

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

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12 **Summary**

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

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34 Introduction

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

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

65 with only small number of sexually reproducing individuals per generation (Bengtsson, 2003).
66 Nonetheless, both empirical and theoretical studies indicate that the key advantage of sexually
67 produced offspring is the ability to colonize unpredictably available new habitats, which is
68 impossible to achieve through asexual reproduction (Longton, 2006; García-Ramos *et al.*, 2007).

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

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

97 Whether dioicy is the ancestral condition of bryophytes and how genetically controlled sex-
98 determination affects the evolution of sexual systems await more investigations. Results from early
99 cytological and cytogenetic studies were largely constrained by uncertainty on the direction of
100 evolution of the sexual systems and on the ploidy level of liverworts (Berrie, 1963; Newton, 1984).
101 To explain these essential questions in reproductive biology, historical approaches combined with
102 systematics must be employed. The former question has been widely discussed and long debated,
103 and either dioicy or monoicy has been assumed to be ancestral for bryophytes (Smith, 1978;
104 Anderson, 1980; Wyatt & Anderson 1984; Newton, 1983, 1986). Limited evidence derived from
105 phylogenetic analyses has given different results for mosses and liverworts, respectively. McDaniel
106 *et al.* (2013b) proposed a high lability of sexual systems in mosses, whereas Laenen *et al.* (2016)
107 suggested dioicy as the putative ancestral state of liverworts, yet the inferred phylogenetic signal
108 associated with the sexual system is significant only at the deepest nodes. Because the evolution of
109 sexual systems may have proceeded differently in different lineages, any analysis attempting for the
110 overall pattern of a group may not be rigorous enough to reveal the true or hidden revolutionary
111 process, therefore different lineages within the group should also be counted separately. The second
112 question on the impact of genetically controlled sex-determination on the evolution of sexual
113 systems in bryophytes has not been explored previously, obviously due to limitation of available
114 data that could be used to address questions as such. At present, despite that genome level evidence
115 on sex chromosomes of bryophytes is available only from two species, it is sufficient to use it and
116 also other evidence to analyze and discuss further on the potential evolutionary mechanisms and
117 environmental influence responsible for the resultant pattern of sexual systems, and to identify
118 profitable ways of further research. Here we will focus our analyses and discussions on liverworts:
119 (1) ancestry and shifts in sexuality in a phylogenetic context, (2) evolution of sex chromosomes and
120 maintenance of haploid dioicy, and (3) environmental impacts on the evolution of monoicism.
121 Directions for future research are proposed.

122 The ancestry, and highly conserved and stable dioicy

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

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

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

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

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

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

180 The central role of sex chromosomes in bryophyte evolution

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

334 Reversal to dioicy, less stable monoicy

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

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

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

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

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

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

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

408 Directions for future research

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

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

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

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

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

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

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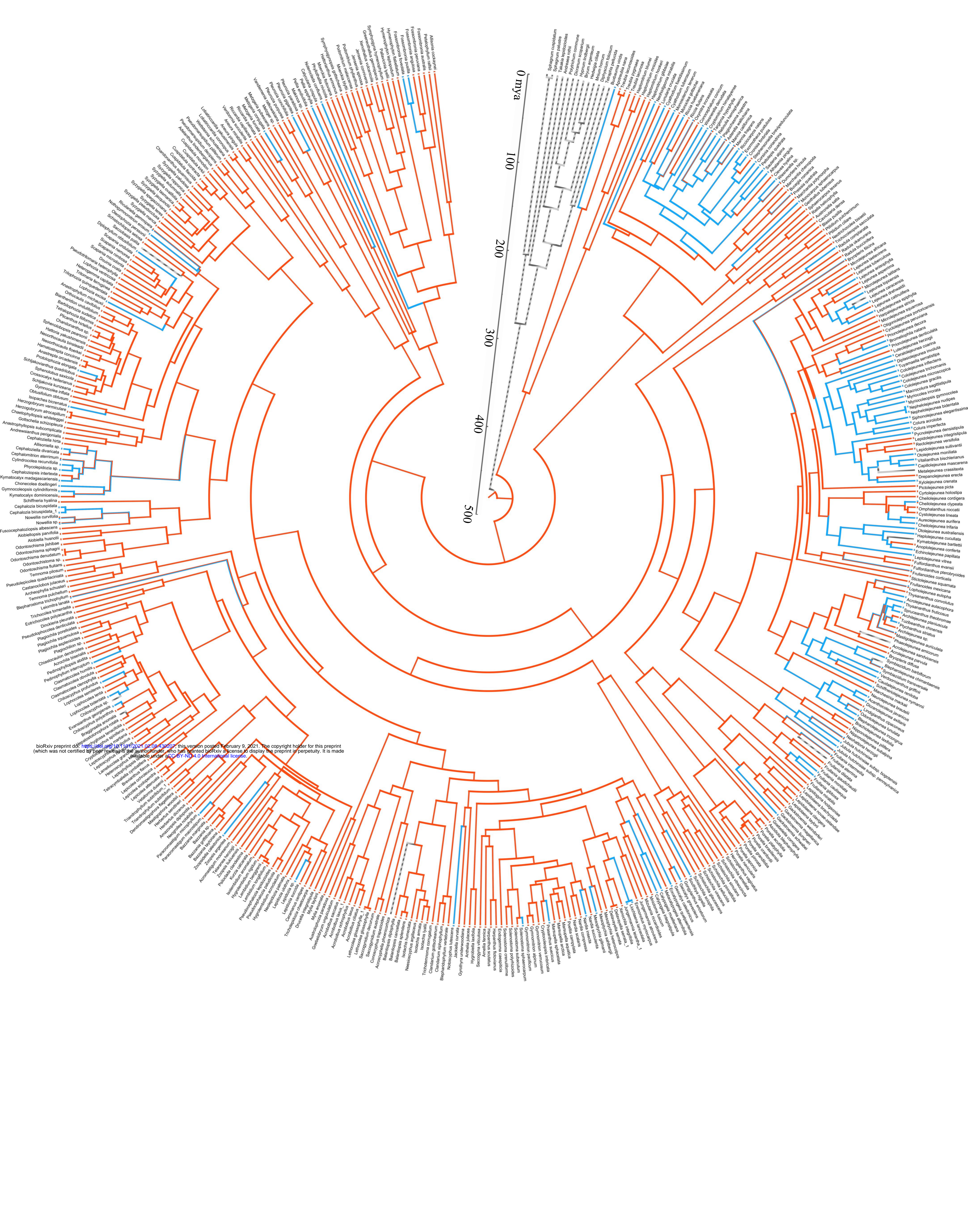
642 Figure legends

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

653 Fig. 2. Evolution of sexual systems of liverworts under different scenarios. Reversal costs were also
654 set higher than gains; being set twice (“[2:1]”), five times (“[5:1]”) and ten times (“[10:1]”) higher
655 than gains. The analysis procedure was applied to five clades of reference: Marchantiophyta (i.e.,
656 the complete tree), Marchantiopsida, Pelliidae, Jungermanniales, and Porellales. Detailed
657 information on the analyses can be found in Supporting material S1.

658 Fig. 3. Fluctuation in the evolution of sexual systems in liverworts across geological time. Seven
659 time-bins were defined based on node ages that could be assigned to the following geologic periods:
660 Palaeozoic (> 252 Myr), Triassic (252-201.5 Myr), Jurassic (201.5-145 Myr), Cretaceous (145-66

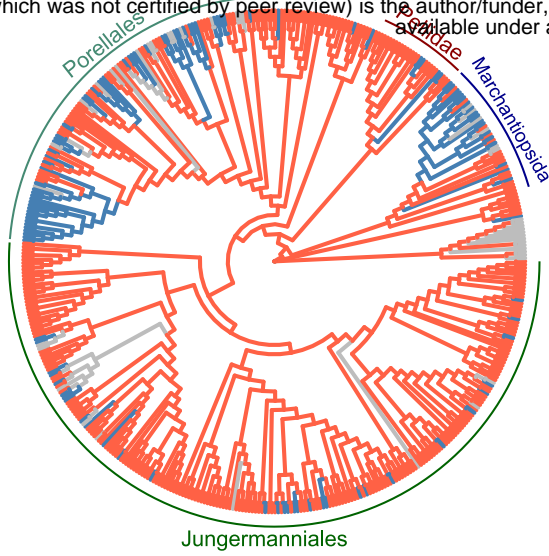
661 Myr), Paleogene (66-23 Myr), Neogene (23-3 Myr) and Quaternary (< 3 Myr). The average number
662 of nodes reconstructed as dioecious (orange) and monoecious (blue) are estimated upon inferring
663 ancestral character states onto the MCC dated phylogeny by using maximum parsimony as
664 optimality criterion (Fig. 1). “Reversals” (changes from monoecy to dioecy) were set equal to
665 (“[1:1]”) or higher than “gains” (changes from dioecy to monoecy; “[2:1]”, “[5:1]” and “[10:1]”).
666 The analysis procedure was applied to five clades of reference: Marchantiophyta (i.e., the complete
667 tree), Marchantiopsida, Pelliidae, Jungermanniales, and Porellales. Detailed information on the
668 analyses can be found in Supplementary Material S1. The result derived from “gains” (changes
669 from dioecy to monoecy; “[2:1]”, “[5:1]” and “[10:1]”) are shown in Supplementary Material Fig.
670 S2.



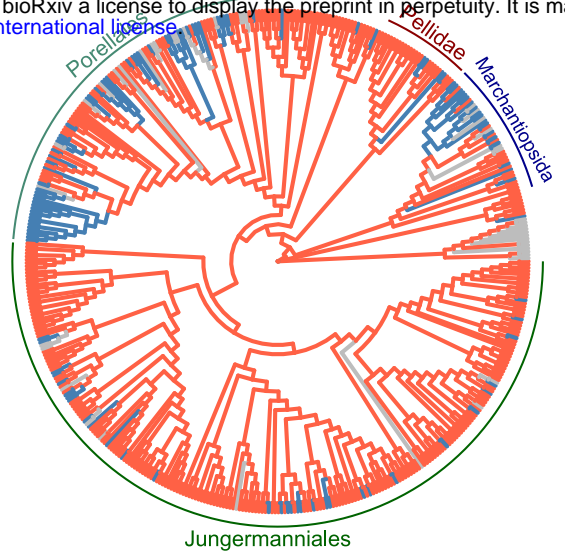
bioRxiv preprint doi: <https://doi.org/10.1101/2021.02.09.430207>; this version posted February 9, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

[1:1]

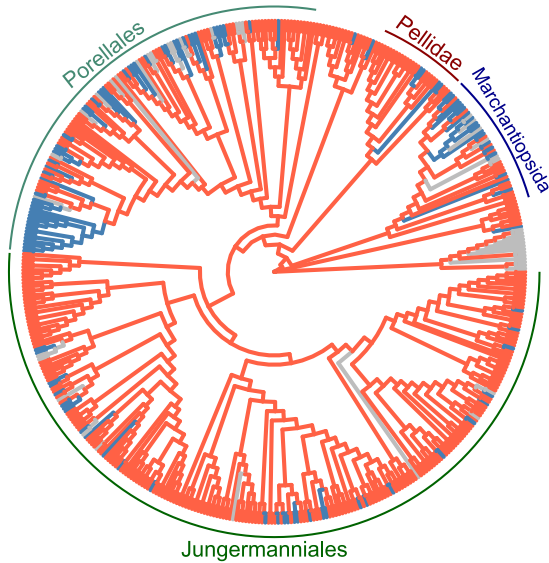
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[2:1]



[5:1]



[10:1]

