

1 **Living apart if you can – how genetically and developmentally controlled sex has shaped the**
2 **evolution of liverworts**

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12 Abstract

13 Sexual differentiation in bryophytes occurs in the dominant gametophytic generation. Over half of
14 bryophytes are dioicous, and this pattern in liverworts is even more profound as over 70% of
15 species are dioicous. However, the evolutionary mechanisms leading to the prevalence of dioicy
16 and the shifts of sexual systems between dioicy and monoicy have remained poorly known. These
17 essential factors in reproductive biology are explored here in light of phylogenetics combined with
18 evidence of genomic characterization of sex chromosomes and sex-determination, as well as
19 cytology. Our analyses and discussions on liverworts are focused on: (1) ancestry and shifts in
20 sexuality, (2) evolution of sex chromosomes and maintenance of haploid dioicy, and (3)
21 environmental impact on the evolution of monoicism. We show that the dioicous condition is
22 ancestral in liverworts, and the evolution of sexual systems is both conserved and stable with an
23 ancient origin, but also highly dynamic in certain more recently diverged lineages. We assume that
24 the haploid dioicy maintained in the course of evolution must be linked to the genetically controlled
25 sex-determination, and transition from genetically to developmentally controlled sex determination,
26 the evolution of monoicism, is driven by ephemeral and unstable environments. Monoicy is less
27 stable in the long-term than dioicy, and thus, ultimately, dioicy is selected in liverworts. It is
28 concluded that sexual dimorphism is maintained through a highly dynamic evolutionary process,
29 sex chromosomes bearing distinct set of evolutionary forces that can have large impacts on genome
30 evolution and may also promote speciation.

31 Key words: bryophytes, cytology, evolution, genomics, haploid dioicy, liverwort, phylogeny, sex
32 chromosome, sex-determination, sexual system

33 Introduction

34 Sexual reproduction and dispersal are two interacting features playing an essential role in the
35 evolution of almost all eukaryotes. This is particularly true for bryophytes due to their unique life
36 cycle characteristics and sexual systems. The life cycle of bryophytes is distinct from vascular
37 plants; in bryophytes the haploid phase consists of a free-living gametophyte responsible for both
38 sexual and asexual functions, and the diploid phase represented by a sporophyte that produces large
39 amounts of haploid resistant spores as dispersal units. Although the sexual stage occurs only in the
40 gametophytes, in terms of distance, gene flow achieved through spore dispersal is orders of
41 magnitude greater than that through sperm dispersal (Wyatt, 1982). Therefore, production of
42 sporophytes has widely been considered as a key factor for measuring success in bryophyte
43 reproduction. Sexual reproduction in bryophytes can be accomplished by either dioicous or
44 monoicous species and infrequently in some species both reproductive modes occur. The sexual
45 condition strongly affects production of sporophytes, with dioicous species producing sporophytes
46 less often than monoicous species, and a fair number of dioicous moss and liverwort species have
47 never been found to produce sporophytes (Schuster, 1966; Longton, 1976).

48 More than half of the bryophytes are dioicous, and this pattern in liverworts is even more profound
49 as dioicy is represented by over 70% of species (Schuster, 1966; Longton, 1976). The only
50 exception is the hornworts, among which 60% of species are monoicous (Villarreal & Renner,
51 2013). The evolutionary mechanisms leading to the prevalence of dioicy in liverworts, and in
52 mosses as well, remain unexplained, but it is believed that outcrossing in promoting and
53 maintaining genetic variability should be selected in bryophytes, as dioicy simply makes
54 outcrossing obligatory (Mishler, 1988; Longton, 2006). However, intra-gametophytic self-
55 fertilization in monoicous bryophytes leading to a totally homozygous sporophyte in one
56 generation, will result no or less inbreeding depression in future generations because of efficient
57 purging of deleterious alleles (Eppley *et al.*, 2007; Taylor *et al.*, 2007; Szövényi *et al.*, 2014).
58 Sexual reproduction in bryophytes has also been considered facultative with genetic variation
59 maintained largely through somatic mutation, because asexual reproduction is effective in rapid
60 spreading of existing populations and production of sporophytes is infrequent in many dioicous
61 species (Mishler, 1988). In contrast, the study of García-Ramos *et al.* (2007) emphasizes the role of
62 asexual reproduction in promoting coexistence of the sexes. For diploid and polyploid organisms,
63 theory predicts that large population with stable reproductive systems can be highly variable even
64 with only small number of sexually reproducing individuals per generation (Bengtsson, 2003).

65 Nonetheless, both empirical and theoretical studies indicate that the key advantage of sexually
66 produced offspring is the ability to colonize unpredictably available new habitats, which is
67 impossible to achieve through asexual reproduction (Longton, 2006; García-Ramos *et al.*, 2007).

68 The genetic basis for dioicism of bryophytes, and the role of sex chromosomes, has been debated
69 for the past century (Lewis, 1961; Smith, 1978; Ramsay & Berrie, 1982). The correlation between
70 dimorphic chromosomes in size and phenotypic sexual expression found in the liverwort genus
71 *Sphaerocarpos* by Charles E. Allen (1917, 1919) was the first direct evidence of the occurrence of
72 sex chromosomes in bryophytes. Other presumed sex chromosomes widely reported in bryophytes
73 in subsequent investigations were mostly based on their presence in one or the other sex and the
74 amount and distribution of heterochromatin, the nuclear material that remains highly condensed
75 within the interphase nucleus (Heitz, 1928). He named the large heterochromatic sex chromosomes
76 of dioicous *Pellia neesiana* ($n = 8 + X/Y$) as macrochromosomes, whereas microchromosomes are
77 for the small heterochromatic sex chromosomes of dioicous *P. endiviifolia* ($n = 8 + x/y$). Tatuno
78 (1941) renamed the macrochromosomes of Heitz as 'H', and the microchromosomes as 'h'.
79 Subsequently, the available cytological evidence suggested that sex chromosomes tend to bear high
80 content of heterochromatin, as in *Sphaerocarpos* and also other animal species (Heitz, 1928, 1933;
81 Lorbeer, 1934; Tatuno, 1933; Segawa, 1965; Newton, 1977). However, Tatuno (1941), who
82 assumed the monoicous condition is primitive in liverworts, noted both H- and h-chromosomes in
83 some monoicous species, indicating that not all H or h chromosomes are sex chromosomes.

84 Mutagenesis experiments whereby female plants could be transformed into male plants, but not vice
85 versa provided evidence that there exists a 'feminizer' locus on the female sex-specific
86 chromosome in *Sphaerocarpos*, *Marchantia* and *Pellia* (reviewed in Bowman, 2016; Heitz, 1949).
87 Likewise, plants possessing a haploid autosomal complement along with both sex chromosomes
88 (A+XY) were functionally female, indicating that the feminizer locus is dominant. Further, as the
89 transformed males (possessing a X chromosome) had immotile sperm suggested that there exist
90 multiple sperm 'motility' loci on the male sex-specific chromosome of *Sphaerocarpos* and
91 *Marchantia* (reviewed in Bowman, 2016; Heitz, 1949). These are the most convincing data on the
92 sex-determining role of specific chromosomes in liverworts. Following these insights, a stasis of
93 several decades ensued until recently, after the sequences of sex chromosomes of a liverwort
94 *Marchantia polymorpha* (Yamato *et al* 2007; Bowman *et al.*, 2017) and a moss *Ceratodon*
95 *purpureus* (McDaniel *et al.*, 2013a; Carey *et al.*, 2020) were characterized.

96 Whether dioicy is the ancestral condition of bryophytes and how genetically controlled sex-
97 determination affects the evolution of sexual systems await more investigations. Results from early
98 cytological and cytogenetic studies were largely constrained by uncertainty on the direction of
99 evolution of the sexual systems and on the ploidy level of liverworts (Berrie, 1960; Newton, 1984).
100 To explain these essential questions in reproductive biology, historical approaches combined with
101 systematics must be employed. The former question has been widely discussed and long debated,
102 and either dioicy or monoicy has been assumed to be ancestral for bryophytes (Smith, 1978;
103 Anderson, 1980; Wyatt & Anderson 1984; Newton, 1983, 1986). Limited evidence derived from
104 phylogenetic analyses has given different results for mosses and liverworts, respectively. McDaniel
105 *et al.* (2013b) proposed a high lability of sexual systems in mosses, whereas Laenen *et al.* (2016)
106 suggested dioicy as the putative ancestral state of liverworts, yet the inferred phylogenetic signal
107 associated with the sexual system is significant only at the deepest nodes. Because the evolution of
108 sexual systems may have proceeded differently in different lineages, any analysis attempting for the
109 overall pattern of a group may not be rigorous enough to reveal the true or hidden revolutionary
110 process, therefore different lineages within the group should also be counted separately. The second
111 question on the impact of genetically controlled sex-determination on the evolution of sexual
112 systems in bryophytes has not been explored previously, obviously due to limitation of available
113 data that could be used to address questions as such. At present, despite that genome level evidence
114 on sex chromosomes of bryophytes is available only from two species, it is sufficient to use it and
115 also other evidence to analyze and discuss further on the potential evolutionary mechanisms and
116 environmental influence responsible for the resultant pattern of sexual systems, and to identify
117 profitable ways of further research. Here we will focus our analyses and discussions on liverworts:
118 (1) ancestry and shifts in sexuality in a phylogenetic context, (2) evolution of sex chromosomes and
119 maintenance of haploid dioicy, and (3) environmental impacts on the evolution of monoicism.
120 Directions for future research are proposed.

121 The ancestry, and highly conserved and stable dioicy

122 Liverworts encompass over 7000 species in nearly 400 genera and 90 families (Söderström *et al.*,
123 2016). Phenotypic variations of the gametophytes of liverworts are the most prominent among
124 bryophytes, represented by three highly distinctive types of body plan referred to as simple thalloid,
125 complex thalloid and leafy organizations, coupled with numerous unique structures. The life span of
126 a gametophyte varies from ephemeral to perennial, and the maturation of a sporophyte can last from
127 a few weeks to nearly one year. The sporophytes present less extensive morphological variation

128 than those of the gametophytes, but their meiotic pattern in sporogenesis is extremely varied
129 (Brown & Lemmon, 2013). Liverworts occupy a vast geographical range in all terrestrial
130 environments on substrates from bare soil on dry land to tall canopies as epiphytes of rainforest.
131 Although they share with other bryophytes the same life cycle characteristics, liverworts differ
132 markedly from mosses in possessing a low number and narrow range in chromosome numbers, with
133 over 85% of species having the basic number $n=8, 9$ or 10 (Berrie, 1960; Newton, 1983, 1988), and
134 with only a rare occurrence of polyploidy (ca. 5% of the species, Laenen *et al.*, 2016).

135 To further understand the evolutionary mechanisms leading to the predominant dioicy, and shifts
136 between sexual systems in liverworts, we performed phylogenetic analyses to reconstruct the
137 evolution of sexual system through time across 80% of the extant liverwort genera and 97% of
138 families (Figs. 1-3). Each major clade was also analyzed separately for detection of any specific
139 pattern (Figs. 2 and 3). In our phylogeny, dioicy is ancestral in liverworts and it has persisted as the
140 dominant condition throughout their evolutionary history (Marchantiophyta, Fig. 3). The same
141 pattern is seen in Pelliidae, which includes the simple thalloid liverworts and a few leafy species,
142 and in Jungermanniales, one of the major leafy liverwort groups. The most frequent shifts of the
143 sexual systems occurred from dioicy to monoicy (e.g., in Marchantiopsida during the Cretaceous;
144 Fig. 3), followed by reversals from monoicy to dioicy (e.g., in Porellales during the Quaternary;
145 Fig. 3). Shifts from dioicy to monoicy were infrequent in Pelliidae and Jungermanniales and
146 occurred mostly in the recently diverged nodes. These monoicous species are associated with
147 polyploidy, such as in the genera *Chiloscyphus*, *Calypogeia* and *Nardia* in the leafy liverworts, and
148 in *Metzgeria* and *Pellia* in the simple thalloid liverworts. We conclude that the shift to monoicy in
149 these two major liverwort clades resulted from autopolyploidy or allopolyploidy, possibly due to
150 somatic doubling involving a failure of mitosis that produces diploid cells in the gametophytes or
151 tetraploid cells in the sporophytes, or diplospory involving a failure of meiosis in the sporophyte
152 (Wyatt & Anderson, 1984). Polyploidy evolved from apospory, commonly presented in mosses is
153 unlikely to happen in liverworts (Smith, 1978; Anderson, 1980), as induced apospory has only been
154 reported for *Blasia pusilla* (Raudzens & Matzke, 1968).

155 In contrast, most of the shifts from dioicy to monoicy in our analyses occurred in the leafy
156 Porellales (Figs. 1-3) and the complex thalloid Marchantiopsida (Figs. 1-3). In Marchantiopsida,
157 monoicy has appeared after the Jurassic and, depending on the transformation cost, the number of
158 nodes reconstructed as monoicous in this clade has surpassed the dioicous ones already by the Early
159 Mesozoic or Early Cenozoic (Fig. 3). In Porellales, monoicy evolved after the Cretaceous and,

160 when transformation costs are equal between sexual systems, the number of monoicous nodes
161 increased beyond the number of dioicous ones during the Neogene followed by a second shift to
162 dioicy (Fig. 3). Based on these results, our explanation on the dynamic changes in sexual systems is
163 that dioicy is selected in liverworts and that monoicy, despite occurring repeatedly, is less stable
164 than dioicy. Our hypothesis on the evolution of monoicy will be discussed in more detail in a later
165 section.

166 We show that evolution of sexual systems in liverworts is complicated, being both conserved as
167 seen in the maintenance of dioicy, but also highly dynamic as seen in the shifts of the sexual
168 system. Because dioicy is highly stable and conserved in numerous lineages through time, and in
169 support with available evidence of cytology, we can only assume that the dioicous condition must
170 be linked to the genetically controlled sex-determination. Such sexual systems likely fit most
171 liverworts and all bryophytes having a gametophyte which enables to reproduce asexually and
172 vegetatively in addition to sexual reproduction. It may also partially explain the slow evolution rates
173 found in bryophytes (Linde, 2019) because time for changes between sexual systems is required;
174 that is, a species may need to accumulate some amount of genetic change over time in order to
175 effectively evolve from one sex phenotype to the other. The time lag between the dioicy and
176 monoicy (Fig. 3) supports this hypothesis. If the chromosomal controlled sexual system is highly
177 conserved, knowledge must be acquired through genomic studies on the sex chromosomes in order
178 to assess how dioicy is maintained in the course of evolution.

179 The central role of sex chromosomes in bryophyte evolution

180 Evolutionary implications of sex chromosomes have been extensively studied in diploid organisms,
181 especially in animals (Muller, 1914; Bull, 1983; Rice, 1984; Charlesworth, 1996). Widely accepted
182 model for sex chromosome evolution in diploid organisms with XY or ZW systems suggests that
183 sex chromosomes evolve from a pair of autosomes, initially by acquisition of a sex-determining
184 locus, with subsequent emergence of sexually antagonistic alleles at loci close to the sex-
185 determining locus select for reduced recombination, leading to degeneration of Y/W (Charlesworth,
186 1996; Bachtrong, 2013). In bryophytes, both empirical and theoretical studies on the evolution of
187 sex-determination and sex chromosomes have been limited. Because of its specific mode of
188 inheritance, the haploid sexual system occurring in bryophytes and also in some macroalgal, as well
189 as fungal systems has been recently designated as UV sex chromosome system (Bachtrog *et al.*,
190 2011; Coelho *et al.*, 2018). In a haploid system, the female U and the male V are either unpaired in
191 the haploid gametophyte and both are exposed to purifying selection, or they are paired in the

192 diploid UV sporophyte. Bull (1978) predicts that the U and V sex chromosomes should show
193 similar characteristics, including similar extent of degeneration, minor degenerations in both,
194 retention of genes on U or V required by the gametophyte and loss of genes required only in the
195 diploid, and that changes in size of U or V should be additions rather than losses. Recent model
196 (Immler & Otto, 2015) predicts that the degeneration is expected to be slower in haploid organisms
197 because the U and V are exposed to haploid selection and also, they do not undergo a marked
198 reduction in the effective population size compared with diploid organisms (one-half for U or V
199 compared to autosomes, but one-quarter for Y/W). However, deleterious mutations in sex-
200 determination genes can be masked if they function in the diploid sporophyte.

201 Empirical data derived from *Marchantia polymorpha* (Bowman *et al.*, 2017) and *Ceratodon*
202 *purpureus* (Carey *et al.*, 2020) agrees largely with the theories in suppressed recombination on both
203 U and V chromosomes with concomitant repetitive element acquisition. The presence of homologs
204 between the U and V, known as gametologs, also supports the prediction that in a haploid organism
205 the essential genes on sex chromosomes are more likely to persist. *M. polymorpha* has a pair of
206 small, heteromorphic sex chromosomes, including a larger U chromosome ($n=8+U$) in the female
207 and a smaller V chromosome ($n=8+V$) in the male, and both sex chromosomes are smaller than the
208 autosomes (Haupt, 1932; Bischler, 1986). Bowman *et al.* (2017) show that *M. polymorpha* exhibits
209 a long-time absence of recombination between the U and V chromosomes pointing to their ancient
210 origin before the split of Marchantiidae and Pelliidae ca. 400 MYA. Likewise, the sex
211 chromosomes have undergone a large degree of degeneration on both sex chromosomes — with a
212 five-fold lower gene density in both U and V than in the autosomes, which does not support Bull's
213 theory (Bull, 1978).

214 The *M. polymorpha* 10-Mb V chromosome is characterized by a number of striking features. It was
215 designated into two segments of YR1 and YR2, both are rich in repeats, but the origins of these
216 repeats are very different (Okada *et al.*, 2001; Yamato *et al.*, 2007). YR1 is composed of copies of
217 repeat sequences consisting of a small number of repeat elements in various arrangements to form
218 an extensive 2-3 Mb V chromosome-specific stretch. A male-specific gene family, ORF162, with
219 an estimation of a few hundred copies, was found embedded in the repeat sequences (Okada *et al.*,
220 2001; Yamato *et al.*, 2007). This unique feature of co-amplification of protein-coding genes with
221 unique repeat sequences may signify the stage of degeneration of the V chromosome and also
222 indicate how the genes required for male functions have been maintained over the course of
223 evolution (Okada *et al.*, 2001; Tanurdzic & Banks, 2004). RY2 of the 6 Mb segment of the V

224 chromosome is composed of repeats and transposable elements accounting for approximately 43%
225 of the segment and is relatively gene rich (Yamato *et al.*, 2007). In the most recent analysis, there
226 are 129 annotated genes on the V chromosome, with 19 of these being gametologs shared with the
227 U chromosome (Bowman *et al.*, 2017; Montgomery *et al.* 2020). Of the 110 genes unique to the
228 male genome many expressed in reproductive organs but not in vegetative thalli, suggesting their
229 participation in male reproductive functions (Yamato *et al.*, 2007; Bowman *et al.*, 2017). Six male
230 reproductive protein-coding genes have homologs in animals but not in angiosperms, possibly
231 involved in spermatogenesis as they encode proteins related flagellar components of other species
232 (Okada *et al.*, 2001; Yamato *et al.*, 2007; Bowman *et al.*, 2017). Many V-specific loci are
233 autosomal genes or gene fragments that have been shown to have recently accumulated into the V
234 chromosome, and the same pattern also occurs for the U, suggesting dynamic evolution of the non-
235 recombining regions of both sex chromosomes (Bowman *et al.*, 2017).

236 The study on chromatin profiling of *M. polymorpha* (Montgomery *et al.*, 2020) shows further that
237 the V chromosome is the most densely packed with transposons belonging to all different classes, as
238 suggested in earlier analyses (Yamato *et al.*, 2007), and with an abundance of silencing histone
239 modifications, a pattern which is in stark contrast to the relatively uniform interspersed of
240 transposons and genes in autosomes. These observations are molecular confirmation of Heitz's
241 (1928) cytological analyses nearly a century earlier. The strong compaction may likely have
242 important evolutionary implications in regulating gene and transposon activity relating to sexual
243 differentiation. In animals, there have been ample examples showing that transposable elements can
244 regulate the expression of sexual development genes (Dechaud *et al.*, 2019).

245 As with the V chromosome, the U chromosome is sparsely populated with genes, with only 74
246 annotated genes, of which 20 have V chromosome gametologs (Bowman *et al.*, 2017). There are
247 only a few functionally U-specific genes, including a presumably feminizer locus that can
248 dominantly determine sex in diploid gametophytes (Haupt, 1932; Bowman *et al.*, 2017). Also
249 similar to the V chromosome, the U chromosome harbors specific repetitive sequences in the form
250 of rDNA clusters with distinct intergenic sequences that evolved independently of that on
251 autosomes (Fujisawa *et al.*, 2003).

252 The gametolog pairs shared between the *M. polymorpha* U and V chromosomes exhibit no synteny,
253 despite these genes presumably being descended from genes on the ancestral autosome that gave
254 rise to the sex chromosomes (Bowman *et al.*, 2017). Analysis of synonymous substitution
255 frequencies between the members of a gametolog pair can provide a rough estimate of their time of

256 divergence, and thus indirect evidence for sex chromosome evolutionary strata that arises by
257 successive rearrangements incorporating sex chromosome regions into the non-recombining region
258 initially localized to the sex determination locus. Such analyses in *M. polymorpha* indicate multiple
259 evolutionary strata, with the oldest predating the Marchantiopsida-Jungermanniopsida divergence
260 (Bowman *et al.*, 2017).

261 Compared with *M. polymorpha*, the sex chromosomes of *C. purpureus* show structural variation
262 between the U and V to a similar extent, and highly differentiated transposable elements
263 accumulation, but with the non-recombining regions younger (McDaniel *et al.*, 2013a; Carey *et al.*,
264 2020). Carey *et al.* (2020) demonstrated that the sex chromosomes of *C. purpureus* expanded via at
265 least two distinct chromosomal fusions to form neo-sex chromosomes and most of the numerous
266 sex-linked genes to the non-recombining U and V are of recent recruitment. The authors suggest
267 that the evolution of sexual dimorphism in bryophytes is largely driven by sexual antagonistic
268 selection through sex chromosome rearrangement, including gene translocations and also sex
269 chromosome translocations in some species (Carey *et al.*, 2020). They further showed that in *C.*
270 *purpureus*, genes involved in sexual development and functions evolved faster than other genes,
271 indicating there is distinct set of evolutionary forces acting on sex chromosomes relative to
272 autosomes. Therefore, sex chromosomes should have profound impact on genome evolution, such
273 as lack of ancient polyploidy in liverworts (Bowman *et al.*, 2017), and likely also on speciation
274 process (Carey *et al.*, 2020). This unique feature of sex chromosomes has remained largely
275 unexplored in bryophytes.

276 While the identity of the U chromosome ‘feminizer’ proposed in early genetics experiments
277 (reviewed in Bowman, 2016; Heitz, 1949) is not yet known, part of the downstream sex-
278 determination pathway of *M. polymorpha* has been elucidated. An autosomal *FEMALE*
279 *GAMETOPHYTE MYB* (MpFGMYB) was identified as a gene specifically expressed in female
280 plants (Hisanaga *et al.*, 2019). Expression of MpFGMYB in males is suppressed by the gene
281 *SUPPRESSOR OF FEMINIZATION (SUF)*, by producing an antisense RNA at the MpFGMYB
282 locus, thus there is a *cis*-acting bidirectional transcription switch controlling sexual dimorphism.
283 The expression of *SUF* is expected to be suppressed by the unidentified feminizer encoded by the U
284 chromosome (Bowman *et al.*, 2017). Whether this sex-determination pathway is *Marchantia*
285 specific, and whether different pathways exist in other liverwort species, more species should be
286 investigated. The autosomal *cis*-acting sexual dimorphism switch MpFGMYB was found to be
287 orthologous to the recently captured U and V-linked MpFGMYB gene copies in the moss

288 *Ceratodon purpureas* (Carey *et al.*, 2020). Therefore, the genomic evidence suggests that the sex
289 chromosomes of *M. polymorpha* is relatively preserved, and their evolution is highly dynamic.
290 These studies mentioned above provide genetic evidence for sex-specific genes on the sex
291 chromosomes, that is, there likely exist multiple 'motility' loci on the V encoding male-specific
292 proteins required for flagellar function and that at least one locus on the U for egg cell development.

293 Theoretical evidence suggests that if sex-antagonistic genes are located on autosomes, sexual
294 antagonistic mutations will be selected to be linked to the sex-specific nonrecombining region
295 through chromosomal rearrangements with autosomes (Charlesworth & Charlesworth, 1980; Rice,
296 1984). Bull's theory on the sex chromosome in haploid dioicy (Bull, 1978) was proposed mostly
297 based on the cytology and genetics of liverwort genus *Sphaerocarpos* possessing a chromosome set
298 $n = 8 (7+U/V)$ (Knapp, 1936; Allen, 1945). His prediction, however, on the equal magnitude of
299 degeneration of the sex chromosomes did not fit the karyotypes of the genus in which the U
300 chromosome is larger than both the autosomes and V chromosome, and the V is smaller than the
301 autosomes. Bull assumed that this discrepancy suggests that there is some fundamental difference
302 between the male and female gametophyte or their gametes which favors these additions in the
303 female but not the male chromosome. However, this is unlikely as the opposite conditions also
304 exist.

305 *Sphaerocarpos* is in many ways different from *Marchantia*. It occurs widely in warm and dry
306 Mediterranean climate and has an ephemeral habit to avoid extreme conditions by having a short-
307 lived gametophyte, and with a sporophyte producing large and resistant spores that lie dormant in
308 the driest months (Schuster, 1992). Some *Sphaerocarpos* species shed their spores in tetrads, hence
309 keeping two males and two females together — thus, even though it lives in an ephemeral habitat, it
310 has not evolved monoicy, but rather evolved another mechanism to ensure that males and females
311 are growing together.

312 The life history of *Sphaerocarpos* implies that much of the resource of the female should be
313 allocated to the sporophyte development, thus suggesting parent-offspring conflict. In his
314 experimental studies on the inheritance of gametophytes of *Sphaerocarpos*, Allen (1919, 1935)
315 reported that none of the mutant genes he found on the gametophytes was borne on a sex
316 chromosome but there was a puzzling amount of linkage of certain mutants with sexuality,
317 therefore, he assumed that certain autosomes tend to be associated with sex chromosomes during
318 meiosis. Although cytological evidence shows that the U chromosome is much larger and more
319 heterochromatic than the V, Meyer and Herrmann (1973) demonstrated using reassociation analysis

320 that about 22% of the DNA of *S. donellii* is repetitive and there is no difference in the repetition of
321 nucleotide sequences between DNA in males and females. Furthermore, in the attempt of
322 identifying sex specific markers in *S. texanus*, surprisingly few markers (three as specific to females
323 and one to males) were found (McLetchie & Collins, 2001). These early findings suggest that the
324 sex chromosomes of *Sphaerocarpos* underwent large scale rearrangements, likely through sex
325 chromosome–autosome fusion to form neo-sex chromosomes. It has been shown that in animal
326 species larger and more heteromorphic sex chromosomes are associated with faster evolution of
327 postzygotic isolation, leading to divergence, thus can contribute to ecological specialization and
328 speciation (Paladino *et al.*, 2019). Therefore, it seems clear that genetically controlled sex-
329 determination in bryophytes is maintained through a highly dynamic evolutionary process, and as in
330 other sexual systems predicted by van Doorn & Kirkpatrick (2007), that accumulation of sex-
331 antagonistic polymorphisms may enhance evolutionary stability of the long-established sex-
332 determination system and at the same time it may also promote speciation.

333 Reversal to dioicy, less stable monoicy

334 Sexual differentiation in monoicous species depends on their immediate environment, and there is
335 no or little constraint on spatial isolation of the sexes, therefore, monoicy must have adaptive
336 advantages, such as enhanced possibility for sexual reproduction, and hence, dispersal. It has been
337 shown that intra-gametophytic selfing, which occurs frequently in monoicous bryophytes, can
338 efficiently prevent the accumulation of deleterious mutations (Szövényi *et al.*, 2014). On the other
339 hand, repeated events of intra-gametophytic selfing may hinder adaptive evolution as predicted by
340 theory (Birky & Walsh 1988; Charlesworth, 2012).

341 As we have shown in the previous section, shifts of sexual system from dioicy to monoicy occurred
342 mainly in recently diverged nodes within Marchantiopsida and Porellales clades, wherein monoicy
343 persisted over time in many genera (Figs. 1-3). Laenen *et al.* (2016) showed that monoicous
344 lineages have higher diversification rates than dioicous lineages in liverworts, stating that increased
345 diversification rate follows the shift to monoicy. Although species of Marchantiopsida and
346 Porellales have a different evolutionary history, distribution and physiology, they share habitats that
347 are often unstable or temporary wherein species should have higher growth rates and be able to
348 complete their life cycles soon after favorable conditions are set. Therefore, monoicy is an
349 adaptation of liverworts to such habitat. Species of both lineages tend to exhibit a higher level of
350 parental investment on sporophyte by producing larger spores, for surviving during unfavorable
351 intervals. Species of Porellales are mostly epiphytes growing on tree trunks and/or living leaf

352 surfaces in humid tropical forests. Their spores undergo precocious germination within the capsule,
353 with their release as a several-celled, chlorophyllose sporeling ready for immediate further
354 development (Schuster, 1983). In the study of sexual system evolution of genus *Radula*, Devos *et*
355 *al.* (2011) showed that transitions to monoicy from the dioicous ancestral condition were
356 phylogenetically significantly correlated with epiphytism and that it is not the sexual system that
357 determines the evolution of epiphytism, but the reverse. In many species of Marchantiopsida that
358 occur in seasonally dry areas, their gametophytes have either evolved short life cycles and with
359 sporophytes producing durable spores, or both the gametophytes and sporophytes become drought
360 resistant linked with morphological adaptations to reduce water loss, for example, the development
361 of ventral parenchymatous tissue and dorsal assimilatory chlorenchyma of the thallus, and their
362 spores with thick and rigid walls in addition to the large size (e.g., in *Mannia*, Schuster, 1992).
363 Therefore, monoicous expression is likely driven by certain life histories.

364 There has been no ancient whole genome duplication (WGD) retained in liverworts (Bowman *et al.*,
365 2017), but so far it is known that one molecular mechanism leading to monoicy is through a
366 polyploidization event resulting in both U and V sex chromosomes being present (Berrie, 1964;
367 Ramsay and Berrie, 1982). However, because some monoicous liverworts appear to be haploid
368 based on cytology (Berrie, 1960) it is likely that monoicy can evolve through sex chromosome
369 rearrangement, for example, by the U chromosome feminizer translocating to an autosome. In
370 monoicous species, genes responsible for controlling sex expression may be dispersed throughout
371 the genome. Note that a monoicous species derived from ancestral dioicous species might retain a
372 chromosome, now autosomal, that descended from a sex chromosome but that has characteristics of
373 an H or h chromosome. Comparative studies on both dioicous and monoicous species, especially
374 closely related species pairs would likely provide insights into the genomic changes associated with
375 transitions between sexual systems.

376 Although monoicy has evolved repeatedly in liverworts, reversal to dioicy did occur in some
377 species of both Marchantiopsida and Porellales. The cause for the resultant pattern is not manifest,
378 but because the time interval for the transition to dioicy from monoicy is short (Fig. 3), we presume
379 that monoicy is less stable in the long-term than dioicy in liverworts. This may also be the case in
380 mosses and hornworts, as it has been shown that in mosses the transition rate from monoicy to
381 dioicy was approximately twice as high as the reverse transition (McDaniel *et al.*, 2013b). To test
382 this hypothesis, the impact of sexual systems of bryophytes on island biogeography can provide
383 some hints. Patiño *et al.* (2013) found that the proportion of monoicous taxa was significantly

384 higher on islands, and that a significant proportion of continental species that are monoicous or
385 dioicous are represented on oceanic islands only by monoicous populations. They further pointed
386 out that the life history traits shifted toward a greater proportion of species producing asexual
387 propagules and smaller proportion of species producing spores, showing weakened advantage of
388 monoicy over time. In other studies, reduced fitness among progeny produced by selfing in
389 monoicous mosses under certain stressful conditions have been suggested (Jesson *et al.*, 2012).
390 Hornworts show an extreme paucity in species diversity with little over 200 species in total, and
391 except for species of the genus *Dendroceros* that have evolved as epiphytes growing on tree trunks
392 and their gametophytes can tolerate drier periods, the rest species lack desiccation tolerance and
393 many of them grow on moist soil in transient habitats as annuals (Wood, 2007; Warny *et al.*, 2012).
394 It is thus not surprising that there are more monoicous species than dioicous ones. Furthermore,
395 Villarreal and Renner (2013) found that the transition rate from dioicy to monoicy was twice higher
396 than in the opposite direction, but monoicous groups have higher extinction rates.

397 Unlike previously assumed that monoicous species should have larger ranges than dioicous species
398 (Longton & Schuster, 1983), Laenen *et al.* (2015) found that sexual systems are not correlated with
399 geographical ranges, and they suggested that monoicous species can also experience severe
400 fertilization constraints under certain conditions. A better understanding on the evolutionary
401 significance of monoicy in liverworts is still necessary. At present, we could argue that the “erratic”
402 or “constant” shifts in monoicy through time (Fig. 3) are reasonable based on Patiño *et al.*’s and
403 Jesson *et al.*’s findings, and they are further supported by Laenen *et al.*’s suggestion. Since monoicy
404 poses constraints on fertilization (Laenen *et al.*, 2015) and offers “weak” advantages over time
405 (Patiño *et al.*, 2013; Jesson *et al.*, 2012), the tendency of the nodes being monoicous fluctuates
406 through evolutionary time, thus, dioicy is ultimately favoured (Fig. 3).

407 Directions for future research

408 A number of bryophytes have long served as model plants in studies of cytology, cytogenetics and
409 genetics, which led to the remarkable findings of plant sex chromosomes and heterochromatin. It
410 was once predicted by the well-known botanist and geneticist of his time, Fritz von Wettstein
411 (1895-1945), that bryophytes would “remain especially favored organisms for many geneticists”
412 (Wettstein, 1932). Unfortunately, Wettstein’s prediction did not turn to be true until the present day
413 mainly because of the lack of sufficient study tools and techniques in the early times. Genetic basis
414 for phenotypic evolution can now be addressed using genome-wide information to further advance
415 our understanding of reproductive biology of bryophytes, which is of vital importance in studies of

416 evolution, biodiversity, systematics, development, ecology, as well as conservation, among many
417 others. Both genetical factors and environmental influence play an important role in the evolution of
418 sexual systems, the implication of the former has been little assessed for bryophytes, therefore, the
419 following points can be put forth for the future studies.

420 First, more genome characterization among key bryophyte species is to be accumulated for allowing
421 comparative studies of the evolution of sexual systems including sex-determination, sexual
422 dimorphism, and testing existing hypotheses and also hypotheses proposed herein, in order to build
423 up evolutionary basis on sexual reproduction. It is expected that sex chromosomes should play a
424 large role in sexual dimorphism, thus can further shape their evolutionary and genomic properties.
425 In a broader scale, UV sexual system can provide substantial contribution to the understanding of
426 the evolution of sex chromosomes. For bryophytes, there is still a lack of knowledge regarding why
427 one sexual system is more favored over the other, and particularly, knowledge about how the
428 reversal of sexuality occurs, i.e., how a monoicous species with autosomes descended from sex
429 chromosomes can evolve back to dioicy and whether this is easier than evolving sex chromosomes
430 de novo or, alternatively, relatively equivalent. These gaps may be filled with further investigations
431 on relevant groups such as families Frullaniaceae, Radulaceae and Ricciaceae.

432 Second, cytological and functional differences between sex chromosomes should be more studied.
433 Because the evolution of the sex chromosomes in bryophytes is much more dynamic than thought
434 before, cytological and cytogenetical studies may likely provide new information on the potential of
435 sex chromosome rearrangement and turnover. Phenotypic evolution of dioicy and monoicy in
436 combination with habitat conditions, and life history traits may be further studied in light of the
437 increased knowledge of nature and evolutionary significance of the sex chromosomes. This will
438 lead to a deeper understanding on the spatial and temporal distribution of the bryological diversity.
439 In the field of taxonomy and systematics, R. M. Schuster realized long ago that taxonomical
440 problems such as species delimitation is expected to be solved with increased knowledge of
441 reproductive biology and biogeography (Schuster, 1988).

442 Third, the above proposed endeavor will also help understand the evolution of polyploidy in
443 bryophytes, thus the species diversity. So far, genetic implications of polyploidization event on the
444 evolution of sex chromosomes, such as whether the rarer occurrence of polyploidy in liverworts
445 than in mosses is related to the stability of dioicy, and to the female dominated expression in the
446 diploid gametophytic phase, wait further studies.

447 Fourth, sexuality of a species should be considered as a key feature if it is subjected for
448 conservation among other life history traits. Dioicous and monoicous species may be susceptible to
449 threats in different way and to different extent, thus, these factors should be considered in the
450 conservation effort. Conservation strategies will become meaningless if information in reproductive
451 biology of the species to be protected is inadequate, as reproduction together with dispersal is the
452 key element affecting whether a species will be sufficiently resilient to climate change or become
453 vulnerable to extinction.

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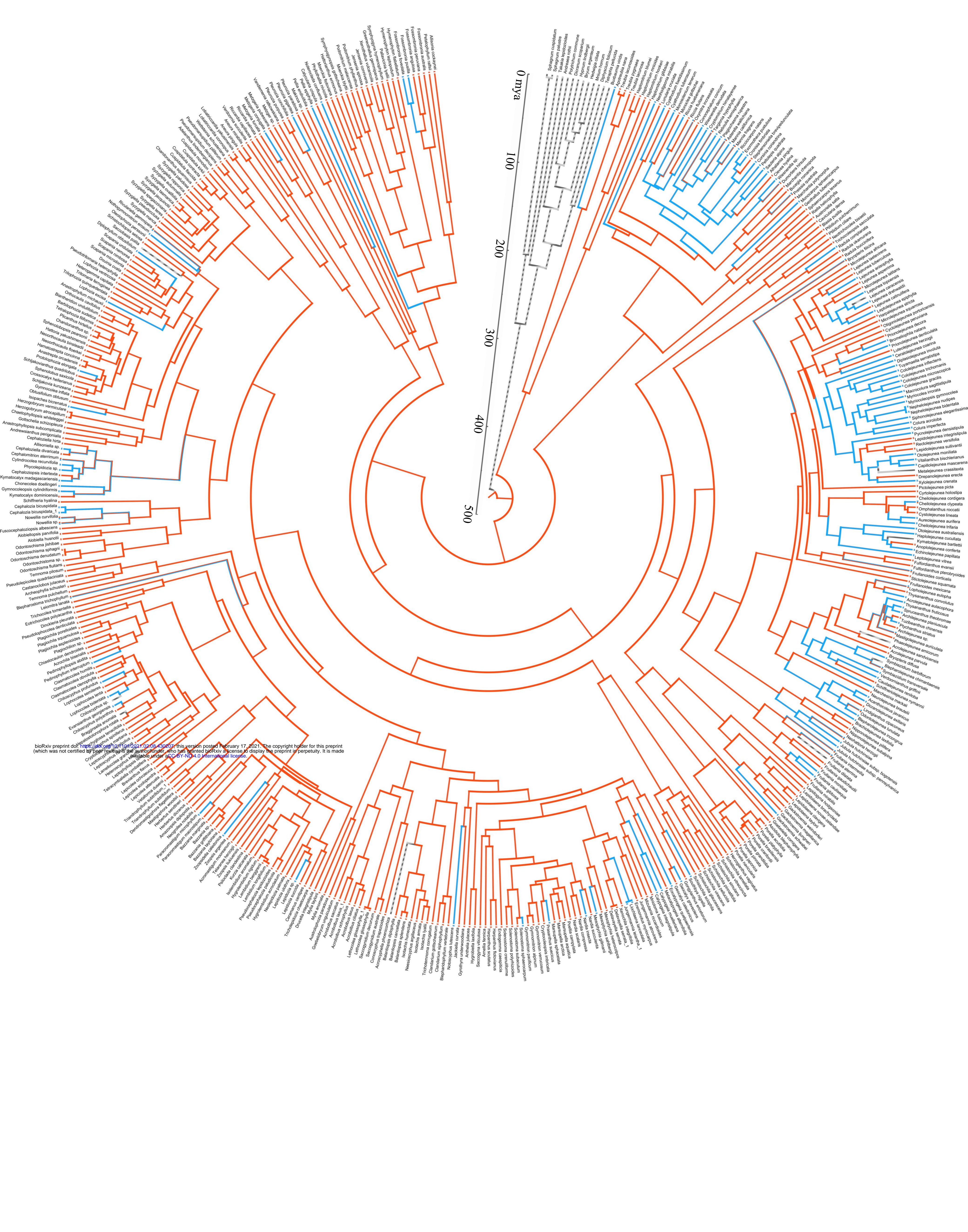
645 Figure legends

646 Fig. 1. Evolutionary pattern of sexual systems of liverworts. The dated phylogenetic reconstruction
647 was performed using 466 species representing 299 liverwort genera and 84 families based on
648 Bayesian Inference, as implemented in BEAST v1.10.4 (Suchard *et al.*, 2018). Of 3232 aligned
649 bases of nucleotides represent three markers (*rbcL*, *rps4* and *trnL-F*). The taxon sampling
650 represents 80% of the extant liverwort genera and 97% of the families (Söderström *et al.*, 2016).
651 The ancestral character state condition for the sexual system was reconstructed throughout the
652 phylogeny on the basis of maximum parsimony in MESQUITE 3.6.1 (Maddison & Maddison,
653 2019). The evolution of the sexual system through time was shown as dioicous (orange) and
654 monoicous (blue). Detailed information on the material and methods and the dated phylogeny can
655 be found in the Supplementary Material as S1 and Fig. S1 respectively.

656 Fig. 2. Evolution of sexual systems of liverworts under different scenarios. Reversal costs were also
657 set higher than gains; being set twice (“[2:1]”), five times (“[5:1]”) and ten times (“[10:1]”) higher

658 than gains. The analysis procedure was applied to five clades of reference: Marchantiophyta (i.e.,
659 the complete tree), Marchantiopsida, Pelliidae, Jungermanniales, and Porellales. Detailed
660 information on the analyses can be found in Supporting material S1.

661 Fig. 3. Fluctuation in the evolution of sexual systems in liverworts across geological time. Seven
662 time-bins were defined based on node ages that could be assigned to the following geologic periods:
663 Palaeozoic (> 252 Myr), Triassic (252-201.5 Myr), Jurassic (201.5-145 Myr), Cretaceous (145-66
664 Myr), Paleogene (66-23 Myr), Neogene (23-3 Myr) and Quaternary (< 3 Myr). The average number
665 of nodes reconstructed as dioicous (orange) and monoicous (blue) are estimated upon inferring
666 ancestral character states onto the MCC dated phylogeny by using maximum parsimony as
667 optimality criterion (Fig. 1). “Reversals” (changes from monoicy to dioicy) were set equal to
668 (“[1:1]”) or higher than “gains” (changes from dioicy to monoicy; “[2:1]”, “[5:1]” and “[10:1]”).
669 The analysis procedure was applied to five clades of reference: Marchantiophyta (i.e., the complete
670 tree), Marchantiopsida, Pelliidae, Jungermanniales, and Porellales. Detailed information on the
671 analyses can be found in Supplementary Material S1. The result derived from “gains” (changes
672 from dioicy to monoicy; “[2:1]”, “[5:1]” and “[10:1]”) are shown in Supplementary Material Fig.
673 S2.

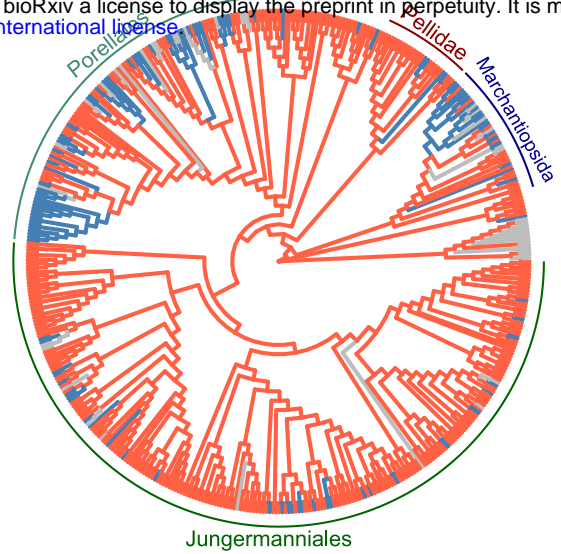
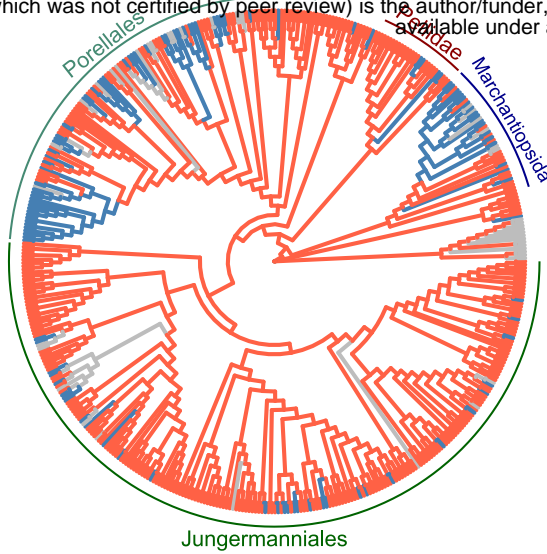


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[1:1]

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