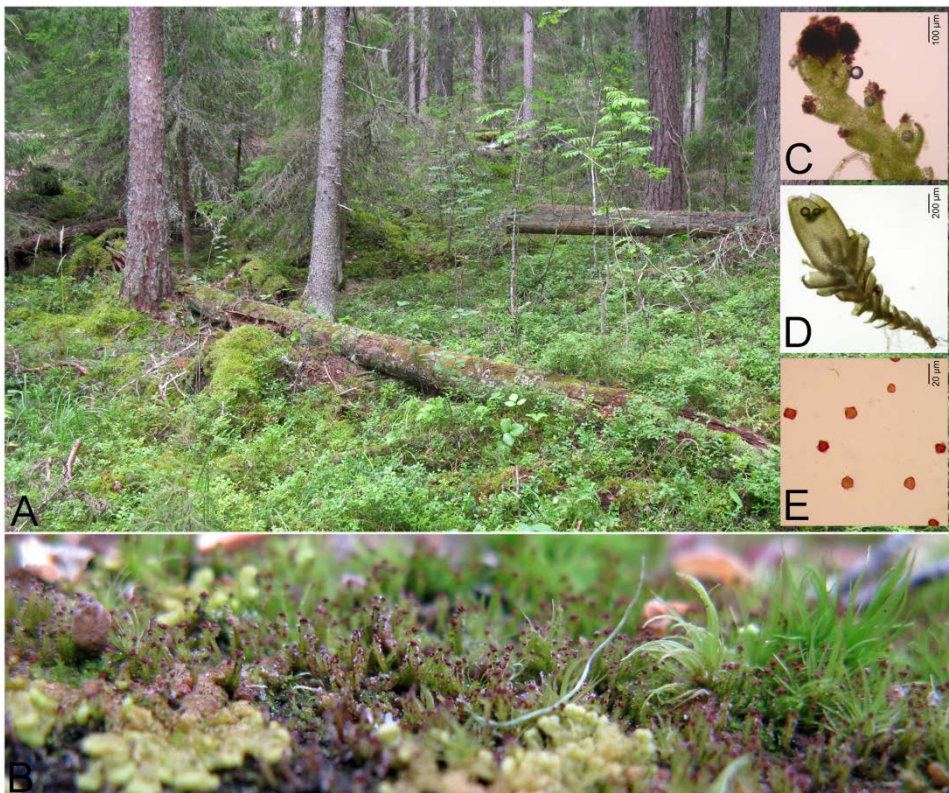


Fig. 1. The studied species *Crossocalyx hellerianus*. Pictures from Vesijako Strict Nature Reserve (A) overgrown log of *C. hellerianus*, (B) *C. hellerianus* in detail. Light microscope pictures (C) gemmiparous shoot, (D) perianth, (E) gemmae.

Material and Methods

Study sites and sampling

Sampling was performed in Finland (FI, 4 populations) and in the Czech Republic (CZ, 6 populations; Table 1 and S1 Fig.). Mean geographic distances among CZ populations amounted to 55 km, those among FI populations 62 km, and the distances among CZ and FI populations averaged 1500 km (S1 Fig.). Studied Finnish populations are located in the boreal zone of southern Finland, representing only a part of regional populations [34]. Czech populations are located in South Bohemia within the temperate zone and represent all known Czech localities as of 2012. The Finnish forests are mainly old virgin forests dominated by spruce with several canopy layers (pines, birches and aspens), characterized by huge amounts of decaying conifer wood, which is reflected in the relatively common occurrence of *Crossocalyx hellerianus*. The Czech forests represent small extant fragments of herb-rich and acidophilous montane mixed old-growth forests with the tree composition and herb vegetation approaching the natural one, dominated mostly by beech with spruce admixtures. The amount of suitable decaying wood is only high in the Boubínský prales National Nature Reserve among the Czech forests. Consequently, *C. hellerianus* is relatively common only in this reserve, while the other Czech localities support only very small populations of the liverwort (Table 1).

In populations, where *C. hellerianus* was abundant (with more than 10 logs supporting the species, further on assigned as ‘large’ populations, Table 1), 8–9 logs were sampled. In smaller populations (‘small’, Table 1), all logs supporting the occurrence of *C. hellerianus* were sampled and surroundings of these logs (up to 0.5 km around) were investigated for possible occurrence.

Approximately 0.5×0.5 cm was sampled from every occurrence of *C. hellerianus* at a minimum distance of 20 cm; the maximum distance depended on the patchy distribution of species on each sampled log (Fig. 2). For detection of genetic structure at the smallest spatial distances, three shoots were taken from four pairs of neighboring patches (one pair on each log) in large populations and three shoots from two pairs of neighboring patches in small populations. One shoot was taken from each of the other patches. Distances among shoots that originated from the same patch were arbitrary equaled to one centimeter and distances among the sampled patches were measured. The small size of the population Nová Bystrice (10×15 cm) allowed for removal of only five shoots.

Table 1. List of study populations with quantitative data.

Locality abbr.	Population	Coordinates [WGS 84]	Country	Population size	Number of sampled logs	Date of sampling (DD.MM.YY)
Z	Boubínský prales National Nature Reserve	48°58'32"N, 13°48'54"E	CZ	LARGE	9	17.11.2012
G	Kamenná hill	48°49'08"N, 13°48'50"E	CZ	SMALL	3	22.11.2012
M	Medvědí hora Nature Monument	48°37'13"N, 14°13'40"E	CZ	SMALL	2	16.09.2012
Y	Milešický prales Nature Reserve	48°59'06"N, 13°50'19"E	CZ	SMALL	5	17.11.2012
R	Nová Bystřice	49°01'13"N, 15°01'16"E	CZ	SMALL	1	08.05.2012
P	Žofínský prales National Nature Reserve	48°40'10"N, 14°42'20"E	CZ	SMALL	2	13.10.2012
N	Nuukio National Park	60°18'36"N, 24°29'57"E	FI	LARGE	8	11.08.2012
S	Sudenpesänkangas Nature Reserve	61°12'15"N, 25°11'49"E	FI	LARGE	8	08.08.2012
K	Kotinen Nature Reserve	61°14'28"N, 25°03'47"E	FI	LARGE	8	08.08.2012
V	Vesijako Strict Nature Reserve	61°21'00"N, 25°06'04"E	FI	LARGE	8	09.08.2012

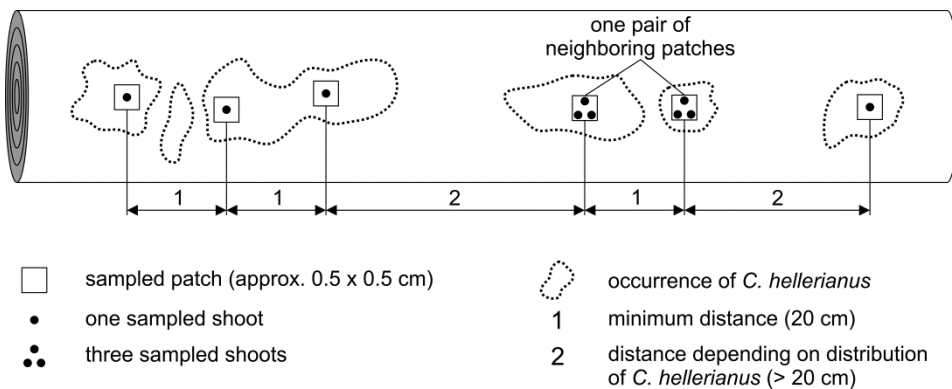


Fig. 2. Schematic illustration of *Crossocalyx hellerianus* sampling on logs.

All studied populations were searched for the production of sporophytes. As these are ephemeral and we were not able to record them at the time of visit, perianths were considered as the indication of the sexual reproduction. Perianths (Fig. 1D) of the leafy liverworts are gametophytic structures of foliar origin around the archegonium which serve the protection of developing capsule. Perianths were searched in all sampled patches, using a stereo-microscope.

Ethics statement

All necessary permits were obtained for field studies to collect species material. Metsähallitus issued the permission for entry into Finish localities and Nature Conservation Agency of the Czech Republic issued the entry permission into Czech localities. No special permission is required for sampling of *Crossocalyx hellerianus* in the respective countries, although it is considered is Endangered (EN) species in the Czech Republic according to IUCN criteria [32], which however does constitute the basis for legal protection in that country (see S1 File).. The species sampled are not listed by CITES (Convention on the International Trade in Endangered Species). All studied localities are on public lands.

Genetic analysis

A SSR-enriched genomic library was constructed using a biotin-streptavidin capture method [36]. Screening of SSR-enriched genomic library was performed using combined approach involving traditional cloning and Sanger sequencing of the library, together with direct 454 pyrosequencing of the library on a GS Junior System (454 Life Sciences, Branford, USA) as described in [37]. Specific primers were designed using Primer3 [38, 39], see Table 2.

Total genomic DNA was extracted from each of the analyzed shoots using the NaOH method [40]. PCRs were performed in a reaction mixture containing 0.5 μ L of genomic DNA, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.3 μ M primers, 0.25 U *Taq* polymerase (Top-Bio, Prague, Czech Republic) in the manufacturer's reaction buffer, and sterile water to make up a final volume of 5 μ L. Amplifications were performed with an initial denaturation of 3 min at 94°C, followed by 45 cycles of 1 min denaturation at 94°C, 30 s at primer-specific annealing temperature (Table 2), 15–30 s extension at 72°C, and a final extension of 10 min at 72°C. PCR products were pooled and analyzed using fragment analysis on an ABI 3730xl DNA Analyser (Applied Biosystems) with GeneScan 600 LIZ (Applied Biosystems, Foster City, USA) as the internal size standard. Microsatellite alleles were scored using GeneMarker v1.80 (SoftGenetics LLC, State College, USA) and were coded as a number of repeats of the SSR motif. Samples in which amplification of more than three loci failed were omitted.

Table 2. Characterization of the nine microsatellite loci developed for *Crossocalyx hellerianus*.

Repeat motif	Ta [°C]	Forward primer (5' - 3')	Reversed primer (5' - 3')	No. alleles	Size range [bp]	GenBank accession no.
(TG)13	58	CCACTTTCATTGTGACCTTT	AGTTTCTTCTCCGCATCA	7	148-160	KM065844
(AC)10	54	GGACGCACAACTCGTTTTCTC	GGTCCAGCATGAGGTTGATT	33	246-314	KM065843
(TG)24	54	TTCTGTCATTTTCGGATTTGG	GTGGGCAACTCTTTGGACT	18	384-426	KM065842
(TC)24	54	TTGGGATGAGAAAAAGTGA	CCTCGTATTGATTGTGGGTAT	24	486-536	KM065838
(GT)10	54	CCTTGCAGCTCATATCTTGTT	CCTTTCGTCCACCATAAGTCC	14	205-237	KM065837
(CA)11	54	CCAAAGCATGAACATAATCCCATC	GCAAAGGTAACACCAAAGTGAG	5	158-172	KM065839
(CA)21	58	TCAAAGAACCTTACATCCAAACC	GCATCACTCACTCCTCACCA	25	307-357	KM065840
(AC)13	54	CGTGAAAGACTGTTGAGGA	GGATTTGAGGCGGAGGATAG	7	173-185	KM065845
(GT)13	54	CAAGCCAACAAGGAGAGAGATT	AAGCCCAATGTGAAGAAGGA	12	226-260	KM065841

Data analysis

Nei's gene diversity (\hat{H}) was calculated using Arlequin v3.5 [41]. Number of genotypes – N_g and number of recurrent genotypes – N_{rg} were calculated in the GenClone 2.0 program [42]. With respect to different sample size of populations, values of N_g were resampled using GenClone 2.0, and HP-Rare software [43] was used for rarefaction of mean number of observed alleles per locus – N_a . For both N_g and N_a calculations, the sample sizes were adjusted to five (the smallest sample in the comparison). The probability that individuals shared the same multilocus genotypes (MLG) were derived from sexual reproduction involving recombination (P_{sex}) calculated in the GenClone 2.0. Samples with missing data were excluded from all above mentioned computations.

In addition to P_{sex} assessment, linkage disequilibrium analysis was performed to assess whether marker distributions resulted from sexual or asexual reproduction. Multilocus linkage disequilibrium was tested using the index of association modified to remove the effect of number of loci analyzed (r_d [44]) and calculated for each population using Multilocus v 1.3. 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 1,000 randomizations.

Hierarchical structure of genetic variation was examined using analysis of molecular variance (AMOVA) in Arlequin v 3.5 [41] with calculations based on the R_{ST} -like method, using the sum of squared size differences. The R_{ST} -like method was preferred because a preliminary allele permutation test performed in SPAGeDi 1.4 software [45] was significant, indicating that an allele size-based statistic was informative for population differentiation and may contain more information than allele identity measures such as F_{ST} , which is likely to provide a biased estimate of gene flow [46]. The following partitioning of genetic variation was tested: between distant geographic regions (Czech Republic and Finland) and among localities within the regions. The analysis based on F_{ST} -like method showed that variation among populations within regions was slightly higher than variation between the two geographic regions (CZ vs. FI). In addition, the pairwise R_{ST} values for all populations were computed. The significance of AMOVA components and of pairwise R_{ST} values was tested using 10,000 permutations.

To reveal the fine-scale spatial genetic structure (SGS), a spatial autocorrelation analysis was conducted in SPAGeDi 1.4 software [45]. Distance

classes with upper boundaries of 0.01, 0.5, 1, 2, 4, 8, 16 and 500 m were used. Multilocus pairwise kinship coefficients (F_{ij}) based on Nason's kinship coefficient [47] were calculated. To test the influence of population size on SGS, populations were further assigned into three groups: small CZ populations, large FI populations and the large CZ population (see Table 1). For each group of populations, mean multilocus pairwise kinship coefficient values were plotted against the upper boundaries of geographic distance classes. Significance of the mean F_{ij} per distance class was tested using 1,000 random permutations of individuals.

The spatial extent of clonal dispersal was quantified using distance classes and population assignment defined as above. The percentage of clones within each of the distance classes was calculated using pairwise comparisons which included identical genotypes and they were plotted against the upper boundaries of classes. In addition, the maximum distance among samples of the same genotype was recorded for each population.

Results

Population genetic analyses

Nine polymorphic microsatellite markers from the liverwort *Crossocalyx hellerianus* were developed (Table 2 and S2 Fig.). All genotyped material was haploid and the microsatellite loci contained between 5 and 33 alleles (Table 2). The final dataset of 393 successfully genotyped samples contained two samples with missing data for three loci, four samples with missing data for two loci, and 52 samples with missing data for one locus, respectively. 243 MLGs were found among the 335 genotyped individuals (without missing data). Identical genotypes were only rarely detected inside large FI and CZ populations, while in small CZ populations recurrent genotypes occurred at higher rates (Fig. 3 and S3 Fig.). Identical genotypes were relatively frequently detected only within individual logs (see below). No identical genotype has been found among populations.

Resampled number of genotypes (N_g), mean number of observed alleles per locus (N_a) after rarefaction, and Nei's gene diversity (\hat{H}) varied from 1.00 to 4.97, 1.00 to 4.45, and 0.233 to 0.995, respectively (Fig. 3). Lower values of N_a , N_g and \hat{H} were detected in small CZ populations; the small CZ population R contained a single MLG.

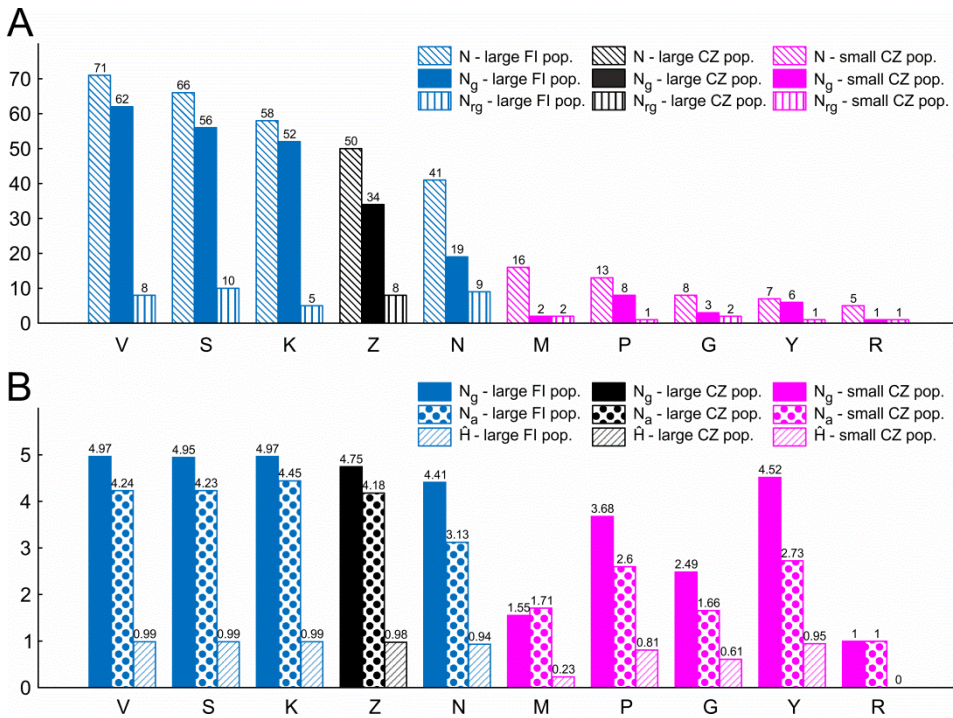


Fig. 3. Genetic diversity indices for *Crossocalyx hellerianus* populations. (A) Sample size (N), number of genotypes (N_g) and number of recurrent genotypes (N_{rg} , i.e. those occurring more than once) computed for all samples in each population. (B) Resampled values of number of genotypes (N_g), mean number of observed alleles per locus (N_a) after rarefaction, and Nei's gene diversity (\hat{H}). Abbreviations of localities correspond to Table 1.

The analysis of molecular variance based on R_{ST} -like method (Table 3) showed that the highest proportion of genetic variation occurred within populations (67.7%), followed by the variation between the two geographic regions (CZ vs. FI; 25.3%), and the variation among populations (7.0%). Separate analyses of both regional datasets found higher rate of variation among CZ populations (18.5%) than among FI populations (6.2%).

The highest pairwise R_{ST} values were usually observed between CZ and FI populations (Fig. 4 and S1 Table), which is in agreement with geographic distances separating both regions (ca. 1,500 km). Nevertheless, considerable divergence was also found among most of the CZ populations, with pairwise R_{ST} values usually higher than 0.1 (11 out of 15 values). On the contrary, the pairwise R_{ST} values between FI populations except the most remote population N did not exceed the value of 0.1. Even in case of population N, the pairwise comparisons with the remaining FI populations (K, S and V) revealed generally lower R_{ST} values than those observed among CZ populations separated by even shorter geographic

distances (the distances between N and other FI populations spanned 106–120 km, whereas 18–92 km separated CZ populations, respectively). The pairwise R_{ST} values among geographically close populations (separated by distances not exceeding 18 km, i.e. CZ populations G, Z, Y, and FI populations S, K, V, respectively) were higher among CZ populations (see S1 Fig.).

Table 3. The distribution of genetic variation based on the analysis of molecular variance (AMOVA).

Source of variation	d.f.	Variance component	Variance %	Fixation index
Between CZ and FI groups of populations	1	53.526	25.3	$F_{CT} = 0.253^{**}$
Among populations within groups	8	14.854	7	$F_{SC} = 0.094^{***}$
Within populations	382	143.298	67.7	$F_{ST} = 0.323^{***}$
Total (CZ and FI)	391	211.678		
Among CZ populations	5	21.949	18.5	$F_{ST} = 0.185^{***}$
Within CZ populations	129	96.781	81.5	
Total (CZ)	134	118.73		
Among FI populations	3	11.796	6.2	$F_{ST} = 0.062^{***}$
Within FI populations	254	180.009	93.9	
Total (FI)	257	191.805		

Significance of F values is marked as *** $P < 0.001$; ** $P < 0.01$.

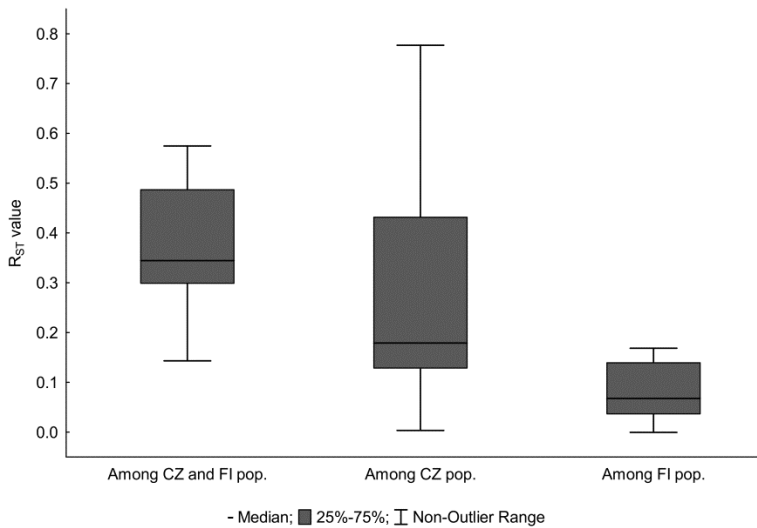


Fig. 4. Genetic differentiation. Genetic differentiation among populations between and within the two geographic areas based on pairwise R_{ST} values.

Significant and high r_d values indicating linkage disequilibrium were found in all small CZ populations (Table 4). Only the value for the large FI population N was comparable to values of small CZ populations. Non-significant or low values of linkage disequilibrium were observed in populations with high N_g , N_a , \hat{H} . These populations also contained a high number of observed patches with perianths, indicating the production of sporophytes.

Table 4. Linkage disequilibrium, maximum distance between the same MLG, % of patches with perianths.

Locality	Linkage disequilibrium (r_d)	Max. distance between samples of the same genotype [m]	% of patches with perianths
Z	0.06***	6.8	7.5
G	0.44***	15	0
M	0.87***	10	8.3
Y	0.20***	0.01	0
R	—	0.01	0
P	0.26***	3.5	0
N	0.22***	50	25.9
S	0.01	20	8.6
K	0.03**	62	24.5
V	0.02	80	25

Linkage disequilibrium (significance of r_d values is marked as *** $P < 0.001$; ** $P < 0.01$) based on data at nine microsatellite loci in *Crossocalyx hellerianus*, maximum distance between samples of the same multilocus genotype, and percentage of patches with perianths. Locality R comprised a single multilocus genotype.

Spatial genetic structure

Kinship coefficient in small CZ populations reached initial values of 0.77 on distances up to 1 cm, and varied from 0.48 to 0.72 on distances between 50 cm and 16 m (Fig. 5). On the other hand, kinship coefficients in large populations were considerably lower, reaching the initial values of 0.47 and 0.37 on distances up to 1 cm, respectively, and varied from 0.02 to 0.28 on distances between 50 cm and 16 m. On distances exceeding 16 m, the kinship coefficient decreased and dropped below zero in all population groups.

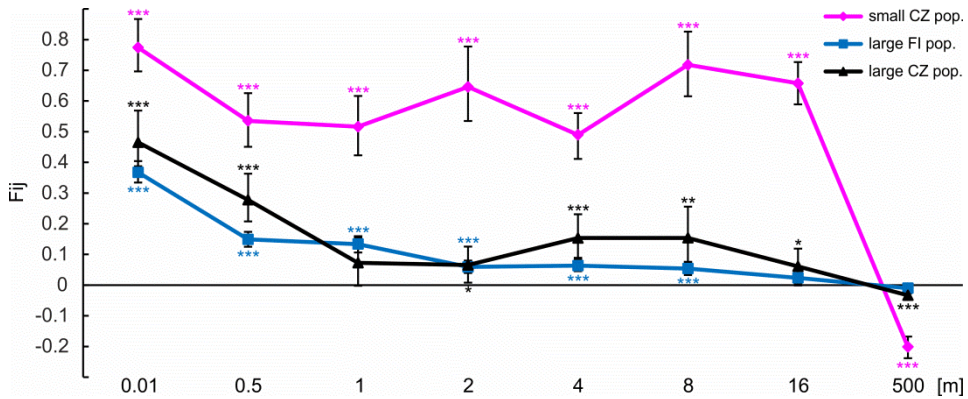


Fig. 5. Spatial autocorrelation analysis based on microsatellite data. Populations of *Crossocalyx hellerianus* were divided into three categories (Table 1): small CZ pop., large FI pop., large CZ pop. The Nason's kinship coefficients (F_{ij}) are positioned along the X-axis at the mean pairwise distance within each distance class. Vertical bars show standard errors. Significance of average F values is marked as *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

The spatial extent of clonal dispersal differed between small and large populations (Fig. 6 and S4 Fig.). In small CZ populations, the percentage of pairwise comparisons with observed identical genotypes sustained high values (31.0–75.9%) for the first six distance classes (1 cm – 8 m), and started to decrease at the distances exceeding 16 m. The pattern found in large FI and CZ populations were rather similar to each other. High initial values of clonality were observed only in the first two distance classes (< 1 and 1–50 cm), then suddenly dropped in the third class (50–100 cm), and decreased more or less gradually at longer distances. However, the percentage of clonality was higher in the large CZ population than in all large FI populations. The probability of sexual origin (P_{sex}) was relatively high for some of the putative clones from small CZ populations, but negligible for majority of individuals from large CZ and FI populations (S4 Fig.).

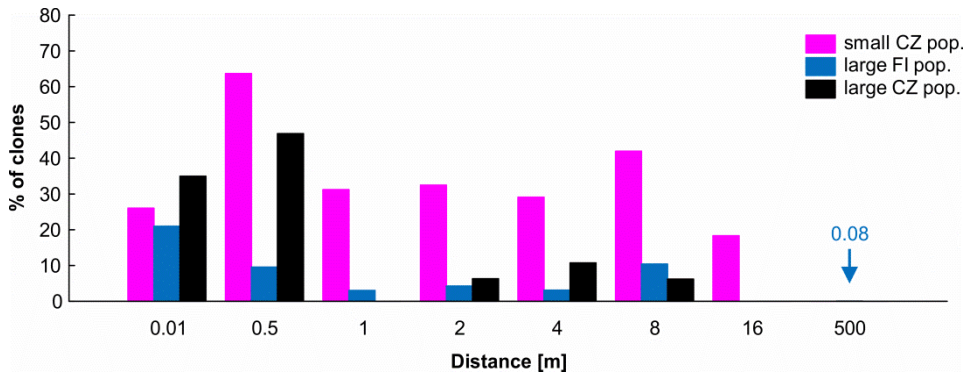


Fig. 6. Percentages of clones within distance classes. Number of all pairwise comparisons in each distance: 0.01 m – 58, 96, 24; 0.5 m – 54, 211, 35; 1 m – 43, 166, 30; 2 m – 31, 274, 39; 4 m – 75, 321, 39; 8 m – 36, 266, 48; 16 m – 87, 168, 48; 500 m – 270, 6558, 948 for small CZ pop., large FI pop., large CZ pop., respectively. Long distances among clones (> 16 m) were found only in all FI populations (blue arrow).

The maximum extent of clonal dispersal was found in the population V, with shoots sharing the same genotype separated by 80 m (Table 4). Nevertheless, considerably long distances among shoots of identical genotypes (≥ 20 m) were found in all FI populations sampled (see Table 4). The maximum distance value for small CZ population was 15 m in population G, with clones always confined to one log.

Discussion

Genetic variability

The observed pattern of genetic variation in studied populations of *Crossocalyx hellerianus*, as documented by values of N_g , N_a and \hat{H} , is congruent with the general assumption that larger populations (here FI populations N, S, K, V and CZ population Z) tend to have bigger pool of genotypes/alleles. In large populations, the majority of the individuals were genetically unique, whereas small populations showed higher ratio between N/N_g . The reduced variation in smaller populations may result from processes such as bottleneck, genetic drift or inbreeding [48, 49]. Nevertheless, several bryophyte studies found no relation between population size and genetic variation [15, 19, 50]. Moderate levels of genetic diversity found in small CZ populations of *C. hellerianus* support the earlier views that even small populations are important sources of genetic variation in bryophytes [19, 50] and that such populations may not be drastically threatened by processes causing loss of genetic variation (genetic risk [51]). Interestingly, genetic diversity

of the small CZ populations Y and P is somewhat higher than those found in other small CZ populations. Possible explanation could include the history, in course of which these populations experienced significant reduction of population size as a consequence of a severe drop in the availability of substrate. It is known that the tree species composition in the Žofín forest (population P) changed significantly from *Abies alba* dominated forest towards broad-leaved forest dominated by *Fagus sylvatica* with only a minor percentage of spruce (*Picea abies* 15% [52]). The population Y could have benefited from the past or recent gene flow from nearby large population Z, as evidenced by the lowest detected genetic differentiation based on pairwise R_{ST} values (S1 Table).

Fully identical individuals were only found within populations. A large diversity of multilocus genotypes within populations appears to be common in both liverworts [19, 20, 23] and mosses [24, 53], irrespective of the prevailing reproductive mode. The unexpected genetic variation found in taxa with rare sexual reproduction or even in asexually reproducing populations [19–21] implies other sources of genetic diversity than recombination events. The authors mostly suggest neutral somatic mutations, originating in various vegetative parts as the probably most important source. According to Weismann's doctrine [54], only the germ line (i.e. cells giving rise to gametes) has evolutionary significance and somatic variation within individuals is not transmitted to progeny [55]. However, this is not the true for majority of land plants including bryophytes, as the sequestration of somatic cells and germ line is incomplete, and the extent to which cells or tissues become irreversibly excluded from propagation is rather low [55]. Both sexual organs and asexual propagules are formed in later ontogenetic phases from somatic stem cells, leading to transmission of mutations originated in somatic tissues directly to gametes and/or asexual gemmae or vegetative fragments. In other words, the nature and relative contribution to novel alleles is basically indistinguishable for both sexual and asexual propagules. The propagation of somatic mutations is further enhanced by consistently greater mutation rates in somatic tissues than in germ lines [56]. In plants, as well as in other clonal or modular organisms, such as aphids, freshwater snails, bryozoans, or reef corals, the somatic cells in bryophytes undergo high number of cell divisions before gametes and/or asexual propagules are formed, providing relatively high probability of mutation during numerous DNA replications [57]. In liverworts, a single apical cell is responsible for the shoot growth, and each somatic mutation in this cell is propagated to all thallus parts, which originated from mitotic divisions following the mutation event. Similarly, any somatic mutation that occurred in leaf cells that gave rise to the asexual propagules (gemmae)

of liverworts, which often are only 1-2 celled, can easily be directly expressed in the progeny.

Other explanations of remarkable genetic diversity in predominantly and/or seemingly asexual bryophytes may involve e.g. population establishment by multiple genotypes, or periodical occurrence of sexual reproduction generating novel recombinant genotypes. Recruitment of new genotypes from neighboring populations seems to be a rather improbable and rare event in the studied system, as no identical MLG were shared among populations, not even between the spatially closest populations Z and Y, distant only 4 km. Occasional and unobserved sexual reproduction, which might be a major source of variation in large populations with stable reproductive system even with only small number of reproducing individuals per generation [58], also probably plays a minor role in generating the genetic diversity of *Crossocalyx hellerianus*, as the frequency of these events is massively outweighed by the gemmae production. The study [59] reported only 32% of bisexual colonies, and only 12% of colonies producing sporophytes and even these numbers are much higher than in studied Central European populations (only two out of six populations producing perianths at all and 8% of perianth-forming patches in these populations; Table 4). Moreover, the estimated gemmae output per square centimeter of *C. hellerianus* colony exceeded the spore production nearly five times, while the ability to germinate in both types of propagules was similar [59]. Prevailing asexual reproduction and absence of recombination in small CZ populations of *C. hellerianus* is also indicated by high values of linkage disequilibrium (or P_{sex} values). Significant and rather high linkage disequilibrium was also found in the large FI population N, although the percentage of patches with perianths (25.9%) was comparable with other large FI populations. Nevertheless, the slightly lower genetic variation as inferred from N_g , N_a and \hat{H} values was congruent with linkage disequilibrium. This pattern could be explained by low portion of gametophytes arising from sexually produced spores or as a result of inbreeding. Mating may occur among haploid siblings originating from the same sporophyte as a result of non-existing mechanism to distinguish among differently related gametes [60]. Inbreeding would further reduce the relative contribution of otherwise rare sexual reproduction for genetic variation in *C. hellerianus*. Especially small CZ populations showed high values of linkage disequilibrium, rather low number of genotypes and aggregation of similar genotypes, which is consistent with the assumption of low recombination efficiency. Therefore, genetic variation in small CZ populations has most likely been caused by somatic mutations, past genetic variation prior to population reduction, and/or establishment by multiple

genotypes, although we cannot rule out the contribution of sexual reproduction with respect to the facts discussed above.

Estimates of genetic differentiation among populations reflect the amount of gene flow between them [61]. Isolation by distance inferred from pairwise R_{ST} values was found in most of the studied populations. Genetic differentiation was rather low among the FI populations (R_{ST} values usually < 0.1), whereas the values among the CZ populations mostly exceeded 0.1 (Fig. 4). This implies greater gene flow among Finnish populations than it is the case in the Czech Republic, which might be explained by the less fragmented landscape of forests with better availability of decaying wood substrate in Finland. Lesser extent of gene flow among CZ populations can be demonstrated in comparison of genetic differentiation between similarly distant FI and CZ populations. The small CZ population G was considerably differentiated from the 18 km distant Y and Z populations, which is in contrast with low R_{ST} values among FI populations V, S and K, respectively, separated by similar spatial distances (7–18 km). We suppose that suitable substrate, enabling step-by-step dispersal [14, 62] supports gene flow among FI populations in contrast to the complete lack of ‘substrate bridges’ among the CZ populations. Our results are in accordance with other studies of genetic differentiation in wood living fungi and beetles [17, 18]. Generally, habitat loss and fragmentation have negative effect on the genetic structure of populations with respect to the restricted level of gene flow. The combination of reduced gene flow among isolated populations and their reduced size leads to genetic drift and the fixation of different alleles, which brings strong genetic differentiation among populations [48, 49].

Spatial genetic structure

Direct observations of propagule dispersal in *Crossocalyx hellerianum* [7] showed that a proportion of propagules deposit within few meters from source colonies but a considerable proportion may disperse over farther distances. In the absence of any specialized dispersal adaptations, the wind probably serves as the main dispersal vector, and the deposition of propagules may be further enhanced by water during rainy days. Dispersal by animal vectors such as the ants, hardly has an important role [23]. Anyway, the direct methods have limited use for large spatial scale studies (few hundreds of meters) or for studies on short timescale. In these cases, indirect methods revealing the spatial genetic structure can bring a reasonable assessment of propagule dispersal.

High values of kinship coefficients observed in most of the small populations provided the evidence for aggregation of similar genotypes. This can be caused by

the relatively low level of genetic diversity resulting from bottleneck and/or founder effect, prevailing asexual reproduction or breeding of related individuals, as discussed above. Spatial distribution reflects both substrate availability and the mode of reproduction. If suitable habitats are evenly distributed and spore production is frequent, allowing effective dispersal at the middle and long distances, randomness in distribution, reflected in the absence of SGS, can be achieved [33]. This is not the case at localities with small populations of *C. hellerianus*, where the amount of decaying wood is generally low and essentially all available substrate is occupied. Random SGS cannot be achieved in the absence of sexual reproduction, evidenced by high values of linkage disequilibrium and absence of perianths in small CZ populations. Asexual reproduction by gemmae represents here the most important and efficient role in maintaining the populations. This is in agreement with previously postulated conclusions in vascular plant studies [63, 64].

Recent investigations of SGS in seed plants, reviewed in [65] showed that its presence is positively correlated with self-compatibility, low population densities, and poorly dispersed seeds. In *C. hellerianus*, large populations possessed less SGS than small populations, with their individuals showing marked decrease in kinship over 50 cm distances and appearing to be without any obvious kinship on distances exceeding 16 m. This result reflects higher population density and more frequent spore production observed in large populations, both allowing more efficient dispersal of different or novel MLGs on farther distances, which reduces the pattern of SGS. Anyway, even in large populations, the plants continue to produce gemmae massively, contributing to aggregation of genotypes and presence of SGS over short distances. Vegetative reproduction by gemmae obviously contributes to economic balance avoiding the costly production of sporophytes [59].

Comparison of SGS shape between studied populations of *C. hellerianus* and the small-scale pattern of SGS in a closely related liverwort species, *Barbilophozia attenuata* [23] shows similar patterns between large CZ and FI populations and the shape for *B. attenuata*, whereas small CZ populations of *C. hellerianus* differed in noticeably strong SGS. Whereas the kinship coefficients reached zero over 8–10 m in *B. attenuata*, they approached zero not earlier than at distance of 16 m and turned negative at distance of 500 m in *C. hellerianus*, reflecting the aggregation of genotypes over larger distances in the latter species. This might infer that *B. attenuata* produces sporophytes more often or the gemmae of *C. hellerianus* have better dispersal capacity. The latter explanation can be supported by the difference in propagule weight, because the smaller gemmae

of *C. hellerianus* have about eight times smaller volume than the gemmae of *B. attenuata*.

In our study, clones, probably arising from gemmae, were detected even at distances of 20, 50, 62, and 80 m. Although some of the identical MLG may have arisen from sexual reproduction, the probability of such events was negligible in large FI and CZ populations (S4 Fig.). Higher frequency of clones distributed over long distances in FI populations thus probably reflects the larger spatial extent of these populations. The observation of clones spanning long distances is consistent with the results of an earlier experiment [7], who found considerable potential for long-distance dispersal of gemmae in *C. hellerianus*. We observed most of clones to be dispersed only within logs at short distances in large populations, whereas small CZ populations showed significant portion of clones dispersed at distances up to 10 m (Fig. 6). The apparently more efficient dispersal of clones in small CZ populations might however rather be the consequence of the absent sporophyte production. On the other hand, the clonal pattern of FI populations seemingly involving long distance dispersal might be a consequence of several successive step-by-step dispersal events over much shorter distances, as the continuous availability of epixylic substratum in space and time at Finnish localities increases the probability of successful establishment.

Conclusions

Genetic diversity in populations of the dioicous epixylic liverwort *Crossocalyx hellerianus* was related to population size but even the small populations were found to be important sources of genetic variation. Recombination connected with sexual reproduction only plays a significant role in generating the genetic diversity in large populations of *C. hellerianus*, whereas smaller populations are maintained by vegetative diaspores and their main source of genetic diversity are probably the somatic mutations. We were able to demonstrate notably low levels of gene flow among populations in Central Europe, where habitat fragmentation poses a significant barrier to dispersal of diaspores. Populations from southern Finland show lower levels of inter-population differentiation at the same distances, which can probably be explained by the presence of step-by-step dispersal. 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, strong

SGS in large populations seems to be reduced by the relatively efficient dispersal of both spores and gemmae.

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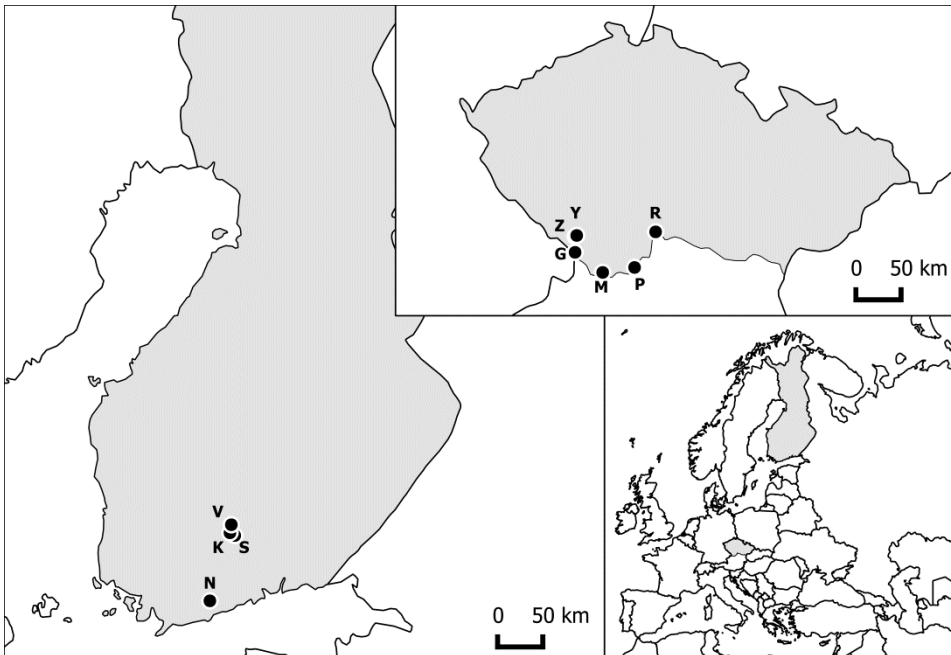
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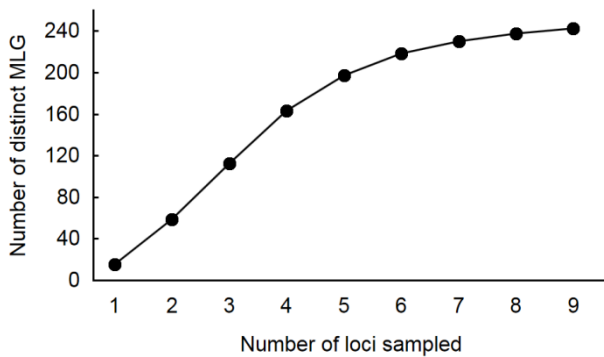
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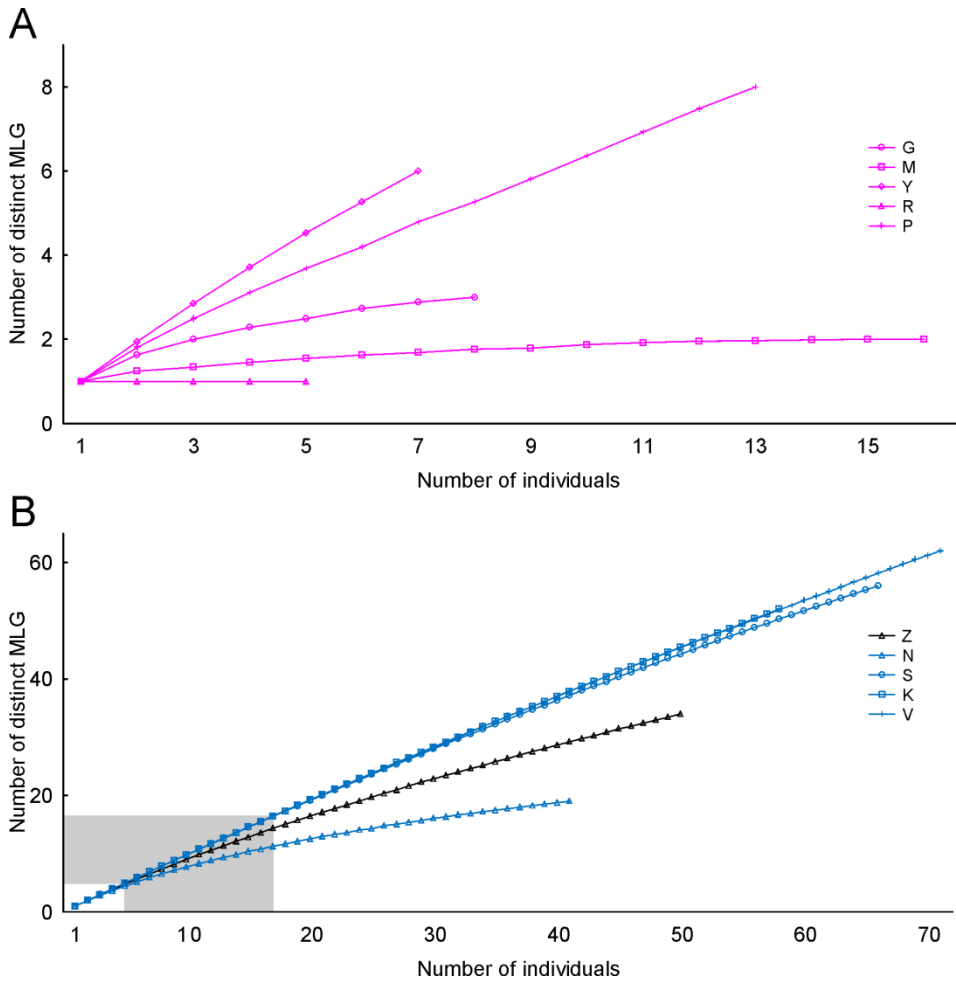
Supporting Information



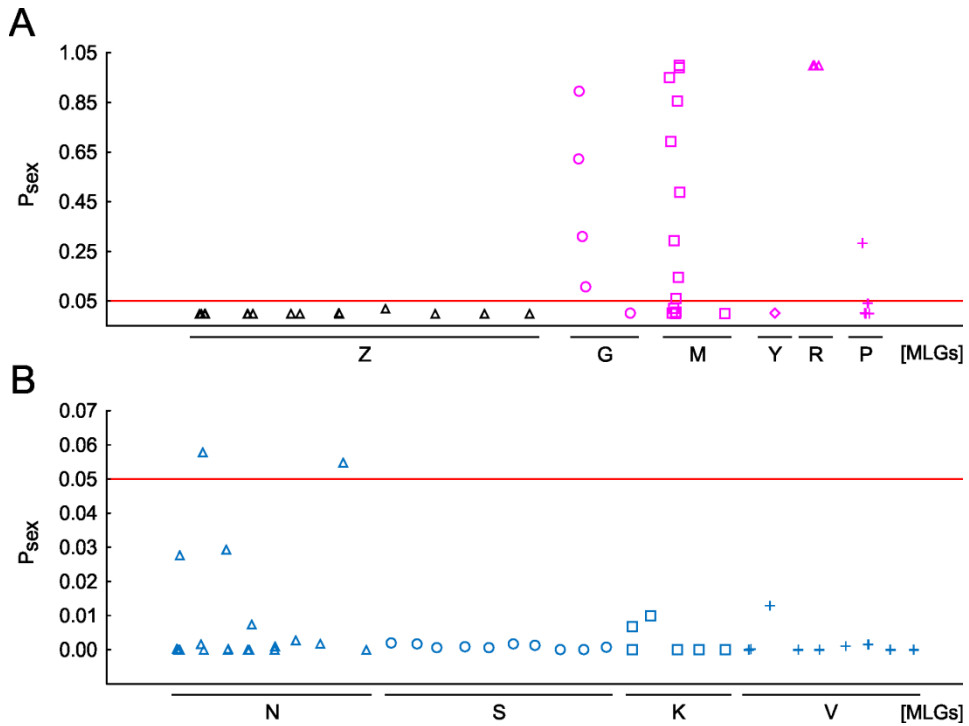
S1 Fig. Sampling sites in the Czech Republic and Finland. Abbreviations of localities correspond to Table 1. Made with Natural Earth. Free vector and raster map data @ naturalearthdata.com.



S2 Fig. Multilocus genotypic resolution of microsatellites in the data set of *Crossocalyx hellerianus*. The plot was generated using 1,000 random samples of 1 to 9 loci. Resampling of loci indicated that our set of nine loci had sufficient haplotypic resolution, as even the use of approximately 7 loci would reveal the majority of MLGs detected in this study.



S3 Fig. Number of distinct multilocus genotypes (MLGs) plotted against the number of individuals (A and B). Plots were generated for each population separately (A) small populations and (B) large populations, using 1,000 random samples of individuals to see if the relationship reached a plateau. Resampling of individuals indicated that increased sampling would yield higher number of MLGs in large populations (B), whereas in small populations the number of MLGs mostly tended to reach a plateau (A). The estimated number of MLGs was substantially lower in small populations (1–8 MLGs) than in large populations (5–15 MLGs, grey part of B) at smaller sampling sizes (N ranging from 5 to 16), corresponding to the maximum sampling size in small populations. Therefore, sampling in small populations was probably rather comprehensive despite lower number of individuals in population, whereas in large populations the clonal diversity estimates could be underestimated.



S4 Fig. The probability of sexual reproductive events (A, B and C). Probability of sexual reproduction (P_{sex}) was plotted against the particular repeated multilocus genotypes (MLG) for populations (A) small CZ populations, (B) large CZ population and (C) large FI populations. If the probability is below significance threshold ($P_{sex} < 0.05$), the respective individual is not likely to be the result of a distinct event of sexual reproduction. Thus we can conclude that individuals with identical genotypes, which occur more than once in the population and their $P_{sex} < 0.05$, were probably established from asexual propagules (predominantly found in large CZ and FI populations – Z, N, S, K and V).

S1 Table. The pairwise R_{ST} values calculated between all populations.

Locality	Z	G	M	Y	R	P	N	S	K
G	0.172***								
M	0.179***	0.415***							
Y	0.003	0.129**	0.176**						
R	0.432***	0.777***	0.656***	0.192					
P	0.076*	0.153**	0.256***	0.051	0.740***				
N	0.396***	0.575***	0.501***	0.279***	0.152*	0.515***			
S	0.328***	0.515***	0.487***	0.235**	0.323**	0.464***	0.169***		
K	0.208***	0.356***	0.367***	0.083*	0.161*	0.317***	0.139***	0.053**	
V	0.186***	0.333***	0.325***	0.080*	0.143*	0.281***	0.083**	0.037*	-0.001


Significance of F values is marked as ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$.

S1 File. Certification of Ethics statement.

24th February 2015

Crossocalyx hellerianus (syn. *Anastrophyllum hellerianum*) is an Endangered (EN) species in the Czech Republic (Kučera et al. 2012¹) according to IUCN criteria, which constitute the basis for legal protection in some countries. However, this does not apply to the Czech republic, where no special species permission is required for sampling of this species.

The list of species protected by law is presented in Regulation No. 395/1992 Coll. to Act No. 114/1992 Coll. (<http://www.ochranaprirody.cz/res/data/107/014856.pdf?seek=1>), no bryophytes are listed.



Mgr. Eliška Horodyská
Species Protection Office
Nature Conservation Agency of the Czech Republic
Kaplanova 1931/1, 148 00 Praha 11
tel.: 283069253
e-mail: eliska.horodyska@nature.cz

¹ Kučera J, Váňa J, Hradělek Z (2012) Bryophyte flora of the Czech Republic: updated checklist and Red List and a brief analysis. *Preslia* 84: 813–850.

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

Eva Holá

Date of birth: 15.1.1983

Nationality: Czech

e-mail: eva.neurazy@gmail.com

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

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- Kučera J., Plášek V., Kubešová S., Bradáčová J., **Holá E.**, Košnar J., Kyselá M., Manukjanová A., Mikulášková E., Procházková J., Táborská M., Tkáčiková J. & Vicherová E. 2014. Mechorosty zaznamenané během podzimních 26. bryologicko-lichenologických dní (2013) v Beskydech [Bryophytes recorded in course of the 26th Bryological and Lichenological Days (2013) in the Beskydy Mts (NE Moravia)]. – *Bryonora* 54: 11-21.
- Štechová T., **Holá E.**, Košnar J. & Vicherová E. 2014. Bryoflóra Novodomského rašeliniště [Bryophyte flora of Novodomské rašeliniště]. – *Sborník muzea Karlovarského kraje* 22: 55-62.
- Štechová T., **Holá E.**, Ekrťová E., Manukjanová A. & Kučera J. 2014. Monitoring ohrožených rašeliništních mechorostů a péče o jejich lokality [Monitoring of endangered mire mosses and care for their habitat]. – *Agentura ochrany přírody a krajiny ČR, Praha*
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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

- Mgr. Jan Kučera, Ph.D., University of South Bohemia, Faculty of Science, Branisovska 1760, CZ-370 05 Ceske Budejovice; e-mail: kucera@prf.jcu.cz
- Dr. Sanna Laaka-Lindberg, Ph.D., Finnish Museum of Natural History LUOMUS, Botany unit, ESCAPE , P.O. Box 7, FI-00014 University of Helsinki; e-mail: sanna.laaka-lindberg@helsinki.fi
- Mgr. & Mgr. Karel Chobot, Ph.D., Nature Conservation Agency of the Czech Republic, Kaplanova 1931/1, CZ-148 00 Praha 11 - Chodov; e-mail: karel.chobot@nature.cz

© for non-published parts Eva Holá

eva.neurazy@gmail.com

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University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 1760
CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201
www.prf.jcu.cz, e-mail: sekret@prf.jcu.cz

