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1 SELECTION DRIVES CONVERGENT GENE EXPRESSION CHANGES
2 DURING TRANSITIONS TO CO-SEXUALITY IN HAPLOID SEXUAL
3 SYSTEMS

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10 **ABSTRACT**

11 Co-sexuality has evolved repeatedly from unisexual (dioicous) ancestors across a wide range of taxa.
12 However, the molecular changes underpinning this important transition remain unknown, particularly in
13 organisms with haploid sexual systems such as bryophytes, red algae and brown algae. Here we explore
14 four independent events of emergence of co-sexuality from unisexual ancestors in brown algal clades to
15 examine the nature, evolution and degree of convergence of gene expression changes that accompany
16 the breakdown of dioicy. The amounts of male versus female phenotypic differences in dioicous species
17 were not correlated with the extent of sex-biased gene expression, in stark contrast to what is observed
18 in animals. Although sex-biased genes exhibited a high turnover rate during brown alga diversification,
19 some of their predicted functions were conserved across species. Transitions to co-sexuality consistently
20 involved adaptive gene expression shifts and rapid sequence evolution, particularly for male-biased
21 genes. Gene expression in co-sexual species was more similar to that in females rather than males of
22 related dioicous species, suggesting that co-sexuality may have arisen from ancestral females. Finally,
23 extensive convergent gene expression changes, driven by selection, were associated with the transition to
24 co-sexuality. Together, our observations provide insights on how co-sexual systems arise from ancestral,
25 haploid UV sexual systems.

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29 INTRODUCTION

30 Eukaryotic organisms exhibit a wide diversity of sexual systems, ranging from separate sexes (referred to
31 as gonochorism in animals and dioecy in plants) to co-sexuality (combined sexes) and several theories
32 have been developed to explain what conditions favour which strategy¹⁻⁷. The evolution of this diversity
33 often involved transitions between sexual systems. For example, separate sexes have evolved from co-
34 sexual ancestors independently many times in several eukaryotic lineages, and the fundamental
35 mechanisms and evolutionary drivers of this important transition have been intensively studied in many
36 organisms (reviewed in ^{2,8}). Frequently, organisms with separate sexes display marked sexual dimorphism
37 in a range of morphological, behavioral and physiological traits. Females and males are nevertheless
38 genetically similar with the exception of the sex-specific regions of their sex chromosomes. While sex-
39 chromosomes necessarily play a role in the expression differences between sexes, most of sex-biased
40 gene expression involves autosomal genes ⁹⁻¹¹. Differences in autosomal gene expression patterns
41 between sexes may be associated with different physiological processes directly linked to the production
42 of male or female gametes (primary sexual dimorphism) or to the consequences of sexual selection
43 and/or sexual specialization (secondary sexual dimorphism) that may occur once separate sexes have
44 evolved ¹².

45 While the emergence of separate sexes from co-sexual ancestors and the evolution of sexual dimorphism
46 have been thoroughly investigated ^{11,13-15}, less attention has been devoted to the opposite transition, i.e.
47 from separate sexes to co-sexuality. Transitions to co-sexuality have occurred frequently during
48 eukaryotic evolution and are relatively common in animals (e.g. ^{13,16-20}). In flowering plants this transition
49 was believed to be rare but recent studies are increasingly providing evidence that dioecy-to-monoecy
50 transitions may have occurred frequently ^{21,22}. Evolutionary models intending to decipher the causes of
51 such transitions invoke the sex-allocation theory ⁵ and the deterministic fate of genetic modifiers causing
52 the acquisition of an opposite-sex function ^{23,24}. However, empirical knowledge on the proximate
53 mechanisms and forces driving the shift from separate sexes to co-sexuality remains largely elusive.

54 Transitions from separate sexes to co-sexuality are also prevalent in eukaryotic lineages other than
55 animals and flowering plants, and in particular those that express sex during the haploid stage of their life
56 cycles. In organisms such as bryophytes, liverworts, green, red and brown algae, male and female sexes
57 are expressed during the haploid (gametophyte) stage ²⁵. The terms 'dioicy' (i.e., separate sexes during
58 the haploid phase of the life cycle, as opposed to 'dioecy' where separate sexes occur in the diploid
59 phase) and monoicy (i.e, co-sexuality during the haploid phase of the life cycle, as opposed to 'monoecy'
60 where co-sexuality occurs in the diploid phase) are used to describe the sexual systems of these
61 organisms ²⁶. Genetic sex determination in dioicous organisms occurs during meiosis (and not at
62 fertilisation as in XY and ZW systems) ²⁷, depending on whether spores inherit a U or V sex-chromosome
63 ^{26,28}. Spores receiving a V chromosome will develop into a male multicellular individual (male
64 gametophyte) and the spores inheriting a U chromosome will grow into females (female gametophytes).
65 Organisms with haploid sex determination may also produce male and female sexual structures in the
66 same (co-sexual) individual (monoicy) ^{29,30}. Despite the prevalence of haploid sexual systems among
67 eukaryotes, the gene expression changes and evolutionary forces underlying transitions from dioicy to
68 monoicy have remained largely unknown.

69 In this context, the brown algae represent a particularly attractive group for studies of the evolution of
70 sexual systems and breakdown of dioicy. The brown algae are a complex multicellular lineage that is part
71 of the stramenopile (or heterokont) supergroup, which also includes diatoms and oomycetes, and they
72 have diverged from the Archaeplastida lineage at the time of the eukaryotic crown radiation³¹. Most
73 brown algae have a haplo-diplontic life cycle, with a haploid gametophyte generation alternating with a
74 diploid sporophyte generation. In these brown algae, sexuality is expressed in the haploid generation,
75 with male and female gametes either produced by the same haploid individual (monoicy) or on two
76 separate haploid individuals (dioicy). Dioicy is the prevalent reproductive system^{29,32}. This situation
77 contrasts markedly with that described for flowering plants, where only about 6% of extant species have
78 separate sexes, and is more similar to that of bryophytes and liverworts³⁰. Dioicous brown algae may
79 exhibit a broad range of levels of sexual dimorphism, both at the level of the gametophytes but also with
80 respect to the difference between male and female gametes size^{29,32}. While the predicted ancestral state
81 in the brown algae is dioicy, transitions to monoicy have occurred frequently and independently in the
82 different clades^{32,33}. The independent emergence of monoicous lineages from dioicous ancestors makes
83 this group particularly interesting to examine the genomic consequences and mechanisms underlying the
84 breakdown of dioicy.

85 Here, we explore multiple, repeated events of loss of dioicy (Figure 1) to investigate the molecular basis
86 and level of convergence of the shifts to co-sexuality. We test the hypothesis that sexually dimorphic
87 algae might be expected to have more sex-biased genes and, because dioicy is ancestral, we predicted
88 that similar gene sets would be sex-biased across all the dioicous species. Contrary to our prediction, we
89 demonstrate a lack of correlation between phenotypic sexual dimorphism and gene expression levels
90 among dioicous brown algae. Ancestral state reconstruction indicated high turnover rates of sex-biased
91 genes, yet independently recruited sex-biased genes shared similar functions across the species. To
92 characterise the molecular changes associated with the evolution of monoicy, we then focused on
93 modifications in gene expression patterns of orthologous genes that are specifically or preferentially
94 expressed in haploid males and females of a dioicous species, when they function in a monoicous context.
95 Male-biased genes were particularly concerned by both adaptive expression shifts and faster evolutionary
96 rates associated with the transition to monoicy. Monoicous species displayed expression profiles that
97 were more similar to those of the female of the closely related dioicous species than to the male. Finally,
98 we identified a pronounced level of convergent gene expression changes associated with the emergence
99 of co-sexuality, which were likely driven by selection.

100 RESULTS

101 The present study examines sex-biased gene expression in dioicous brown algae and the gene expression
102 changes associated with the transition from dioicy to monoicy. We based our analysis on transcriptomes
103 sequenced from pairs of dioicous-monoicous species in four major clades of brown algae spanning app.
104 200 million years of evolution³⁴. The transitions are predicted to have occurred at different times in the
105 past (between 20 and 88 MY; Figure 1). Each pair represents an independent transition from dioicy to
106 monoicy. We chose dioicous species with different levels of gamete dimorphism, reflecting the diverse
107 levels of gamete dimorphism occurring across brown algae.

108 *SEX-BIASED GENE EXPRESSION IN DIOICIOUS BROWN ALGAE*

109 Gene expression patterns in gametophytes of the eight brown algal species were measured by deep
110 sequencing (RNA-seq) of cDNA from male, female and co-sexual gametophytes. Transcript abundance
111 (measured as transcripts per million, TPM) was strongly correlated between biological replicates with r^2
112 ranging from 0.89 to 0.99 (Supplementary Table 1). Counts of expressed genes (TPM>5th percentile
113 counts across all genes in at least one sample) identified a number of expressed genes that ranged from
114 13,180 to 27,391 (Supplementary Table 1).

115 Deseq2 was used to identify genes that were differentially expressed in each of the sexes of the dioicous
116 species³⁵. The analysis retained only genes that displayed at least a 2-fold change expression level
117 between sexes (FC>2, p_{adj} <0.05). Note that sex-linked genes (genes located in the sex-specific regions on
118 the V (male) and U (female) sex chromosomes; see methods), were removed from the set of sex-biased
119 genes and thus excluded from further analysis.

120 All four dioicous brown algae displayed substantial sex-biased gene expression, at least compared with
121 plants and other brown alga^{13,15,36} ranging from 12.71 % of the expressed genes in *S. rigidula* to 33.17% in
122 *S. firma* (Figure 2A-2B, Supplementary Tables 2-3). We found similar proportions of male-biased
123 compared with female-biased genes for the majority of the studied species (Figure 2A-2B) with the
124 exception of *S. polyschides*, where male-biased genes were more abundant than female-biased genes
125 (16.51% male-biased genes versus 9.39% female-biased genes; Chi²-test $P < 2.2 \times 10^{-16}$).

126 *SEX-BIASED GENE EXPRESSION AND PHENOTYPIC SEXUAL DIMORPHISM*

127 To investigate the link between sex-biased gene expression and the level of sexual dimorphism, we
128 carried out morphometric measurements of male and female gametophytes complemented with
129 literature searches. These measurements allowed us to quantify the amount of phenotypic dimorphism
130 present in each of the four dioicous species (Supplementary Table 3, Figure 2C). In all dioicous species,
131 gamete size dimorphism was coherent with sexual differences in terms of gametophyte cell size
132 (Supplementary Table 3). For example, *D. herbacea* gametophytes presented marked sexual dimorphism
133 both at level of gamete size and gametophyte cell length, whereas *S. firma* was the species with least
134 sexual difference both in terms of gametophyte morphology and gamete size (Supplementary Table 4,
135 Figure 2C-D).

136 In animals, sexual differences at the phenotypic level are correlated with levels of sex-biased gene
137 expression^{14,37}, but this correlation has not been found in plants³⁶. We compared the differences in
138 gametophyte cell size between males and females with the proportion of sex-biased genes in each of the
139 four dioicous brown algal species. We detected no correlation between phenotypic sexual dimorphism
140 (gametophyte cell size) and the number of sex-biased genes (Figure 2E). For instance, *S. firma* was the
141 species that exhibited the highest level of sex-biased gene expression and nonetheless presented the
142 lowest level of phenotypic sexual dimorphism. Taken together, our observations indicate a considerable
143 level of sex-biased gene expression in the four dioicous species studied here, but the level of sex-biased
144 gene expression did not reflect the level of morphological dimorphism between males and females.

145 *EVOLUTION OF SEX-BIASED GENE EXPRESSION IN DIOICIOUS SPECIES*

146 We next investigated how sex-biased gene expression has evolved by comparing the four dioicous brown
147 algal species. Orthofinder identified a total of 14,017 orthogroups (OGs) across the dioicous species, of
148 which 2,098 contained only one gene per species and therefore represented the set of 1:1:1:1 OGs. An
149 additional 2,778 OGs had a single member in each of three of the studied species (i.e., the gene was
150 missing in the fourth species). We considered that these 1:1:1:0 OGs, which likely represent single copy
151 ancestral genes that were lost in one of the species, also provide useful information about conservation
152 of sex-biased gene expression. Note that the 1:1:1:0 OGs could also represent OGs where one of the
153 genes is missing from one of the genome assemblies, particularly the draft genome assembly for *S.*
154 *rigidula*. Furthermore, we also included 1,085 orthogroups with a duplicated gene in a single species
155 (1:1:1:2 OGs) that aligned along more than 60% of their length, resulting in 5,961 'dioicous single-copy
156 orthologs' (DSOs; Supplementary Table 5, Extended Data Figure 1).

157 We then used maximum likelihood approaches to infer the ancestral states of sex-biased gene expression
158 across these dioicous species (Figure 3B). Our analysis identified very few genes that were predicted to be
159 ancestrally sex-biased, with the vast majority having evolved sex-bias at some point along the branches.
160 Among the 2,116 sex-biased DSOs in at least one species, only 43 (2.03%) were inferred to be sex-biased
161 in the last common ancestor of the four brown algal species (Figure 3). Accordingly, no DSOs were
162 consistently sex-biased across the four species (not different from what is expected by chance, exact test
163 multi-set intersection $P = 0.506$). A total of 139 OGs exhibited a bias in one species that was inconsistent
164 with the direction of bias observed in at least one other species (Supplementary Table 5).

165 Although the above analysis showed that sex-bias genes were not conserved among the four species, we
166 examined if sex-biased genes in different species were involved in similar functions, by comparing gene
167 ontology (GO) terms of sex-biased genes across species using Blast2Go³⁸. We detected significant
168 enrichment of GO terms for biological processes related to 'ion- and transmembrane transport' and
169 'cilium' often associated to male-biased genes across dioicous species. Conversely, the sets of female-
170 biased genes of all the studied species were enriched for GO terms related to oxidation/reduction
171 (Extended Data Figure 2, Supplementary Table 6). Taken together, our results indicate that whilst sex-
172 biased genes exhibited a high turnover rate during brown alga diversification, some of their predicted
173 functions were conserved across dioicous species.

174 We also asked whether sex-biased gene expression emerged in dioicous species as a result of random
175 expression evolution under low selective pressure for non-pleiotropic genes³⁶ or rather as a consequence
176 of sexual selection. To distinguish between these two possibilities, we computed phylogenetic
177 independent contrasts (PICs) of sex-biased genes across species in which those genes are not sex-biased
178 versus unbiased genes (Extended Data Figure 3). We found that PICs differed slightly between unbiased
179 genes and orthologs of sex-biased genes in species in which those genes are not sex-biased (Mann-
180 Whitney Rank test $P = 0.0495$). This result indicates that genes that evolved sex-bias may have done so
181 because they already experienced low constraints on their expression levels, possibly due to lower
182 pleiotropic expression patterns^{36,39}, although we cannot exclude that sexual selection may be also
183 involved in the emergence of sex-biased gene expression in brown algae.

184 *SEX-BIASED GENE EXPRESSION FATE DURING TRANSITION TO MONOICY*

185 To study changes in sex-biased gene expression that accompany the transition from dioicy to monoicy,
186 we first identified single-copy orthologous genes for each of the four dioicous-monoicous sister species
187 pairs (pairwise single-copy OGs; PSOs, Figure 4A). We were able to infer between 6,109 and 11,953 PSOs
188 for each of the four pairs of species (Figure 4A; Supplementary Tables 7-11). PSOs were classified as being
189 sex-biased or unbiased by comparing male and female expression in each dioicous species ($FDR < 0.05$,
190 $FC > 2$). We then examined the patterns of expression of male-, female- and unbiased PSOs in dioicous
191 males and females and in the corresponding monoicous species.

192 In three out of the four species pairs, the levels of expression of sex-biased genes in the monoicous
193 species were similar to the values measured for orthologs in females of the corresponding dioicous
194 species (Figure 4A, Extended Data Figure 4). In these three pairs, male-biased genes were downregulated
195 in the monoicous species compared with males, and they displayed similar expression levels to male-
196 biased genes in females of the dioicous species, suggesting that de-masculinisation of gene expression of
197 the monoicous species counterpart occurred frequently. Female-biased genes were expressed at similar
198 mean levels in *S. firma* females compared with the corresponding monoicous species *C. linearis*. In the *S.*
199 *polyschides-S. dermatodea* pair of species, female-biased genes had a similar pattern in males and
200 monoicous. Both female- and male-biased genes in *D. herbacea* showed significantly different mean
201 expression levels compared with *D. dudresnyi*. In the *S. rigidula/H. paniculata* species pair, no significant
202 difference was detected between the expression of sex- biased and unbiased genes between the two
203 species. Note, however, that results for *S. rigidula/H. paniculata* were more difficult to interpret, as the
204 low number of sex-biased among the PSOs precluded robust statistical analysis.

205 We next investigated the gene expression profiles of monoicous species in order to test whether their
206 transcriptional patterns resemble those of their male or female dioicous counterparts. We computed the
207 Pearson product-moment coefficient of regressions of gene expression profiles (in $\log_2(TPM+1)$) of males
208 or females compared with that of the monoicous species within each species pair. We compared Pearson
209 correlation coefficients for both sex-biased genes and unbiased genes in both males or females,
210 considering sex-biased genes in males and females as independent groups. We also compared the
211 correlations of expression profiles with the orthologs of sex-biased and unbiased genes in the monoicous
212 species, separately for males and females. These groups of sex-biased versus unbiased genes being
213 expressed within the same individuals, we considered them as dependant groups in the *cocor* package⁴⁰.
214 Altogether, these analyses indicated that, with the exception of the *S. rigidula-H. paniculata* species pair,
215 the gene expression profiles of the monoicous species were significantly more similar to those of females
216 than they were to male profiles (Figure 4B; Extended Data Figure 5). Moreover, the close association
217 between female and monoicous expression profiles was observed for both sex-biased and unbiased
218 genes specifically in *Sacchoriza* and *Desmarestia* species pairs (Figure 4B, black asterisks at the top;
219 Extended Data Figure 6).

220 Interestingly, with the exception of the Ectocarpales species pair (*S. firma- C. linearis*), sex-biased gene
221 expression profiles diverged significantly less from the monoicous species than did that of the unbiased
222 genes (Figure 4B, S5). Overall, the expression profile similarity observed between females and monoicous

223 individuals were mainly driven by expression patterns of male-biased genes, with the exception of the
224 *Desmarestia* species pair (Figure 4A, S5). We also noted that the highest similarity indexes for within
225 species pairs were found for the species with the lowest level of sex-biased gene expression (*S. rigidula*),
226 and the lowest similarity was observed for *S. firma*, the species with the highest level of sex-biased gene
227 expression (Figure 4B). The relatively high proportion of sex-specific genes present in *S. firma* (Figure 2A)
228 is unlikely to be the cause of the observed low similarity, because only 29 (0.49%) of the sex-specific
229 genes are among the PSOs used for the Pearson similarity analysis.

230 Taken together, the above observations suggest that gene expression profiles of monoicous species tend
231 to be more closely related to the females of the related dioicous species, and this similarity appears to be
232 driven by sex-biased genes, in particular male-biased genes. The tendency to reproduce the female
233 transcriptome in the monoicous species was repeatable in independent transitions to co-sexuality.

234 *IS SELECTION INVOLVED IN EXPRESSION CHANGES DURING TRANSITION TO MONOICY?*

235 To examine whether changes in gene expression during transition to co-sexuality were the result of
236 selective or neutral forces, we computed the degree of directional selection using Δx . This parameter
237 evaluates the divergence in expression level in relation to the variation in expression level seen across
238 replicates^{11,36,41}. We computed Δx of the PSO sets, separately for each pair of species and reported the
239 proportions of orthologs with an absolute $\Delta x > 1$, i.e., orthologs whose expression shift is attributable to
240 directional selection (Supplementary Table 11, Figure 4C). Depending on the species pair, between 10.8%
241 and 40.1% of unbiased genes exhibited expression shifts attributable to selection ($|\Delta x| > 1$)
242 (Supplementary Table 12). We then asked whether male- and female-biased genes were preferentially
243 concerned by adaptive expression shifts during transitions to monoicy compared with unbiased genes.
244 Figure 4C illustrates the proportion of orthologs with sex-bias displaying $|\Delta x| > 1$ (in other words, under
245 putative directional selection) and how sex-biased genes are more likely to display $|\Delta x| > 1$ in comparison
246 to the unbiased orthologs. Fisher's exact tests (asterisks in Figure 4C) showed that for three out of four
247 species pairs, male-biased genes were indeed more likely to display $|\Delta x| > 1$ than unbiased genes (Figure
248 4C). This was also the case for female-biased genes in *S. polyschides*-*S. dermatodea* pair of species (Fisher
249 exact tests, $P < 2.2 \times 10^{-16}$ in both sexes) and *D. herbacea*-*D. dudresnaji* pair (Fisher exact tests, $P = 3.9 \times 10^{-3}$
250 and $P = 1.8 \times 10^{-5}$ in females and males, respectively). In *S. polyschides*-*S. dermatodea* and *S. rigidula*-*H.*
251 *paniculata*, female-biased genes showed lower levels of adaptive evolution of expression compared with
252 unbiased genes (Supplementary Table 11, Figure 4C). Taken together, our observations indicate that
253 male-biased genes preferentially exhibit a shift in expression during the transition to monoicy that may be
254 explained by directional selection.

255 We also assessed if evolution of gene expression during the transition to monoicy has been driven by
256 DNA sequence evolution, by using measures of sequence divergence (d_N/d_S). We computed d_N/d_S for
257 male-biased, female-biased and unbiased genes for each of the dioicy-monoicy species pairs. For all four
258 pairs, male-biased genes consistently exhibited higher evolutionary rates than female-biased and
259 unbiased genes, although this difference was significant only for the *S. polyschides*-*S. dermatodea* species
260 pair (Figure 4D, Supplementary Table 13). As this is the 'youngest' species pair (Figure 1), it appears that
261 the level of sequence divergence during transition to monoicy is not associated with the age of transition.

262 Taken together, our observations indicate that shifts from dioicy to monoicy involved modifications to
263 transcriptional patterns (expression divergence) mostly at male-biased genes that were likely driven by
264 selection but also coding sequence evolution.

265 *CONVERGENT GENE EXPRESSION CHANGES DURING TRANSITION TO MONOICY*

266 In order to assess the extent to which gene expression changes occurring during the transition to
267 monoicy were shared across the four species pairs, we focused on the single-copy orthologs across the
268 eight species, herein termed 'All Single-copy Orthologs' (ASO). We found a total of 1,708 ASO (following
269 the same approach as for DSO, see methods).

270 Among the 1,708 ASOs, 718 were sex-biased in at least one dioicous species (Supplementary Tables 14,
271 15). Sex-biased genes were not over-represented among ASOs (Fisher exact test, $P = 0.097$). Sixty one
272 percent of the ASOs (1,043 out of 1,708) exhibited a conserved pattern of expression across all
273 monoicous species compared to the dioicous species. This proportion was significantly different from
274 what was expected by chance (permutation tests, $P = 0.0255$, 10,000 permutations) suggesting
275 convergent gene expression changes during transition to monoicy across all studied pairs of species.
276 Decomposition of variance components for the 1,708 ASOs detected a clear pattern of grouping of
277 monoicous species, further illustrating the extensive convergence of gene expression during the
278 transition from dioicy towards monoicy (Figure 4E). Functional analysis of genes that are convergently
279 expressed during the transition to monoicy highlighted terms such as nucleic acid metabolic processes
280 and transmembrane transport (Extended Data Figure 7).

281 About half (527) of the 1,043 genes that were consistently differentially expressed in monoicous versus
282 dioicous species had a $|\Delta_x| > 1$, which is significantly more in proportion than among the rest of the ASO
283 (290 genes with $|\Delta_x| > 1$ among 665 ASO, Fisher exact test $P = 0.00543$). This observation indicates that
284 convergent gene expression changes may be associated with directional selection during the switch to
285 monoicy.

286 We next tested whether sexual selection potentially occurring in males and females of dioicous species
287 would be relaxed in monoicous individuals. This would translate by a reduction of purifying selection
288 resulting in increased sequence divergence (increased d_N/d_S). Convergent genes (i.e., genes exhibiting a
289 convergent pattern of gene expression in monoicous species) tended to exhibit faster divergence rates
290 compared with non-convergent genes although the difference was not significant (permutation t -test, $P =$
291 0.0566 ; Extended Data Figure 8). Noteworthy, male-biased (but not female-biased) genes showed
292 significantly higher d_N/d_S than unbiased genes (Supplementary Table 16).

293 A likelihood ratio test of branch models (after Benjamin-Hochberg correction for multiple testing),
294 identified 689 orthologs under positive selection on monoicous branches, 404 of which exhibited
295 convergent gene expression changes. Orthologs under positive selection were over-represented among
296 genes with convergent gene expression (Fisher exact test, $P = 0.025$). Taken together, these observations
297 suggest that directional selection plays a role in driving changes in expression patterns during transitions
298 to co-sexuality.

299 DISCUSSION

300 *SEXUAL DIMORPHISM AND SEX-BIASED GENE EXPRESSION ARE UNCOUPLED*

301 Morphological and physiological differences between males and females are ultimately due to
302 divergences between sex chromosomes in species with genetic sex determination²⁷, but the majority of
303 morphological sexual dimorphism is thought to be associated with autosomal sex-biased gene expression
304 ^{9–11}. Thus, it would be expected that species showing more prominent differences in morphology
305 between male and female would also be characterised by high levels of sex-biased gene expression, as
306 has been shown to be the case in birds ³⁷. Our study, in contrast, revealed no correlation between the
307 level of sex-biased gene expression and the degree of phenotypic sexual dimorphism in the brown algae
308 studied here. Therefore, the link between gene expression evolution and sexual selection is uncertain for
309 these organisms, and sexual selection is likely not to be the main driver of the sex-biased gene expression
310 evolution. This observation may reflect a lower degree of sexual selection in the brown algae compared
311 with animals. Brown algae have relatively low levels of sexual dimorphism ^{15,29} and are broadcast
312 spawners so the opportunities for mate choice and/or mating competition are mainly constrained to
313 interactions involving male and female gametes ⁴². Consistent with the idea that gamete sexual selection
314 may occur, it has been shown recently that in the absence of males, female gametes of brown alga
315 populations lose their sexual morphological characteristics, e.g. female gametes produce lower levels of
316 pheromone and engage in parthenogenesis more rapidly ⁴³. Noteworthy, sex-biased genes found in male
317 and female gametophytes of the model brown alga *Ectocarpus* show more rapid rates of divergence
318 across species (measured as dN/dS) compared to unbiased genes, and their accelerated evolution has
319 been, at least partly, attributed to positive selection ¹⁵. These observations suggest that sexual selection
320 plays a role in the evolution of sex-biased genes in brown algae, but may not be the only driver of sex-
321 biased gene expression in this group of organisms.

322 *SEX-BIASED GENES EXHIBIT FUNCTIONAL CONVERGENCE*

323 Although dioicy is predicted to be the ancestral sexual system in brown algae ³² our results clearly indicate
324 that sex-bias in the expression of individual genes is neither ancestral nor convergent. We found a very
325 limited level of shared (ancestral) sex-biased gene expression across the studied brown algal species, and
326 instead our data is consistent with lineage-specific recruitment of sex-biased genes. Our observations
327 emphasize therefore a substantial turnover of sex-biased expression among brown algal genes.

328 Interestingly, our study suggests that sex-biased expression may have emerged on genes that were
329 experiencing lower selective constraints on their expression level, possibly due to lower pleiotropy, in
330 addition to the potential effect of sex-specific selection occurring after the evolution of separate sexes. A
331 similar situation has been described recently in plants ³⁶ and animals ³⁹.

332 Although the dioicous brown algal species studied here shared very few sex-biased genes, we found some
333 level of convergence in terms of sex-biased gene function, at least for a subset of the sex-biased genes.
334 These include biological functions that were previously found to be enriched in *Ectocarpus* gametophytes
335 ^{15,44}, further underscoring the conservation of sex-biased gene function and supporting primary sexual
336 dimorphic roles. These functions may be associated with sex-specific biological processes. For example,

337 enrichment in oxidation-reduction functions may relate to the more conspicuous growth of female
338 gametophytes producing larger gametes which secrete a sperm-attracting pheromone, whereas cilia and
339 ion transport functions are likely associated to the production of fast-swimming, bi-flagellated sperm by
340 male gametophytes. Considering that brown algae share an ancestral sex chromosome, and that genes
341 within the non-recombining sex determining region play a role in sex ⁴⁵, one possibility is that sexual
342 characteristics in these UV systems mainly involve genes within the SDR together with a relatively limited
343 number of autosomal genes involved in primary sexual dimorphisms. In other words, differences between
344 sexes arise mainly from the different physiological processes directly linked to the production of male or
345 female gametes rather than extensive sexual selection, sexual specialization and/or sexual antagonism (i.
346 e, secondary sexual dimorphism) ¹².

347 *FATE OF SEX-BIASED GENE EXPRESSION DURING TRANSITION TO MONOICY*

348 Our sampling of species distributed across the brown algae phylogeny, associating pairs of related
349 dioicous and monoicous species, allowed us to trace the fate of sex-biased gene expression during
350 independent events of transition from dioicy to monoicy. With the exception of one species pair, sex-
351 biased genes exhibited adaptive expression shifts during the transition to monoicy. Male-biased genes,
352 specifically, were the main drivers of gene expression changes during the transition to monoicy, while
353 unbiased genes exhibited limited changes in pattern of expression with the switch in sexual system. In the
354 model brown alga *Ectocarpus*, RNA-seq analysis of multiple tissues and life cycle stages indicated that sex-
355 biased genes have restricted patterns of expression, which is a proxy for limited pleiotropy ¹⁵. Pleiotropy
356 is known to restrict gene evolution, imposing stricter functional constraints on pleiotropic genes ^{39,46}. The
357 reduced pleiotropy of sex-biased compared with unbiased genes may increase their propensity to
358 adaptively shift towards their evolved optimal expression profile during evolutionary transitions, in this
359 case the transition to monoicy ^{10,39,47}.

360 Sex-biased genes in dioicous brown algae such as *Ectocarpus* sp. typically display high evolutionary rates
361 compared to unbiased genes due either to directional selection or relaxed purifying selection ¹⁵. With the
362 transition to monoicy, increased relaxation of sex-specific purifying selection acting on sex-biased genes
363 may be expected, leading to increased rates of sequence evolution. Accordingly, male-biased genes for all
364 species pairs presented faster evolutionary rates (although not significant for all species) during the
365 switch to monoicy, compared with female-biased or unbiased genes. This observation points to a shared
366 process of sexually antagonistic selection within dioicous species, especially in males, that allowed for
367 faster evolutionary rates of male-biased genes when relaxed during the transition from dioicy to monoicy.

368 *CONVERGENT CHANGES DURING BREAKDOWN OF DIOICY*

369 Convergent evolution, where a similar trait evolves in different lineages, provides an opportunity to study
370 the repeatability of evolution. In the brown algae, co-sexuality has repeatedly emerged from uni-sexual
371 ancestors ³². We found that more than half (61%) of the orthologs across the four pairs of species
372 displayed similar expression shifts concomitant with the transition to monoicy, indicating that common,
373 independently acquired mechanisms are associated with co-sexuality. Remarkably, a substantial number
374 of these convergent genes (38.7%) were under positive selection, underlying the idea that convergent
375 changes associated with the shift of sexual system may be driven by comparable evolutionary pressures

376 across these distant species. Monoicous gametophytes were more closely related to females of the
377 corresponding dioicous species counterpart, suggesting, as proposed in volvocine algae⁴⁸, that monoicy
378 may have arisen from ancestral females.

379 In our study, the expression profiles of gametophytes of all four monoicous species resembled those of
380 the female gametophytes of their dioicous counterpart. Moreover, sex-biased genes tended to maintain
381 the level of expression they had in dioicous species, suggesting that they retained their ancestral function
382 in the context of derived monoicy. When their expression shifted, sex-biased genes, and especially male-
383 biased genes showed signs of selection acting on their expression level to a greater extent than it acted
384 on unbiased genes. Together, our results demonstrate that common mechanisms underlie the transition
385 to monoicy across distant brown algal lineages and suggest that independent events of loss of dioicy may
386 have involved acquisition of genes related to male development by a female gametophyte. The work
387 presented here establishes therefore a framework for understanding at the genomic level how co-sexual
388 systems arise from ancestral haploid UV sexual systems in the brown algae.

389 MATERIALS AND METHODS

390 *SAMPLE PREPARATION, RNA EXTRACTIONS AND SEQUENCING*

391 The algal strains used and sequencing statistics and BioProject accession number are listed in
392 Supplementary Table 1. Gametophytes of all eight species were cultured at 13 °C in autoclaved natural
393 sea water (NSW) supplemented with half-strength Provasoli solution (PES;⁴⁹) with a light:dark cycle of
394 12:12 h (20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) using daylight-type fluorescent tubes⁵⁰. All manipulations were
395 performed under a laminar flow hood in sterile conditions. Immature gametophytes (i.e., absence of sex-
396 specific reproductive structures, oogonia or antheridia) of each strain were frozen in liquid nitrogen and
397 kept at -80C until RNA extraction.

398 RNA from male and female pools was extracted from triplicate samples (each containing at least 800
399 individual gametophytes; except for *S. polyschides* and *S. dermatodea* where two replicates were used)
400 using Qiagen RNA extraction Plant Mini kit. RNA quality and quantity were assessed using an Agilent 2100
401 bioanalyzer, associated with an RNA 6000 Nano kit. For each replicate, the RNA was quantified and cDNA
402 was synthesized using an oligo-dT primer. The cDNA was fragmented, cloned, and sequenced by FASTERIS
403 (CH-1228 Plan-les-Ouates, Switzerland), using Illumina Hi-seq2000 for *Saccorhiza* and *Desmarestia*
404 species; by Genome Quebec using an or Nextgen6000 for *Halopteris* and *Chordaria* species; and by
405 Genoscope using Illumina Hi-seq 4000 for *Sphacelaria* and *Sphaerotrichia* species (see Supplementary
406 Table 1 for details).

407 *TRANSCRIPTOME ASSEMBLIES AND GENE SET PREDICTIONS*

408 Predicted gene sets were constructed for each species base on genome and transcriptome assemblies. In
409 order to filter out potential contamination, first round assembled contigs were blasted against the NCBI
410 non-redundant (nr) protein database using diamond v 0.9.21⁵¹ and reads that mapped on contigs with
411 non-eukaryotic taxons were removed using blobtools v 1.0.1⁵². *De novo* transcriptomes were assembled
412 using Trinity (*Saccorhiza polyschides*, *Saccorhiza dermatodea*, *Desmarestia dudresnayi*, *Desmarestia*

413 *herbacea* female, *Halopteris paniculata*, *Sphacelaria rigidula*) or rnaSPADES v 3.12.0 (*Chordaria linearis*,
414 *Sphaerotrichia firma*) with kmer size of 55.

415 All genomes were soft-masked using Repeatmasker v 4.0.9 after building a *de novo* transposable
416 elements and repeats database with RepeatModeler v 1.0.8⁵³. BRAKER2⁵⁴ and PASA (for *Desmarestia*
417 *herbacea*⁵⁵, using input predicted protein from the reference species *Ectocarpus* sp.
418 (EctsiV2_prot_LATEST.tfa⁵⁶, were used to predict gene sets used for all downstream analyses.

419 The final assemblies are available in NCBI (BioProject accession number PRJNA733856). Transcriptome
420 completeness was assessed using BUSCO v3 eukaryote gene set as reference (Odb9). Transcripts that had
421 DNA data support for only one sex (potentially sex-linked) were tested with PCR using at least 4 males and
422 4 females per species and removed from the sex-biased gene analysis. PCR primers are detailed in
423 Supplementary Table 17.

424 *EXPRESSION QUANTIFICATION AND INFERENCE OF SEX-BIASED GENES*

425 RNAseq reads adaptors were trimmed using trimmomatic v0.38⁵⁷ which was also used for read-quality
426 filtering: reads were removed if the leading or trailing base had a Phred score <3, or if the sliding window
427 Phred score, averaged over four bases, was <15. Reads shorter than 36 bases were discarded (as well as
428 pair of reads, if one of the pair was <36 bases long). Trimmomatic-processed RNAseq reads from each
429 library were used to quantify gene expression with kallisto v 0.44.0⁵⁸ using 31 bp-long kmers and
430 predicted transcript of each species. RNAseq libraries were composed of stranded (--fr-stranded or --rf-
431 stranded option) single-end reads (--single option) or paired-end reads (Supplementary Table 1). A gene
432 was considered expressed in a given species and/or a given sex when at least one library displayed an
433 expression level (in TPM) above the 5th percentile of TPM distribution across all genes and libraries within
434 a species and sex. Following⁵⁹, transcript abundances were then summed up within genes and multiplied
435 by the total library size, using the tximport package³⁵ to obtain the expression level for each gene in
436 transcripts per million reads (TPM).

437 Estimates of sex-biased gene expression in dioicous species were obtained using read count matrices as
438 input for the DESeq2 package (Love et al., 2014) in R 3.6.3. *P*-values were corrected for multiple testing
439 using Benjamini and Hochberg's algorithm in DESeq2, applying an adjusted *P*-value cut-off of 0.05 for
440 differential expression analysis. In addition, only genes with a minimum of 2-fold change expression level
441 between sexes were retained as sex-biased.

442 *QUANTIFICATION OF PHENOTYPIC SEXUAL DIMORPHISM*

443 Individual gametophytes from each of the strains were isolated in sea water and observed using an
444 inverted transmitted light microscope DMi8 (Leica) with the LAS X software. Between 269 and 556 cells
445 (348 cells on average per sex and per species) across five different gametophytes per species were
446 individually measured using Fiji⁶⁰. We used *t*-tests to compare cell length between groups. The
447 difference in mean cell length between sexes of dioicous species was computed and used as a proxy for
448 phenotypic sexual dimorphism. To investigate the relationship between phenotypic sexual dimorphism
449 and extent in sex-biased expression, phenotypic dimorphism was regressed against the fraction of sex-

450 biased genes in R, corrected for phylogeny using phylogenetic generalized least square method as
451 implemented in the nlme R package⁶¹.

452 *ORTHOLOGY AND EVOLUTIONARY RATES WITHIN SPECIES PAIRS*

453 We inferred pairwise single-copy orthologs (PSO) within the four species pair using Orthofinder with
454 default parameters⁶². We used kallisto v 0.44.0 to quantify expression levels for PSO within species pairs.

455 In order to infer the potential role of selection in expression changes between dioicous and monoicous
456 species we computed Δ_x . To summarize, we calculated $\Delta_x = d / r$ with d and r respectively given by:

$$457 \quad d = \text{Mean } X_{\text{dioicous}} - \text{Mean } X_{\text{monoicous}} / \text{Mean } X_{\text{dioicous}}$$

458 and

$$459 \quad r = [X_{\text{dioicous}}]_{\text{high}} - [X_{\text{dioicous}}]_{\text{low}} / \text{Mean } X_{\text{dioicous}}$$

460 where X is the expression level measured in TPM, 'High' and 'Low' represent the maximum and minimum
461 values. Δ_x was computed separately for females and males of the dioicous species, and for male-biased
462 genes, female-biased genes and unbiased genes also separately. Orthologs with $|\Delta_x| > 1$ and a minimum
463 expression fold-change between sister species of 1.5 were considered to have had a significant
464 evolutionary expression shift. Fisher exact tests were computed to detect whether female-biased genes
465 (FBG) and male-biased genes (MBG) were more likely to show an absolute value of $\Delta_x > 1$ compared to
466 unbiased genes.

467 Orthologous proteins between species pairs were aligned with MAFFT v7.453⁶³ the alignments were
468 curated with Gblocks v0.91b⁶⁴ and back-translated to nucleotides using translatorX⁶⁵. We used these
469 nucleotide alignments as input for phylogenetic analysis by maximum likelihood (PAML4, CodeML,⁶⁶) to
470 infer pairwise d_N/d_S (ω) with F3x4 model of codon frequencies. We retained orthologs with $0 < d_S < 2$ as
471 valid for further analysis. We compared species and sexes evolutionary rates separately for female-
472 biased, male-biased and unbiased genes, using permutation t -tests in R with 100,000 permutations.

473 *EVOLUTION OF SEX-BIASED GENE EXPRESSION*

474 We inferred a single orthologous gene set for the four dioicous species (DSO) using Orthofinder with
475 default parameters. Following the methods used in⁶⁷ we included in the DSOs the orthogroups genes that
476 were 1:1:1:0, likely due to situations in which a single-copy ancestral gene was lost in a single species. To
477 further account for gene prediction errors, we also included orthogroups with a single species presenting
478 two-genes that aligned on more than 60% of their length as duplicate genes. In the latter case, the
479 longest duplicated sequence was retained for further analysis.

480 A well resolved phylogeny of the Pheophyceae was used as reference gene tree³⁴ to infer where sex-
481 biased gene expression evolved along the phylogenetic tree. We coded DSO as either male-biased,
482 female-biased or unbiased for each species and used the ape package⁶⁸ in R to reconstruct the discrete
483 ancestral state *via* maximum likelihood. Proportions of ancestral genes in each category were plotted as
484 pie-charts on tree nodes and gain/loss of bias were reported on each branch. We further tested the

485 significance of overlap between sex-biased genes identified within dioicous species with exact multi-set
486 intersection test implemented in the SuperExactTest package v 1.0.7 in R⁶⁹.

487 We computed absolute standardized phylogenetic independent contrasts (PICs) among dioicous species,
488 using the *ape* package in R. Mean PICs were compared using Mann-Whitney Rank tests, between
489 unbiased genes and sex-biased genes with their expression measured in species in which they were not
490 sex-biased.

491 We inferred expression profile similarity index between monoicous species and males and females of
492 dioicous species within pairs as the Pearson correlation coefficient of PSO expression levels in $\log_2(\text{TPM} +$
493 $1)$. This analysis was performed for all expressed genes, and separately for MB, FB and unbiased genes.
494 We compared Pearson coefficients of regression within each species pair, using the *cocor* package⁴⁰,
495 considering gene expression profiles of males and females as independent gene sets. We also compared
496 SBG with unbiased genes within sexes, considering these gene sets as dependent. We report the *P*-value
497 based on Fisher's *z* or, when possible, Silver, Hittner and May's modification of Dunn and Clark's *z*.
498 Pearson's coefficients were plotted for each species pair.

499 *CONVERGENT EXPRESSION CHANGES*

500 Convergent changes associated with transitions to monoicy were investigated on single-copy orthologs
501 inferred across the eight studied species (termed 'All Single-copy Orthologs', ASO) following the same
502 methods as those used for the DSOs. Using this data set, we quantified gene expression with kallisto as
503 described above, and DESeq2 was used to infer orthologs significantly affected by sexual system but not
504 species pair (*lfcShrink* with "ashr" method, sexual system contrast⁷⁰). Significance of the number of
505 convergent expression changes was tested with permutation tests (100,000 permutations). We used the
506 *ComplexHeatmap* package in R to visualize gene expression for each replicate. Orthogroups with
507 inconsistent sex-bias across different species (*n*=139) were removed from the dN/dS analysis of
508 convergent gene evolution.

509 Intersects between genes across PSO, DSO and ASO were represented using the *UpSetR* package v1.4.0
510⁷¹.

511 *ASO EVOLUTIONARY RATES*

512 Following the same process described for pairwise orthologs, we aligned and studied molecular
513 sequence divergence for all species orthologs (ASO) using CodeML. We used a 'two-ratio' branch
514 model (model = 2, Nssites = 0) to specifically study divergence on monoicous branches (foreground
515 branches). We compared ω values separately between sex-biased (male-biased and female-biased
516 genes) and unbiased genes with permutation *t*-tests (10,000 permutations). We also ran two
517 branch-site models in PAML to detect positive selection in foreground branches (model=2, Nssite=2,
518 $\omega=1$ fixed (Null) or allowed to vary). Likelihood-ratio tests were used to compared the model of
519 selection with the null model in order to detect orthologs with sites under positive selection in the
520 monoicous branches. LRT *P*-values were corrected for multiple testing using Benjamini and
521 Hochberg's algorithm⁷².

522 FUNCTIONAL ANNOTATION ANALYSIS

523 Predicted genes and orthogroups were blasted against the NCBI non-redundant (nr) protein database
524 with blast (v2.9.0). Functional annotation was performed using BLAST2GO³⁸, as well as the InterProScan
525 prediction of putative conserved protein domains⁷³. Gene set enrichment analysis was carried out
526 separately for each gene set using Fisher's exact test implemented in the TopGO package, with the
527 *weight01* algorithm⁷⁴. Values were corrected for multiple testing using the Benjamini-Hochberg method
528 in order to control the false discovery rate. We investigated enrichment in terms of biological process
529 ontology and reported significant GO-terms with *P*-value < 0.01. All statistical analyses were performed in
530 R 3.6.3, plots were produced with ggplot2 in R (<https://ggplot2.tidyverse.org/>).

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539 DATA

540 Raw reads have been deposited in the SRA. BioProject accession number is PRJNA733856. Accession
541 codes are given in Supplementary Table 18.

542 AUTHORS CONTRIBUTIONS

543 S.M.C designed the study. Z.C conducted DNA and RNA extractions. C.C. prepared sequencing libraries.
544 O.G assembled transcriptomic gene sets of *S. rigidula*, *S. firma*, *C. linearis* and *H. paniculata*. A.L
545 assembled transcriptomic gene sets and identified V-linked genes of *D. herbacea*. G.G.C. assembled
546 transcriptomic gene sets of *D. dudresnayi*, *S. polyschides* and *S. dermatodea*. G.G.C conducted
547 bioinformatic and statistical analyses. S.M.C. and J.M.C contributed with resources. G.C. and S.M.C wrote
548 the manuscript. All authors approved the final version of the manuscript.

549 COMPETING INTERESTS

550 The authors declare no potential competing interests.

551 FIGURE LEGENDS

552 Figure 1. Diagram of the phylogeny of the eight species of brown algae investigated. Approximate
553 estimated age of nodes is based on³⁴; O. de Clerck pers. communication). A schematic view of typical
554 gamete size differences (female in red, male in blue) per species pair is presented. Dioicous species (D)
555 are marked in brown and monoicous species (M) in black.

556 Figure 2. Patterns of sexual dimorphism in dioicous brown algae. A) Pie charts representing the fractions
557 of sex-biased genes among expressed genes (female-bias in red, male-bias in blue) in the four dioicous
558 species. Gradients of colors represent the intensity of expression fold-change (FC), from 2FC difference to
559 more than 15FC. The percentages are calculated based on the total number of expressed genes averaged
560 across sexes. B) Comparison of gene expression levels, in $\log_2(\text{TPM}+1)$, between males and females
561 within dioicous species. Colour patterns follow the ones used in panel A, except for grey points which
562 represent unbiased genes that presented a $\text{FC} > 2$. C) Scatterplots of the lengths of cells of immature
563 gametophytes of dioicous species. The mean (point) and standard deviations (whiskers) are plotted per
564 sex per species. Stars indicate significant difference between mean cell length, tested with two-sided t-
565 tests. * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$. D) Representative micrographs of male and
566 female immature gametophytes viewed under an inverted light microscope for each dioicous species
567 investigated. Micrographs show individual algae, representative of between 100-200 individuals grown in
568 petri dishes. E) Linear regressions of the fraction of female- and male-biased genes (in red and blue,
569 respectively) among the mean number of expressed genes across both sexes, against the mean difference
570 in cell length recorded between the sexes (in μm), in the four dioicous species investigated. Linear
571 regressions were fitted through phylogenetic generalized least square method, implemented in the R
572 package “nlme”. We report values of adjusted r^2 calculated with ANOVA.

573 Figure 3. Reconstruction of ancestral sex-biased gene sets across the four dioicous species. The number
574 of inferred sex-biased genes (female-bias in red, male-bias in blue) at ancestral nodes as well as the
575 inferred gain and loss of sex-biased genes along branches are displayed.

576 Figure 4. Evolution of sex-biased genes during transitions to monoicy. A) Comparison of gene expression
577 levels within species pairs, in $\log_2(\text{TPM}+1)$, using PSO gene sets. F: females. M: males. Mo: monoicous.
578 The number of female-biased (FBG) and male-biased genes (MBG) among PSO are displayed. Note that
579 only the sex-biased genes with a single-copy ortholog in the corresponding monoicous specie are
580 displayed in the plots (in other words, the SBG represented in the plots are a subset of the SBG identified
581 within each dioicous species). Boxes represent the interquartile range (25th and 75th percentiles) of the
582 data, the line inside the box represents the median, whiskers represent the largest/smallest value within
583 1.5 times interquartile range above and below the 75th and 25th percentile, respectively. Statistical tests
584 are permutation t-tests using 100,000 permutations. Paired two-sided t-test were used for comparisons
585 between sexes of the same species (dioicous species). B) Comparisons of similarity index values (Pearson
586 coefficients) between expression profiles (in $\log_2(\text{TPM}+1)$) of pairwise single copy orthologs (PSOs)
587 between monoicous and dioicous species pairs. The figure represents male versus female similarity
588 indexes in relation to the monoicous expression profiles. Note that similarity index are represented
589 separately for sex-biased genes in females (red) and in males (blue), as well as for unbiased genes
590 averaged across sexes in the dioicous species (black). Pearson coefficients were plotted for each species
591 pair in increasing order of the proportion of SBG among expressed genes of dioicous species (x axis). Stars
592 in the top panel represent significant differences between Pearson coefficients, taking into account the
593 correlations between compared gene sets, using the cocor package in R. Red and blue stars indicate a
594 significant difference between female (red stars) or male sex-biased genes (blue stars) Pearson
595 coefficients with unbiased genes coefficients. Top panel black stars indicate a significant difference of

596 Pearson coefficients of unbiased genes between males and females. Significant differences of coefficients
597 between sex-biased genes in females and males are indicated directly on the plot. * : $0.01 < P < 0.05$; ** :
598 $0.001 < P < 0.01$; *** : $P < 0.001$. See also Figure S4 and S5. C) Fraction of female-biased genes (red),
599 male-biased genes (blue) and non-biased genes (grey) with an absolute value of $\Delta X > 1$ and a fold-change
600 > 1.5 , calculated within species pairs (on PSO). The percentages are calculated on the total number of
601 orthologs in each category. Down-regulated genes in the monoicous species are represented below the
602 $y=0$ line ($\Delta X < -1$), upregulated genes in the monoicous species are represented above the $y=0$ line ($\Delta X > 1$
603). Stars indicate a significant over-representation of female-biased or male-biased genes with an absolute
604 $\Delta X > 1$ compared with the proportion of unbiased genes with $\Delta X > 1$, tested using Fisher exact tests. D)
605 Sequence divergence, measured as dN/dS (ω), between dioicous and monoicous species calculated
606 within species pairs (PSO). Boxes represent the interquartile range (25th and 75th percentiles) of the
607 data, the line inside the box represents the median, whiskers represent the largest/smallest value within
608 1.5 times interquartile range above and below the 75th and 25th percentile, respectively. Statistical tests
609 are permutation two-sided t -tests using 100,000 permutations, p-values are displayed in parentheses. *
610 $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$. E) Principal component analysis (PCA) plot of all the
611 RNA-seq samples, using ASOs. Monoicous species are plotted in orange, female samples in red and male
612 samples in blue.

613

614 SUPPLEMENTARY TABLE LEGENDS

- 615 Supplementary Table 1. Brown algae species used in the study and summary of gene expression data sets.
- 616 Supplementary Table 2. Summary statistics of sex-biased gene expression. Fractions of sex-biased genes
617 are calculated over the number of expressed genes in the sex of bias.
- 618 Supplementary Table 3. Gene expression values in transcripts per million (TPM; from kallisto) and
619 expression bias (from Deseq2) for each of the genes across the four dioicous species.
- 620 Supplementary Table 4. Summary of sexually dimorphic traits in the four dioicous brown algae species
621 investigated in the present study.
- 622 Supplementary Table 5. Orthogroups belonging to the dioicous single-copy orthologs gene set (DSO) and
623 their corresponding gene and bias per species.
- 624 Supplementary Table 6. Description of GO-term enrichment of sex-biased genes identified in each
625 dioicous species. *P*-values correspond to Fisher exact tests (FDR < 0.05). Terms found in two or more
626 species are highlighted in bold.
- 627 Supplementary Table 7. Summary description of all pairwise single-copy orthologs (PSO).
- 628 Supplementary Table 8. Orthogroups belonging to the pairwise single-copy orthologs genes et (PSO)
629 within the Tilopteridales species pair. Mean expression level in TPM across replicates as well as the bias
630 status in the dioicous species are reported.
- 631 Supplementary Table 9. Orthogroups belonging to the pairwise single-copy orthologs gene set (PSO)
632 within the Desmarestiales species pair. Mean expression level in TPM across replicates as well as the bias
633 status in the dioicous species are reported.
- 634 Supplementary Table 10. Orthogroups belonging to the pairwise single-copy orthologs gene set (PSO)
635 within the Sphacelariales species pair. Mean expression level in TPM across replicates as well as the bias
636 status in the dioicous species are reported.
- 637 Supplementary Table 11. Orthogroups belonging to the pairwise single-copy orthologs gene set (PSO)
638 within the Ectocarpales species pair. Mean expression level in TPM across replicates as well as the bias
639 status in the dioicous species are reported.
- 640 Supplementary Table 12. Summary statistics of Δ_x within the four species pairs. Male-biased genes (MBG)
641 and female-biased genes (FBG) that were significantly more likely or less likely to present $|\Delta_x| > 1$ were
642 highlighted in green and purple, respectively (Fisher's exact tests).
- 643 Supplementary Table 13. *P*-values of permutation *t*-tests (100,000 permutations) of sequence divergence
644 data (dN/dS) calculated within species pair, between female-, male-biased and unbiased genes.
- 645 Supplementary Table 14. Summary description of all single-copy orthologs (ASO)

646 Supplementary Table 15. Orthogroups belonging to the all single-copy orthologs gene set (ASO). Mean
647 expression level in TPM across replicates as well as the bias status in the dioicous species are reported.

648 Supplementary Table 16. *P*-values of permutation *t*-tests (10,000 permutations) of sequence divergence
649 data (dN/dS), calculated specifically for monoicous branches (branch model) across ASOs, between
650 female-, male-biased and unbiased genes. Significant difference of divergence with unbiased genes are
651 put in bold.

652 Supplementary Table 17. Primers used to test candidate sex-linked contigs in the different brown algal
653 species.

654 Supplementary Table 18. Accession references.

655

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