Sexual Dimorphism and the Evolution of Sex-Biased Gene Expression in the Brown Alga *Ectocarpus*

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Abstract

Males and females often have marked phenotypic differences, and the expression of these dissimilarities invariably involves sex differences in gene expression. Sex-biased gene expression has been well characterized in animal species, where a high proportion of the genome may be differentially regulated in males and females during development. Male-biased genes tend to evolve more rapidly than female-biased genes, implying differences in the strength of the selective forces acting on the two sexes. Analyses of sex-biased gene expression have focused on organisms that exhibit separate sexes during the diploid phase of the life cycle (diploid sexual systems), but the genetic nature of the sexual system is expected to influence the evolutionary trajectories of sex-biased genes. We analyze here the patterns of sex-biased gene expression in *Ectocarpus*, a brown alga with haploid sex determination (dioicy) and a low level of phenotypic sexual dimorphism. In *Ectocarpus*, female-biased genes were found to be evolving as rapidly as male-biased genes. Moreover, genes expressed at fertility showed faster rates of evolution than genes expressed in immature gametophytes. Both male- and female-biased genes had a greater proportion of sites experiencing positive selection, suggesting that their accelerated evolution is at least partly driven by adaptive evolution. Gene duplication appears to have played a significant role in the generation of sex-biased genes in *Ectocarpus*, expanding previous models that propose this mechanism for the resolution of sexual antagonism in diploid systems. The patterns of sex-biased gene expression in *Ectocarpus* are consistent both with predicted characteristics of UV (haploid) sexual systems and with the distinctive aspects of this organism's reproductive biology.

Key words: **sex-biased gene expression, haploid–diploid life cycle, brown algae, UV sex chromosomes.**

Introduction

In many animal and plant species, males differ markedly from females in morphology, physiology, and behavior. Most of these phenotypic differences are mediated by differential gene expression in the two sexes (Ellegren and Parsch 2007) and this differential gene expression may involve a significant proportion of the genome, as much as 75% in *Drosophila* for example (Assis et al. 2012). These sexually dimorphic patterns of gene expression evolve as a consequence of different selection pressures acting on males and females.

The advent of new generation sequencing has allowed comparative transcriptomic studies of males and females from a range of different species with separate sexes including *Drosophila* (e.g., Perry et al. 2014), birds (e.g., Pointer et al. 2013; Uebbing et al. 2013), cichlid fishes (Bohne et al. 2014), guppies (Sharma et al. 2014), nematodes (Albritton et al. 2014), moths (Smith et al. 2014), the pea aphid (Jaquiery et al. 2013), and brown algae (Lipinska et al. 2013; Martins et al. 2013). A general theme that has emerged from these studies across diverse species is that a significant proportion of the genes in the genome exhibit sex-biased expression, indicating that the expression of sexual dimorphism is associated with marked genetic reprogramming. In most cases, however, there are marked morphological differences between male and female individuals of the species that were studied and analyses of species displaying different degrees of sexual dimorphism would be useful to test the correlation between this character and level of sex-biased gene expression.

Studies such as those listed above are starting to provide a comprehensive overview of sex-biased gene expression in a broad range of species, but the evolutionary causes and consequences underlying the patterns of sex-biased gene expression have been examined in only a small subset of these systems. Most of our knowledge of how sex-biased genes

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evolve comes from work with *Drosophila* and birds (reviewed in Parsch and Ellegren 2013), although some studies have also looked at hermaphrodite species and have provided evidence for sexual selection in these systems (Whittle and Johannesson 2013; Gossmann et al. 2014). Evolutionary analyses have identified several unusual features of sex-biased genes. For example, in gonochoristic/dioecious systems, male-biased genes typically evolve more rapidly at the protein level than female-biased or unbiased genes (e.g., Zhang et al. 2004; Haerty et al. 2007; Assis et al. 2012; reviewed by Ellegren and Parsch 2007; see also Mank et al. 2007). This is believed to result from sex differences in selective pressures on genes; the rapid divergence of male-biased genes resulting from sexual selection due to male–male competition or female choice, natural selection, and/or relaxed purifying selection arising from gene dispensability or reduced functional pleiotropy (Ellegren and Parsch 2007; Mank and Ellegren 2009; Parsch and Ellegren 2013).

The genetic nature of the sexual system can also have an influence, both on the distribution of sex-biased genes in the genome and on their patterns of evolution. In XY sexual systems, for example, X chromosomes spend twice as much time in females as they do in males. Depending on the dominance/recessivity of the male-beneficial allele, this can lead to demasculinization of (i.e., loss of male-biased genes from) the X chromosome (e.g., Arunkumar et al. 2009; Bachtrog et al. 2010; Leder et al. 2010) or to enrichment of male-specific genes on the X (e.g., Khil et al. 2004; Bellott et al. 2010; Jaquiery et al. 2013). Moreover, adaptive fixation of recessive beneficial mutations in X-linked genes (Charlesworth et al. 1987), mutational biases associated with dosage compensation (Begun et al. 2007), or the smaller effective population size (Ne) of sex chromosomes (Vicoso and Charlesworth 2009) may cause genes located on the X (and Z) to evolve more rapidly, the so called faster X effect, and this phenomenon has been observed experimentally, at least in some systems (Presgraves 2008; Mank et al. 2010; Kayserili et al. 2012; Meisel et al. 2012; Avila et al. 2014; Campos et al. 2014; Kousathanas et al. 2014).

These latter effects have not yet been investigated in so-called UV sexual systems, commonly found in mosses and many algae, in which sexuality is expressed during the haploid phase of the life cycle (Bachtrog et al. 2011). There are several important differences between UV systems and the more intensely studied XY and ZW systems and these are expected to have consequences for the evolution of sex-biased genes and for the expression patterns of these genes. For example, in XY and ZW systems recombination is suppressed only for the Y or W chromosome. The X and Z chromosomes can recombine because they are homozygous in one of the sexes. In contrast, in UV systems neither the U nor the V recombines. Moreover, despite the fact that they do not recombine, U and V chromosomes are expected to degenerate less markedly than Y and W chromosomes because they function in a haploid context where both the U and the V are directly exposed to purifying selection (Bull 1978). Finally, the effective population sizes of sex chromosomes differ across different sexual systems and this can have a marked effect on the evolution of the genes carried by these chromosomes. Both the U and the V chromosome have half the effective population size of autosomes (all else being equal) whereas in XY and ZW systems the Y/W and X/Z chromosomes have a quarter or three quarters the population size of autosomes, respectively. As far as sex-biased genes are concerned, masculinization or feminization of sex chromosomes is expected in UV systems only at regions very closely linked to the nonrecombining region because the male and female sex-determining region (SDR) haplotypes function in independent, haploid, male and female individuals. Similarly, a phenomenon similar to the faster X effect is not expected because there is no equivalent of the X chromosome, which recombines but is hemizygous in half of the individuals. Moreover, recent transcriptomic studies from a diverse range of species and tissues (reviewed in Mank 2013) suggest that incomplete or imperfect dosage compensation may be responsible for an important proportion of sex-biased gene expression. Dosage compensation is not expected to occur in UV systems because the U and V chromosomes determine sex during the haploid phase and thus gene dosage is the same for the sex chromosomes and the autosomes.

On the other hand, other features are anticipated to be shared by both diploid (XY and ZW) and haploid (UV) sex-determination systems. For example, in any sexual system resolution of sexual antagonism is expected to be one of the processes that lead to the emergence of sex-biased gene expression. Theoretical models predict that sexually antagonistic alleles should accumulate in the pseudoautosomal regions (PARs) of sex chromosomes, because even partial linkage to the SDR can be adaptive, allowing alleles to be at least partially restricted to the sex for which they are best adapted (Otto et al. 2011; Charlesworth et al. 2014). This effect is expected not only for the PARs of Y and W chromosomes but also for the PARs of U and V chromosomes. This accumulation of sexually antagonistic genes (Charlesworth et al. 2014; Kirkpatrick and Guerrero 2014) might be expected to lead to the PARs becoming enriched in sex-biased genes, although note that there is evidence that the relationship between sexual antagonism and sex-biased gene expression may be quite complex (Innocenti and Morrow 2010; Parsch and Ellegren 2013).

This study focused on sex-biased gene expression in the model brown alga *Ectocarpus*. Brown algae are a group of multicellular photosynthetic organisms that have been evolving independently of both animals and green plants for more than a billion years (Cock, Coelho, et al. 2010). As a group, the brown algae are of considerable interest for investigating the origins and evolution of sexual systems because they have a remarkable variety of levels of sexual dimorphism, reproductive system, types of life cycle, and sex chromosome systems. *Ectocarpus* is a small, filamentous alga that exhibits limited levels of sexual dimorphism, male and female individuals of the sexual phase of its haploid–diploid life cycle, the gametophyte, are morphologically similar organisms and both produce small flagellated gametes (Luthringer et al. 2015). Sex determination in this organism was recently

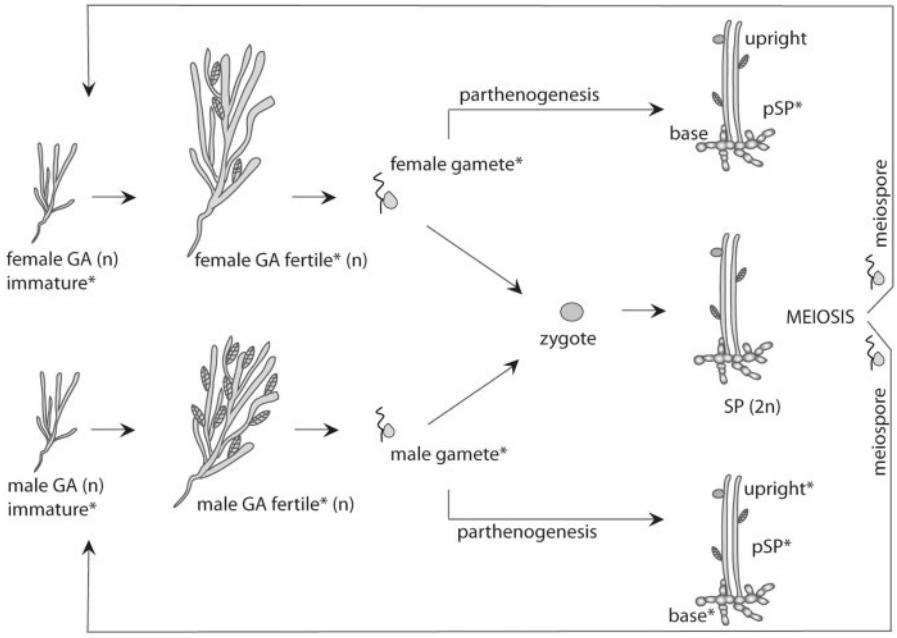

Fig. 1. The *Ectocarpus* life cycle. The life cycle of *Ectocarpus* sp. involves alternation between two independent multicellular generations, the gametophyte (GA) and the sporophyte (SP). Sporophytes produce meiotic spores (meiospores) that develop into haploid gametophytes, which are either male or female (dioecious). After approximately 3 weeks, gametophytes become fertile and produce gametes in reproductive structures (plurilocular gametangia). After release into the water column, male and female gametes strongly differ in their behavior and physiology. Female gametes settle rapidly and release a pheromone to attract male gametes, which then fuse with the female gametes to form zygotes (syngamy). Zygotes develop to produce diploid sporophytes, completing the cycle. Gametes that fail to fuse are able to develop parthenogenetically into haploid parthenosporophytes (pSP). Parthenogenesis is depicted for both male and female gametes. This is observed in some strains but in the majority of *Ectocarpus* species only the females are capable of parthenogenesis. Partheno-sporophytes are morphologically and functionally indistinguishable from diploid sporophytes. Life cycle stages used for transcriptomic analysis are marked with an asterisk.

shown to involve a UV sex chromosome system (Ahmed et al. 2014). In the present study, the level of sexual dimorphism in *Ectocarpus* was precisely quantified using morphometric methods and RNA sequencing (RNA-seq) was used to characterize sex-biased expression. Several unusual features were noted, compared with previously characterized sexual systems. First, fewer than 12% of *Ectocarpus* genes exhibited sex-biased expression, consistent with the low level of sexual dimorphism in this species. Second, both male and female sex-biased genes showed accelerated rates of evolution compared with unbiased genes, with male- and female-biased genes evolving at a similar pace. This balanced rate of evolution is also consistent with the low level of sexual dimorphism, which presumably provides limited scope for asymmetric sexual selection. Gene duplication has played a significant role in the generation of sex-biased genes in *Ectocarpus* and the evolution of these genes has been shaped by both positive selection and relaxation of purifying selection. We identified no clear effects of the UV sexual system on the genomic distribution of sex-biased genes but the PAR was found to be enriched in female-biased genes expressed during the mature gametophyte stage.

Results

Ectocarpus Exhibits a Low Level of Sexual Dimorphism

Sex is determined genetically during the haploid gametophyte generation of the *Ectocarpus* haploid–diploid life cycle (fig. 1) by a UV sexual system (Müller 1975; Ahmed et al. 2014). Meiosis occurs during the sporophyte generation, producing meiospores, which develop into either male or female gametophytes. The gametophyte generation produces either male or female gametes, depending on its sex, in sexual structures called plurilocular gametangia.

Morphometric analysis showed that male gametophytes were significantly smaller than female gametophytes at fertility but that they produced significantly more reproductive structures (plurilocular gametangia) despite their smaller size (fig. 2*A*, Student's *t*-test, $P < 0.0001$). Consequently, male gametophytes presumably produce more gametes than females, because they produce a larger number of plurilocular gametangia per individual.

Ectocarpus gametes have been described as being morphologically isogamous and physiologically anisogamous (Schmid et al. 1994). The physiological anisogamy refers to the behavior of the two types of gamete during the fertilization process. The female gametes settle rapidly after release from the plurilocular gametangia, retract their flagella, and then produce a pheromone to attract male gametes. Male gametes swim for longer and are attracted to the immobile female gametes by the pheromone. We used flow cytometry to precisely measure male and female gamete size in three different species of *Ectocarpus*. This analysis, based on measurements of more than 1,000 gametes, showed that male gametes not only exhibit physiological and behavioral differences

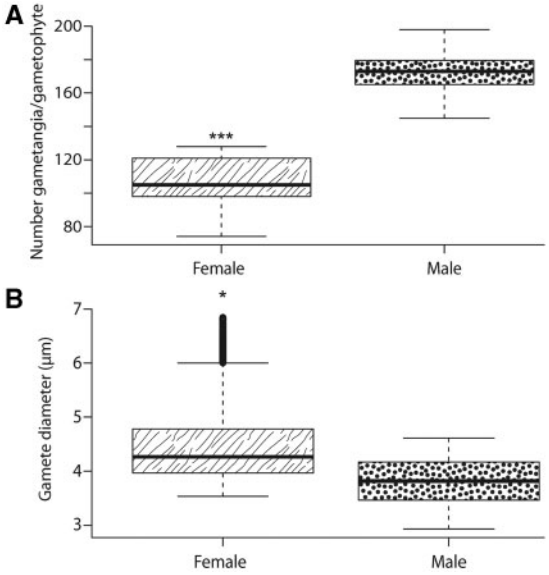

FIG. 2. Sexual dimorphism in *Ectocarpus* gametophytes. (*A*) Number of reproductive structures (plurilocular gametangia) per female ($n = 6$) and male ($n = 8$) gametophyte. Males produced significantly more reproductive structures (Student's *t*-test, $P < 0.0001$). Error bars show standard errors. The number of plurilocular gametangia for each female gametophyte was 128, 109, 74, 121, 101, 98 and for each male gametophyte 176, 145, 198, 178, 169, 170, 181, 161. (*B*) Mean diameters (µm) of female ($n = 5{,}668$) and male ($n = 5{,}619$) gametes. Female gametes (mean diameter 4.46 µm) were significantly larger (Mann–Whitney *U* test, $P < 0.0001$) than male gametes (mean diameter 3.83 µm). Error bars show standard errors. Mean gamete sizes for male and female individuals of other *Ectocarpus* species are provided in supplementary figure S5, Supplementary Material online.

compared with female gametes but they are also slightly, but significantly, smaller (fig. 2*B*, Mann–Whitney *U* test, $P < 0.0001$).

Taken together, these analyses identified sexual dimorphisms at both the gametophyte and gamete stages that had not been previously described. *Ectocarpus* therefore clearly exhibits sexual dimorphism, but the differences between males and females are subtle.

Analysis of Gene Expression during the Development of the Sexual Generation, the Gametophyte

Gene expression patterns during sexual differentiation were measured by deep sequencing (RNA-seq) of cDNA from haploid male and female gametophytes of *Ectocarpus* at two different sexual developmental stages: In juvenile immature gametophytes before the formation of the sexual structures (approximately 10 days after meiospore settlement) and at sexual maturity, when sexual structures were visible (fig. 1). Transcript abundances, measured as RPKM (reads per kilobase per million mapped sequence reads), were strongly correlated between biological replicates of each sex and life cycle stage, with *r* ranging from 0.91 to 0.99 ($P < 2\,e^{-16}$).

Counts of expressed genes (RPKM $>$ 1) identified 13,102 and 12,660 genes that were expressed at the immature stage (male and female, respectively) and 13,941 and 13,663 genes that were expressed at maturity (male and female, respectively). This indicates that about 88% of the protein-coding genes in the genome are transcribed during the gametophyte generation (supplementary fig. S1, Supplementary Material online).

Sex-Biased Gene Expression

Fewer than 12% of *Ectocarpus* genes showed sex-biased expression during the gametophyte generation (including both immature and fertile stages). This is considerably less than the numbers identified in previously characterized systems with more marked morphological sexual dimorphism such as *Drosophila* (e.g., Jiang and Machado 2009) and birds (Pointer et al. 2013) but coherent with the low level of morphological sexual dimorphism in *Ectocarpus*.

Unexpectedly, the number of genes that were differentially transcribed between males and females was higher during the immature gametophyte stage than at gametophyte fertility (fig. 3*A* and *B*). Male-biased genes were more numerous than female-biased genes at both developmental stages, although the numbers for the most strongly differential genes (fold change [FC] $>$ 10) were comparable for the two sexes (fig. 3*A* and *B* and supplementary table S1, Supplementary Material online). The majority of the sex-biased genes showed significant sex-biased expression in only one of the two developmental stages analyzed; only 12% of the male- and 3% of the female-biased genes were differentially expressed in both immature and fertile gametophytes (supplementary fig. S2, Supplementary Material online). Moreover, 3% of the genes that showed male-biased expression in the immature gametophytes were female-specific at maturity. Transitions from female-biased to male-biased were not detected. As females produce fewer plurilocular gametangia than males, we cannot exclude that differences in tissue complexity between male and female fertile gametophytes explain, at least in part, the slight excess of male-biased to female-biased genes (supplementary figs. S1 and S2, Supplementary Material online). Note, however, that comparison of immature gametophytes (where reproductive structures are absent) also identified a slight excess of male-biased over female-biased genes.

To examine the relationship between degree of sex-biased expression and transcript abundance (expression level), the sex-biased genes were grouped according to the FC difference between male and female samples and mean expression level in males and in females plotted for each group (fig. 3*C*). This analysis indicated that when genes exhibited a high degree of female-biased expression, this was predominantly due to downregulation of these genes in males. This was observed at both immature and fertile gametophyte stages. The results obtained for male-biased gene were more complex. In immature gametophytes, the situation was similar to that observed for the female-biased genes in that a high degree of male-biased expression appeared to be correlated with downregulation in females. In contrast, in mature gametophytes, when genes exhibited a high degree of male-biased expression this appeared to be due to a combination of both decreased expression in females and upregulation in males. We also noted

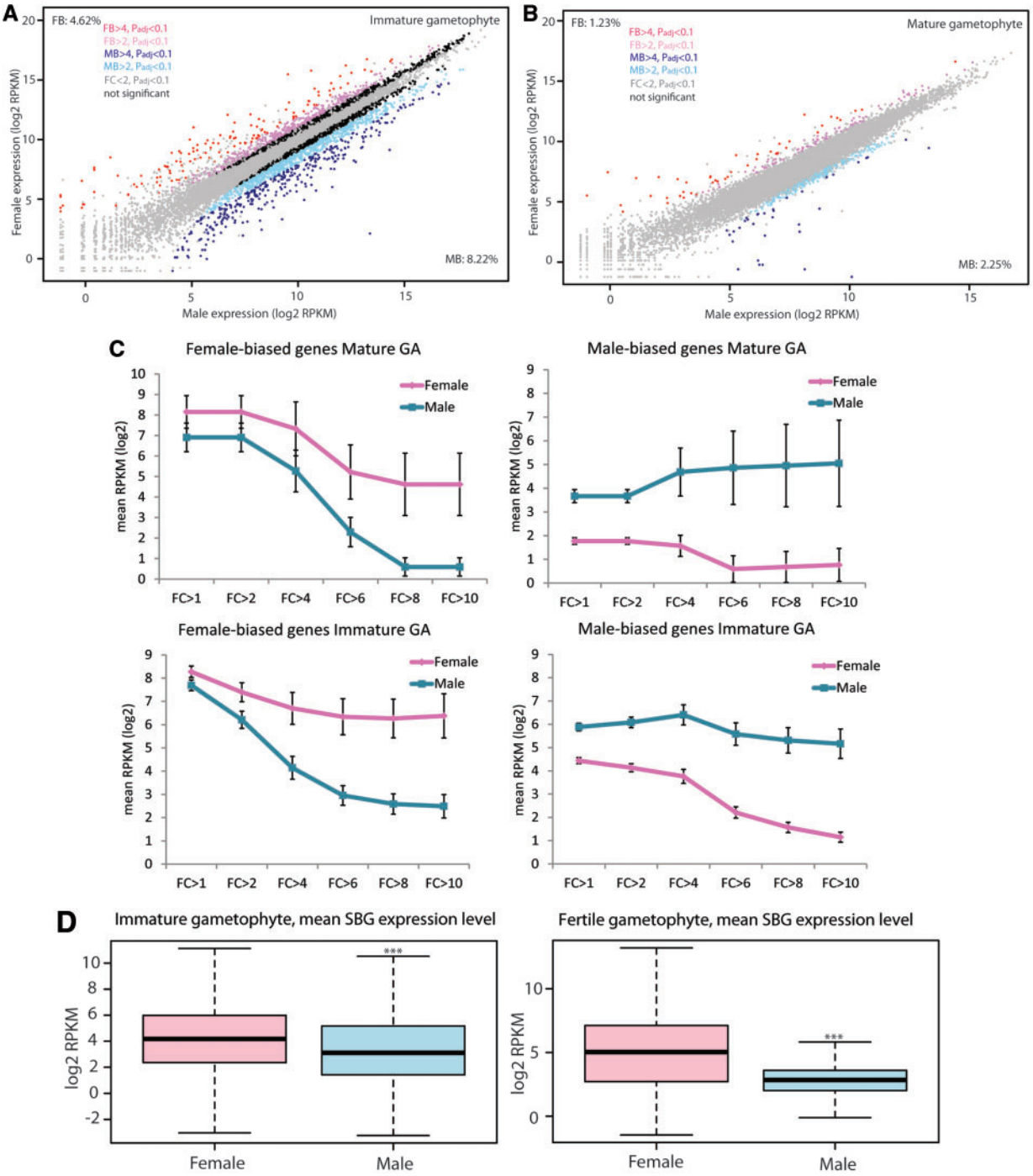

Fig. 3. Sex-biased gene expression. (*A*) Comparison of gene expression levels in male and female immature gametophytes. (*B*) Comparison of gene expression levels in male and female mature gametophytes. Colored dots indicate genes that exhibited significantly different levels of transcript abundance (sex-biased genes). Percentages in each panel indicate genes that were at least 2-fold female-biased (FB; upper left) and male-biased (MB; lower right). FC (fold change); *P*-adjusted (P_{adj}). Unbiased genes were defined as $P_{adj} > 0.1$ or less than 2-fold difference between the sexes. See also table 1. (*C*) Mean gene expression levels (RPKM) at several degrees of sex-bias (from FC > 1 to FC > 10) for female- (pink) and male-biased (blue) genes in fertile and immature gametophytes. Genes located in the SDR were excluded from this analysis. Error bars represent standard errors. (*D*) Boxplot showing the mean expression levels (RPKM) of female- and male-biased genes for immature and fertile gametophytes.

that, on average, female-biased genes were expressed at significantly higher levels than male-biased genes in both fertile and immature gametophytes (Mann–Whitney *U* test, $P < 2\,e^{-16}$) (fig. 3*D*).

Breadth of Expression of Sex-Biased Genes

The breadth of expression of a gene, that is, the extent to which its expression is limited to specific tissues or developmental stages, is a key determinant of its speed of evolution

Table 1. Relative Gene Expression for Male and Female Gametophytes.

		No. Genes	% of Expressed Genes
Immature gametophytes			
Female-biased ($P_{adj} < 0.1$)	**FC > 2**	**585**	**4.62%**
	FC > 4	**131**	**1.03%**
	FC > 10	**68**	**0.54%**
	Total expressed genes (RPKM > 1)	**12,661**	
Male-biased ($P_{adj} < 0.1$)	**FC > 2**	**1,077**	**8.22%**
	FC > 4	**295**	**2.25%**
	FC > 10	**78**	**0.60%**
	Total expressed genes (RPKM > 1)	**13,102**	
Fertile gametophytes			
Female-biased ($P_{adj} < 0.1$)	**FC > 2**	**168**	**1.23%**
	FC > 4	**61**	**0.45%**
	FC > 10	**29**	**0.21%**
	Total expressed genes (RPKM > 1)	**13,660**	
Male-biased ($P_{adj} < 0.1$)	**FC > 2**	**314**	**2.25%**
	FC > 4	**54**	**0.39%**
	FC > 10	**32**	**0.23%**
	Total expressed genes (RPKM > 1)	**13,937**	

NOTE.—Categories of immature or fertile gametophyte sex-biased genes with different levels of FC between the two sexes indicated both as number of genes (N. genes) and as a percentage of the total number of genes expressed (% of expressed genes) in the immature or fertile gametophyte of the corresponding sex.

(Duret and Mouchiroud 2000; Zhang et al. 2004). In the moss *Funaria hygrometrica*, which also has a haploid–diploid life cycle, the effect of breadth of expression was shown to be stronger than the masking effect associated with expression during the diploid phase (Szovenyi et al. 2013). In organisms with haploid–diploid life cycles, the breadth of expression of sex-biased genes is restricted because they tend to be preferentially expressed during the haploid phase (sexuality is only expressed during this phase of the life cycle). This restricted pattern of expression is expected to have a significant effect on their evolutionary rates.

When determining the breadth of expression of *Ectocarpus* genes, we integrated both spatial (tissue) and temporal (developmental and/or life cycle stage) information to obtain meaningful estimates because this species exhibits only a limited level of tissue differentiation during development. We determined the breadth of expression of the sex-biased genes using the specificity index (τ) (see Materials and Methods) and gene expression data collected both for different tissues (upright filaments vs. prostrate tissues during the sporophyte generation; fig. 1) and for different stages of the life cycle (parthenosporophyte, immature and fertile gametophyte and gamete stages; fig. 1). Male and female sex-biased genes had significantly higher τ values compared with unbiased genes, indicating that the former have a greater tendency to be expressed specifically in particular tissues or stages of the life cycle. However, no difference in breadth of expression was observed when the male- and female-biased gene sets were compared with each other (fig. 4). Note that the decrease in the breadth of expression of SBGs was not solely due to their sex-biased pattern of expression; when τ was calculated with a data set in which the male and female samples had been pooled, the male and female SBGs still showed a significantly lower breadth of expression than unbiased genes (Kruskal–Wallis test, $P < 10^{-4}$).

Functional Analysis of Sex-Biased Genes

An analysis of gene ontology (GO) terms associated with the sex-biased genes was carried out using BLAST2GO (Conesa and Gotz 2008) to search for enrichment in particular functional groups and to relate gene function to phenotypic sexual dimorphisms. Significant enrichment of specific GO categories was only detected for fertile male gametophyte and immature female gametophyte sex-biased genes. The set of male-biased genes in mature gametophytes was enriched for "microtubule" and "calcium binding-related" processes. These genes may be involved in the production of flagellated gametes inside plurilocular gametangia. Note that the same GO categories were enriched in the set of sex-biased genes expressed in male gametes identified by Lipinska et al. (2013). The set of female-biased genes in juvenile gametophytes was enriched for "photosynthesis" GO terms, consistent with the more extensive growth phase in the female gametophyte.

A test was also carried out to identify GO terms enriched in the expressed gene sets of the immature compared with the fertile developmental stage of the gametophyte, irrespective of sex. Genes involved in posttranslational regulation of gene expression, cellular component biogenesis, and photosynthesis were significantly enriched in immature compared with fertile gametophytes (FDR < 5%), whereas genes predicted to be involved in signaling, microtubule-based processes, and energy metabolism were significantly enriched in mature compared with immature gametophytes (FDR < 5%) (supplementary table S2, Supplementary Material online). The enriched gene GO terms were coherent overall with the transition from vegetative growth to reproductive function, particularly the production of flagellated gametes, between these two stages of development.

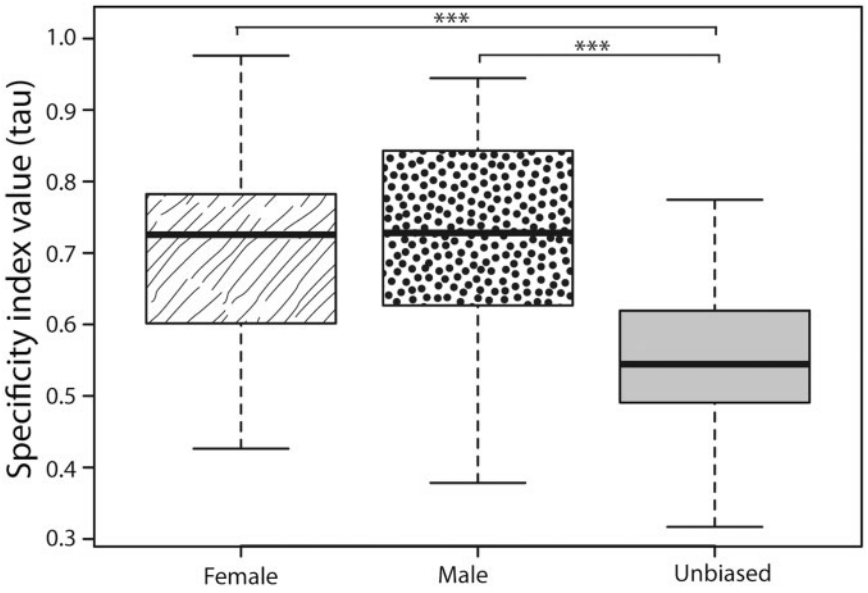

Fig. 4. Breadth of expression of the sex-biased genes as determined using the specificity index. Comparison of specificity index values (τ) for unbiased and for male- and female-biased genes. Male- and female-biased genes had significantly larger specificity index values (i.e., lower breadth of expression) compared with unbiased genes (Kruskal–Wallis test, $P < 10^{-5}$).

Genomic Locations of Sex-Biased Genes

An analysis of the genomic distribution of sex-biased genes expressed in fertile gametophytes found that the PAR region of the sex chromosome was enriched in female-biased genes expressed at this stage compared with the rest of the genome (Chi-squared test, $P < 0.01$) (supplementary fig. S3, Supplementary Material online). Moreover, when RPKM values were used to determine the ratios of transcript abundances in fertile female gametophytes compared with fertile male gametophytes for all the PAR genes, a significant bias toward expression in the female was detected, compared with all the autosomal genes (Kruskal–Wallis, $P < 0.001$) (fig. 5). These tendencies were not observed for sex-biased genes expressed in immature gametophytes. These observations suggest that the PAR and the autosomes are not evolving under the same selection pressures during the fertile gametophyte stage of the life cycle.

Evidence of a Role for Gene Duplication in Resolving Sexual Antagonism

Gene duplication is thought to have played a significant role in the evolution of sex-biased gene expression in *Drosophila* (Connallon and Clark 2011; Wyman et al. 2012). Duplication of a gene can release one or both of the duplicated products from selective constraints allowing the evolution of modified patterns of expression or of new gene functions. Gene duplication therefore represents a potential means to resolve sexual antagonism. The simplest mechanism would be the generation, after duplication, of one male- and one female-biased gene with male- and female-optimized functions, respectively. Other alternatives are possible, however. For example, it may be sufficient for only one member of a duplicated pair to evolve sex-specific functions to resolve a

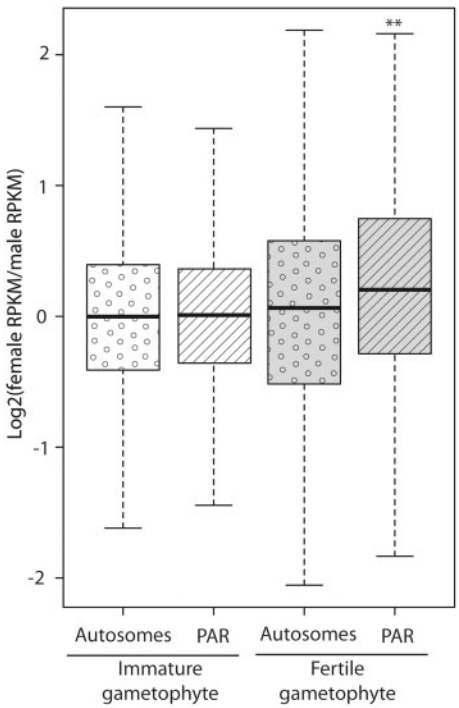

Fig. 5. Ratios of female-to-male expression level in immature and fertile gametophytes for genes on autosomes and genes on the PAR. The figure shows log2 of female/male RPKM ratios for autosomal and PAR genes during the immature and fertile gametophyte stages. Outliers were removed from the plot.

sexual antagonism. In such cases, gene duplication could help resolve sexual conflict for genes with ontogenetic or pleiotropic constraints by allowing one of the duplicated paralogs to evolve sex-biased expression whereas other maintains a general, sex-independent function (Gallach and Betran 2011;

Wyman et al. 2012). It is also possible that duplication of a gene that is already sex-biased may allow one of the duplicates to evolve an even stronger sex-biased function (Wyman et al. 2012).

Genome-wide analysis detected a total of 879 duplicated gene pairs in *Ectocarpus*. Of these, 174 pairs included at least one sex-biased gene. Only 3 of these 174 pairs included both a male-biased and a female-biased gene. These three duplicated gene pairs were autosomal and sex-biased expression was detected during the immature gametophyte stage. Comparisons with sequence data sets for other Ectocarpales species identified orthologs for only one of the genes from these three autosomal gene pairs (Esi0002_0006) but this locus did not show any signatures of positive selection. The other sex-biased, duplicated gene pairs included 143 pairs in which only one member of the pair exhibited sex-biased expression and 28 pairs where both members exhibited sex-biased expression, but in the same sex. The 143 duplicated gene pairs in which only one member exhibited sex-biased expression potentially correspond to events where gene duplication has released one member of the gene pair from selective constraints allowing it to evolve a sex-specific function. This hypothesis is supported by the fact that the specificity index (τ) values for the non-sex-biased members of these pairs are significantly lower than those of the sex-biased members (Kruskal–Wallis test with Dunn's posttest, $P < 10\,e^{-8}$) and are not significantly different from values for randomly selected single copy unbiased genes (fig. 6*A* and *B*).

No evidence has been found for whole genome duplication events having occurred in the lineage leading to *Ectocarpus* (Cock, Sterck, et al. 2010), suggesting that the 879 duplicated gene pairs in the genome of this species arose as a result of small-scale duplication events. When the proportion of the genome corresponding to sex-biased genes is taken into account (1,947 of 16,262 genes), duplicated gene pairs containing at least one sex-biased gene are overrepresented in the total set of 879 duplicated gene pairs (Chi-squared test, $P = 1.5\,e^{-12}$). This overrepresentation was also detected if only male-biased (Chi-squared test, $P = 8.77\,e^{-6}$) or only female-biased genes (Chi-squared test, $P = 2.47\,e^{-5}$) were considered. The results of these tests suggest that the resolution of sexual conflict was one of the forces driving gene duplication in this genome and support a role for gene duplication in the generation of sex-biased genes in this species.

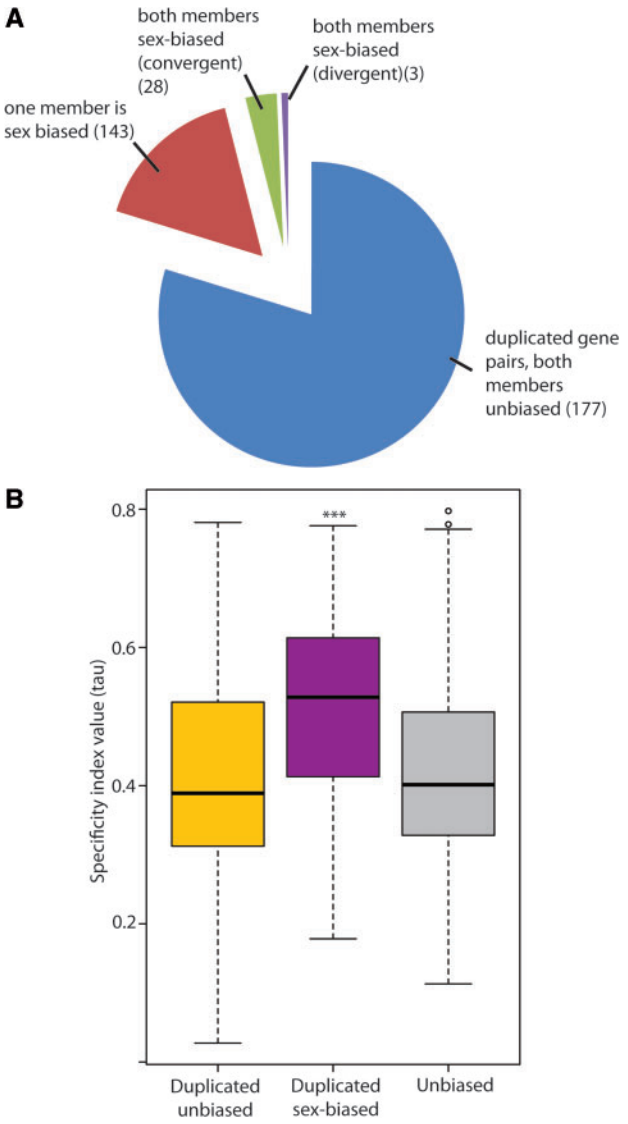

FIG. 6. Duplicated sex-biased genes in *Ectocarpus*. (*A*) Distribution of sex-biased genes among the duplicated gene pairs. (*B*) The sex-biased members (Duplicated sex-biased) of the 143 duplicated gene pairs that include one sex-biased and one unbiased member have a narrower breadth of expression than the unbiased members of these pairs (Duplicated unbiased). A random sample of unbiased single copy genes (Unbiased) is included for comparison. Comparison of breadth of expression is presented using the specificity index (τ). The median for unbiased members of duplicated pairs was significantly lower than the median for sex-biased paralogs (Kruskal–Wallis test with Dunn's posttest, $P < 10\,e^{-8}$) but was not significantly different from the median for single copy unbiased genes.

Sex-Biased Genes Are Evolving More Rapidly

To test for differences in rates of evolutionary divergence between different categories of sex-biased and unbiased genes, we calculated levels of nonsynonymous (d*N*) and synonymous (d*S*) substitution using pairwise comparisons with orthologs from the sister species *Ectocarpus fasciculatus*.

The results of this analysis indicated that genes that exhibited sex-biased expression patterns (either male- or female-biased expression) in fertile gametophytes had evolved significantly faster (i.e., had higher d*N*/d*S* values) than had unbiased genes (Mann–Whitney *U* test, $P < 0.01$). A similar, but weaker, pattern was observed for genes that were male-biased in immature gametophytes (Mann–Whitney *U* test; $P < 0.01$) but the rates of evolution of female-biased genes identified at this developmental stage were not significantly different from those of unbiased genes (fig. 7*A*). Therefore, although the evolution rates of male and female sex-biased genes were similar overall, differences were detected when the developmental stage at which the genes were expressed was taken into account. These differences suggest not only that the average selection pressure

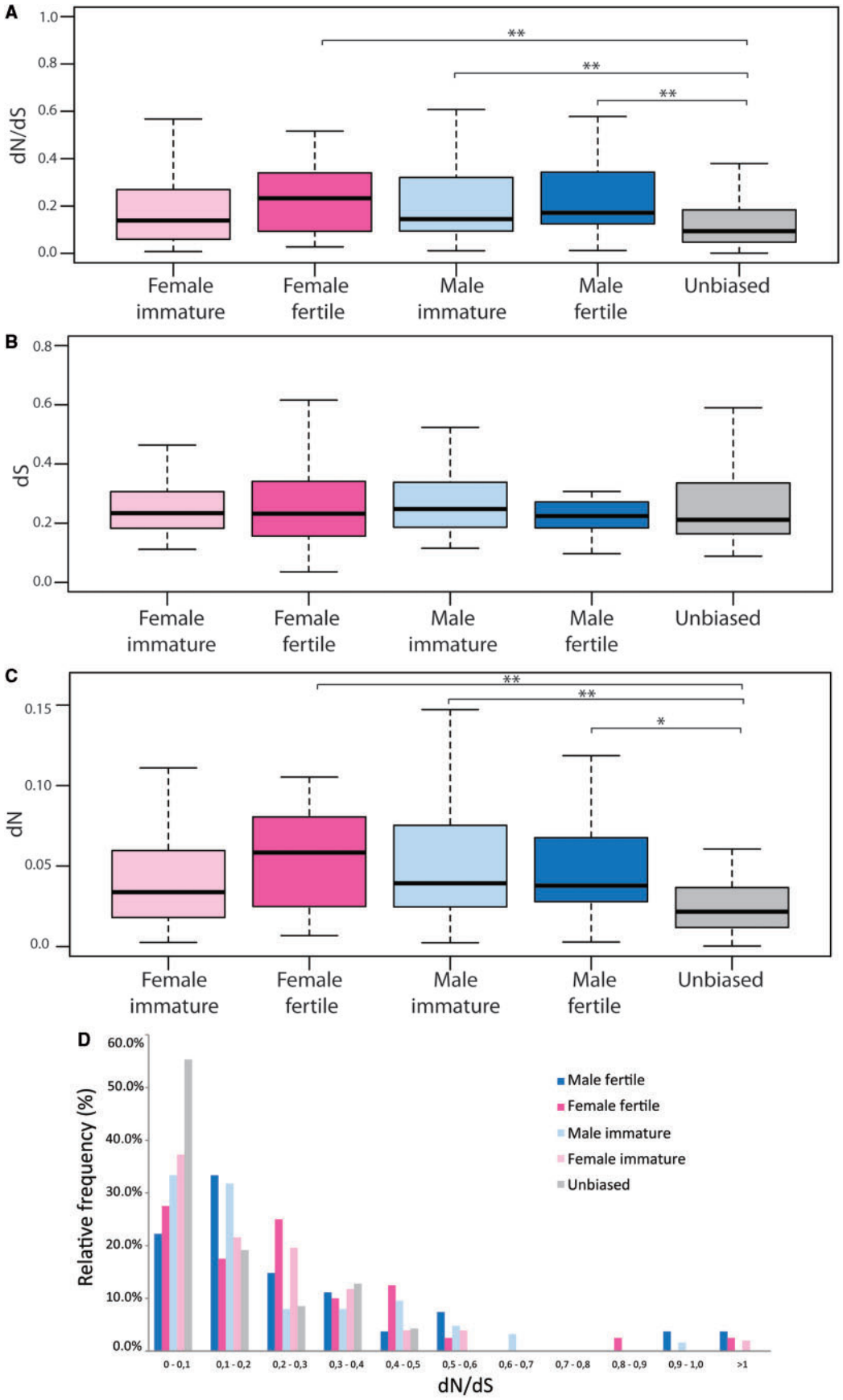

Fig. 7. Rates of evolution of female-biased, male-biased, and unbiased genes. Pairwise d*N*, dS, and d*N*/dS ratios were calculated by comparing orthologous gene sequences from *Ectocarpus* sp. lineage 1c Peru and *Ectocarpus fasciculatus*. (*A*) Ratio of nonsynonymous to synonymous substitutions (d*N*/dS). (*B*) and (*C*) Nonsynonymous substitutions (d*N*) and synonymous substitutions (dS). (*D*) Frequency of classes of d*N*/dS ratio in unbiased genes

(continued)

may vary during development but also that there may be some asymmetry in the evolution rates of male- and female-biased genes that are expressed at particular developmental stages. Concerning this latter point, however, it is possible that the stage at which the comparison was carried out is not directly comparable in males and females because the immature females delay reproduction in order to prolong growth. The comparison is therefore between a stage in males where there may already have been a cryptic transition toward the reproductive phase, as indicated by the greater overlap between the male-biased gene sets identified in immature and fertile individuals, and a stage in females which is equivalent in terms of timing but which corresponds to a continuation of the prereproductive growth phase.

The elevated d*N*/d*S* values for sex-biased compared with unbiased genes were due to significantly higher levels of nonsynonymous substitution (Mann–Whitney *U* test, $P < 0.05$) and not to a reduction in the synonymous substitution rate (fig. 7*B* and *C*). Analysis of the distribution of d*N*/d*S* values indicated that the different groups of sex-biased genes (i.e., male- or female-biased, expressed in immature or fertile gametophyte) tended to be enriched in genes with high d*N*/d*S* values, including values of 1 or more, and to contain fewer genes under strong selective constraint (d*N*/d*S* < 0.1) compared with the group of unbiased genes (fig. 7*D*). No correlation was detected between the degree of sex-bias (FC calculated by DESeq) and the rate of evolution (d*N*/d*S*) of the tested genes (Spearman's $\rho = 0.166$, $P = 0.0516$).

Analysis of specificity index (τ) values indicated that the rates of evolution of the sex-biased genes were only weakly correlated with breadth of expression (Spearman's $\rho = 0.1395$, $P = 0.0229$). This suggests that the effect of sex-biased expression on evolution rate was not solely an indirect effect of restricting gene expression patterns.

Expression bias in sexual tissues has been associated with optimal codon usage, a feature that promotes efficient translation (Duret 2000; Duret and Mouchiroud 2000). For instance, optimal codons occur less frequently in male-biased than in female-biased sexual genes in *Drosophila* (Hambuch and Parsch 2005), suggesting that adaptive protein evolution has modified selection on codon usage. Calculations of the Effective Number of Codons (ENC) and the Codon Adaptation Index (CAI) indicated that selection to maintain codon usage bias in *Ectocarpus* sex-biased genes is globally preserved (supplementary fig. S4*A* and *B*, Supplementary Material online).

As expected, codon usage bias was strongly correlated with the level of gene expression in *Ectocarpus* (CAI vs. $\log_2$RPKM, Spearman's $\rho = 0.623$, $P = 3.76\,e^{-06}$). A slight decrease in CAI was observed in female-biased compared with unbiased genes (Mann–Whitney *U* test, $P = 0.02$) but there was no significant difference in codon usage parameters (CAI and ENC) either between the male-biased genes and unbiased genes or between male and female sex-biased genes.

Evidence for Positive Selection of Sex-Biased Genes

To assess whether differences in divergence rates were due to increased positive selection or relaxed purifying selection, we used sequence data from several Ectocarpales species (supplementary table S3, Supplementary Material online) to estimate direction of selection. We tested 137 sex-biased genes (65 female-biased and 72 male-biased; including 12 genes with d*N*/d*S* > 0.5) and 137 randomly selected unbiased genes using the paired nested site models (M1a, M2a; M7, M8) implemented in PAML4 (CODEML) (Yang 2007). The second model in each pair (M2a and M8) is derived from the first by allowing variable d*N*/d*S* ratios between sites to be greater than 1, making it possible to detect positive selection at critical amino acid residues. This analysis detected evidence of positive selection for 5 of the 12 sex-biased genes with d*N*/d*S* values of greater than 0.5, including both male- and female-biased genes. Moreover, evidence of positive selection was also found for 12 of the remaining 125 sex-biased genes with lower d*N*/d*S* values based on either one or both pairs of models (M1a–M2a, M7–M8) (supplementary table S4, Supplementary Material online). In contrast, only 5 of the 137 unbiased genes had signatures of adaptive evolution, indicating that the set of sex-biased genes was significantly enriched in genes that were under positive selection (Fisher's exact test, $P = 0.0149$).

Discussion

A Complex Relationship across Sexual Species between the Proportion of the Transcriptome Showing Sex-Biased Expression and the Degree of Sexual Dimorphism

Analyses of sex-biased gene expression in *Drosophila* have shown that a large proportion of the transcriptome is differentially expressed in the two sexes (Ellegren and Parsch 2007; Jiang and Machado 2009; Assis et al. 2012). A similar observation was made for turkeys, where it was further shown that male-biased gene expression is significantly enhanced, across the genome, in dominant compared with subordinate males (Pointer et al. 2013). Given that dominant males exhibit stronger secondary sexual characteristics than subordinates, these studies indicate a correlation between the degree of sex-biased gene expression and the extent of sexual dimorphism. However, there is also evidence that the relationship between the level of sex-biased gene expression and the degree of sexual dimorphism may be more complicated. For example, in *Drosophila* more sex-biased genes were detected during the

Fig. 7. Continued

and male- and female-biased genes expressed in immature and fertile gametophytes. Outliers were removed from the plot. Pairwise statistical significance between the four groups of sex-biased genes on the one hand and the unbiased genes on the other was calculated for panels (*A*)–(*C*), only statistically significant differences are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

juvenile stage than in adults, despite the lower degree of observable sexual dimorphism during the former phase of development (Mank et al. 2010; Perry et al. 2014). Further studies are therefore required to investigate the exact relationship between these two parameters.

Ectocarpus represents an interesting system in this respect because the studies that have been carried out to date have focused on species that exhibit very marked sexual dimorphism. In contrast, we show here that this brown alga exhibits a limited degree of sexual dimorphism, restricted to subtle growth-habit and fertility differences during the gametophyte stage and a small difference in male and female gamete sizes. Accordingly, less than 12% of the genes in the genome were found to be differentially regulated between sexes, supporting the hypothesis that the overall degree of sex-biased gene expression and the level of phenotypic sexual dimorphism are correlated.

Analysis of the expression of *Ectocarpus* sex-biased genes during development revealed a more complex relationship between the expression patterns of these genes and the manifestation of sexually dimorphic traits. As observed with *Drosophila*, more sex-biased genes were detected during the sexually immature stage than in fertile, sexually mature individuals, despite the fact that the former exhibited less marked sexual dimorphism. Similarly, male and female gametes have been shown to exhibit high levels of sex-biased expression despite limited phenotypic sexual dimorphism (Lipinska et al. 2013). Thus, there is evidence in both *Drosophila* and *Ectocarpus* that the correlation between the level of sex-biased gene expression and the level of observed sexual dimorphism breaks down to some extent when the relationship is examined over the course of development. As *Ectocarpus* and *Drosophila* are two phylogenetically distant organisms with very marked differences in their levels of sexual dimorphism, these observations suggest that the lack of correlation between sex-biased gene expression and sexual dimorphism in immature individuals may be a general feature of sexual systems, but further studies on diverse sexual organisms are required to confirm this. One possible reason for this could be that part of the sex-biased gene expression is related to differences at the cellular level that do not have any effect on morphology.

Analysis of predicted gene functions indicated that about 12% of the male-biased genes expressed during the immature stage were also expressed in fertile gametophytes, but there was less overlap between female-biased genes expressed at the two stages (3% of the female-biased genes). This suggests that immature females were principally carrying out processes unrelated to those engaged at maturity, such as filamentous growth for example, whereas reproductive processes were already initiated to some extent in immature males, before any phenotypic change could be detected. Somewhat paradoxically, therefore, one of the roles of sex-biased genes in females may be to suspend reproductive functions to allow more extensive vegetative growth during the juvenile phase.

As far as the mechanism of evolution of the sex-biased genes in *Ectocarpus* is concerned, the set of sex-biased genes in this species is enriched in genes that are members of duplicated pairs indicating that neo- or subfunctionalization following gene duplication is one of the mechanisms through which sex-biased genes evolve in this brown alga. Gene duplication has been proposed to be one of the means of resolving sexually antagonistic conflict in other systems (Connallon and Clark 2011; Gallach and Betran 2011; Wyman et al. 2012).

Symmetrical Evolution Rates of Male- and Female-Biased Genes in *Ectocarpus*

In general, sex-biased genes tend to evolve at faster rates than unbiased genes and this effect is usually significantly more marked for male-biased genes than for female-biased genes (reviewed in Ellegren and Parsch [2007]). The faster evolution rate is thought to be due, at least in part, to positive selection acting on the sex-biased genes, the most likely underlying causes being sexual selection and/or sexual antagonism. The sex-biased genes in *Ectocarpus* also exhibit faster evolution rates than unbiased genes but this system is unusual in that, overall, male- and female-biased genes have evolved at similar rates. There are several possible explanations for this symmetry. The most obvious explanation, which is consistent with the low level of sexual dimorphism in this system, is that male- and female-biased genes are under similar levels of sexual selection. Both male and female gametes are small, motile cells that are produced in large numbers in plurilocular gametangia by male and female gametophytes, respectively. It is not known whether gamete competition occurs during fertilization under natural conditions but, if it does occur, the mechanism involved affords scope for both male and female competitions. Male gametes may compete to find and fertilize the settled female gametes, but the abundant female gametes may compete for optimal niches in which to settle and then compete with each other to attract male gametes through pheromone production. It is therefore quite possible that selection pressures on males and females are very similar in this organism.

Sex-biased genes in *Ectocarpus* are expressed during the haploid phase of the cycle and therefore directly exposed to purifying selection (Kondrashov and Crow 1991; Orr and Otto 1994; Gerstein et al. 2011). Another possible explanation for the symmetric evolution rates of male- and female-biased genes in *Ectocarpus* may be that haploid phase purifying selection is strong enough to mask any effects of sexual selection or sexual antagonism. This seems unlikely, however, as land plants also possess a haploid gametophyte generation and selection-driven evolution suggestive of sexual selection has been detected in this group of organisms (Arunkumar et al. 2013; Gossmann et al. 2014).

Another possible factor affecting evolution rate is breadth of expression pattern, as broadly expressed genes tend to be more constrained and therefore to evolve less rapidly than genes with restricted patterns of expression (Hastings 1996; Duret and Mouchiroud 2000). In *Drosophila* one of the reasons that female-biased genes evolve less quickly than male-biased genes may be that, in general, they tend to have broader patterns of expression (e.g., Meisel 2011; Grath and

Parsch 2012). Our analysis, based on RNA-seq analysis of multiple life cycle stages and tissues, indicated that, in contrast, both male- and female-biased genes in *Ectocarpus* tend to have restricted patterns of expression compared with unbiased genes (fig. 4). This parallel reduction in breadth of expression may be one of the factors underlying the symmetrical accelerated evolution of male- and female-biased genes in this species. However, we noted that there was only a weak positive correlation between expression breadth (τ) and evolutionary rate (d*N*/d*S*), suggesting that other factors have also influenced evolutionary rates.

In summary, therefore, possible explanations for the symmetrical rates of evolution of male- and female-biased genes in *Ectocarpus* include limited sexual selection, impacting similarly males and females, due to a low level of sexual dimorphism and comparable levels of breadth of expression pattern.

Sexual Selection Is One of the Forces that Drives the Evolution of Male- And Female-Biased Genes in *Ectocarpus*

The mean d*N*/d*S* value for sex-biased genes in *Ectocarpus* was more than twice as high as that of unbiased genes. This difference, which was particularly marked for genes expressed in fertile gametophytes, was due to a significantly higher rate of nonsynonymous changes compared with the unbiased genes. A test for adaptive evolution detected evidence for positive selection in a significant proportion of the sex-biased genes with the highest d*N*/d*S* values (>0.5). Similar observations have been made for sperm-specific genes in *Arabidopsis thaliana* (Arunkumar et al. 2013) and for gametophyte-specific genes in the moss *Funaria hygrometrica* (Szovenyi et al. 2013). The evidence that positive selection acts on a considerable number of *Ectocarpus* sex-biased genes indicates that sexual selection may be one of the forces driving their evolution. Note however that positive selection only affects a subset of the *Ectocarpus* sex-biased genes and a significant proportion appear to be under relaxed selection. One important consideration in this respect is that a gene that is expressed in only one sex will experience half as much purifying selection because selection can only act on the gene when it is in the appropriate sex (Barker et al. 2005).

Patterns of Genomic Distribution of Sex-Biased Genes

In XY and ZW systems, the pattern of segregation of the sex chromosomes can have a measurable influence on the distributions of sex-biased genes on this linkage group. For XY systems, for example, X chromosomes spend twice as much time in females as they do in males. Male beneficial mutations can either accumulate or be purged from this chromosome depending on whether they are recessive or dominant (Rice 1984). There is no equivalent to this phenomenon in UV systems because the sex chromosomes function in the haploid generation. However, UV systems may share other features with XY and ZW systems that affect the distribution of sex-biased genes. In particular, even partial linkage to the SDR can be beneficial for genes with sexually antagonistic alleles, allowing alleles to segregate preferentially to the sex for which they are most adaptive (Otto et al. 2011; Jordan and Charlesworth 2012). This is predicted to lead to the accumulation of sexually antagonistic genes in the PAR, which in turn could lead to an accumulation of sex-biased genes in this region because sex-biased expression is one of the possible mechanisms of resolving sexual antagonism. There is some experimental evidence for this mechanism from work on the ZW sexual system of the emu, which has shown that the PARs of the homomorphic sex chromosomes of this species are enriched in male-biased genes (Vicoso et al. 2013). As expected, this effect was most pronounced for genes expressed in older embryos with fully developed gonads.

For UV systems, in the absence of any additional selective pressure favoring genes of one sex or the other, this effect of linkage to the SDR would not be expected to lead to a preferential accumulation of male-biased genes compared with female-biased genes or vice versa, but it might be expected to result in a general excess of sex-biased genes in the PAR. We did not observe any such excess in *Ectocarpus*, the proportion of sex-biased genes in the PAR was not significantly different from the proportion in the autosomes. However, compared with the autosomes, the *Ectocarpus* PAR was found to be significantly enriched in genes that exhibited female-biased expression during the fertile gametophyte stage. One possible explanation for this enrichment in female-biased genes may be a combination of an effect of linkage to the SDR together with stronger selection for female-biased genes during the fertile gametophyte stage.

There is accumulating evidence that gene duplication has played a significant role in the evolution of sex-biased genes in animals (Connallon and Clark 2011; Gallach and Betran 2011; Wyman et al. 2012) and the data presented here indicate that this has also been the case for *Ectocarpus*, suggesting that similar mechanisms may be operating to generate sex-biased genes across diverse eukaryote sexual systems.

Materials and Methods

Biological Material

Ectocarpus strains were cultured at 13 °C in autoclaved natural sea water (NSW) supplemented with half-strength Provasoli solution (PES; Starr and Zeikus 1993) with a light:dark cycle of 12:12 h (20 µmol photons m^{-2} s^{-1}) using daylight-type fluorescent tubes. All manipulations were performed under a laminar flow hood in sterile conditions. Near-isogenic lines, Ec602 female and Ec603 male, were prepared by crossing brothers and sisters for eight generations. This produced male and female strains with essentially identical genetic backgrounds apart from the sex locus. Supplementary table S3, Supplementary Material online, describes the *Ectocarpus* species used in this study. Note that currently only three species are recognized within the genus *Ectocarpus* (*E. siliculosus, E. fasciculatus,* and *E. crouanorium;* Peters et al. 2010) but there is increasing evidence that the taxon *E. siliculosus* represents a complex of several species. As the type specimen for *E. siliculosus* was collected in England, we prefer to refer to the non-European strains related to

E. siliculosus (such as the Peruvian and Greenland strains) as "*Ectocarpus* sp."

Male and female gametophytes of *Scytosiphon lomentaria* were collected at Asari, Japan in March 2012. *Scytosiphon lomentaria* has been described as exhibiting near-isogamy, with the male gametes being slightly smaller than the female gametes (Nagasato and Motomura 2002). The male and female gametophytes are morphologically similar and no sexual dimorphism has been described at this stage. *Scytosiphon lomentaria* was cultured in NSW with full strength PES. Two different light conditions were required to complete the life cycle. Short-day conditions, with a light:dark cycle of 10:14 h (20 µmol photons m^{-2} s^{-1}), were used to produce unilocular sporangia from a diploid sporophyte. After a month approximately 100 young gametophytes were isolated. The gametophytes were then subjected to long-day conditions with a cycle of 14:10 h to induce gametophyte maturation. Gametophytes became fertile after approximately 4 weeks and were frozen in liquid nitrogen. Each individual was sexed by crossing with male and female tester lines.

Measurement of Gamete Size

Male and female gamete size was measured in three different *Ectocarpus* species (see Stache-Crain et al. 1997 for a description of the lineage structure of the genus *Ectocarpus*): Isogenic male and female strains of *Ectocarpus* sp. lineage 1c Peru (Ec602 and Ec603), *E. siliculosus* lineage 1a Naples, and *Ectocarpus* sp. lineage 4 New Zealand. Synchronous release of gametes from 3- to 4-week-old cultures was induced by transferring ten gametophytes to a humid chamber in the dark for approximately 14 h at 13 °C followed by the addition of fresh PES-supplemented NSW medium under strong light irradiation. Gametes were concentrated by phototaxis using unidirectional light, and collected in Eppendorf tubes. Gamete size was measure by impedance-based flow cytometry (Cell Lab QuantaTM SC MPL, Beckman Coulter). Values of gamete size shown represent the mean ± SE of each gamete and measurements were taken for at least three biological replicates. A *t*-test ($\alpha = 5\%$) was performed using GraphPad Prism software to compare female and male gamete size.

Measurement of Gametophyte Size and Fertility

For the analysis of gametophyte habit and fertility, male and female near-isogenic strains (Ec602 and Ec603; supplementary table S3, Supplementary Material online) were placed in culture conditions as described above at constant density (ten individuals per 140-mm Petri dish). In each Petri dish, all ten gametophytes grew synchronously and attained approximately the same size. The gametophytes attained sexual maturity (production of plurilocular gametangia) after 3–4 weeks in culture. The number of plurilocular gametangia, each containing approximately 300 gametes, was counted under an inverted microscope for one individual randomly taken from each Petri dish. It was not possible to accurately weigh a single gametophyte, so ten gametophytes were pooled, weighed and the individual weight estimated by dividing by 10. Results shown correspond to the mean ± SE for six biological replicates for Ec602 and eight biological replicates for Ec603. Significant differences were tested using a corrected *t*-test with R software ($\alpha = 5\%$).

Generation of RNA-seq Data

RNA-seq analysis was carried out to compare the relative abundances of gene transcripts at different developmental stages of the life cycle (fig. 1). For the gametophyte generation, synchronous cultures of gametophytes of the near-isogenic male and female lines Ec603 and Ec602 were grown under standard conditions and frozen at early stages of development (about 10 days after meiospore release) and at fertility (presence of plurilocular gametangia). For each stage, total RNA was extracted from 2 bulks of 400 male individuals and 2 bulks of 400 female individuals (two biological replicates for each sex) using the Qiagen Mini kit (http://www.qiagen.com) as previously described (Coelho et al. 2012). Two biological replicates of basal parthenosporophyte filaments from strain Ec32 (which carries the V chromosome) were frozen in liquid nitrogen 10 days after settlement of gametes. Similarly, two biological replicates of upright filament tissue were isolated 15 days after settlement of gametes.

Two biological replicates for each sex of *S. lomentaria* were prepared by pooling between 8 and 12 individuals per sample. RNA from male and female pools was extracted using the protocol described by Apt et al. (1995). RNA quality and quantity was assessed using an Agilent 2100 bioanalyzer, associated with an RNA 6000 Nano kit.

RNA Sequencing

For each replicate, the RNA was quantified and cDNA was synthesized using an oligo-dT primer. The cDNA was fragmented, cloned, and sequenced by Fasteris (CH-1228 Plan-les-Ouates, Switzerland) using an Illumina Hi-seq 2000 set to generate 100-bp single-end reads. Supplementary table S5, Supplementary Material online, shows the statistics for the sequencing and mapping. Data quality was assessed using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and the reads were trimmed and filtered using a quality threshold of 25 (base calling) and a minimal size of 60 bp. Only reads in which more than 75% of the nucleotides had a minimal quality threshold of 20 were retained.

Filtered reads were mapped to the *Ectocarpus* sp. genome (Cock, Coelho, et al. 2010) (available at ORCAE; Sterck et al. 2012) using TopHat2 with the Bowtie2 aligner (Kim et al. 2013). More than 90% of the sequencing reads for each library could be mapped to the genome. The mapped sequencing data were then processed with HTSeq (Anders et al. 2014) to obtain counts for sequencing reads mapped to exons. Expression values were represented as RPKM and a filter of RPKM > 1 was applied to remove noise and genes with very low expression levels. This resulted in a total of 14,302 genes with expression values above the threshold (supplementary fig. S1, Supplementary Material online). The SRR accession

numbers for the raw sequence data are SRR1660827, SRR1660828, SRR1660829, and SRR1660830.

Differential expression analysis was performed with the DESeq package (Bioconductor) (Anders and Huber 2010) using an adjusted *P*-value cut-off of 0.1 and a minimal fold-change of 2 (supplementary fig. S2, Supplementary Material online). Full lists of sex-biased genes can be found in supplementary table S1, Supplementary Material online.

The sex-biased genes were also analyzed for the presence of duplicated genes to determine whether duplications might have arisen to resolve sexual conflict. Duplicated gene pairs were detected as described in Cock, Sterck, et al. (2010). Briefly, each *Ectocarpus* protein was compared with the entire set of *Ectocarpus* proteins using BLASTp and duplicate genes were defined as two sequences from different loci with a maximal *E* value of $e.10^{-4}$. The clustering analysis was performed using the MCL algorithm (Markov Cluster Algorithm; Li et al. 2003) with the inflation value fixed at 3.0.

Measurement of Synonymous and Nonsynonymous Mutation Rates

To estimate rates of evolution of sex-biased gene sequences, we searched *E. fasciculatus* transcriptome data (Gachon CM, unpublished data) for orthologs of sex-biased and unbiased control genes (the latter was a random subset of 47 genes without differences in expression levels between males and females) by retaining best reciprocal BLASTn matches with a minimum *e* value of $10\,e^{-10}$. The orthology of genes derived from duplications in *Ectocarpus* sp. was further evaluated by calculation of phylogenetic trees using *E. siliculosus* and *E. fasciculatus* sequences, along with *S. lomentaria* sequences as outgroups. MEGA6 (Larkin et al. 2007; Tamura et al. 2013) was used for maximum-likelihood analyses and branch support was assessed with by bootstrapping (1,000 replicates).

Putative orthologs were aligned using ClustalW implemented in MEGA6 (Larkin et al. 2007; Tamura et al. 2013) and manually curated. Sequences that produced a gapless alignment that exceeded 100 bp were retained for pairwise dN/dS (ω) analysis using Phylogenetic Analysis by Maximum Likelihood (PAML, CODEML, F3x4 model, runmode = -2) implemented in the PAL2NAL suit (Suyama et al. 2006; Yang 2007) Genes with saturated synonymous substitution values ($dS > 1$) and genes located in the SDR were excluded from the analysis.

The ENC and the CAI were calculated for all sex-biased and unbiased genes in this study using CAIcal server (http://genomes.urv.es/CAIcal/) (Puigbo et al. 2008).

Positive Selection Analysis

We used transcriptomic and genomic data from four different *Ectocarpus* species and another Ectocarpales species, *S. lomentaria* to detect positive selection (supplementary table S3, Supplementary Material online). *Ectocarpus* sp. lineage 1c Greenland, *E. fasciculatus*, and *S. lomentaria* transcriptome data were generated using Illumina HiSeq v3 paired-end technology (2×100 bp) and quality filtered using either the FASTX toolkit or Trimmomatic (http://www.ncbi.nlm.nih.gov/pubmed/24695404) (Gachon CM, unpublished data). Transcriptome assemblies were generated using the Trinity de novo assembler (Grabherr et al. 2011) with default parameters and using normalized mode. Transcripts were filtered for isoform percentage (>1) and RPKM (>1). *Ectocarpus siliculosus* lineage 1a genomic data were aligned to the reference genome and consensus sequences of coding regions with at least 10× coverage were recovered using the CLC Assembly Cell (www.clcbio.com).

Orthologs of *Ectocarpus* sp. lineage 1c Peru sex-biased and unbiased genes were identified in *E. siliculosus* lineage 1a, *Ectocarpus* sp. lineage 1c Greenland, *E. fasciculatus*, and *S. lomentaria* by selecting transcripts that could be aligned over at least 100 bp using a best reciprocal BLASTn approach (*E* value cutoff of 10^{-10}). Nucleotide alignments for genes identified from at least four of the five species were made using ClustalW implemented in MEGA6 (Larkin et al. 2007; Tamura et al. 2013) curated manually when necessary and transformed to PAML4 format using perl fasta manipulation scripts (provided by Naoki Takebayashi, University Alaska Fairbanks).

Levels of nonsynonymous (dN) and synonymous (dS) substitution were estimated by the maximum-likelihood method available in the CODEML program (PAML4 package) using the F3x4 model of codon frequencies and a user tree specified according to the phylogeny (Stache-Crain et al. 1997). CODEML paired nested site models (M0, M3; M1a, M2a; M7, M8) (Yang 2000, 2007) of sequence evolution were used and the outputs compared using the likelihood ratio test. Empirical Bayes methods allowed for identification of positively selected sites a posteriori (Yang 2000, 2007).

Breadth of Gene Expression

RNA-seq data corresponding to complete organisms from seven different stages of the life cycle (male and female gametes, parthenosporophytes, immature and fertile male and female gametophytes) and to two different tissue types (basal structures and upright filaments) were used to estimate breadth of gene expression. The gamete transcriptomic data (Lipinska et al. 2013) were converted to RPKM in order to make them comparable with the other libraries. The specificity index (τ) (Yanai et al. 2005) was used as a measure of breadth of expression for each gene, using the following formula

$$\tau = \frac{\sum_{i=1}^{N}(1 - x_i)}{N - 1}.$$

For each gene, we calculated x_i as the expression profile in the given library *i* normalized by the maximal expression value across all analyzed tissues and life cycle stages (*N*). τ index values range from 0 to 1, where 1 corresponds to strong tissue/life cycle stage specificity (low expression breadth).

Analysis of Predicted Gene Functions

InterProScan (Zdobnov and Apweiler 2001) and BLAST2GO (Conesa and Gotz 2008) were used to recover functional annotations for *Ectocarpus* proteins. For BLAST2GO, a Fisher exact test with an FDR corrected *P* value cutoff of 0.05 was used to detect enrichment of specific GO-terms in various groups of sex-biased genes.

Genomic Location of Sex-Biased Genes

A Chi-squared test of observed and expected distribution of sex-biased genes across the *Ectocarpus* linkage groups (Heesch et al. 2010) was used to test whether sex-biased genes were randomly distributed throughout the genome. The expected distribution was calculated with the assumption that the sex-biased genes were randomly distributed and therefore that representation on a particular chromosome should have been proportional to the number of genes on that chromosome. The Chi-squared test was performed in Excel 2010 (Microsoft, Redmond, WA). All other statistical analyses were performed in RStudio (R version 3.0.2).

Supplementary Material

Supplementary figures S1–S5 and tables S1–S5 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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