Research

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Evolutionary dynamics of sex-biased gene expression in a young XY system: insights from the brown alga genus *Fucus*

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Summary

- Sex-biased gene expression is considered to be an underlying cause of sexually dimorphic traits. Although the nature and degree of sex-biased expression have been well documented in several animal and plant systems, far less is known about the evolution of sex-biased genes in more distant eukaryotic groups.
- Here, we investigate sex-biased gene expression in two brown algal dioecious species, *Fucus serratus* and *Fucus vesiculosus*, where male heterogamety (XX/XY) has recently emerged.
- We find that in contrast to evolutionary distant plant and animal lineages, male-biased genes do not experience high turnover rates, but instead reveal remarkable conservation of bias and expression levels between the two species, suggesting their importance in sexual differentiation. Genes with consistent male bias were enriched in functions related to gamete production, along with sperm competition and include three flagellar proteins under positive selection.
- We present one of the first reports, outside of the animal kingdom, showing that male-biased genes display accelerated rates of coding sequence evolution compared with female-biased or unbiased genes. Our results imply that evolutionary forces affect male and female sex-biased genes differently on structural and regulatory levels, resulting in unique properties of differentially expressed transcripts during reproductive development in *Fucus* algae.

Introduction

Males and females can display striking differences in morphology, physiology, and behavior. Evolution of these sexually dimorphic traits is thought to be rooted in anisogamy and shaped by sex-specific selection (Hedrick & Temeles, 1989; Connallon & Knowles, 2005; Ellegren & Parsch, 2007; Schärer *et al.*, 2012). Ultimately, the sexes are defined by the gamete size they produce (either many small or fewer larger gametes) and sexual selection is predicted to act differently regarding these two distinct reproductive strategies (Kokko & Jennions, 2008; Schärer *et al.*, 2012). Due to the disparity of resources and energy invested by males and females into their reproductive cells, it is hypothesized that sexual selection will be stronger in the sex that makes the smaller, more abundant, and relatively 'cheaper' to produce gametes, resulting in higher levels of selection on male-biased genes (Darwin, 1871; Bateman, 1948; Parker, 1979; Schärer *et al.*, 2012; Andersson, 2019). Because males and females share most of their genomic sequence, the expression of sexually dimorphic traits relies largely on the regulation of sex-biased gene (SBG) expression (Ellegren & Parsch, 2007; Parsch & Ellegren, 2013; Grath & Parsch, 2016).

Sex-biased gene expression has been well documented across a wide number of animal species such as insects (Zha *et al.*, 2009; Perry *et al.*, 2014; Papa *et al.*, 2017), mammals (Yang *et al.*, 2006; Blekhman *et al.*, 2010; Naqvi *et al.*, 2019), birds (Mank *et al.*, 2007; Mank & Ellegren, 2009; Harrison *et al.*, 2015), and recently also in plants (Zemp *et al.*, 2016; Darolti *et al.*, 2018; Cossard *et al.*, 2019; Sanderson *et al.*, 2019; Feng *et al.*, 2020; Scharmann *et al.*, 2021) and brown algae (Martins *et al.*, 2013; Lipinska *et al.*, 2015; Monteiro *et al.*, 2019; Müller *et al.*, 2021). It has been shown that SBG expression can vary in strength throughout development, can be detected already at juvenile stages (Thoemke *et al.*, 2005; Magnusson *et al.*, 2011; Ingleby *et al.*, 2014; Perry *et al.*, 2014; Lipinska *et al.*, 2015), and can constitute a large proportion of the transcriptome, with up to 90% in extreme cases (Ranz *et al.*, 2003; Ayroles *et al.*, 2009). Genome-wide expression studies have found that the properties of sex-biased genes differ between the sexes, where male-biased genes show stronger bias, more rapid turnover rates and, at least

in animals, greater evidence of relaxed purifying selection compared with female-biased genes or unbiased genes (Parisi *et al.*, 2003; Ranz *et al.*, 2003; Yang *et al.*, 2006, 2016; Voolstra *et al.*, 2007; Zhang *et al.*, 2007; Martins *et al.*, 2013; Parsch & Ellegren, 2013; Harrison *et al.*, 2015). In dioecious plants, sex-biased genes experienced faster evolution of gene expression levels and high turnover rates between species, but no evidence of higher divergence rates of protein-coding sequences has been found so far (Zemp *et al.*, 2016; Cossard *et al.*, 2019; Sanderson *et al.*, 2019; Feng *et al.*, 2020; Scharmann *et al.*, 2021). Moreover, studies in willow (*Salix viminalis*) found reduced rates of sequence evolution in male-biased genes compared with unbiased genes, which was attributed to haploid purifying selection (Darolti *et al.*, 2018). In turn, male-biased genes in animal species were found to evolve rapidly due mainly to relaxed selective constraint rather than adaptive evolution (Gershoni & Pietrokovski, 2014; Harrison *et al.*, 2015; Sayadi *et al.*, 2019). By contrast, female-biased genes often evolve at similar or slower rates compared with unbiased genes possibly due to larger pleiotropic constraints (Ellegren & Parsch, 2007; Zhang *et al.*, 2007; Assis *et al.*, 2012). Altogether, these observations suggest that male traits experience stronger sexual selection and sexual conflict arising from anisogamy (Ranz *et al.*, 2003; Connallon & Knowles, 2005; Hayward & Gillooly, 2011; Janicke *et al.*, 2016). However, our knowledge about the evolution of sex-biased expression is limited, mainly, to the animal species with conspicuous sexual dimorphism and where separate sexes evolved a long time ago.

Here, we study the evolution of sex-biased gene expression in two brown algal species from the order Fucales, which has recently evolved separate sexes (Serrão *et al.*, 1999; Coyer *et al.*, 2006; Heesch *et al.*, 2021). Brown algae are an interesting group to study the evolution of sexual systems and sex-biased expression because they have been evolving independently of organisms such as animals, fungi, and plants for over a billion years (Baldauf, 2003). The majority of brown algal species engage in a haploid–diploid life cycle where sex is expressed during the haploid gametophyte generation and controlled by haploid sex chromosomes (UV system; Coelho *et al.*, 2018). In that respect, Fucales are unique among the brown algae as they represent the only group that underwent a recent shift toward a diplontic life history, in which the short-lived male sperm and female egg are the only haploid stages (Coelho *et al.*, 2019). Moreover, the conversion to diploidy imposed a switch from the haploid UV (via a hermaphroditic intermediate) to the diploid sex-determination system, in several families of Fucales *c.* 17.5 Ma (million years ago) (Heesch *et al.*, 2021). While the transition to diploid sex determination from the haploid system seems to be irreversible, further transitions toward hermaphroditism within the diploid lineages are still possible and occurred independently in several genera of the Fucaceae (Heesch *et al.*, 2021).

Fucus species have a rather simple structure with the vegetative body consisting of a holdfast, a thallus, and the fronds. The fronds contain reproductive receptacles which in dioecious species bear either antheridia (producing motile sperm) or oogonia (producing immotile, large eggs; Serrão *et al.*, 1999; Coyer *et al.*, 2006; Cánovas *et al.*, 2011; Fig. 1). The eggs produce pheromones which facilitate gamete–gamete recognition by attracting sperm within a very short distance (Müller & Gassmann, 1985) and fertilized zygotes usually settle within one to two meters of the parent (Arrontes, 1993; Serrão *et al.*, 1997). The different reproductive structures are the only visible sexually dimorphic trait in *Fucus* in the absence of detailed morphometric measures, so that dioecious species are sexed solely by the presence of male or female gametes (Coyer *et al.*, 2002). In the case of hermaphrodite species, the same receptacle encloses both, antheridia and oogonia, at the same time (Whitaker, 1931).

In this study, we focused on the dioecious species of two distinct lineages, *Fucus serratus* and *Fucus vesiculosus* (Supporting

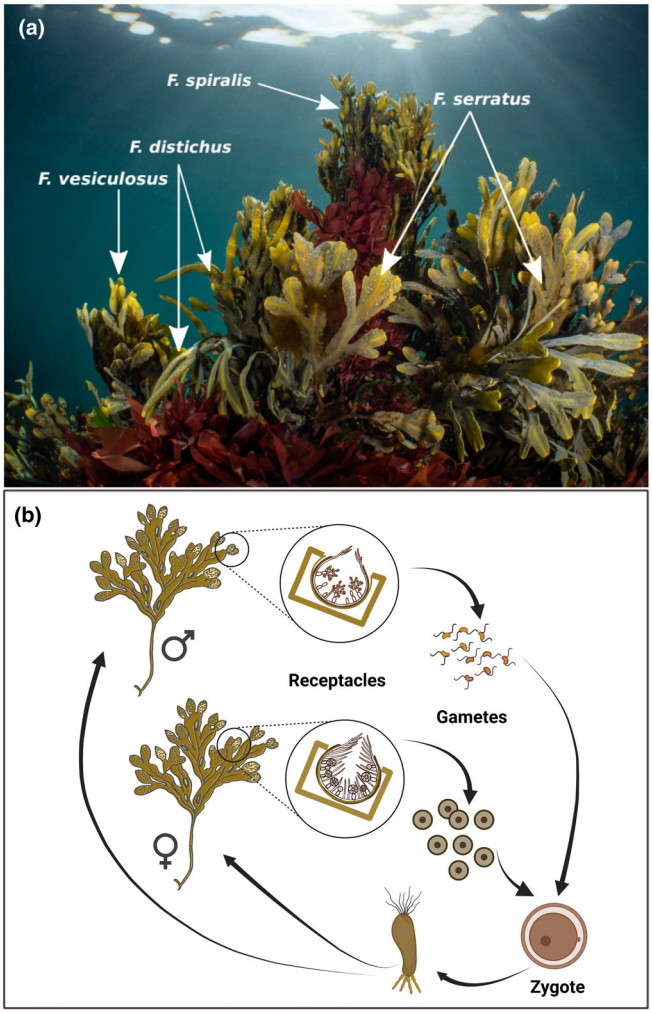

Fig. 1 *Fucus* species co-occurring in their natural habitat. (a) *Fucus spiralis* (top), *Fucus distichus* (center-left), *Fucus serratus* (center-right), and *Fucus vesiculosus* (bottom-left) living in sympatry. (b) Diplontic life cycle of dioecious *Fucus*. Gametes are produced in the receptacles of males and females from which they are then released into the water column. Fertilization is external, the developing zygote attaches to the substrate and the germlings develop into male and female individuals. Diplontic life cycles occur within the Fucales, whereas in most other brown algae with haploid–diploid life cycles, a free-living diploid stage (sporophyte) alternates with a free-living haploid stage (gametophyte). Photo credit G. Hoarau (a); image created with BioRender (b).

Information Fig. S1), that dominate the rocky intertidal North Atlantic shoreline. The two lineages evolved *c.* 0.9–2.25 Ma, and both contain hermaphroditic species, including *Fucus distichus* and *Fucus spiralis* (Fig. S1; Serrão *et al.*, 1999; Coyer *et al.*, 2006; Hoarau *et al.*, 2007). All four species often occur intertwined with one another (Fig. 1a), and molecular studies have shown that hybridization is common, involving dioecious-hermaphrodite species pairs within each lineage, but hybrids of dioecious species are almost never found (Coyer *et al.*, 2002, 2007; Wallace *et al.*, 2004; Billard *et al.*, 2005; Hoarau *et al.*, 2015). Character mapping analysis suggested dioecy as the most likely ancestral sexual system in the *Fucus* genus; however, the direction of transition between hermaphroditism and separate sexes within the two lineages remains ambiguous (Heesch *et al.*, 2021; Fig. S1).

Field observations and laboratory crosses of *F. serratus*–*F. distichus* hybrids allowed the identification of the type of sexual system in dioecious species as a male heterogamety (XX/XY; Coyer *et al.*, 2002). Combined with the low levels of selfing, almost 100% fertilization success in dioecious species and effective polyspermy block (Bolwell *et al.*, 1977; Brawley, 1992; Pearson & Brawley, 1996; Serrao *et al.*, 1996; Coyer *et al.*, 2002), these observations suggest that the targets of reinforcement and speciation in *Fucus* involve gamete attraction and/or recognition genes. Moreover, high levels of sperm competition in marine free spawners like *Fucus* imply there is strong selection pressure on the males for reproductive success as species in sympatry have increased sperm specificity (Hoarau *et al.*, 2015).

In this work, we explore male and female transcriptomic data of *F. serratus* and *F. vesiculosus*, which recently evolved dioecy, to elucidate the early stages of the evolution of sex-biased gene expression. We study evolutionary dynamics of sex-biased transcriptome expression, investigate the correlation of gene expression patterns between the two algal species, and identify sex-biased genes with signatures of positive selection in this relatively young XX/XY system.

Materials and Methods

Sampling

Reproductively mature *F. serratus* Linnaeus, *F. vesiculosus* Linnaeus, *F. distichus* Linnaeus, and *F. spiralis* Linnaeus were collected from the intertidal shoreline at Mjelle, Norway (67°24′47.3″N, 14°37′49.3″E) in May 2017 (Table S1). The dioecious species were sexed by confirming the presence of antheridia (male) or oogonia (female) in the receptacles. Receptacles and small segments of vegetative tissue were dissected from both hermaphroditic and dioecious individuals and stored at −80°C, and then freeze-dried using a VirTis Bench Top K Freeze Dryer before RNA extraction.

RNA extraction, library preparation, and sequencing

Heterogeneous tissue and variation in cellular composition can impact RNA abundance between groups of samples and contribute to large differences in gene expression that could be misinterpreted as regulatory differences (Montgomery & Mank, 2016; Hunnicutt *et al.*, 2022). Specifically, inferences from comparative bulk RNA-Seq approaches obtained from homogenized whole bodies can introduce biases in inferred differential expression profiles. To circumvent these biases, we reduced sample complexity and dissected the reproductive organs from vegetative tissue to detect sex-biased genes and reproductive tissue genes with more confidence.

Total RNA was extracted from 5 mg of freeze-dried sample from reproductive and vegetative tissue from three different male and female individuals of both dioecious species *F. serratus* and *F. vesiculosus* and from three *F. spiralis* and *F. distichus* individuals as described in Pearson *et al.* (2006). Samples were purified with the ZR-96 RNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA), and potential PCR inhibitors were removed with the OneStep-96™ PCR Inhibitor Removal Kit (Zymo Research). RNA concentrations were quantified with the Qubit RNA Assay Kit (Life Technologies, Paisley, UK) and tested for both quantity and integrity using RNA screen tape (Agilent Technologies, Waldbronn, Germany) on the Agilent 2200 Tapestation.

Libraries were prepared from 1 μg RNA using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and sequenced on the Illumina NextSeq 500 (150-bp pair-end reads), using the NextSeq 500/550 High Output Kit v.2.5 (300 Cycles).

RNA-Seq analysis and *de novo* reference transcriptome assembly

Sequencing data were demultiplexed using the Bcl2Fastq Conversion Software (v.2.20; Illumina, San Diego, CA, USA). Raw sequences were adapter- and quality-trimmed with Trimmomatic (v.0.33; Bolger *et al.*, 2014), followed by a quality check using FastQC (v.0.11.4; Andrews, 2010). Before *de novo* transcriptome assembly, the reads were normalized to reduce redundancy of overrepresented sequences, using Trinity's *in silico* read normalization (v.2.8.5). A reference transcriptome per species was generated (all replicates and conditions combined), using Trinity's *de novo* assembly (Grabherr *et al.*, 2011; Haas *et al.*, 2013). Isoforms were collapsed into single gene sequences using a Trinity_gene_splice_modeler.py script (Trinity toolkit).

The predicted genes generated from the *de novo* assembly were then blasted against a custom bacterial/reference genomes database to identify and eliminate bacterial contamination. The longest open reading frames (ORFs) were constructed using Transdecoder (v.5.5.0; Haas *et al.*, 2013). The ORFs were then blasted against an in-house heterokont database and a standard UniProt and Pfam database to keep the most likely ORFs. Transdecoder. Predict was used to predict the best coding regions with homology search results (Pfam and heterokont results) and genes without a coding region of at least 100 bp were removed from the dataset. Trinity's Cd-hit-est (v.4.6; Li *et al.*, 2001) clustered genes with predicted ORFs to further reduce the number of redundant sequences, thus

generating the final reference gene sets for each species. Transcript abundances were then quantified using KALLISTO (Bray *et al.*, 2016) with 1000 bootstraps and represented as TPM (transcript per million). Genes with $\log_2(\text{TPM} + 1) < 1$ were considered not expressed.

ORTHOFINDER (v.2.3.3; Emms & Kelly, 2019) was used to find orthologous genes between all four *Fucus* species (Table S1). We used orthogroups with single and/or multicopy-genes to study global patterns of conservation of sex-biased expression in the dioecious species pair; and orthogroups with strictly single copy genes for the evolutionary and comparative expression analyses. Orphan genes (i.e. taxonomically restricted genes) were defined as genes present in the reference transcriptome of only one species and having no BLASTP match (10 − 04e-value cutoff) in the other *Fucus* species.

Differential gene expression analysis

Differential gene expression within species (between sexes and tissue types) was tested with the DESEQ2 (BIOCONDUCTOR v.3.9; Love *et al.*, 2014). Genes with fold change (FC) ≥ 2 and FDR-adjusted *P*-values $P_{adj} < 0.05$ were considered significantly differentially expressed.

Phylogenetic analysis

Phylogenetic trees of the four *Fucus* species, *Pelvetia canaliculata* and *Ascophyllum nodusum* were generated using a set of 32 nuclear protein-coding genes used previously to construct a Phaeophycean species tree (Akita *et al.*, 2022). CLUSTAL-OMEGA (v.1.2.4) was used to align the sequences which were then quality checked for missing data (> 90%) and converted to nexus format using a custom python script. IQ-TREE (v.1.6.1) was used to infer phylogenetic trees (−bb 1000). ASTRAL (v.5.7.1) was then used to search for the tree with the highest consensus in both bootstrap trees and maximum likelihood trees and were then visualized using FIGTREE (v.1.4.4).

Evolutionary analysis

Amino acid sequences of the single-copy orthologs of *F. serratus*–*F. distichus* and *F. vesiculosu*–*F. spiralis* were aligned using MAFFT (v.7.450; Katoh *et al.*, 2002) and translated back to nucleotide alignments using PAL2NAL (v.14; Suyama *et al.*, 2006). The alignments were trimmed using Gblocks with a minimum block length of 20. In order to remove poorly aligned sequences that could bias the evolutionary analysis, we realigned all the FASTA files with EMBOSS WATER (v.6.6.0; Madeira *et al.*, 2019) and removed alignments with < 80% similarity. The remaining high-quality, gapless alignments exceeding 100 bp in length were retained for pairwise dN/dS (ω) analysis using YN00 method in PAML4 (F3x4 model of codon frequencies; Yang & Nielsen, 2000; Yang, 2007). The difference in mean dN/dS value between SBGs and unbiased genes was assessed by 10 000 permutations using a custom R function (R Core Team, 2020).

The positive selection analysis was carried out using CODEML (PAML4, F3x4 model of codon frequencies) using single-copy orthologs of the four *Fucus* species and two other brown algal species (*Ectocarpus* sp.; Cock *et al.*, 2010) and *Sacchraina japonica* (Ye *et al.*, 2015). Gapless alignments longer than 100 bp containing sequences from all six species were retained for subsequent analysis. We applied two branch-site models implemented in CODEML PAML4 (Yang, 2007): a null model (H0, model = 2, NS sites = 2, fix_omega = 1), in which the branch of interest (foreground branch) may have different proportions of sites under neutral selection than the background (i.e. relaxed purifying selection), and an alternative model (H1, model = 2, NSsites = 2, fix_omega = 0), in which the foreground branch may have a proportion of sites under positive selection. The outputs of the two models (H0 and H1) were compared using the likelihood ratio test. *P*-values under chi-squared distribution with the degree of freedom equal 1 and FDR correction were calculated using P_{chisq} and *P*.adjust functions in R (R Core Team, 2020).

Euclidean distances were estimated for all single-copy orthologs between *F. serratus* and *F. vesiculosus* following the approach of (Pereira *et al.*, 2009). The following formula was used:

$$\text{EucD} = \sqrt{\sum_{j=1}^{k} \left(x_{1j} - x_{2j}\right)^2}$$

where x_{ij} is the expression level of the gene under consideration (TPM) in species i (i.e. species 1 or species 2) during stage j and k is the total number of stages (i.e. four, male and female individuals, reproductive and vegetative tissues). All statistical analyses were performed using RSTUDIO (R v.3.6.3).

Gene ontology analysis

EGGNOG v.5.0 (Huerta-Cepas *et al.*, 2019) was used to perform functional annotation of *F. serratus* and *F. vesiculosus* genes. We used TOPGO package in R (Alexa & Rahnenfuhrer, 2020) to detect enrichment of specific GO terms in sex-biased genes (Fisher's exact test with a *P*-value cutoff of 0.05).

Results

Transcriptome assembly and analysis of gene expression

We sequenced reproductive and vegetative tissue from males and females of dioecious *F. serratus* and *F. vesiculosus* and hermaphroditic *F. distichus* and *F. spiralis*. We obtained a total of 478 million reads from two sequencing runs with an average of over 21 million reads per tissue type and species (Table S1). The *de novo* assembled reference transcriptome for each species contained 29 610 genes for *F. vesiculosus* and 39 009 genes for *F. serratus* (Table S1, see the 'Materials and Methods' section for details) after filtering out the transcripts with low expression or high similarity to other transcripts. BUSCO v.3 (Waterhouse *et al.*, 2018) estimated completeness of each reference transcriptome at 88.8% for *F. vesiculosus* and 92.4% for *F. serratus* (Table S1).

Sex-biased gene expression

Genes with significant sex-biased expression (FC ≥ 2, $P_{adj} < 0.05$ (FDR-adjusted *P*-value)) were identified in two comparisons, male reproductive vs female reproductive tissue and male vegetative vs female vegetative tissue, using the DESeq2 R package (Love *et al.*, 2014; Tables S2, S3). As expected, the greater number of sex-biased genes (SBGs) was found in the reproductive tissue when male vs female receptacles were compared (2993 and 2772 genes in *F. serratus* and *F. vesiculosus*, respectively; Fig. 2a). By contrast, in vegetative tissues, only 20 and 22 genes were sex-biased in *F. serratus* and *F. vesiculosus*, respectively (Tables S2, S3). Since the sex-biased genes from the vegetative tissue overlapped largely with those from the reproductive tissue, we decided to focus on the latter in all consecutive analyses on sex-biased gene expression.

We found more male-biased genes (MBGs) than female-biased genes (FBGs) in both species (2315 MBGs vs 678 FBGs in *F. serratus*; and 2025 MBGs vs 747 FBGs in *F. vesiculosus*; Fig. 2a). Noteworthy, more than half of the MBGs were also male-specific (55% in *F. serratus* and 58% in *F. vesiculosus*), meaning their expression in female reproductive tissue fell below the detection threshold ($\log_2(TPM + 1) < 0$; Fig. 2a). By contrast, the majority of female-biased genes were also expressed in male receptacles, and female-specific genes constituted a smaller fraction of the female sex-biased gene (FBG) pool (17%, *F. serratus*; 3%, *F. vesiculosus*; Fig. 2a; Table S3).

To further examine the relationship between the expression levels and the degree of sex bias, we grouped the genes according to the fold change (FC) difference between males and females and plotted their mean expression levels in each sex (Fig. 2b). We observed that the highest fold changes (FC > 20) were a result of very low expression or silencing ($\log_2(TPM + 1) < 0$) of the given gene in the other sex (Fig. 2b). Interestingly, between 60% and 90% of female-biased genes featured moderate expression bias (2 < FC < 6) (416 in *F. serratus*; and 674 in *F. vesiculosus*), whereas the majority of male-biased genes were silent in females and exhibited very high fold changes (FC > 20; 61% or 1416 genes in *F. serratus* and 59% or 1201 genes in *F. vesiculosus*), which is consistent with the high proportion of male-specific SBGs (Fig. 2a,b).

We also noted that female-biased genes were highly expressed and ubiquitously present in both sexes and both tissue types, including male receptacles (Fig. 2c). Conversely, MBGs showed a strong signal of expression only in the male reproductive tissue and had significantly lower expression levels compared with unbiased genes in male and female vegetative and female reproductive tissues in both species (Fig. 2c, $P < 2e\text{-}16$ in all pairwise Wilcoxon tests).

Tissue-biased gene expression

We analyzed transcript abundance in the reproductive vs vegetative tissues within each sex and species to identify genes with tissue-biased expression (FC ≥ 2, $P_{adj} < 0.05$ (FDR-adjusted *P*-value); Tables S2, S3; Fig. 3a). Males of both *Fucus* species displayed higher tissue-bias than females, and more of these tissue-biased genes were over-expressed in the reproductive organs compared with vegetative tissue (Fig. 3a). To identify sex-biased genes that were predominantly expressed in the reproductive tissue, we compared the tissue-biased data set with that of the male and female sex-biased genes identified above. Not surprisingly, most of the male reproductive tissue-biased genes overlapped with MBGs (72% and 88% in *F. serratus* and *F. vesiculosus*, respectively), whereas FBGs were more uniformly expressed across the female body (only 18% and 7% localized specifically in the reproductive tissue of *F. serratus* and *F. vesiculosus*, respectively; Fig. 3a, shaded area). Noteworthy, the SBGs showed significantly higher degrees of sex bias in the reproductive tissue than in the nonreproductive tissue in both sexes and species (Fig. 3b, Wilcoxon test, $P < 1.4e\text{-}06$).

Common patterns in male-biased expression among *Fucus* species

Using Orthofinder, we found 20 077 orthogroups that comprised 85 430 genes (72.6% of all the genes), out of which 14 818 orthogroups contained genes from both dioecious species (*F. vesiculosus* and *F. serratus*). In addition, we searched for single-copy orthologs within each lineage (*F. distichus*–*F. serratus* and *F. spiralis*–*F. vesiculosus*) as well as between the two dioecious species (*F. serratus*–*F. vesiculosus*). We found 9401 and 8758 one-to-one orthologs between *F. distichus*–*F. serratus* and *F. spiralis*–*F. vesiculosus*, respectively, and 9778 one-to-one orthologs in the dioecious pair (Table S4). Up to 35% of genes in each species were 'orphans', meaning species-specific genes, without any intra- or interspecific orthologs.

First, we analyzed the conservation of sex bias among all orthogroups, including orthogroups with multi-copy genes per species, provided that at least one of the paralogs exhibited sex-biased expression. Comparisons of orthogroups comprising the sex-biased genes of *F. serratus* and *F. vesiculosus* revealed that the male-biased genes were highly conserved between the two species (Table S4). As much as 65–75% of the orthogroups containing male-biased genes were common between *F. serratus* and *F. vesiculosus*. By contrast, only 20–26% of orthogroups with female-biased genes were shared between these species (Fig. 4a). Interestingly, the low number of female-biased genes shared between the lineages was not caused by the presence of orphan genes among FBGs, but rather gain/loss of female bias in existing, orthologous genes. In fact, the proportions of sex-biased genes among the orphan genes were significantly lower than expected in both species and sexes (χ^2 test, $P < 2.4e\text{-}23$, Table S5). Taken together, we observed high conservation of male sex-biased expression and higher variation in female-biased genes between *F. serratus* and *F. vesiculosus*.

To further analyze the common patterns of the sex-biased expression, we focused on genes for which there was a clear one-to-one relationship across *F. serratus* and *F. vesiculosus*. Out of the 9778 orthogroups with single copy genes, 21% (2070 orthogroups) contained genes with sex-biased expression in at least one of the two species (Table S4). Again, male sex bias was

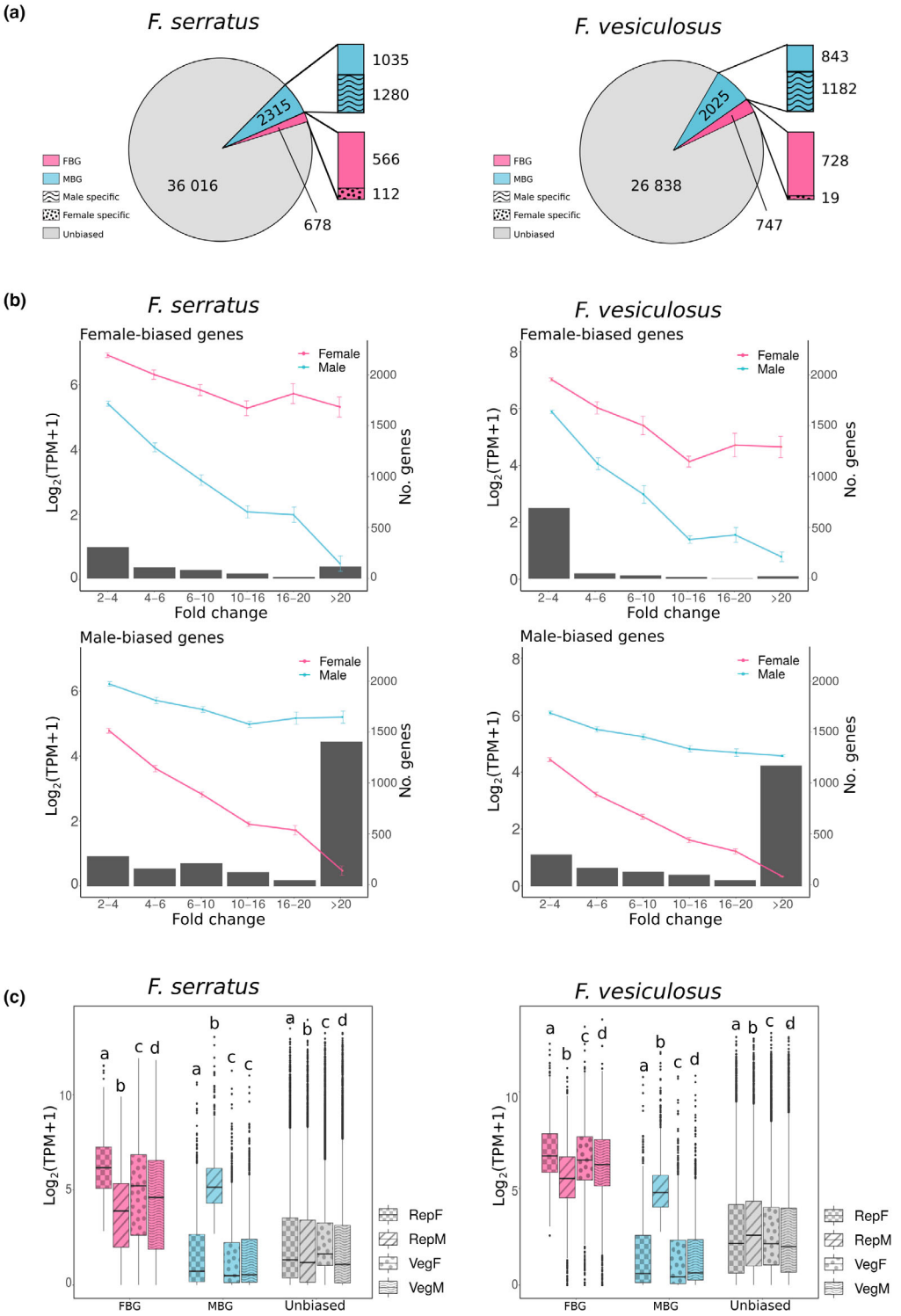

Fig. 2 Sex-biased gene expression. (a) Number of sex-biased genes (MBG, male-biased; FBG, female-biased) in *Fucus serratus* and *Fucus vesiculosus* reference transcriptomes. Unbiased genes were defined as $P_{adj} > 0.05$ or showing less than twofold difference between the sexes. Bars represent the proportion of sex-specific genes among the sex-biased genes in each species. (b) Mean expression levels (log_2(TPM + 1)) of female-biased and male-biased genes at several degrees of sex bias (fold change) in the female (pink) and male (blue) reproductive tissues. Error bars represent SE. Bar plots indicates the number of genes in each fold change (FC) category. (c) Boxplots showing the mean expression levels across the replicates (log_2(TPM + 1)) of female-biased (pink), male-biased (blue), and unbiased (gray) genes in male and female reproductive and vegetative tissues indicated by the hashed pattern (check, female reproductive tissue; angled lines, male reproductive tissue; dots, female vegetative tissue; waves, male vegetative tissue). The letters above the plots indicate significant differences within each gene group (pairwise Wilcoxon test, $P < 0.05$). Horizontal bars, median; lower whiskers, Q1 − 1.5×(interquartile range); upper whiskers, Q3 + 1.5×(interquartile range); outliers, single data points with >1.5× value of the upper quartile or <1.5× value of the lower quartile. RepF, female reproductive tissue; RepM, male reproductive tissue; VegF, female vegetative tissue; VegM, male vegetative tissue.

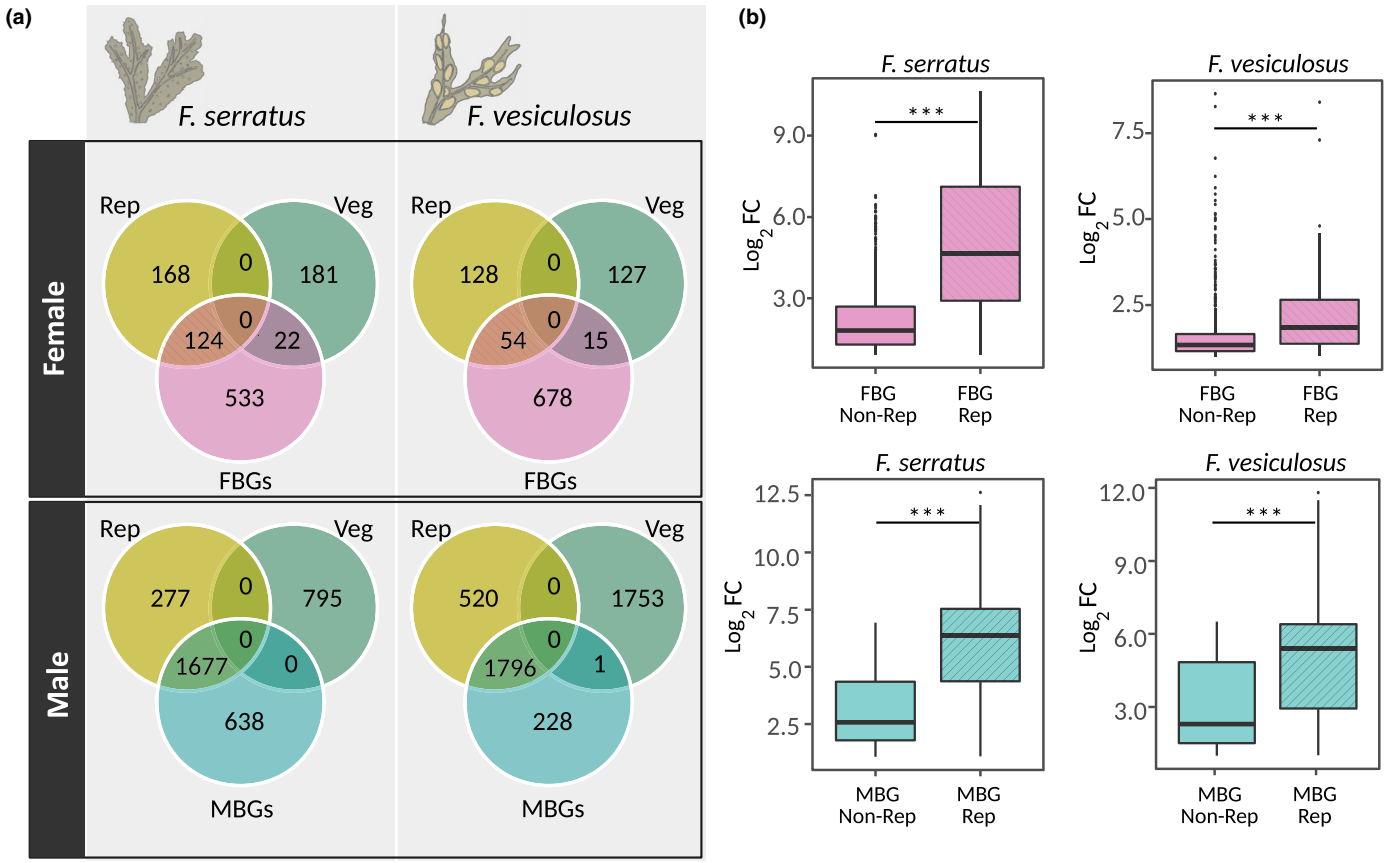

Fig. 3 Sex-biased genes (SBGs) are over-expressed specifically in the reproductive tissue. (a) Venn diagram shows numbers of significantly differentially expressed genes between reproductive (Rep) and vegetative (Veg) tissues of males and females from *Fucus serratus* and *Fucus vesiculosus* (FC > 2, P_{adj} < 0.05). The shaded overlap highlights female-biased genes (FBGs, upper panel) and male-biased genes (MBGs, lower panel) that were over-expressed in reproductive tissue. (b) Overall levels of sex-biased expression (log_2FC) of SBGs up-regulated in reproductive (Rep) or vegetative tissue (Non-Rep; Wilcoxon test, P < 1.4e-06). Horizontal bars, median; lower whiskers, Q1 − 1.5×(interquartile range); upper whiskers, Q3 + 1.5×(interquartile range); outliers, single data points with >1.5× value of the upper quartile or <1.5× value of the lower quartile. ***, P < 1.4e-06.

strongly correlated across the two lineages and applied to *c.* 70–80% of MBGs with one-to-one orthologs, contrary to 25–16% of shared FBGs in *F. serratus*/*F. vesiculosus* (Fig. 4b).

The patterns of expression of common and species-specific SBGs showed similar trends in *F. serratus* and *F. vesiculosus* (Fig. 4c). Genes with common sex bias had significantly higher average expression levels in reproductive tissue than the species-specific SBGs (genes biased toward one sex in one species but not the other; Fig. 4c, Wilcoxon test, P < 0.001). Interestingly, this was also true for the FBGs shared between the lineages in the vegetative tissue (Wilcoxon test, P < 0.01), whereas shared male-biased genes exhibited significantly lower expression levels in the vegetative tissue compared with species-specific MBGs (Wilcoxon test, P < 0.001). In short, male-biased genes shared by the dioecious species were primarily expressed in reproductive tissue and constituted almost half of the male-biased genes found in the receptacles (42% in *F. serratus* and 48% in *F. vesiculosus*).

The tissue specificity of male-biased genes was further highlighted in the hierarchical clustering of the one-to-one orthologs based on expression levels within and among the *F. serratus and F. vesiculosus* species (Fig. 5). For the sex-biased genes (when at least one or both orthologs are SBGs), the male reproductive samples formed a separate cluster from all the other samples (Fig. 5a), which grouped primarily by phylogenetic relatedness, with female reproductive tissue appearing more similar to that of male and female vegetative tissue (Fig. 5a). For unbiased genes (when neither of the orthologs showed sex-bias), the samples clustered by phylogeny and tissue types (Fig. 5b).

Evolution of sex-biased genes

To investigate the role of selection on coding sequence evolution, we calculated pairwise divergence of the one-to-one orthologs within lineages (*F. serratus–F. distichus* (7759 orthologs); *F. vesiculosus–F. spiralis* (7103 orthologs)) using the YN00 package in PAML4 (Yang, 2007; Table S6).

In both dioecious species, female-biased genes showed similar rates of nonsynonymous to synonymous substitutions (dN/dS) to that of unbiased genes (Fig. 6a, permutation test, P > 0.07). By contrast, the average dN/dS was significantly higher for male-biased than unbiased genes (Fig. 6a, permutation test, P < 0.02) and did not depend on the magnitude (FC) or conservation (universal vs species-specific) of the sex-biased expression patterns

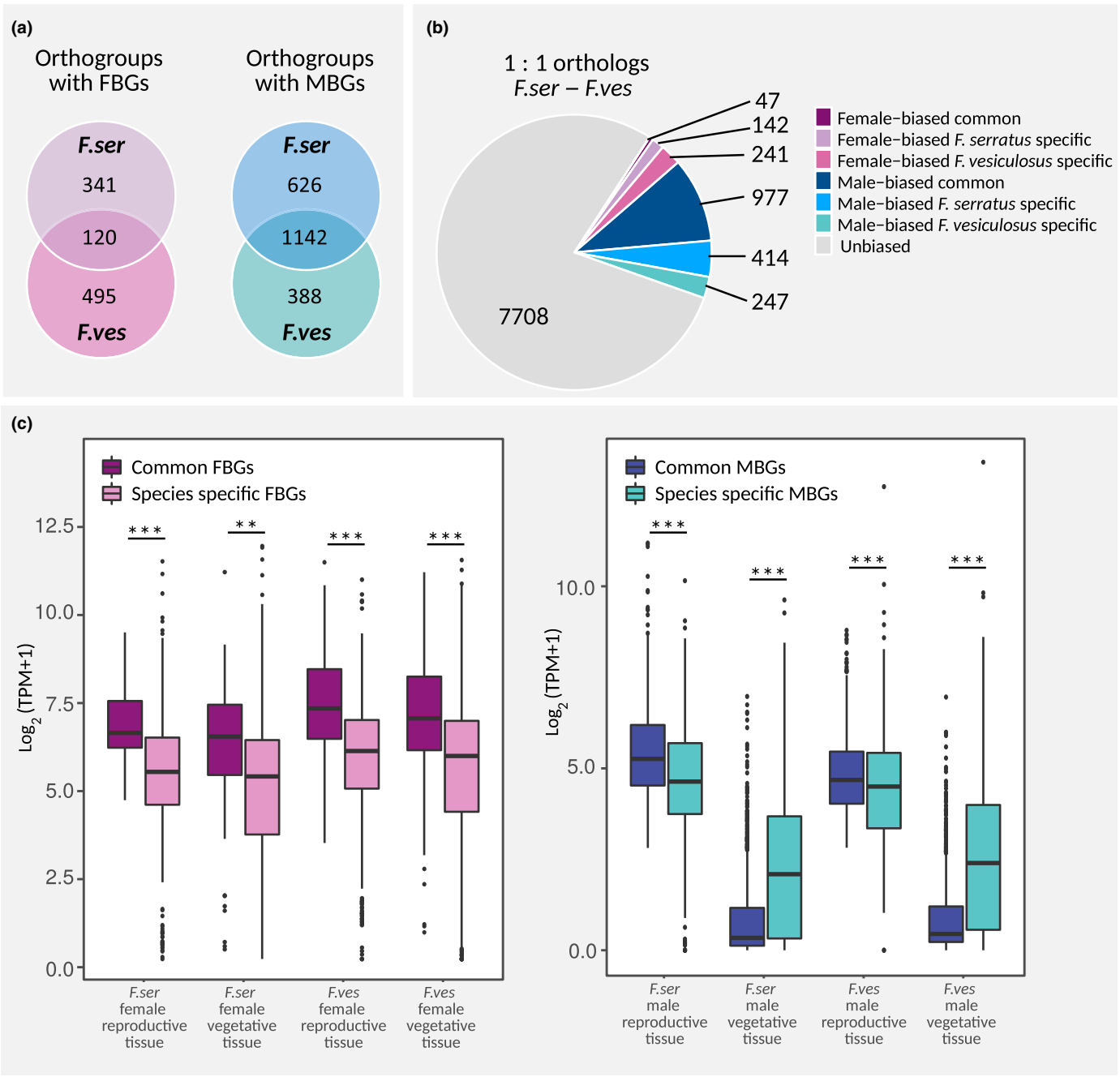

Fig. 4 Conservation of sex-biased gene (SBG) expression across *Fucus serratus* and *Fucus vesiculosus* species. (a) Numbers of orthogroups with female (pink, FBGs) and male (blue, MBGs) sex-biased genes shared between dioecious species. Orthogroups with multi-copy genes of a species were included if at least one of the paralogs exhibited sex-biased expression. (b) Conservation of sex-biased expression among single copy, one-to-one orthologs between *F. serratus* and *F. vesiculosus*. (c) Mean expression levels (log_2(TPM + 1)) of conserved and species-specific SBGs with single-copy orthologs in *F. serratus* and *F. vesiculosus* across different tissue types. Wilcoxon test: **, $P < 0.01$; ***, $P < 0.001$. *F.ser*, *Fucus serratus*; *F.ves*, *Fucus vesiculosus*. Horizontal bars, median; lower whiskers, Q1 − 1.5×(interquartile range); upper whiskers, Q3 + 1.5×(interquartile range); outliers, single data points with >1.5× value of the upper quartile or <1.5× value of the lower quartile.

(Table S7, Wilcoxon test, $P > 0.11$). In addition, we found a significant difference in dN/dS ratios between male and female SBGs in both dioecious species (Fig. 6a, permutation test, $P < 2e\text{-}16$).

To assess whether increased protein divergence rates were due to increased positive selection or relaxed purifying selection, we performed a maximum likelihood analysis using a branch-site model implemented in CODEML in PAML4 (Yang, 2007). The branch-site models allow ω to vary both among sites in the protein and across branches on the tree and aim to detect positive selection affecting a few sites along particular lineages (called foreground branches). We used sequences from the four *Fucus* species (*F. vesiculosus*, *F. serratus*, *F. distichus*, and *F. spiralis*) and two other brown algae (*Ectocarpus* sp. (Cock *et al.*, 2010) and

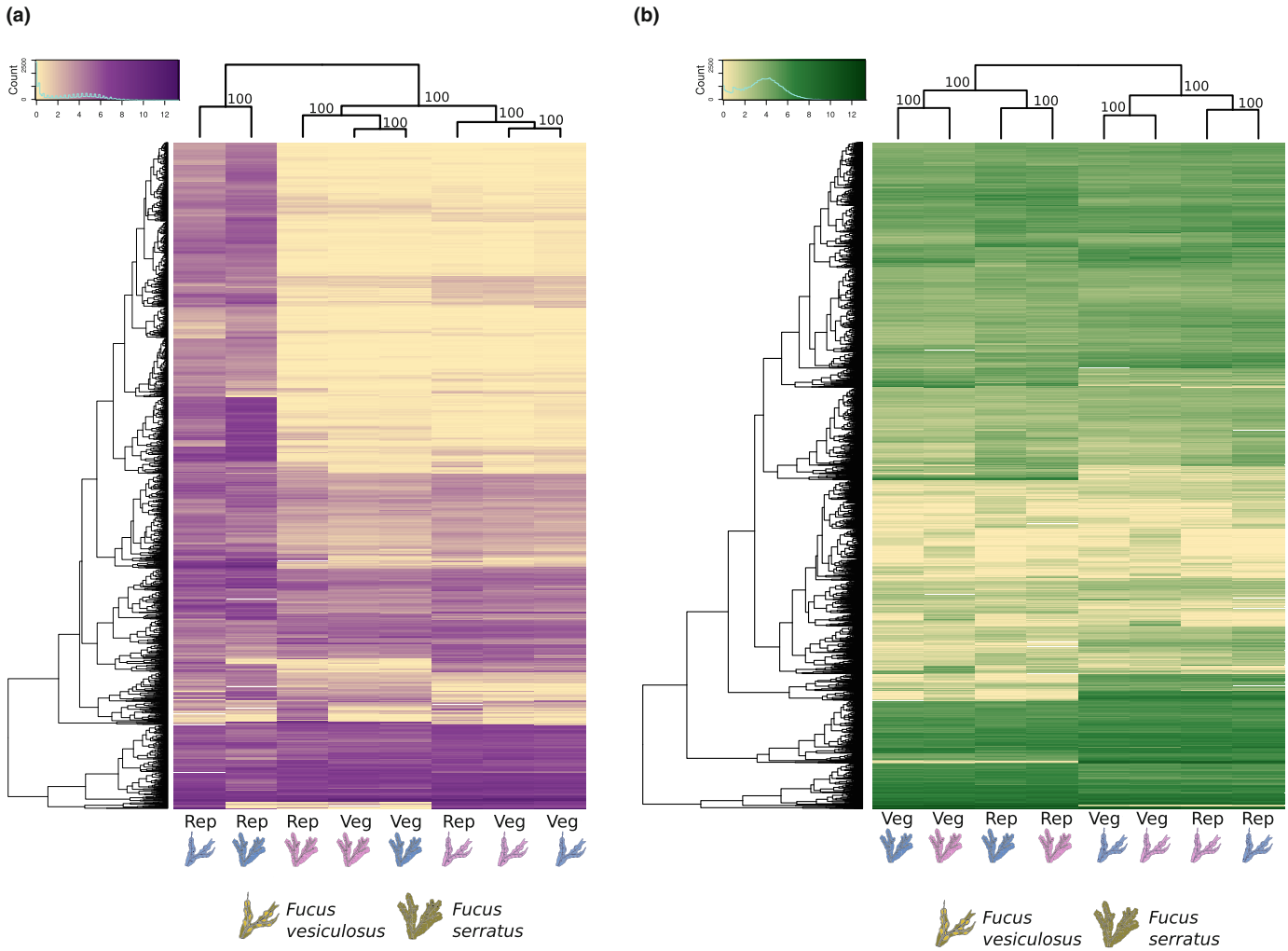

Fig. 5 Heatmaps and hierarchical clustering of gene expression levels (log_2(TPM + 1)) for all single-copy orthologs among *Fucus serratus* and *Fucus vesiculosus*. The dendrogram was generated using hierarchical clustering with 1000 bootstraps (PVCLUST package, R). (a) Sex-biased genes (at least one sex-biased gene in one of the studied species); (b) unbiased genes (none of the genes was sex-biased). The diagrams under the heatmap indicate the species (*F. vesiculosus* or *F. serratus*), the sex of an individual (blue, male; pink, female) and tissue type (Rep, reproductive tissue; Veg, vegetative tissue).

Saccharina japonica (Ye *et al.*, 2015)) to find 561 conserved single-copy orthologs. Among those, 57 orthologs exhibited male-biased expression and 13 exhibited female-biased expression in at least one of the *Fucus* species (Table S8). Each alignment was tested for the direction and magnitude of selection on amino acid changes, comparing the average of foreground ω values (branches leading to either *F. serratus* or *F. vesiculosus*) with the average of background ω values. We also performed the same test choosing forward branches leading to: all four *Fucus* species; *F. serratus–F. distichus* lineage; and, *F. vesiculosus–F. spiralis* lineage, to identify genes with evidence for positive selection specific to the dioecious species (Table S8). After filtering out the genes under selection on the internal branches, we detected evidence for adaptive evolution (FDR < 0.05) in 94 genes (eight male-biased genes, two female-biased genes, and 84 unbiased genes) in *F. serratus* and 119 genes (nine male-biased genes and 110 unbiased genes) in *F. vesiculosus* (Table S8). We found no significant enrichment of genes under positive selection among the sex-biased genes compared with unbiased genes (χ^2 test, $P > 0.05$), which is consistent with the idea that sex-biased genes are evolving predominantly under relaxed selective constraint. Finally, we compared the dN/dS analysis with gene expression divergence measured as Euclidean distances for the one-to-one orthologous pairs between *F. serratus* and *F. vesiculosus*. Female-biased genes showed the highest divergence in expression patterns compared with male-biased or unbiased genes (Fig. 6b, Wilcoxon test $P <$ 2e-16). These results are in line with the FBGs being more liable (a given gene has a female bias in one species but it is unbiased in the other species). By comparison, male-biased genes presented highly conserved expression, with the universal MBGs having overall the most stable expression patterns among all SBGs (Figs S2, S3; Wilcoxon test $P < 0.003$).

Functional analysis

The Gene Ontologies (GO) associated with female-biased genes in *F. serratus* and *F. vesiculosus* were enriched in biological processes related to cell wall synthesis, translation, transmembrane

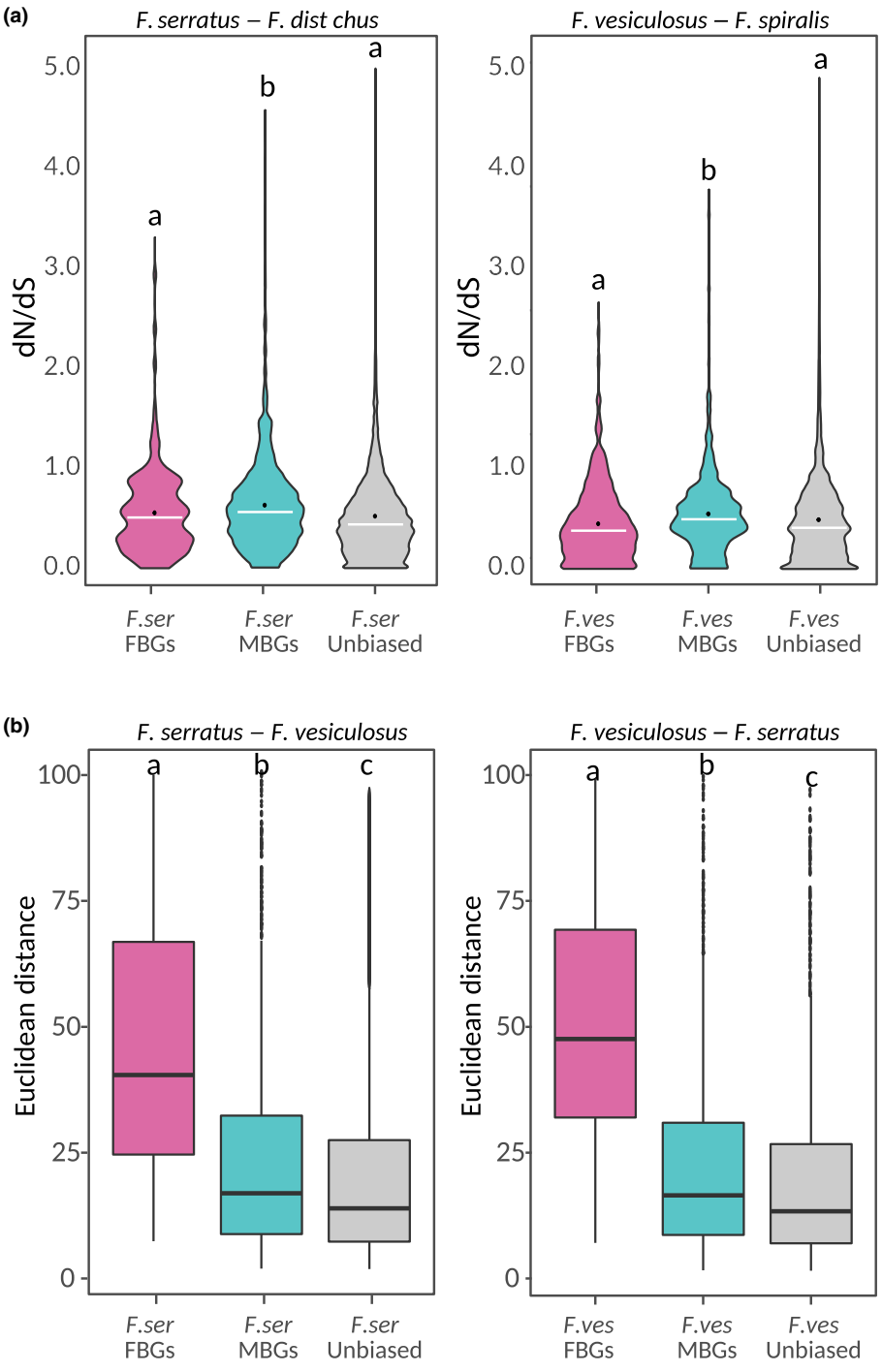

Fig. 6 Evolution of sex-biased genes. (a) Evolutionary rates measured as dN/dS between species pairs (*Fucus serratus*/*Fucus distichus* and *Fucus vesiculosus*/*Fucus spiralis*) for unbiased, female-biased, and male-biased genes in the two dioecious *Fucus* species. White bar, median; black dot, mean. Different letters above the plots indicate significant differences in mean dN/dS (10 000 permutations test; $P < 0.05$). (b) Expression divergence measured as Euclidean distances between single-copy orthologous genes of *F. serratus* and *F. vesiculosus*. Horizontal bars, median; lower whiskers, Q1 – 1.5×(interquartile range); upper whiskers, Q3 + 1.5×(interquartile range); outliers, single data points with >1.5× value of the upper quartile or <1.5× value of the lower quartile. Different letters above the plots indicate significant differences (pairwise Wilcoxon test: $P < 4.3e\text{-}10$). FBGs, female-biased genes; MBGs, male-biased genes.

transport, receptor signaling, photosynthesis, cell homeostasis, and establishment of cell polarity (Fisher exact test, $P < 0.05$, Table S9). Interestingly, analysis of male-biased genes of both species identified GO terms related to spermatogenesis and sperm competition in addition to microtubule and flagellar movement categories, as well as photo- and chemotaxis (Fisher exact test, $P < 0.05$, Table S9). Furthermore, three consistently male-biased flagellar-associated proteins were found to evolve under positive selection (Table S8). These results are coherent with the reproductive functions of males and females, with MBGs being predominantly involved in male germ cell differentiation, sperm motility, and response to pheromones produced by the egg, whereas FBGs being related to the development of a future embryo.

Discussion

Brown algae are excellent models to study the evolution of sexual systems, as their extraordinary divergence in sex-determination mechanisms and sexual dimorphism (ranging from isogamy to oogamy) sets them apart from other eukaryotic groups (Silberfeld

et al., 2010; Coelho *et al.*, 2019). In this work, we asked whether there are similarities in sex-biased gene expression patterns between two *Fucus* species, which recently evolved separate sexes after the transition to a diploid life history. We investigated the proportion of the transcriptome that evolved sex-biased expression in this relatively young XX/XY system with modest sexual dimorphism. We also examined whether the evolutionary patterns of sex-biased genes in *Fucus* are convergent with the ones found in well-established XY or ZW systems.

Sex-biased expression in dioecious *Fucus* species

While very few genes were differentially expressed between male and female vegetative tissue, thousands of genes (*c.* 8–9% of *F. serratus* and *F. vesiculosus* transcriptomes, respectively) were differentially expressed in the reproductive tissues. A similar fraction of the genome displayed tissue-biased expression between receptacles and the rest of the body within each sex, allocating the majority of tissue- and sex-biased expression to the reproductive organs. These findings agree with the general trend found in animals and plants where reproductive tissues show the highest expression divergence between sexes (animals: Yang *et al.*, 2006; Yang, 2007; Pointer *et al.*, 2013; Harrison *et al.*, 2015; Allen *et al.*, 2018; plants: Song *et al.*, 2017; Darolti *et al.*, 2018; Sanderson *et al.*, 2019). This could be expected in *Fucus*, as sexes are morphologically identical except for their receptacles. The overall moderate levels of SBG expression in *Fucus* (8–9%), compared with many model organisms (Grath & Parsch, 2016), may be explained by the low levels of sexual dimorphism, external fertilization, and, accordingly, more narrow range of sexual selection in both *F. serratus* and *F. vesiculosus* (Luthringer *et al.*, 2014). In birds, the proportion of SBGs corresponded with the strength of selection and the extent of phenotypic dimorphism between males and females (Harrison *et al.*, 2015). Similarly, in a male feminized mutant strain of the brown alga *Macrocystis*, sex-specific phenotypes (male, female, or feminized male variant) showed sex-specific transcriptomic patterns (Müller *et al.*, 2021). However, a cross-genus study of SBG expression in *Leucadendron* plants with varying levels of sexual dimorphism found no correlation between levels of morphological differences and percent of sex-biased genes (Scharmann *et al.*, 2021).

It is worth noting that the proportion of SBGs in the oogamous *Fucus* (reproduction involving a small motile male and large immobile female gametes) much exceeded that of the near-isogamous *Ectocarpus* (motile male and female gametes of similar sizes; Lipinska *et al.*, 2015). *Ectocarpus* is a filamentous brown alga with low levels of sexual dimorphism between the male and female gametophytes, has a haploid–diploid life cycle, and produces morphologically similar, small, flagellated male, and female gametes (Luthringer *et al.*, 2014; Lipinska *et al.*, 2015). In brief, phenotypic sexual dimorphism in *Ectocarpus* is imperceptible, with < 4% (658) of *Ectocarpus* genes being sex-biased during the reproductive stage in contrast to 8% (2993) in *F. serratus* and 9% (2772) in *F. vesiculosus* in this study. Furthermore, in oogamous kelp *Macrocystis*, where male and female gametophytes have visibly distinct morphologies (Müller *et al.*, 1979), sex-biased gene expression analysis found 24% (5442) of genes with male/female bias (Müller *et al.*, 2021). In summary, our results suggest that the evolution of anisogamy alone, without the other morphologically dimorphic characters, has triggered a significant increase in sex-biased gene expression.

Excess of male-biased genes in the *Fucus* transcriptome

In both systems, *Ectocarpus* with UV, and *Fucus* with XX/XY sex chromosomes, we identified an excess of male-biased over female-biased genes. Sex-biased genes were also more commonly male-biased in dioecious plants like *Silene* and asparagus (Harkess *et al.*, 2015; Zemp *et al.*, 2016), but not in poplar (Sanderson *et al.*, 2019). However, in *Fucus* species, male overexpression was much more pronounced, exceeding more than three times the number of FBGs (400 MBGs vs 258 FBGs in *Ectocarpus*; 2315 MBGs vs 678 FBGs in *F. serratus*; 2025 MBGs vs 747 FBGs in *F. vesiculosus*). Globally, male-biased genes featured extreme expression bias (FC > 20) with more than half of the male-biased genes being male-specific, expressed explicitly in male receptacles, and at significantly higher levels than unbiased genes in the vegetative tissue. This transcription profile may result from adaptive changes in males, and, as predicted for anisogamy, implies that males experience stronger selection on gene expression than females (Darwin, 1871; Bateman, 1948; Parker, 1979; Schärer *et al.*, 2012; Andersson, 2019). Excess of male-based expression has been found in many other species and could be due to the relative expression of male sexual traits, female choice, and male–male competition (Connallon & Knowles, 2005; Pointer *et al.*, 2013; Harkess *et al.*, 2015; Zemp *et al.*, 2016). Although female choice in the 'classical' understanding does not exist in free-spawning species like *Fucus*, it could still occur at the level of gametes or postfertilization. Evidence for 'gamete-mediated mate choice' and the evolutionary significance of non-random interactions among gametes to the evolutionary origins of more definite forms of mate choice was recently reviewed (Kekäläinen & Evans, 2018). Moreover, sperm competition would be facilitated in the water column, where ejaculates from different males mix and compete for fertilization of the egg.

To test the hypothesis that the sex-biased expression in *Fucus* was associated with increased sexual selection in males, we would need to compare our data with transcriptomic data from closely related hermaphrodite species. For example, gene expression data from the two Fucales families that remained hermaphroditic (Sargassaceae and Notheiaceae) could serve as a baseline to assess the direction of changes in expression that led to sex bias in *F. serratus* and *F. vesiculosus* (Heesch *et al.*, 2021).

By contrast to MBGs, female-biased genes seemed to be uniformly and highly expressed throughout the female and male body. This overall homogeneous expression pattern of FBGs became apparent when vegetative and reproductive tissue within each sex were compared (so-called tissue-biased expression, as opposed to sex-biased expression, where the same tissue types are compared between the two sexes). The majority of FBGs did not show tissue-biased expression in females (79% in *F. serratus* and 91% in *F. vesiculosus*), and only 20 and 22 genes showed sex bias in vegetative tissue in *F. serratus* and *F. vesiculosus*, respectively. To summarize, sex-biased

gene expression in *Fucus* appears to arise from the down-regulation of expression of pleiotropic female genes in male receptacles and by restricting the expression of MBGs to the male reproductive tissue, resulting in tendentiously male-biased transcriptomes as previously reported for the giant kelp *Macrocystis* (Müller *et al.*, 2021).

High conservation of male-biased expression

Male-biased genes are largely shared between the two *Fucus* species, which contrasts with the overall trends found in other species. Male-biased genes in *Fucus* presented not only the equivalence of bias, but also of expression levels (measured as Euclidean distance), which resulted in clustering of the male reproductive samples by sex rather than by species. The changes in male-biased gene regulation may have risen in the common ancestor of *F. serratus* and *F. vesiculosus* and shared ancestry could be, therefore, responsible for the observed correlation. This would further support a hypothesis that dioecy was the ancestral state in the *Fucus* genus and hermaphroditism in *F. distichus* and *F. spiralis* is a derived state. However, previous reports have shown that the targets of sex-biased expression can change over a short evolutionary time and that a small fraction of genes show parallel changes in recently diverged species (Ranz *et al.*, 2003; Harrison *et al.*, 2015; Huylmans *et al.*, 2017). Similarly, studies on *Leucadendron* plants failed to find genes that were consistently sex-biased but, instead, concluded that the sex-biased gene expression evolved independently in each species (despite dioecy being most likely the ancestral state in this genus; Scharmann *et al.*, 2021). Furthermore, global patterns of evolution of sex regulation in dioecious plants found more differences than similarities in both sex-determining genes and downstream pathways (Feng *et al.*, 2020).

Given the relatively young evolutionary age of our system, phenotypic differences accumulated between and within species may be insufficient to drive the turnover of sex-biased genes. However, this is unlikely since the number of single-copy orthologs with male-biased expression (in both species) exceeded four times the number of unbiased genes with one-to-one orthologs, suggesting that the MBGs are selectively maintained to perform a role in male reproduction. Functional analysis of male-biased genes further support this assumption, as MBGs were consistently enriched in ontologies related to male fertility, sperm production, and motility. In contrast to MBGs, FBGs showed more variability and had species-specific expression patterns indicated by significantly increased Euclidean distances, compared with both unbiased and male-biased genes. Taken together, if intralocus conflict (expression of sexually antagonistic alleles that increase fitness in one sex but move the other sex from its phenotypic optimum) is the main driver of sex-biased expression, our results suggest that the targets of this conflict are fixed in males, but not in females of *Fucus*.

Evolution of sex-biased genes

Sex-biased genes tend to evolve faster than unbiased genes in animal species (Meiklejohn *et al.*, 2003; Harrison *et al.*, 2015; Lipinska *et al.*, 2015; Darolti *et al.*, 2018). Nevertheless, no evidence for faster evolution of male-biased genes has been found in plants (Zemp *et al.*, 2016; Cossard *et al.*, 2019; Sanderson *et al.*, 2019; Scharmann *et al.*, 2021). Although male-biased genes displayed conserved expression between *Fucus* species, they presented higher rates of protein evolution compared with unbiased genes. Both positive selection in males or relaxed selection in females may be responsible for rapid DNA sequence evolution of MBGs (Zhang *et al.*, 2004; Dyken & Wade, 2010; Gershoni & Pietrokovski, 2014; Gossmann *et al.*, 2014; Mank, 2017). The fraction of MBGs under selection was, however, not significantly different to that observed for unbiased genes, indicating that adaptive evolution is not the main driver of the elevated substitution rates in MBGs. Interestingly, three of the 21 male-biased genes under positive selection were associated with the sperm flagella, suggesting that at least a proportion of male-biased genes could experience adaptive evolution resulting from stronger sexual selection driven by, *for example*, sperm competition in *Fucus*.

Alternatively, other aspects of genetic architecture could be contributing to the rapid evolution of male-biased genes. For example, MBGs could be less constrained by pleiotropy, because their expression is predominantly confined to male reproductive tissue, which is often associated with patterns of faster sequence evolution (Meisel, 2011; Grath & Parsch, 2012; Darolti *et al.*, 2018). In line with this, female-biased genes in *Fucus* are expressed in both vegetative and reproductive tissue in male and female gametophytes, and show lower rates of synonymous to nonsynonymous substitutions. Interestingly, high tissue specificity of male-biased genes in animals was accompanied by high rates of turnover, consistent with differential selection pressures (Harrison *et al.*, 2015; Catalán *et al.*, 2018; Whittle & Extavour, 2019). This was not the case in *Fucus*, as we observed accelerated rates of protein divergence linked to low pleiotropy of MBGs, but also high conservation of the magnitude of sex bias and gene expression levels. Furthermore, the rate of evolution could be determined by the genomic location of MBGs, specifically the sex-chromosome linkage. Elevated rates of coding sequence evolution on the sex chromosome relative to autosomes have been reported for several species, consistent with the theoretical prediction of fast-X or fast-Z evolution (Kirkpatrick & Hall, 2004; Mank *et al.*, 2010; Belleghem *et al.*, 2018). In *Fucus*, male-biased genes show high expression levels only in the male reproductive tissue, and the fast-X theory predicts that genes highly expressed in the hemizygous sex should be especially prone to fast-X evolution (Meisel *et al.*, 2012). This interesting aspect of MBGs evolution should be revisited in the future when the genome sequences of *F. serratus* and *F. vesiculosus* become available. Finally, the set of MBGs could be enriched for young genes, which are known to evolve more rapidly in plant gametophytes (Gossmann *et al.*, 2016). However, to assess the evolutionary age of *Fucus* sex-biased genes, additional data from closely related species are needed.

In summary, MBGs and FBGs in *Fucus* seem to follow different evolutionary paths and are under different selective pressures. Male-biased genes evolve faster at the level of the protein sequence, but their expression levels remain very similar

between *Fucus* species. By contrast, FBGs do not show accelerated rates of coding sequences evolution, but rather higher diversification of their expression levels. Because the changes in coding and changes in regulatory sequences are often decoupled, it has been suggested that they play different evolutionary roles in the evolution of morphological and physiological characters (Connallon & Knowles, 2005; Wray, 2007; Tirosh & Barkai, 2008; Liao *et al.*, 2009; Martin *et al.*, 2013; Loehlin *et al.*, 2019). Both types of changes (morphological or physiological) could be under selection due to reinforcement, since members of both lineages (*F. serratus–F. distichus* and *F. vesiculosus–F. spiralis*) show signatures of ongoing or past hybridization, and hybrids of the dioecious *F. serratus–F. vesiculosus* are extremely rare (Coyer *et al.*, 2002, 2007; Wallace *et al.*, 2004; Billard *et al.*, 2005; Hoarau *et al.*, 2015). Additionally, hybridization in *Fucus* species usually occurs asymmetrically, with the sperm of the dioecious species fertilizing the eggs of the hermaphrodite species. As a result of asymmetric hybridization, male and female-biased genes could experience different selection pressures from reinforcement. Furthermore, studies of geographical hybrid zones of *F. serratus* and *F. distchus* show signatures of reinforcement of prezygotic isolation, namely decreasing rates of hybridization and interspecific fertilization success, with increasing duration of sympatry (Hoarau *et al.*, 2015). Further studies are needed to characterize the genetic basis of reproductive isolation in *Fucus* as well as the connection between prezygotic barriers to fertilization and within-species sexual selection.

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Competing interests

None declared.

Author contributions

GH, AOJ, JAC, APL and WJH planned and designed the research. WJH, MK, GH, AOJ and APL performed experiments, conducted fieldwork, and analyzed data. WJH, GH, AOJ, SMC, JAC and APL wrote the manuscript.

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Data availability

Sequencing data have been deposited in the National Center for Biotechnology Information database under BioProject ID PRJNA731608. The Transcriptome Shotgun Assembly projects have been deposited at DDBJ/ENA/GenBank under the accession nos. GJHE00000000, GJHF00000000, GJHR00000000, and GJHG00000000. The versions described in this paper are the first versions.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Phylogenetic relationships between the four *Fucus* species used in this study.

Fig. S2 Sex-biased gene expression among single-copy orthologs in *Fucus serratus* and *Fucus vesiculosus*.

Fig. S3 Expression divergence measured as Euclidean distances between single-copy orthologous genes of *Fucus serratus* and *Fucus vesiculosus*.

Table S1 Sequencing and assembly summary.

Table S2 Number of sex-biased and tissue-biased genes in *Fucus serratus* and *Fucus vesiculosus*, *DESeq2* (FC $\geq$ 2, $P_{adj} < 0.05$).

Table S3 Expression levels ($\log_2$(TPM + 1)) and fold change (logFC) of sex-biased and tissue-biased genes in *Fucus serratus* and *Fucus vesiculosus*, and tissue-biased genes in hermaphrodite *Fucus distichus* and *Fucus spiralis*.

Table S4 Gene orthology statistics.

Table S5 Orphan genes among the sex-biased genes in *Fucus serratus* and *Fucus vesiculosus*.

Table S6 Evolutionary rates measured as dN/dS (YN00 method, PAML4) between species pairs (*Fucus serratus*/*Fucus distichus* and *Fucus vesiculosus*/*Fucus spiralis*) for unbiased, female-biased, and male-biased genes.

Table S7 Evolutionary rates measured as dN/dS between species pairs (*Fucus serratus*/*Fucus distichus* and *Fucus vesiculosus*/*Fucus spiralis*) for unbiased, female-biased, and male-biased genes in relation the fold change of expression between males and females.

Table S8 Positive selection analysis.

Table S9 Gene Ontology enrichment of the sex-biased genes in *Fucus serratus* and *Fucus vesiculosus*, Fisher exact test, $P < 0.01$.

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