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The brown algal genus Fucus: A unique insight into reproduction and the evolution of sex-biased genes

William John Hatchett

FACULTY OF BIOSCIENCES AND AQUACULTURE

NORD University

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Nord University
N-8049 Bodø
Tel: +47 75 51 72 00
www.nord.no

Preface

This doctoral thesis is submitted in fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University. The studies compiled in this dissertation are original research studies conducted at Nord University, Bodø.

The core project team consisted of the following members:

William John Hatchett, MSc, FBA, Nord University – PhD Fellow

Galice Hoarau, Professor, FBA, Nord University – Primary supervisor

Alexander Jueterbock, Research, FBA, Nord University – co-supervisor

Agnieszka Lipinska, Researcher, Max Planck Institute – co-supervisor

Acknowledgements

My entire life has changed since I started my PhD over 6 years ago and I have been tested in ways that I did not think possible. Anybody who has known me throughout this time knows that it has not been easy with many obstacles that have got in the way of progress in my PhD. Without those special people in my life, I never would have been able to persevere and finally get to this point and I would like to take this opportunity to thank them now.

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could push through these last few months and finish this PhD. I am forever grateful for your support, belief and prayers.

To my big boy **Edward**, we have been through so much together and our little team has never been broken. I hope that with the completion of this PhD we can start a new chapter in our lives once again full of fun times and happiness.

To my little boy **George**-052, you have been my sunshine in the dark days, your smile and laughter has made life so much brighter. I hope to one day tell you how important you were to completing this PhD. Thank you for being you, my little spartan.

And last but definitely not least, to **Aashi** - there are so many words that I could write about how you have helped me, I could probably write a book. My guardian angel, who came into mine and the boys lives in a desperate state and took care of us and loved us with your entire being. The support you have given me is indescribable and no one has come close to what you have done for me. I thank my lucky stars that I met you and I couldn't have asked for a better partner in crime. I thank you with all of my being and I look forward to starting the rest of my life with you.

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Paper II: Hatchett, W. J., Jueterbock, A., Kopp, M., Coyer, J. A., Coelho, S. M., Hoarau, G., and Lipinska, A. P. (2022). Evolutionary dynamics of sex-biased gene expression in a young XY system: Insights from the brown alga genus *Fucus*. *New Phytologist*, accepted December 2022.

Paper III: Hatchett, W. J., Wagner, C. I., Kopp, M., Jueterbock, A., and Hoarau, G. (2022). Novel genomic approach using target capture sequencing for the Genus *Fucus* (Ochrophyta, Fucales). (Manuscript)

Abstract

The brown algal genus *Fucus* (Heterokontophyta, Fucales), also known as rockweed, are a familiar objects both on the shore and in salt marshes and are important primary producers in the North Pacific and the North Atlantic. Historically, *Fucus* was one of the earliest species to be researched in marine biology stations due to its abundance and availability. While the majority of brown algae have a haplo-diploid life cycle, *Fucus* has recently undergone a shift to a diplontic life cycle. *Fucus* species have two lineages, each containing hermaphroditic and dioecious species. Additionally, morphological variability is legendary in *Fucus* with multiple morphs, hybrids, ecads and cryptic species. Hybridisation within each lineage is common and often asymmetrical between hermaphroditic and dioecious species. These unique aspects of *Fucus* were reviewed, providing an intriguing model organism for research in fields of speciation, life cycle evolution, reproduction and the evolution of XY systems.

The resources and methods required to investigate sexual dimorphism at a molecular level and to establish the genus *Fucus* as suitable model organisms were evaluated. RNAseq from different tissue types and sexes was used to assemble transcriptomes for four *Fucus* species and investigate sex-biased gene expression. Male and female sex-biased genes of *F. serratus* and *F. vesiculosus* experience different selection pressures and have their own evolutionary paths. Male-biased genes evolve faster at a protein sequencing level and have conserved levels of expression between species. They were overexpressed and appear to be male limited and mainly found in the male reproductive tissue. Female-biased genes on the other hand, do not show accelerated rates of coding sequence evolution and are not conserved across species. They were uniformly highly expressed and have diverse levels of expression with no tissue biased.

Finally, target capture sequencing probes were successfully developed for *Fucus*. This novel genomic approach allowed the genotyping of tens of thousands of genome wide SNPs in species of both lineages. This method can facilitate population/evolutionary genomics studies, involving a large number of individuals and even in species with limited availability of genomic resources. The probes were successfully used to analyse closely related *Fucus* species and showed no loss in efficiency with increased genetic distance from the focal species. The future now looks promising for *Fucus* as a model organism, which now has the genomic resources and new tools in which to answer key questions about its unique evolutionary history, its life cycle transitions and the genes that play key roles in reproduction and those that limit it, resulting in speciation.

Abstrakt – Sammendrag på norsk

Brunalgeslekten *Fucus* (Heterokontophyta, Fucales), også kjent som tang, er en kjent art langs kysten og i saltvannssumper og er viktige primærprodusenter i nordlige Stillehav og i Nord-Atlanteren. Historisk sett har *Fucus* vært en av de tidligste artene som ble forsket på ved marinbiologiske stasjoner på grunn av dens overflod og tilgjengelighet. Mens flertallet av brunalger har en haplo-diploid livssyklus, har *Fucus* nylig gjennomgått et skifte til en diplontisk livssyklus. *Fucus*-arter har to avstamninger, som hver inneholder både arter som er hermafroditter og som er særbo. I tillegg er morfologisk variasjon legendarisk i *Fucus* med flere morfer, hybrider, "ecads" og kryptogamer. Hybridisering innenfor hver avstamning er vanlig og ofte asymmetrisk mellom hermafroditter og særbo. Dette arbeidet har sett på disse unike aspektene ved *Fucus*, og funnet at den er en spennende modellorganisme for forskning innen fagfeltene artsdannelse, livssyklusevolusjon, reproduksjon og utviklingen av XY-systemer.

Ressursene og metodene som kreves for å undersøke seksuell dimorfisme på et molekylært nivå og for å etablere slekten *Fucus* som passende modellorganismer ble evaluert. RNAseq fra forskjellige vevstyper og kjønn ble brukt til å sette sammen transkriptomer for fire *Fucus*-arter og undersøke kjønnsskjeve genuttrykk. Mannlige og kvinnelige kjønns-relaterte gener av F. serratus og F. vesiculosus har forskjellige seleksjonstrykk og sine egne evolusjonære veier. Mannlige gener utvikler seg raskere på et proteinsekvenseringsnivå og har konserverte nivåer av genuttrykk mellom arter. Overekspresjon av disse genene ser ut til å være begrensede til hanner og finnes hovedsakelig i det mannlige reproduksjonsvevet. Kvinnelige gener på den annen side, viser ikke akselererte hastigheter for kodende sekvensutvikling og er ikke bevart på tvers av arter. De ble jevnt høyt uttrykt og har forskjellige nivåer av genuttrykk uten å være knyttet til noe spesielt vev.

Til slutt ble en nylig utviklet genomiske metode tatt i bruk, som tillater genotyping av titusenvis av genetiske basepar, såkalte SNP-er. Metoden ble tatt i bruk på arter av begge avstamninger. Denne metoden kan lette populasjons-/evolusjonsgenomiske studier, som involverer et stort antall individer og til og med på arter med begrenset tilgjengelighet av genomiske ressurser. Metoden ble vellykket brukt til å analysere nært beslektede *Fucus*-arter og viste ingen tap i effektivitet med økt genetisk avstand fra fokal-arten. Fremtiden ser nå lovende ut for *Fucus* som en modellorganisme, siden vi nå har de genomiske ressursene og nye verktøyene for å svare på nøkkelspørsmål om dens unike evolusjonshistorie, dens livssyklusoverganger og genene som spiller nøkkelroller i reproduksjon og de som begrenser den, noe som resulterer i nye artsdannelser.

1 Introduction

1.1 Evolution of *Fucus*

Strewn across the seashore after a storm, nestled in the rocks beaten by the changing tide, rotting in the sun, or frozen under sheets of ice in a rock pool, brown algae is a common sight worldwide (Fig 1.). Think of the many who have played, walked or sat on the beaches of the world and not wondered for more than a moment what stories could be told by the inconspicuous brown algae, lying flat at their feet. Over 300 years ago, a marine biologist published an illustration of *Fucus* (rockweed) in his book, labelling it as "the flowers and seeds of various *Fucus* and some other physical observations of these same plants" (Réaumur 1711).

Figure 1: Sample site for *Fucus* species, Mjelle, Norway.

Subsequent investigations necessarily focused on the taxonomy of a genus characterized by morphological variability both within and among species, as evident by the 717 described names, 10 of which are currently accepted as species and 402 retaining infraspecific designations (see https://www.algaebase.org/search/genus-/detail/?genus_id=71). As culture techniques and microscopy advanced, investigations were able to address basic life history and fertilization/development. Further advancements in instrumentation lead to physiological and autecological studies. Beginning in the 1960s, the number of publications of *Fucus* species began a hyperbolic increase aided by advances in molecular techniques that were used to address, physiology, phenotypic plasticity, cytogenetics, phylogeny, phylogeography, hybridization, and sex-biased genes (Evans 1962; Scott and Hardy 1994; Rousseau et al. 1997; Coyer et al. 2002a; Pearson et al. 2006; Martins et al. 2013; Jueterbock et al. 2014; Almeida et al. 2022).

The genus *Fucus* is a member of the class Phaeophyceae (phylum Ochrophyta, class Phaeophyceae, order Fucales) which consists of 16 orders, 285 genera and 2100 species (Guiry and Guiry 2022). The class is within one of four supergroups in the TSAR lineage (Telonemia, Stramenopila, Alveolata, and Rhizaria) (Burki et al. 2020), which diverged from plants and animals more than a billion years ago (Fig. 2) (Coelho and Cock 2020). The SAR portion of the TSAR lineage forms nearly half of all eukaryote species diversity and includes unicellular, filamentous, colonial, and multicellular organisms. In general, the unicellular heterotrophs are known as protozoans and the photosynthetic organisms are considered algae (del Campo et al. 2014; Burki et al. 2020).

The earliest ancestral links of the SAR lineage is with Archaeplastida in which an ancestral endosymbiotic event of phagocytosis resulted in shared plastids originating from a secondary or a tertiary endosymbiotic event and is now present in most algae within the SAR supergroup (Janouškovec et al. 2010; Burki et al. 2016; Irisarri et al. 2021; Strassert et al. 2021). The Stramenopile clade is a large group of an estimated

100,000 species, diverging ~1025–1077 Mya (million years ago), but has a complicated history of classification and the true evolutionary history remains highly debated (Yoon et al. 2009). Stramenopiles acquired chloroplasts by secondary or tertiary endosymbiosis from a unicellular red alga and can be either photoautotrophs or heterotrophic (Tengs et al. 2000; Takishita 2004; Petersen et al. 2006; Cavalier-Smith and Chao 2006).

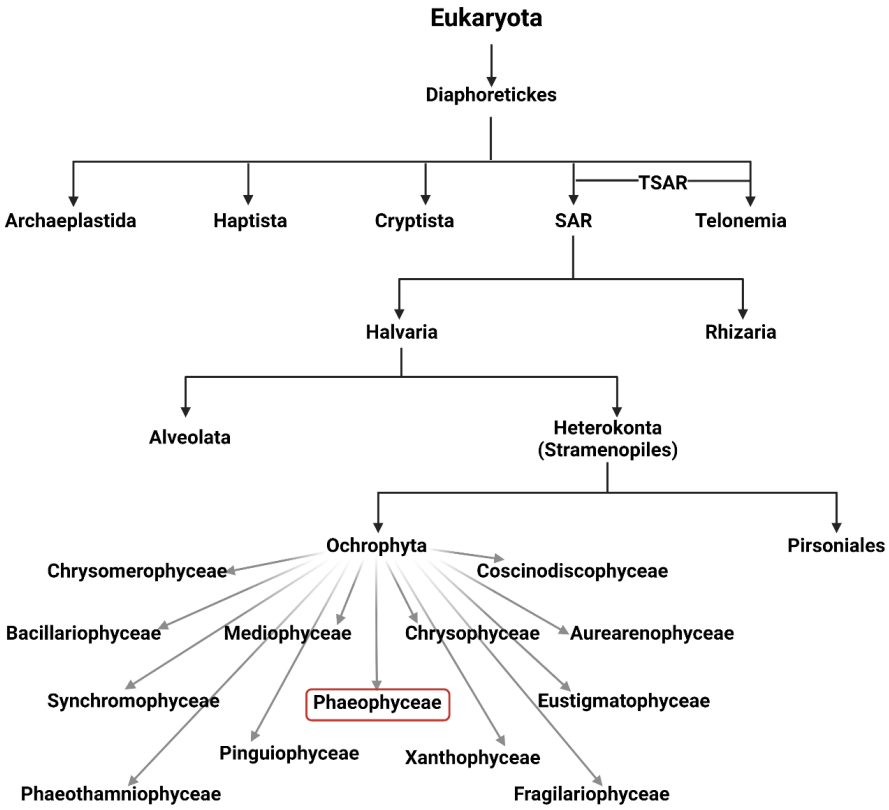

Figure 2: Evolutionary tree of Phaeophyceae within the Eukaryotes.

Ochrophyta are a phylum that consists of mostly photosynthetic stramenopiles (or heterokonts) (Fig. 2) however, despite their ecological and evolutionary importance, their phylogeny still remains unresolved. It is one of the most diverse groups of photosynthetic eukaryotes (Fig. 2), but also including some heterotrophic species (Silberfeld et al. 2014; Adl et al. 2019; Dorrell et al. 2019; Kayama et al. 2020). Phaeophyceae are multicellular brown algae which support coastal ecosystems and provide habitat for a huge variety of aquatic life (Steneck et al. 2002; Teagle et al. 2017). They are situated within one of three lineages in Ochrophyta, which emerged during the late Paleozoic period (∼310 Mya) (Bringloe et al. 2020). In a relatively short period of time (<250Mya), brown algae have evolved a huge variety of species and morphologies, and are one of five eukaryotic lineages that have independently evolved multicellular complexity (Fig. 3) (Kawai et al. 2015). Brown algae are relatively recent in geological records. With limited and tenuous fossil interpretations due to a lack of hard tissue and their typical habitat of rocky shores renders fossilization difficult. One of the earliest fossilised species was preserved in the early Cretaceous period ~ 145–100 Mya (*Padina*-like) and additional fossils, presumably of kelps, were dated ~ 13-17 Mya (Parker and Dawson 1965; Rajanikanth 1989). Although it is difficult to calibrate phylogenetic trees with the limited fossil record (Silberfeld et al. 2010), some evidence suggests that the brown algal orders diversified in the Mesozoic Era ~ 252-66 Mya (Bringloe et al. 2020).

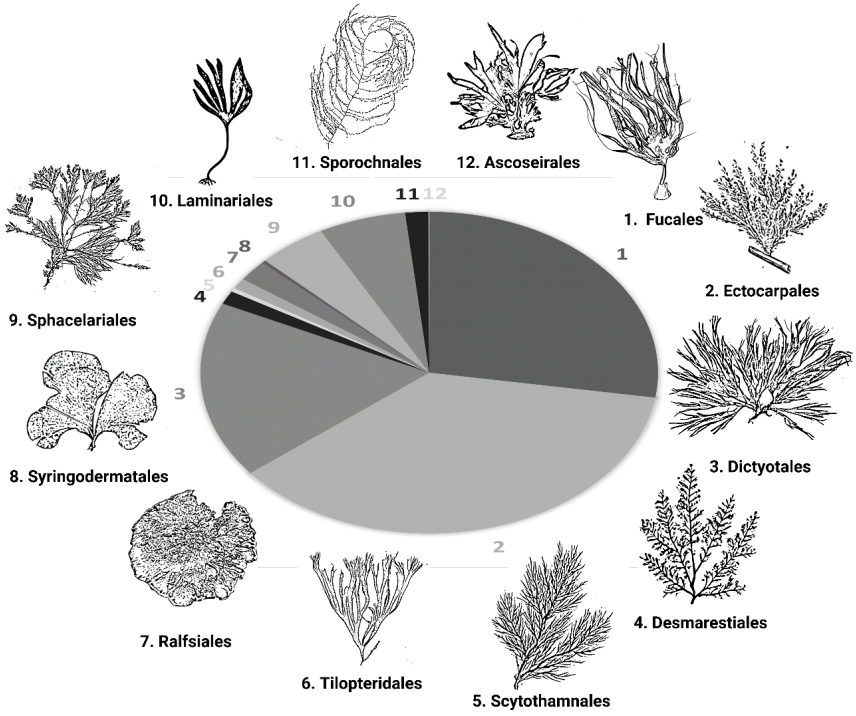

Figure 3: Relative proportion of species within each Order of the Phaeophyceae. Images are representatives from each order
(see https://www.algaebase.org/search/genus/detail/?genus_id=71).

Fucales are a large order of 9 families and more than 500 species. It is thought to have diverged around 39-16 mya (Yip et al. 2020). Very little is known about the phylogenetic history and diversification of this group with only 1 fossil from this order dating to 17-13 mya (Parker and Dawson 1965; Silberfeld et al. 2010). The genus *Fucus*, located within the Fucales originated in the North Pacific and diverged around 2.3-5.5 mya (Coyer et al. 2006a; Hoarau et al. 2007). Two lineages are present within the genus: Lineage 1 consisting of *F. distichus* and *F. serratus*; and Lineage 2 consisting of eight species (*F. vesiculosus, F. spiralis, F. radicans, F. macroguiryi, F. ceranoides, F. chalonii, F.virsoides* and *F. cottonii*) (Serrão et al. 1999; Coyer et al. 2006a).

1.2 *Fucus* Ecology and Distribution

All *Fucus* species are crucial ecosystem engineers that collectively provide shelter and food for a large variety of invertebrate and fish species, as well as create substrates for attachment of epibionts (Connell 1972; Lüning 1990; Jones et al. 1994; Chapman 1995; Drakard et al. 2021). Furthermore, detached and senescent tissue (drift), becomes an important source of food for detritivores, suspension feeders, and microbes as well as a means of carbon sequestration to the deep sea. (Lucas et al. 1981; Dunton and Schell 1987; Duggins et al. 1989). The general morphology of *Fucus* species is relatively simplistic. The thallus, which is the body of *Fucus* organisms can grow to a maximum size of around 2m (Fig. 4) (Wehr 2015). A holdfast, anchors the alga in place on the substrate upon which it grows, preventing the organism from being carried away by currents and tides (Fig. 4). Unlike root systems, it is not the primary source of water intake and does not absorb nutrition from the substrate it is attached to. The stipe, which grows near the base of the organism, is a stem-like structure which provides support to the thallus (Fig. 4) (Pennington 1937). The majority of the biomass is found in leaf-like structures known as fronds. The shape and structure of fronds can vary between species, but usually consist of a broad wing like lamina that run continually alongside a branched midrib and are usually attached directly to the stipe or to the holdfast (McCook and Chapman 1992; Ang and Wreede 1993; Malm et al. 2001). The fronds of *Fucus* are where the reproductive tissue known as receptacles are found, usually at the tip of the leaf-like structure (Fig 4.). Additionally, growth occurs at the tips of the structure as a result of single/rows of apical cell. Branching and other lateral structures occur when the apical cells divide and produce two new apical cells. Some species of *Fucus* produce numerous gas-filled pneumatocysts to increase buoyancy (Fig 4.). These are found in the lamina as spherical bladders or take the shape of the lamina in which it develops. The elongated gas-filled regions help the organism stay buoyant and nearer the water surface to receive more light for photosynthesis (Bringloe et al. 2020).

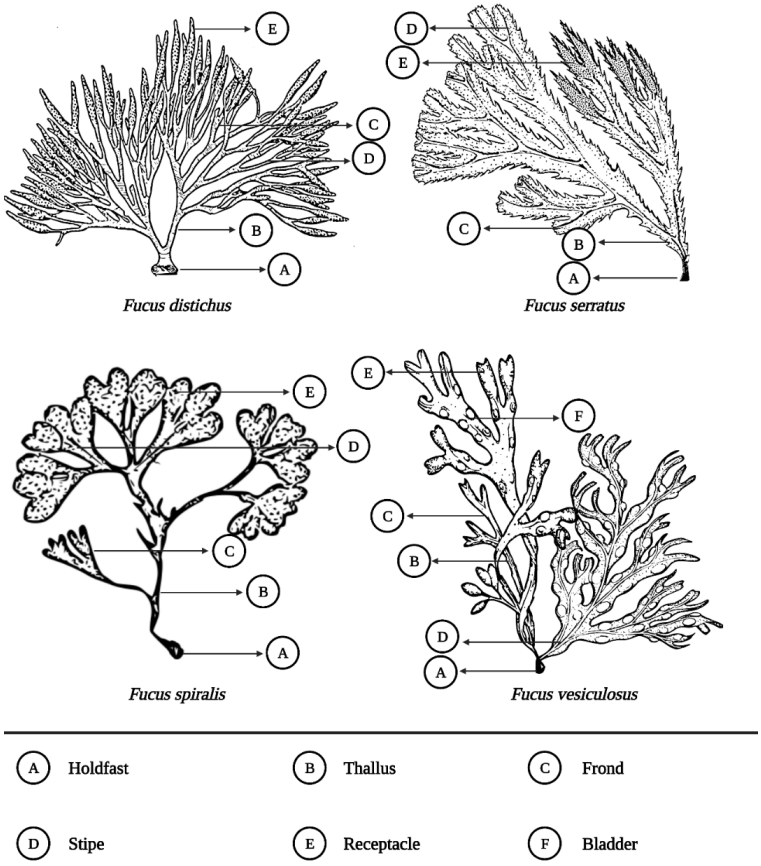

Figure 4: Main morphological features for four *Fucus* species.

Historically, *Fucus* was one of the earliest species for research at Europe's first marine biology stations, due to its abundance and easy accessibility (Breyne et al. 2010). Ten *Fucus* species are present in the intertidal and shallow subtidal of North Atlantic shores from Morocco to Svalbard, across the central Atlantic islands to Nova Scotia, Canada then south to Rhode Island, USA. In the North Pacific, *F. distichus* ranges from Hokkaido Japan northeast to the Aleutians and Alaskan coast and south to Monterey Bay, California USA (Abbott and Hollenberg 1976; Lüning 1990). *Fucus distichus* is the only *Fucus* species to occur in both the North Atlantic and North Pacific,

although *F. spiralis* is a recent introduction to British Columbia (Canada) and Washington State (USA) (Coyer et al. 2011). Some species (e.g., *F. vesiculosus*, *F. spirais*) are particularly plastic in their distribution, inhabiting both sheltered and moderately-exposed rocky shores in fully marine salinities, as well as rocky shores and marshes in sheltered brackish conditions.

Figure 5: Global distribution of *Fucus* species from *https://www.gbif.org/species/7832266*

1.3 Evolution of diplontic life cycle in Fucales

Fucales belong to the clade BACR (brown algal crown radiation) and radiated throughout the Cretaceous period 145-66 Mya (Bringloe et al. 2020). The last common ancestor of this clade had a heteromorphic life history with oogamous fertilisation which evolved from an isomorphic life history (Silberfeld et al. 2014). This evolutionary switch in life history is thought to have only occurred twice, once in Syringodermatales and once in the common ancestor of BACR. Within BACR, several orders revert from a heteromorphic to an isomorphic life cycle and adopt anisogamous or isogamous fertilization (Silberfeld et al. 2010). The last common ancestor of Fucales had an

independent transition from haploid to diploid sex determination about 74.5 My ago which coincided with a transition from dioicy to monoecy and a loss of parthenogenesis (Heesch et al. 2021).

Fucales thus became one of the only orders with a diplontic life cycle and oogamous fertilisation with oogonia and spermatangia, which are found in specialised tissues known as receptacles (Evans et al. 1982; Silberfeld et al. 2010). Later, around 17 Mya Fucales evolved dioicy once again with separate sexes, followed by further transitions to monoecy. However, the transition to diplontic life cycle is thought to be irreversible (Heesch et al. 2021). To fully understand the evolution of different life cycles and how they co-exist, one must understand the importance of ecological niches, survival/fertility and the role they play on haploid and diploid individuals (Hughes and Otto 1999; Rescan et al. 2016; Scott and Rescan 2017). Very little is known about how different ecological niches affect the fitness of haploid and diploid phases, which is only compounded by the fact that within species variation of brown algal life cycles also occurs. However, different life cycle generations have been shown to have different ecological niches (Valero et al. 1992; Mable and Otto 1998; Pacheco-Ruíz et al. 2011; Couceiro et al. 2015) During the time period in which Fucales transitioned to a diplontic life cycle, sea levels rose. This dramatic change in habitat could have opened new ecological niches, providing the conditions required for the emergence of new evolutionary novelties (Schiel and Foster 2006).

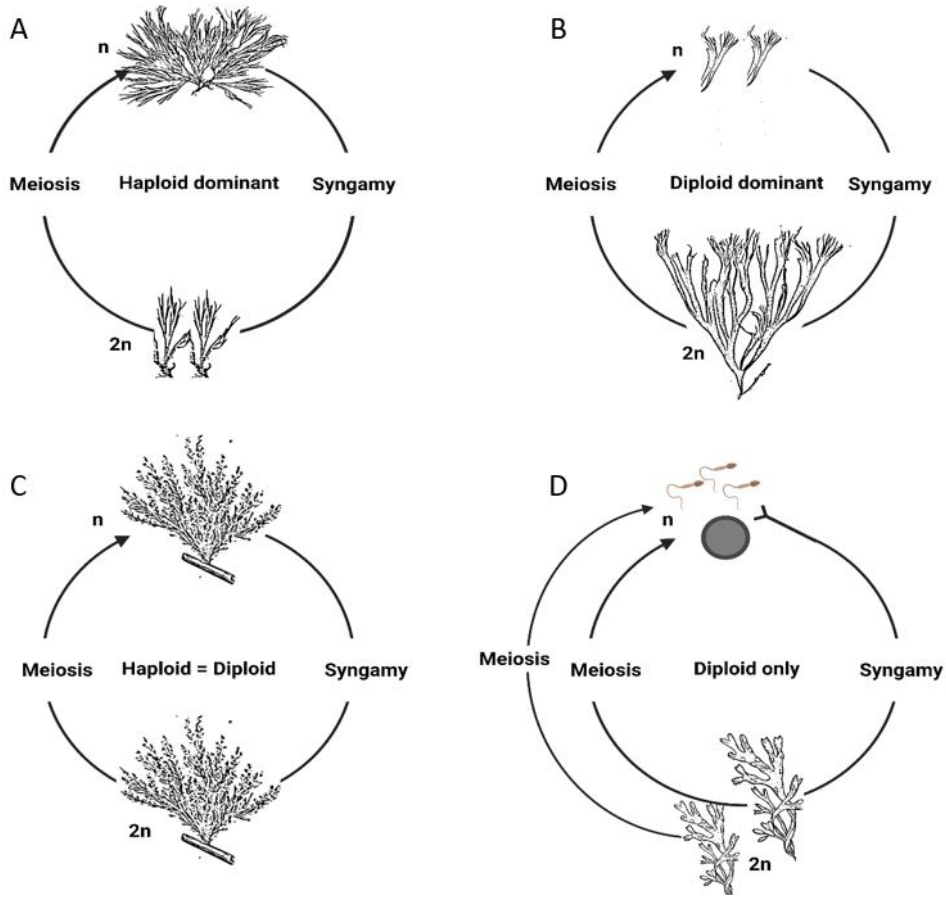

Figure 6: Range of life cycle traits found in brown algae. n=haploid phase and 2n=diploid phase. A-C are diplohaplontic and D is diplontic. A. Haploid dominant life cycle has both haploid and diploid mitosis, with a dominant gametophyte generation which is haploid. B. Diploid dominant life cycle with both haploid and diploid mitosis, with a dominant sporophyte generation which is diploid C. Haploid = Diploid life cycle with both haploid and diploid mitosis, with equal dominance of both gametophyte and sporophyte generations D. Diploid only life cycle with no haploid mitosis and the only haploid phase is limited to the gametes. This is the life cycle which is found in Fucales. Created with biorender.com.

1.4 Life Cycle of *Fucus* species.

The majority of brown algal species display a diplohaplontic life cycle, alternating between the multicellular gametophyte and sporophyte generations. Sex is expressed in the haploid gametophyte generation and is controlled by haploid sex chromosomes

(UV system) (Coelho et al. 2018). Gametophytes produce either isogamous (gametes have the same morphology) or anisogamous (gametes that differ in size and/or form) gametes which fuse to form the diploid sporophyte. Mature sporophytes produce unicellular haploid spores by meiosis, which mature to male or female gametophytes, thereby completing the life cycle (Fig 6A, B & C). The gametophyte and sporophyte can be morphologically indistinguishable (isomorphic) or different (heteromorphic) (Thornber 2006; Lipinska et al. 2019; Heesch et al. 2021)

Fucus along with all Fucale species differ from other brown algal lineages and have evolved to have a diplontic life cycle, in which the sperm and eggs are single cell gametophyte stages produced in numerous conceptacles within each of numerous receptacles at branch tips of mature individuals (Fig. 4, Fig. 6D). Dioecy is when an individual bears receptacles with antheridia (sperm) or oogonia (eggs); hermaphroditism is when an individual has both antheridia and oogonia in a single conceptacle (Fig. 7) (Otto and Marks 1996; Heesch et al. 2021). Gametes generally are synchronously released from adjacent individuals in calm conditions on a rising tide in full sunlight and the relatively heavy eggs sink to the substrate within a few meters of the parent (Brawley 1990, 1992; Pearson et al. 1998; Berndt et al. 2002; Monteiro et al. 2016). The short-lived sperm are attracted to an egg-produced pheromone (fucoserratene, (Müller and Jaenicke 1973)) that is effective only within μm to mm distances (Arrontes 1993; Serrao et al. 1997; Engel et al. 2005; Muhlin et al. 2008).

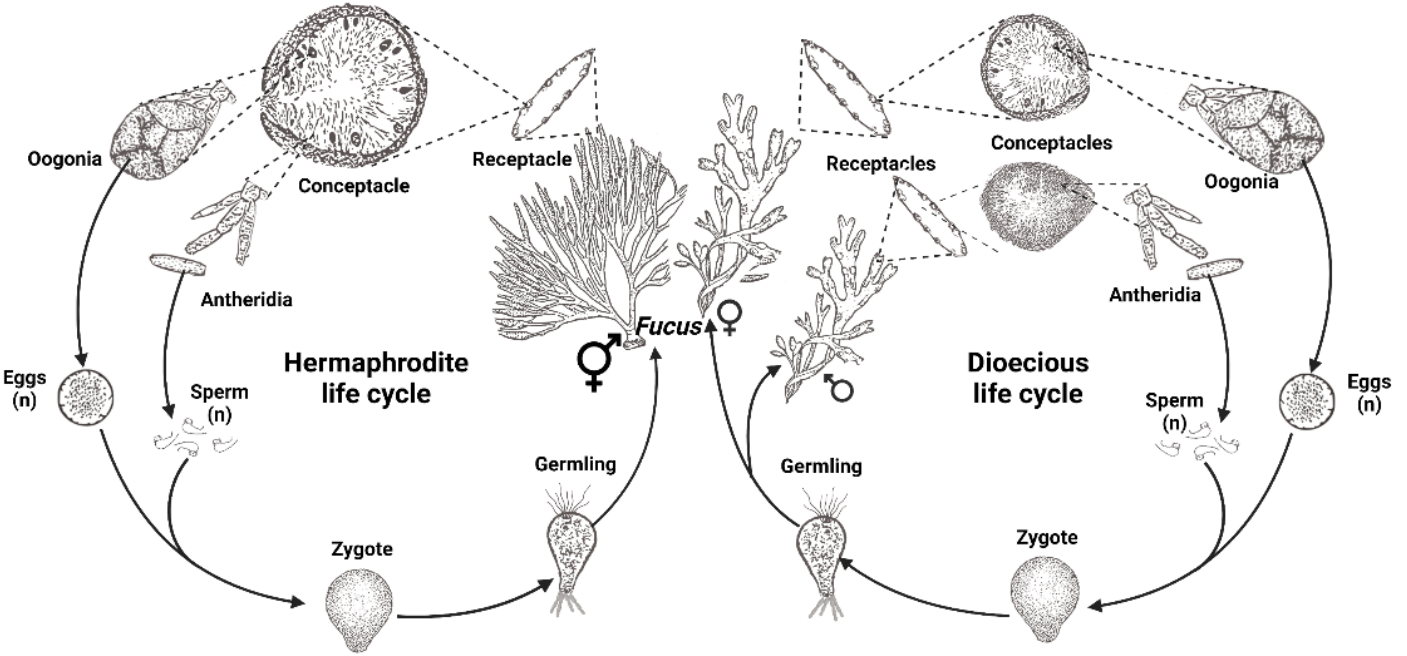

Figure 7: Hermaphroditic and dioecious life cycles in *Fucus* species. Created with biorender.com.

1.5 Selfing

Selfing is the fusion of two gametes that originate from the same individual. Given that one species in each of the two *Fucus* lineages is a simultaneous hermaphrodite, it is no surprise that selfing readily occurs. Selfing has been demonstrated with *in vitro* fertilization experiments in *F. spiralis* (Pollock 1970; Vernet and Harper 1980; Müller and Gassmann 1985) and is supported by genetic investigations in *F. spiralis, F. guiryi*, and *F. distichus* (Coleman and Brawley 2005; Engel et al. 2005; Billard et al. 2005, 2007; Perrin et al. 2007; Coyer et al. 2007, 2011; Whitaker et al. 2017; Almeida et al. 2022).

It is commonly believed that selfing is deleterious because; 1) it increases the probability that recessive maladaptive genes will become homozygous and subsequently decrease adaptation and fitness (e.g., 'dead end' of Stebbins (1974)); 2) genetic recombination is limited and further reduces genetic potential to enhance survival and reproduction in a changing environment; and 3) effective population size may be reduced. However, the notion of selfing being a 'dead end' over the long term is unclear (Wright et al. 2013). Selfing has some advantages (see, Wells 1979, and references therein). For example, in angiosperms, it is possible that environmental conditions will inhibit pollen dispersal, thereby leading to extinction unless selfing is employed. Additionally, when pollinators and/or mates are rare, or when sperm is limiting (or only self-sperm is available for fusion with eggs due to phenological incompatibility; (Engel et al. 2005)) and outcrossing is uncertain, selfing offers reproductive assurance (see references in; (Vernet and Harper 1980; Perrin et al. 2007; Wright et al. 2013). Selfing also allows transmission of a whole genome through both the male and female functions to the next generation (Fisher 1941) .

Furthermore, selfing is a viable means of colonization, requiring only one fertile individual and can be an advantage in a mixed population of two species that produce sterile hybrids. Prolonged selfing also can lead to purging of deleterious homozygotes and reduce inbreeding depression (Schoen 2005; Igic et al. 2006) in unchanging environments. And finally, high-fitness selfed individuals with low rates of

recombination at adaptive loci may facilitate colonization by locally-adapted genotypes (Eriksson and Rafajlović 2021).

1.6 Hybridization

Hybridization is when two divergent lineages (e.g., species), with independent evolutionary histories and are not reproductively isolated, interbreed and form a hybrid. Natural hybridization is an important source of genetic variation and a mechanism of speciation (Genovart 2009). The resulting hybrid may be morphologically, genetically and/or physiologically different and will be subjected to natural selection. Hybrid fitness will determine the fate of a hybrid and three scenarios are possible: 1) if there is no selection against the hybrids and introgression (backcrossing with one of the parental species) is extensive, all individuals become hybrids; 2) if introgressed individuals become established and/or are adapted for new habitats, new lineages can evolve; and 3) if hybrids are less fit, pre-zygotic isolating barriers can evolve to strengthen selection against formation of hybrids (=reinforcement), as less fit hybrids can be viewed as a waste of resources (summarized in Hoarau et al. 2015). Evidence suggests that scenario 3 is most likely in *Fucus*. Other factors can influence hybridization in *Fucus*. For example, receptacles in unsuitable condition and with reduced health, can have diminished species-specific barriers and can increase the levels of hybridization (Bolwell et al. 1977; Edwards 1999). Another factor is timing of gamete release: asynchronous release of gametes may have evolved as a pre-zygotic barrier to reduce hybridization (Cánovas et al. 2011; Monteiro et al. 2012, 2016).

Crossing experiments began as early as 1854 when Thuret crossed various dioecious *Fucus* species, (*F. serratus, F. vesiculosus* and *Ascophyllum nodosum,* (the latter erroneously considered as *Fucus*) that simultaneously released gametes. No crossings were successful beyond a few cells. Nearly 80 years later, Kniep (1925) successfully crossed a diecious species (*F. vesiculosus*) with a hermaphroditic species (*F. spiralis*) and suggested the possibility of natural hybridization. Numerous studies

have since documented field individuals with a morphology intermediate between two co-existing species (see references in Scott and Hardy 1994). With the advancements in sequencing technologies it is now possible to investigate hybridization with greater detail and accuracy. The first study to use molecular techniques in *Fucus* hybrids, used nuclear rDNA-ITS1 sequence, Rubisco spacer in chloroplasts, and *nad*11gene in mitochondria (Coyer et al. 2002a; Coyer et al. 2002b). The authors confirmed field individuals as true hybrids, as well as laboratory crosses between the two Lineage 1 species. Hybridization was asymmetrical, with the dioecious *F. serratus* contributing sperm and the hermaphroditic *F. distichus* the egg. Later investigations targeted loci in mixed populations and showed that recent hybrid zones had higher levels of F_1 hybrids and low hybrid fitness (Coyer et al. 2007), but older contact zones had few to no F_1 hybrids (Hoarau et al. 2015). The pattern is likely due to reinforcement: when hybrids are less fit, pre-zygotic isolating barriers can evolve to strengthen selection against the formation of hybrids that are less fit (Marshall et al. 2002).

RNA-seq data have shown that hermaphroditic species within Lineage 2 also hybridize with dioecious species in the lineage (as is the case for the two Lineage 1 species); in some cases hybrid frequencies of 13% in sympatric populations and 7% in parapatric populations (Engel et al. 2005; Billard et al. 2007; Moalic et al. 2011; Almeida et al. 2022). As for the two Lineage 1 species, hybridization among hermaphroditic and dioecies species in Lineage 2 was asymmetrical, with the former contributing eggs and the latter sperm. The underlying causes for this asymmetrical hybridization in both lineages still have not been explained, however, could be a result of sexual dimorphism. As the gametes (and as a result the receptacles) are the only sexually dimorphic traits within and between the species pairs.

1.7 Sexual dimorphism

Sexual dimorphism is defined as the presence of distinct differences between males and females within the same species. These dimorphisms can include morphological, behavioural, and physiological variation and have stimulated research

at molecular, ecological, and behavioural levels (Leutenegger and Kelly 1977; Ranz et al. 2003; Cooper 2010; Han and Dingemanse 2017; Pearson et al. 2019). Collectively, sexual dimorphism has been described as complex interactions of sex hormones, genetic variability, and the environment (Yang et al. 2006; Stillwell et al. 2010; Quinn et al. 2014; Pearson et al. 2019).

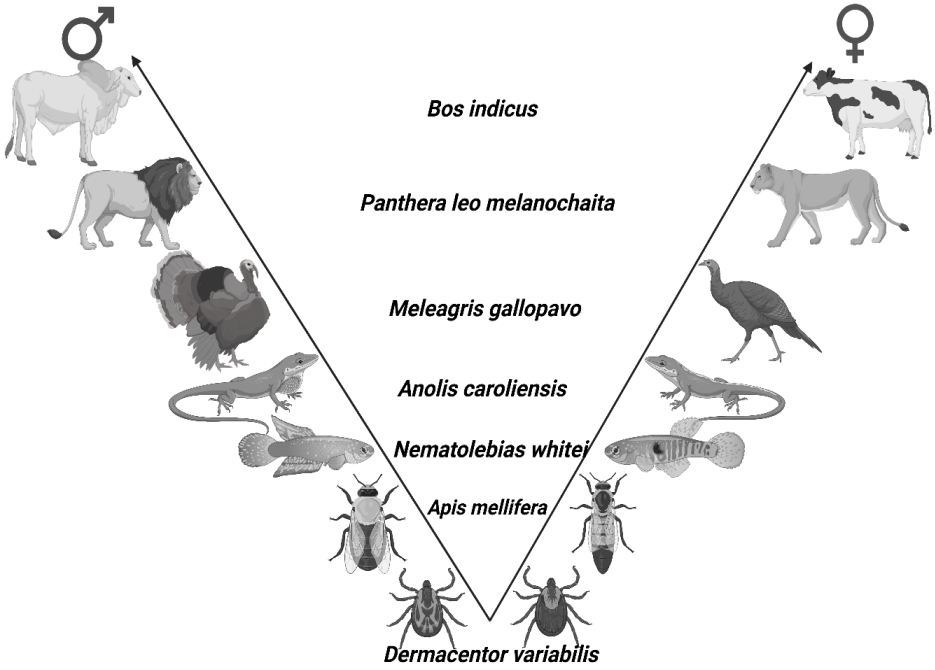

Figure 8: Examples of sexual dimorphism. Created with biorender.com.

Any trait which differs between sexes on average is considered sexually dimorphic. In some species, sexes are visually distinct due to their sexually dimorphic characters, whereas in other species they are indistinguishable (Cox and Calsbeek 2009; Mainguy et al. 2009; Quinn et al. 2014; Luthringer et al. 2014; Mori et al. 2017). Depending on the influence of sexual dimorphism on reproduction, sexual dimorphism can be further characterised as primary or secondary. Primary sexual dimorphism

directly influences reproduction, for example traits that effect the reproductive organs or gametes such as egg size or sperm size. Traits attributed to secondary sexual dimorphism are not essential for reproduction to occur, but do increase the chance of reproductive success (Cox 2010; Mori et al. 2017).

Sexual dimorphism is usually a product of natural and/or sexual selection, but probably evolved through sexual selection. Sex-specific natural selection occurs when a trait increases the survival and/or reproductive success for a specific sex, while sexual selection is more specific and generally favours traits which are only involved in mating/fertilization success. (Ellegren and Parsch 2007). Despite large phenotypic differences between sexes, the majority of the genome is identical between males and females, with the most distinct genetic differences between sexes found on sex-specific chromosomes or associated with gamete production (Parisi et al. 2003; Skaletsky et al. 2003; Malone et al. 2006; Tukiainen et al. 2017). However, with advancements in sequencing technology, emerging evidence suggests that the majority of variation between males and females is a result of differential gene expression. Thus, sex-biased gene expression underlines the majority of intra-species variation observed in nature and allows males and females to avoid the constraints of a shared genome in order to cope with different selection pressures (Ellegren and Parsch 2007; Cox and Calsbeek 2009; Grath and Parsch 2016).

1.8 Sex-biased genes

Charles Darwin's theory of sexual selection explained the evolution of sexual dimorphic traits by stating that phenotypes which are sex-specific and increase reproductive success, are preserved within a species even if survivability is reduced (Darwin 1871). Conflicting selective pressures from sexual selection, or sexual antagonism, can lead to the selection of genes which are beneficial to one sex but are harmful to the other. Emerging bodies of data suggest that sexual antagonism is an important factor in the evolution of sex-biased genes (van Doorn 2009; Pennell and Morrow 2013; Maklakov and Lummaa 2013).

In an ideal example of sexual antagonism, the two sexes will adapt different traits, even though both sexes are under the constraint of a shared genome which controls trait expression. The 'tension' produces high levels of intersexual genetic correlation which makes it difficult for each sex to reach its specific fitness optimum (Lande 1980). One key factor of sexual antagonism is if ongoing conflicts are conflicts that cannot be resolved or are a transitional evolutionary stage before resolution has been achieved. Evidence has shown that genetic variation can allow sexes to independently reach optimal trait values, however genetic barrier and varying selection pressures can continue to constrain each sex and preventing resolution of ongoing conflicts.

Artificial selection regimes have been used to demonstrate ongoing sexual antagonism (Mokkonen et al. 2012) Studies on reduced fitness in offspring of the opposite sex, showed sex-biased genotypes with positive fitness can be less fit when expressed in the opposite sex. For example, in ground crickets (*Allonemobius socius*), males with the highest fitness produce males with high fitness but females of low fitness (Fedorka and Mousseau 2004).

Despite sexual antagonism being widespread across organisms, the effects on fitness and distribution of antagonistic loci throughout the genome remains relatively unknown. It is predicted that despite the ability for any allele to exist on any chromosome, the vast majority of sexually antagonistic alleles will be found on the X chromosome (Fry 2009; Innocenti and Morrow 2010). Rice (1984) suggested that X-linked recessive alleles that increase fitness in males are always expressed in males as they are hemizygous in XY systems while expression only occurs in 50% of females. As a result, there is weak selection against females as the benefits for males are exposed to selection twice as often as the negative effects in females. Additionally, dominant alleles that increase fitness in females will also accumulate on the X chromosome as they are expressed in 67% of females, but only in 33% of males (Rice 1984).

These patterns of expression could allow for the selection of sexually antagonistic alleles, despite the cost it infers on one sex (Pennell and Morrow 2013). For mammals,

birds and insects, X-and Z-linked genes have increased rates of evolution compared to autosomal genes (Torgerson 2003; Khaitovich et al. 2005; Baines and Harr 2007; Mank et al. 2007; Baines et al. 2008; Grath and Parsch 2012). The pattern has been termed faster-X or faster-Z evolution and has been found at a gene expression level in mammals (Brawand et al. 2011), *Drosophila* species (Meisel et al. 2012; Llopart 2012; Kayserili et al. 2012) and birds (Dean et al. 2015).

The underlying cause of faster-X(Z) evolution is not fully understood, but a possible explanation is positive selection acting on the heterogametic sex, where recessive advantageous alleles are more likely to go to fixation as there is only one copy of each sex chromosome (Charlesworth et al. 1987). A second reason could be caused by a reduction in relative effective population size when comparing sex chromosomes to autosomes, leading to a reduction in the effectiveness of selection on the sex chromosome (Caballero 1995; Laporte and Charlesworth 2002). Thirdly, dosage compensation and other unique aspects associated with sex chromosomes could also contribute to a faster-X(Z) evolution (Begun et al. 2007).

A significant signal of a faster-X(Z) evolution is present in the *Drosophila* genome when comparing less-stringent sex-biased expression for all genes across all tissues, but was not male-biased (Ávila et al. 2015). Overall, genes on sex chromosomes show greater expression divergence between species than genes on autosomes and when comparing the ratio of expression divergence to expression polymorphism, a considerable proportion of expression divergence is adaptive (Laporte and Charlesworth 2002; Meisel et al. 2012; Kayserili et al. 2012; Dean et al. 2015). However, it is thought that the low effective population size of the Z-chromosome, relative to that of autosomes, reduces the adaptive role of the Z chromosome and its role in encoding sexually selected traits (Wright et al. 2015). The difference in rates of molecular evolution for Z linked genes compared to that of autosomes is more likely due to neutral and non-adaptive processes (Vicoso et al. 2013). This relaxed

purification selection was considered main contributor to faster-X(Z) in birds and snakes (Mank et al. 2007; Vicoso et al. 2013; Wright et al. 2015).

Relaxed selection constraints have also been suggested as a cause for the increased level of divergence in male-biased genes that accumulate more neutral mutations than female-biased genes. The neutral mutations would could only be explained if male-biased genes were less constrained by pleiotropy, such as high tissue specificity or purification selection in males but not in females. Sex-biased gene expression is influenced by tissue type, with some sex-biased genes isolated to specific tissue types. The specificity of sex-biased genes could increase their rates of evolution as it is thought that pleiotropy reduces the probability of a mutation being beneficial and limit adaptive evolution (Fisher 1958).

Genes that are expressed in a limited number of tissues and are highly tissue specific are referred to having low expression breadth. Whereas, genes that are expressed in multiple tissue types and have low tissue specificity are referred to having a broad expression breadth. Genes with low expression breadth are expected to evolve faster than those with broad expression breadth. This is due to accelerated rates of adaptive substitutions, or the replacement of one mutation (or allele) by another with greater fitness (Duret and Mouchiroud 2000; Zhang and Li 2004; Larracuente et al. 2008). An alternative explanation for the increased rates of evolution in genes with low gene expression breadth is that they are under relaxed selective constraints. This, along with the gene expression breadth and sex-limited tissues such as reproductive organs, must be considered when deciphering the cause of rapid evolution of sex-biased genes.

1.8.1 Sex-biased gene expression

Sex-biased gene expression likely accounts for the majority of sexually dimorphic traits between males and females of the same species. Sex-biased gene expression occurs in three ways: 1) the less common sex-specific expression, when only one sex expresses a specific gene, 2) the more common sex-enriched expression, when one sex expresses a specific gene in higher quantities than that of the other sex, or, 3) through

complete sex-linkage, when a gene or allele is physically restricted to the sex chromosome of just one sex (Connallon and Knowles 2005; Yang et al. 2006; Cox and Calsbeek 2009; Mank 2009).

Sex-biased gene expression can be further categorised as male-biased or female-biased, depending on which sex has higher expression (Fig. 9) and as unbiased genes, when there is equal expression in both sexes. Sex-biased gene expression plays a key role in understanding evolutionary, epigenetic, developmental and medical questions (Baker et al. 2011; Stamboliyska and Parsch 2011; Parsch and Ellegren 2013; Gilks et al. 2014; Morrow 2015). For example, female mosquitos are a vector for malaria transmission, one of the leading causes of death worldwide, and understanding the causes of this particular sexual dimorphism in mosquitoes may provide better methods for biological control (Baker et al. 2011; Stamboliyska and Parsch 2011).

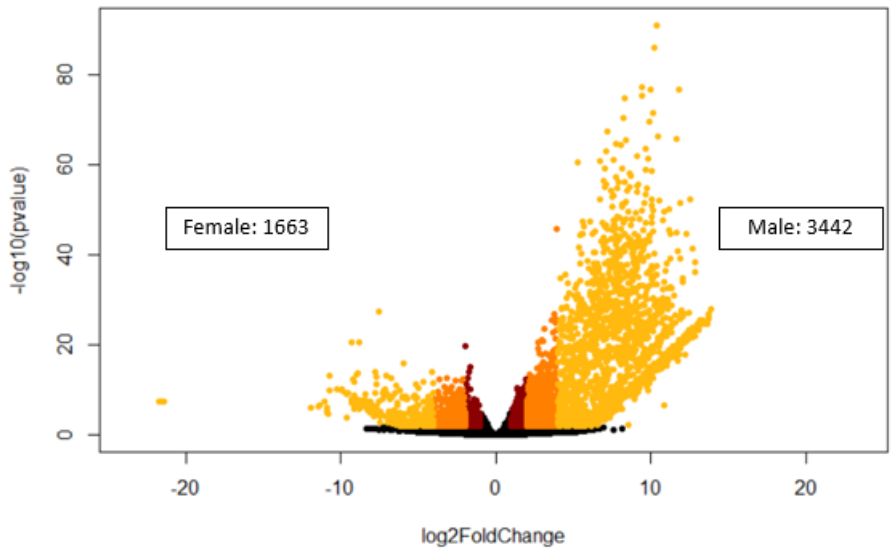

Figure 9: Example of sex-biased gene expression found in *Fucus serratus*. Sex and the number of significantly differentially expressed genes Padj <0.05 are labelled in the diagram. Log2fold change are colour coded by values; Padj <0.05 <1 Black , Padj <0.05 >1 Red, Padj <0.05 >2 Orange and Padj <0.05 >4 Yellow.

Research on sex-biased gene expression started in the early 2000s with the introduction of DNA microarrays. Using this method, one of the first sex-biased gene expression models was *Drosophila melanogaster*. Using DNA microarrays, the expression of thousands of genes from two samples were compared and showed that sex-biased gene expression had a larger impact on expression variation than either age or genotype (Jin et al. 2001). Further development using next-generation sequencing (NGS) technology made it possible to run high throughput RNA sequencing, which provided a high number of accurate digital outputs of expression levels for each gene in a genome. Unlike microarray methods, NGS output the molecular sequence for each gene, which allowed for comparative gene expression analysis on non-model organisms. However, due to the highly plastic nature of gene expression, the bias of a gene is limited to a single observation of the sample being investigated. As a result, a gene can have sex-biased gene expression in one sample, but show no bias or even a bias towards the opposite sex in another.

1.8.2 Factors that affect sex-biased gene expression

The levels of sex-biased gene expression can be influenced by a multitude of different factors: 1) the species and/or population being studied, 2) the individual tissue type under investigation, 3) the developmental stage, 4) sex chromosomes, 5) environmental and biological factors of the individual, and 6) experimental methodology and statistical criteria used to define differential expression. Sex-biased gene expression has been documented across a wide number of different organisms 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 and Ellegren 2009), plants (Darolti et al. 2018; Cossard et al. 2019), fish (Yang et al. 2016) and brown algae (Martins et al. 2013; Lipinska et al. 2015). Levels of sex-biased expression vary across all species and can comprise a large proportion of the genome, with up to 90% in some extreme cases (Ranz et al. 2003; Ayroles et al. 2009).

In early studies, RNA was extracted from the entire organism of small bodied model species such as *Drosophila* (Jin et al. 2001; Parisi et al. 2003; Ranz et al. 2003; Reinke et al. 2004) and when males and females were compared, ~ 57% of all genes showed sex-biased expression (Ranz et al. 2003). Although giving an initial insight into levels of sex-biased gene expression, it was later shown that sex-biased expression can vary greatly among tissue types, which cannot be detected when using the whole organism (Parisi et al. 2003; Goldman and Arbeitman 2007; Huylmans and Parsch 2014). This is because the abundance of RNA is affected by heterogeneous tissue and variations in cellular composition between groups of samples, resulting in large differences in gene expression easily misinterpreted as regulatory differences (Montgomery and Mank 2016; Hunnicutt et al. 2022).

The extent to which each sex-biased gene is found in different tissues is known as gene expression breadth and more genes with sex-biased expression are likely to be found as more tissue types are investigated (Yang et al. 2006). The highest levels of sex-biased gene expression were usually found in the gonads and then in the brain, while lower levels were expressed and heart (Parisi et al. 2003; Goldman and Arbeitman 2007; Mank et al. 2008b; Catalán et al. 2012; Pointer et al. 2013; Wong et al. 2014; Harrison et al. 2015). In plants, sex-biased expression is isolated primarily in floral tissue and nearly absent in leaf tissue (Zemp et al. 2016; Sanderson et al. 2019). RNA-seq analysis of multiple tissues in the brown algae *Fucus vesiculosus* showed higher levels of sex-biased genes in reproductive tissue than vegetative tissue (receptacles *vs.* vegetative tips (Fig. 4)) (Martins et al. 2013).

Starting as early as sexual differentiation in the zygote, sex-biased gene expression tends to increase as development progresses, with low levels in embryonic stages and high levels in sexually mature adults (Thoemke et al. 2005; Mank et al. 2010; Magnusson et al. 2011; Perry et al. 2014; Ingleby et al. 2015). Levels of sex- biased gene expression in an organism can vary throughout development and can fluctuate during particular developmental stages then lost in later stages. For example, in the *D.*

melanogaster gonad, 10% of genes exclusively show sex-biased expression in adults, 11% exclusively in the larval stages and 30% across all developmental stages (Perry et al. 2014). Similarly, in chicken gonads, 35% of genes show sex-biased expression in adults, 5% during embryonic stages and only 1% consistent across all developmental stages. And in the plant *Mercurialis annua,* males and females differed in their gene expression early in development but peaked just prior to, and after, flowering (Cossard et al. 2019).

Other examples exist in brown algae. For example, the number of sex-biased genes in the kelp *Saccharina japonica* was always higher in mature stages compared to that of the immature stage (Zhang et al. 2021). In *Ectocarpus*, the majority of sex-biased genes were only expressed in one of the two developmental stages with only 12% of male-biased genes and 3% of female-biased genes expressed in both the immature and fertile gametophytes. Additionally, 3% of male-biased genes in immature gametophytes transitioned to female-biased genes in the fertile gametophyte stage (Lipinska et al. 2015).

Clear genomic differences exist between sexes in species with specialised sex chromosomes. In mammals, where males are heterogametic (XY) and females are homogametic (XX), genes located on the Y chromosome can only be expressed in males. Likewise, in birds when females are heterogametic (ZW) and males homogametic (ZZ). For genes located on chromosomes found in both sexes, the levels of expression are more complex as there are different copy numbers for homogametic and heterogametic sexes. In ZW systems, male-biased expression is usually associated with the Z linked genes. This is not the case for the XY systems due to a process called dosage compensation that has evolved to regulate expression of the X chromosome (Disteche 2012; Mank 2013; Ercan 2015; Lucchesi and Kuroda 2015; Malviya et al. 2016).

Dosage compensation in mammals is expressed by inactivating one copy of the X chromosome in females, whereas in *Drosophila*, the single copy of the X chromosome

is upregulated (Mank 2013). In plants with separate sexes, four families have been described with dosage compensation. For example, in *Coccinia grandis,* there was an excess of sex-biased genes on the sex chromosome, with a reduction of 40% in Y gene expression which were compensated for by elevated expression of corresponding genes on the X (Fruchard et al. 2020). In UV systems such as the brown algae *Ectocarpus,* dosage compensation is not expected to occur because the U and V chromosomes determined sex during the haploid phase. This means that the gene dosage is the same for the sex chromosomes and the autosomes. Additionally, sex-biased genes on the sex chromosomes are only expected in regions closely linked to the non-recombining region as both the male and female sex-determining region haplotypes only occur in independent haploid male and female individuals (Lipinska et al. 2015).

Environmental factors also play an important role in sex-biased gene expression. An organism's diet can influence the level of sex-biased expression, suggesting that expression is a costly investment for an organism. Diet was used as an indicator for an individual's condition in *D. melanogaster* males and females, where individuals fed a high-quality diet had a greater level of sex-biased expression than those fed a low-quality diet (Wyman et al. 2010). Another environmental factor that influences sex-biased expression is the social structure. For example, the social structure of turkeys is composed of dominant breeding males and subordinate non-breeding males. Subordinate males display a reduction in male-biased genes and an increase in female-biased gene expression (Pointer et al. 2013).

In the kelp *Saccharina latissima,* temperature influenced sex-biased gene expression and only a small percentage of sex-biased genes remained consistent. Female gametophytes had a stronger response to higher temperatures than males, suggesting that males are more heat tolerant than females (Monteiro et al. 2019). Finally, an often overlooked factor when considering sex-biased gene expression analysis is the way sex-biased gene expression is detected and portrayed. This can

depend on different technical aspects, for example, comparing the difference between NGS methods and microarrays to measure expression. The number of replicates, RNA quality and availability also play an important role. Furthermore, detection of sex-biased genes depends on methods of data collecting, processing, and statistical analyses used to detect and determine sex-biased expression.

Two approaches have been used to determine sex-biased expression. Firstly, a multiple test-corrected statistical threshold is set to determine a false discovery rate of 5%. The method detects a large number of sex-biased genes, but many have only a slight difference in expression. A second method uses a fold-change threshold, for example a two-fold difference in expression between the two sexes. Although the method is highly dependent on the number of replicates and variability between replicates, it will identify genes with statistically significant levels of sex bias (Ellegren and Parsch 2007; Grath and Parsch 2016).

With many complex factors influencing the expression and evolution of sex-biased genes, it is no surprise that detailed research has been limited to a very few taxa. However, modern genomic tools can expand from the classical model organisms to include less studied phylogenetic groups. Brown algae, a group characterized by a multitude of reproductive traits, is posed to provide model species for research in key evolutionary events such as the evolution of sex and sex-biased genes (Coelho and Cock 2020)

1.8.3 Sex bias of gene expression in *Fucus*

Fucus provides an interesting system in which to study the evolution of sex-biased genes, as it is a member of the only order (Fucales) within the Phaeophyceae to evolve a diplontic life cycle which resembles that of animals. *Fucus* has two distinct lineages with at least two transitions between dioecious species and hermaphroditic species and reciprocal diecious and hermaphroditic species with asymmetrical hybridization and signs of reinforcement. With this unique set of evolutionary history, wide distribution, high abundance, simple morphology and lack of behavioural

characteristics, *Fucus* provides an all-purpose model for the research in sex-biased genes and reproduction as a whole.

Nevertheless, only one publication has investigated sex-biased genes in *Fucus*. Martins et al. (2013) compared male and female reproductive tissue transcriptomes to vegetative tissue in *F. vesiculosus*. They estimated that sex-biased gene expression was higher in males (14%) than in females (9%) relative to vegetative tissue. Additionally they found that male-biased genes were less constrained than female-biased genes possibly due to pleiotropy , similar to patterns found other XY systems (Parisi et al. 2004; Ellegren and Parsch 2007; Mank et al. 2008a; Parsch and Ellegren 2013).

Functional annotation showed that uniquely male-biased genes were associated with sperm development and function, transduction, signal perception and flagella proteins. Female sex-biased genes were associated with a carbohydrate modifying enzyme most likely associated with zygote cell wall biogenesis (Martins et al. 2013). Since 2013, sequencing technology has developed rapidly, opening doors and possibilities that were not possible at the time. With an ever increasing volume of data and efficiency what are now the best methods for studying sex-biased gene expression on different scales?

1.9 Whole Genome Sequencing and Reduced Representation Sequencing.

The genomic revolution (1st, 2nd, and now 3rd generation sequencing techniques) has progressed through stages that have dramatically increased the quality, reduced the cost and increased efficiency, at rates doubling every five months (Stein 2010; Heather and Chain 2016). As a result, the ability to produce whole genome sequencing data vastly overtakes the ability to process these data. In view of the lag between production and processing, more and more studies, especially those using non-model organisms, use alternative methods such as reduced representation sequencing (Therkildsen and Palumbi 2017). This method generates genome-wide data and

genotyping of a large amount of genetic polymorphisms, but without the use of whole genome sequencing (van Tassell et al. 2008; Förster et al. 2018).

1.9.1 Reduced Representation Sequencing

Reduced representation sequencing has become a common technique for investigations of population genetics, phylogenies, molecular marker development, genetic map construction, phylogeography, and QTL mapping (Pante et al. 2015; Andrews et al. 2016; Xuereb et al. 2018; Fang et al. 2018; Christiansen et al. 2021). Much like the switch from amplified-fragment length-polymorphisms to microsatellites, changing to a novel method requires detailed research and understanding to avoid the potential pitfalls (Christiansen et al. 2021).

Reduced representation sequencing requires initial fragmentation of the genome into smaller segments, which can then be sequenced using high-throughput sequencing platforms (Davey et al. 2011). Subsequent alignment of the fragments to either a reference genome or by *de novo* assembly allows the detection of genetic variation or single nucleotide polymorphisms (SNPs) (Rochette et al. 2019). The data produced from reduced representation sequencing have been used to produce microsatellites, microhaplotypes, and copy number variants (Jansson et al. 2016; Baetscher et al. 2018; Dorant et al. 2020). Three of the most commonly used reduced representation sequencing methods are transcriptome sequencing (RNA-seq), restriction-site associated DNA sequencing (RAD-seq), and target capture sequencing (TCS).

1.9.2 Transcriptome sequencing

Transcriptome sequencing (RNA-seq) targets specific transcriptomic expression in a specific tissue type in a certain state. Using RNA-seq messenger RNA (mRNA), fragments are converted into complementary DNA (cDNA) which encodes various proteins. Using high throughput sequencing technologies RNA-seq has become the basis of most expression analysis studies such as the transcription structure of genes (splicing patterns, 5′ and 3′ terminals, and post-transcriptional modifications) and

expression levels during development. The sequences that are produced can be mapped to a reference genome or assembled via *de novo*. Reads that are mapped to a reference genome can be used to detect genetic variation in coding regions or used in structural annotation of the transcriptome.

RNA-seq is highly sensitive, can be used on non-model and model organisms without the design of probes for known sequences and is useful for sequencing organisms with large and complex genomes. However, the presence of high abundance RNAs can require additional processing to reduce the relative abundance of the transcripts and genes sequenced depend upon the tissue or organ that is sequenced, thus producing a time-tissue snapshot of transcriptomic expression. The method, despite being cheaper, quicker and produce less data than whole genome sequencing, remains expensive and labour-intensive and produces large amounts of data that requiring a lot of time for data analysis.

RNA-seq has been used extensively to address questions involving *Fucus,* specifically ecological research, developmental biology, population genetics, evolutionary dynamics, and expression analysis. In *F. vesiculosus*, for example, the number of sex-biased genes were higher in male reproductive tissue than in female reproductive tissue and the expression levels of these male-biased genes were higher than that of female-biased genes (Martins et al. 2013). This observation is thought to be a result functional pleiotropic constraint in females and/or male competition (Ellegren and Parsch 2007). This supports the theory that males possess a large array of genes that regulate male fitness, similar to that of the evolutionarily distant heterogametic animal models (Ranz et al. 2003; Meiklejohn et al. 2003; Yang et al. 2006; Small et al. 2009; Parsch and Ellegren 2013). High levels of differentiation in gene expression involved in salinity acclimation mechanisms also were detected between populations (Rugiu et al. 2020) and may demonstrate a high level of fixation and expression levels related to local adaptation to salinity. An embryo development RNAseq dataset was generated for *F. vesiculosus* to investigate differential gene

expression during development, revealing for example, cell wall-driven cellular expansion mechanisms similar to plants and a cleavage-type cell proliferation similar in both plants and animal (Linardić et al. 2020). These results suggest a conserved element in developmental phenomena across all branches of multicellular life. RNA-seq has also been used to address the complex morphological variation in *Fucus* species, which has stymied taxonomic studies for centuries (Akita et al. 2022; Almeida et al. 2022).

1.9.3 Restriction Site Associated DNA sequencing

Restriction Site Associated sequencing (RAD-seq), digests genomic DNA with a single or many restriction enzymes, to create genomic libraries for subsequent sequencing of loci at high or low coverage (Baird et al., 2008). RAD-seq was initially developed for use on non-model organisms with no reference genome (Davey and Blaxter 2010) and has become a staple in population genomics because of the flexible nature and cost-effectiveness (Christiansen et al. 2021).

To date, RAD-seq methods have not been widely used in *Fucus* species. The technique requires a large amount of high quality DNA (50–500 ng of DNA in ~ 50µL) that is free from contaminants (Graham et al. 2015). DNA extraction from macroalgae is challenging as there are high levels of both polysaccharides and phenolics which contaminate the DNA and often inhibit downstream enzymatic reactions (Snirc et al. 2010; Fort et al. 2018). The only use of RAD-seq in *Fucus* so far has been to show the responses to salinity stress in different populations of *F. vesiculosus* in the Baltic Sea (Kinnby et al. 2020).

1.9.4 Target capture sequencing

Target capture sequencing (TCS) or target capture enrichment methods, sequence specifically selected fragments or subset of genes and/or regions of interest, to produce relatively short (typically <1000 base-pair) sequencing templates. A high level of flexibility allows selection of millions of areas of interest. Several methods have been developed, such as: multiplexed PCR amplification reactions (Tewhey et al. 2009),

DNA hybridization to capture (Albert et al. 2007; Gnirke et al. 2009), and DNA capture via molecular inversion probes that can be retrieved using magnetic beads (Porreca et al. 2007; Rohland and Reich 2012). One of the main differences between RAD-seq and TCS is that TCS requires a reference for probe design. The reference does not need to be an entire genome or transcriptome and could simply be a single gene/transcript of interest and can come from another closely related species.

TCS has yet to be used in *Fucus* which is partly due to a lack of any detailed genome or transcriptome. However, there have been many recent advancements in the use of TCS such as target capture of ultra-conserved elements, anchored hybrid enrichment and genotyping (Lemmon et al. 2012; Faircloth et al. 2015; Campbell et al. 2015). As TCS has been successfully used to examine microevolution/macroevolution, phylogenetics, morphological evolution and gene expression in non-model organisms (Choquet et al. 2019; Martuscello et al. 2022; Mengual et al. 2022; Ortiz-Sepulveda et al. 2022), it is just a matter of time before TCS will address questions in *Fucus* species.

2 Aims of the Dissertation

The overall goal of this dissertation is to use the genus *Fucus* as model organisms to understand general aspects of reproduction, speciation and the evolution of sex-biased genes.

In **Paper I**, I review the many unique features of reproduction in *Fucus* species to establish why and how their study will promote a more general understanding of speciation and evolution of sex-biased genes.

In **Paper II**, I examine evolutionary dynamics of sex-biased gene expression in *Fucus,* a phylogenetically young XY system, and assemble transcriptomes of different sexes and tissue types of the four most studied *Fucus* species.

In Paper III, I develop a novel genomic approach using target capture sequencing in *Fucus* for population and evolutionary genomics studies.

3 General Discussion

One of the challenges facing evolutionary biologists is understanding the evolution of a wide variety of eukaryotic life cycles. The different life cycle types (haplontic, diplontic and haplodiplontic) have evolved independently multiple times in different eukaryotic groups. Understanding the evolution of these traits is sorely lacking in most eukaryotic groups and currently limited in focus (Villarreal and Renner 2013; Hanschen et al. 2018). Similarly, knowledge on the evolution of sex-biased genes and sex-biased gene expression is scarce, with current research limited to a small number of taxa (Wyckoff et al. 2000; Swanson et al. 2001; Mank and Ellegren 2009; Lipinska et al. 2015; Zemp et al. 2016).

3.1 *Fucus*, as a model organism for reproduction and sexual evolution

Brown algae, particularly members of the genus *Fucus*, are an excellent system in which to examine broad questions of the evolution of life cycles, sex-biased gene expression and how sex-biased genes have evolved. *Fucus* species as model organisms, is not a new concept. The zygotes of *F. serratus* and *F. vesiculosus* have been used as a standard model for cell polarisation and asymmetric cell division for a long time (Brownlee and Bouget 1998). The ease with which gametes and zygotes could be collected, the abundance of *Fucus* species and their resilient nature, provide researcher with the perfect subject. (**Paper I**).

Indeed, hundreds of years of research into *Fucus* reproduction have set up the foundations for a model organism in reproductive biology and the evolution of life history (**Paper I**). *Fucus* mating system has undergone at least two independent transitions between dioecy and hermaphroditism (Cánovas et al. 2011). The majority of brown algal species have a haploid-diploid life cycle, where sex is only expressed in the haploid gametophyte generation and is controlled by haploid sex chromosomes (UV system) (Coelho et al. 2018); whereas *Fucus* has undergone a recent shift to a diplontic life cycle (Silberfeld et al. 2010). This is expected to require an intermediate

stage with epigenetic sex determination due to the difference in genetic mechanisms of UV chromosome sex determination (where sex is determined during meiosis) and that of XY (where the sex is determined post-fertilisation) (Beukeboom and Perrin 2014). This makes *Fucus*, a perfect model to study the evolutionary drivers and consequences of these transitions, which still remains obscure as the transition to diplontic life cycle is very recent (Silberfeld et al. 2010).

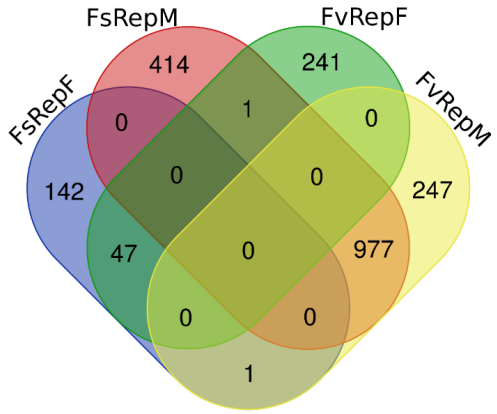

Figure 10: 1 -1 Ortholog comparison between *Fucus serratus* (Ser) and *Fucus vesiculosus* (Ves) of sex-biased genes not isolated to reproductive tissue in males(M) and females(F). Unpublished data.

When investigating the evolution of sex-biased genes in *Fucus* species, we found that a majority of male-biased genes had male-limited expression and were found in male reproductive tissue; unlike female-biased genes which were found in both reproductive and vegetative tissue and expressed in both sexes (**Paper II**). These patterns of male-biased gene expression are usually associated with high turnover rates and fast sequence evolution, as genes with low expression breadth are expected to evolve faster than those with broader expression breadth (Duret and Mouchiroud 2000; Larracuente et al. 2008; Grath and Parsch 2012). This is due to a reduced probability of a mutation being beneficial for all tissue types, thereby limiting adaptive

evolution (Fisher 1958). This is not quite the case in *Fucus,* as we also observed high convergence of male-biased expression in orthologs between *Fucus* species in male-biased genes (**Paper II**). This is unexpected as male-biased genes should be associated with higher turnover rates and evolve faster at a protein-coding sequence level, so male-biased genes in different *Fucus* species are expected to be further diverged from each other. Instead, we found more male-biased gene orthologs in the hermaphrodites within each lineage and in the dioecious species between different lineages (Fig. 10 & 11). This could be an indication of how the transition between dioecy and hermaphroditism occurs.

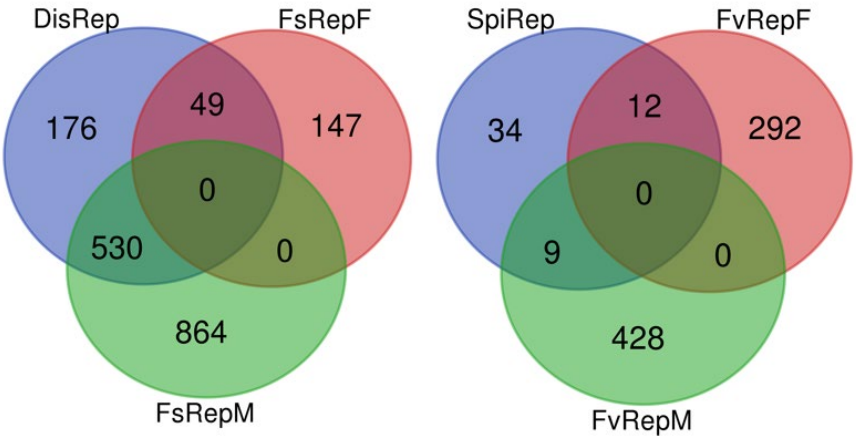

Figure 11: 1 -1 Ortholog comparison between sex-biased genes in *F. serratus*(Fs) & *F. vesiculosus*(Fv) male(M) and female(F) individuals and *F. distichus*(Dis) *F. spiralis*(Spi) reproductive tissue(Rep) biased genes. Unpublished data.

In plants, no convergence at the gene expression level is found and, the transcriptomes from dioecious plants cluster by species rather than by sex (Scharmann et al. 2021). Similar patterns have also been seen in animals which also clustered by species first and showed little evolutionary conservation of sex-biased gene expression (Harrison et al. 2015; Naqvi et al. 2019). However, the convergence of sex-biased gene expression has also been investigated in 8 other brown algal species in the context of

changing sexual systems (Cossard et al. 2022). Dioecy is predicted to be the ancestral sexual system in brown algae, although there have been multiple transitions back to monoecy/hermaphroditism (Fig. 12) (Heesch et al. 2021). The results from an investigation of four dioecious-monoecious species pairs in four major brown algal clades showed that sex-biased expression was neither ancestral nor convergent, with limited levels of shared ancestral sex-biased gene expression across the 8 brown algal species (Cossard et al. 2022). More than half of the orthologs across the four pairs of brown algal species were more consistent with lineage specific recruitment and quick turnover of sex-biased genes among brown algal species. A significant proportion of sex-biased genes were under positive selection and were associated with lower selective constraints on expression levels, possibly due to lower pleiotropy and sex-specific selection after evolving separate sexes (Cossard et al. 2022). The convergent changes in gene expression driven by selection seen in these brown algal species, may be due to the shift in sexual system and lower selective constraints, which is comparable across these distant species.

Additionally, the monecious gametophytes were more closely related to females of the corresponding dioecious species, which is also contradictory to our results where male-biased genes were more closely related to the corresponding hermaphroditic species (Fig. 11). From this and the similar patterns seen in volvocine algae, it is suggested that monoecy may have arisen from ancestral females, which in *Fucus* species would imply that monoecy may have evolved from males (Yamamoto et al. 2021; Cossard et al. 2022). However, we would need more data on other closely related Fucales, which have also transitioned between reproductive methods, to test this theory.

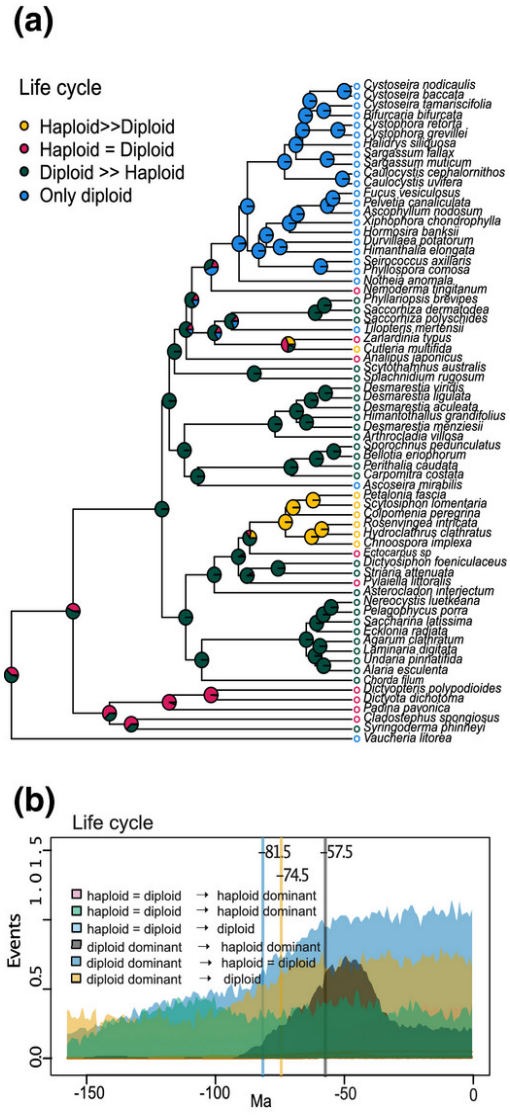

Figure 12: Maximum likelihood ancestral state reconstructions for the four-brown algal life-history traits. Pie charts and colors at each node represent the probabilities for each state. Colors at the tips represent the species states (a). Estimated number of transitions through time for the corresponding four life-history traits (b). Colored densities identify the mean number of events for each possible transition. Vertical lines and numbers denote the minimum age as the point in time, where at least one transition is recorded in 60% of the reconstructions. (a and b) Male and female gamete size. Adapted from Heesch et al. 2021.

One other variation that is yet to be considered is the transition from a UV system to an XY system. In a study on the evolution of Y-chromosome content across fly species, it has been found that young Y-chromosomes still show clear evidence of their autosomal origins (Mahajan and Bachtrog 2017). This could explain why we find many conserved orthologs in the males of *Fucus* species (Fig. 10 & 11). The young neo-Y chromosome (1.5myo) found in fly species, still shows substantial homology with the X chromosome, whereas in the old Y chromosome (15myo), almost all traces of a shared ancestry have been eroded (Mahajan and Bachtrog 2017). Fucales which diverged around 19.5-7 mya, significantly later than those of Laminariales, Ectocarpales, Desmarestiales, and Dictyotales, could still be in the process of erosion (Paper II; Liang et al. 2022). Finally, the XY system in *Fucus* is young and the new X and Y sex chromosomes are still probably homomorphic, which could explain frequent transitions to hermaphroditism. With the multiple switches in sexual systems in *Fucus* and Fucales, this could have further prolonged the erosion process.

Another important effect that came with the transition to a diplontic life cycle is the variation between male and female gametes. Anisogamy is a core element of sexual dimorphism, which can explain phenotypic differences between sexes. The variation in gametes between males and females evolved through sexual selection, reproductive strategies and parental investment to optimize the individual's fecundity (Levitan 1996; de Lisle and Rowe 2015). Like most other brown algae, *Fucus* does not have any phenotypic differences between male and female individuals but still presents with a moderate level of sex-biased gene expression (8-9%) (**Paper II**). This is especially clear when compared to other brown algal species like *Ectocarpus* (>4%), kelp *Macrocystis* (24%) and *Saccharina latissima* (34%) (Lipinska et al. 2015; Monteiro et al. 2019; Müller et al. 2021). The higher proportion of sex-biased genes found in *Macrocystis* and *Saccharina latissima* is the likely result of higher levels of phenotypic sexual dimorphism between male and female gametophytes, which have clearly distinct morphologies (Lüning 1981; Müller et al. 2021). In *Fucus* and *Ectocarpus*, no large phenotypic differences between sexes is observed, but is expected to increase as

the number of sex-biased genes increase (Ellegren and Parsch 2007; Pointer et al. 2013). Ectocarpales have near-isogamous gametes whereas Fucales have clearly morphologically different gametes (Paper II; Luthringer et al. 2014; Lipinska et al. 2015; Müller et al. 2021). Our results suggest that the evolution of anisogamy alone, without any other phenotypical sexually dimorphic characteristics, could have triggered a significant increase in sex-biased gene expression (**Paper II**).

3.2 *Fucus*, as a model organism for speciation

Fucus species are also becoming a suitable model for research on speciation. The mechanisms of speciation have always been of central interest in evolutionary biology. However, no agreement has emerged about how genetic, geographical, ecological, evolutionary and environmental factors interact to create two species from one (Slatkin 1993; Lee and Mitchell-Olds 2011; Wang 2012; Shafer and Wolf 2013; Orsini et al. 2013; Sexton et al. 2014; Wang and Bradburd 2014). Speciation is becoming increasingly viewed as a continuum (speciation continuum), with local adaptation on one end of the spectrum and reproductive isolation on the other (Seehausen et al. 2014). The investigation of reproductive barriers and their timing has become a central task for speciation geneticists in order to establish the differences between the causes and the consequences of speciation (Seehausen et al. 2014). In most species, reproductive isolation results not from a single isolating factor, but from different pre- and postzygotic barriers and their complex interactions. These barriers can be produced by divergent selection, such as ecological or sexual selection that result in an extrinsic reproductive isolation (Price; Panhuis et al. 2001; Boughman 2002; van der Sluijs et al. 2008; Maan and Seehausen 2011; Nosil 2012). Additionally, evolution of genetic incompatibilities via genetic drift, selection or genomic conflict, can produce intrinsic reproductive isolation (Gavrilets 2004; Abbott et al. 2013). *Fucus* could provide a useful model for research in the speciation continuum with its high abundance, morphological plasticity, ecosystem variations and hybridization **(Paper I; Paper III)**.

Reproductive barriers between hybridizing populations are more or less permeable, allowing for the transfer of genetic information from one species to another through hybridization, also called 'introgression' (Abbott et al. 2013).Hybrid zones thus provide an ideal natural experiment to study the selective forces driving reproductive isolation, and ultimately speciation. Hybridisation is quite common among *Fucus* species **(reviewed in Paper I)**. Reproductive isolation in externally fertilising taxa can be produced by either temporal and spatial timing of gamete release, and/or the evolution of gametic incompatibility mechanisms (Palumbi 1994; Vacquier 1998; Swanson and Vacquier 2002; Levitan et al. 2004; Ladah et al. 2008; Lessios 2011; Monteiro et al. 2012; Hoarau et al. 2015). The rapid evolution of proteins which are involved in gamete-gamete recognition in free spawning invertebrates, have been widely studied and are important in species recognition and speciation (Swanson and Vacquier 2002; Lessios 2011). Egg and sperm proteins may be engaged in an arms race driven by several processes resulting in sexual dimorphism. In the case of *Fucus* species the fraction of male biased genes under selection was not significantly different to that observed for unbiased genes, which would indicate that adaptive evolution is not the cause for the elevated substitution rates we found in male biased genes (**Paper II**). However, three male-biased genes that were under positive selection were associated with the sperm flagella. This could suggest that at least some of the male biased genes could be experiencing adaptive evolution in the form of sperm competition. In *Fucus* early work established some of the key characteristics of sperm-egg recognition, the egg membranes of *Fucus serratus* use a glycoprotein that reacts with the sperm surface and a complimentary lectin-like protein on sperm which acts as an egg receptor (Bolwell et al. 1979, 1980). However, further progress has been limited by to the lack of genomic information.

The sister species pair *F. serratus* and *F. distichus* provide an ideal model system to study reproductive isolation mechanisms. Three hybrid zones consist of a natural zone in Northern Norway with around 10,000 years of sympatry, and two zones where either species has been introduced **(reviewed in Paper I)**. *F. serratus* was introduced

to Iceland with around 100 years of sympatry and *F. distichus* was introduced to the Kattegat which also has around 100 years of sympatry (Coyer et al. 2002b, 2006b). It has been shown that there is strong indications for reinforcement in these hybrid zones (Hoarau et al. 2015). Reinforcement is a mode of speciation where natural selection acting on prezygotic barriers increase reproductive isolation between two species. This usually occurs as a result of selection against the formation of hybrid individuals with low fitness **(reviewed in Paper I)**. Using microsatellites and cpDNA, it has been shown that hybridization and introgression was significantly decreased with increased time of sympatry (Coyer et al. 2007). Cross-fertilization experiments have suggested that increased gametic incompatibility explain the negative correlation between the age of the hybrid zone and the degree of hybridization (Hoarau et al. 2015). Until now, all of the studies looking at *Fucus* hybrid zones have been limited to few (10s) of molecular markers, which is hardly representative of the genomes. The identification of genes involved in reproduction and potentially under selection (**Paper II**) now offers us a set of candidate genes for the study of reproductive isolations. The inclusion of such genes (1,991) as well as "neutral" genome wide ones (1,007) into a successful target capture design **(Paper III)**, opens to genome population genomic studies of these hybrid zones with 10s thousands SNPs. Such studies will undoubtedly shed more lights of speciation mechanism in this fascinating genus.

Although the specific genes underlying reinforcement in *Fucus* remains unknown, using new reduced representation sequencing methods and the newly available genomes and transcriptomes (see Phaeoexplorer: https://phaeoexplorer.sb-roscoff.fr/home/) we are able to investigate which specific genes could be under selection (**Paper II; Paper III**). This will allow us to elucidate some of the more complex forms of speciation, such as reinforcement that is still lacking both theory and data from a variety of organisms and is mainly isolated to a small number of *Drosophila* species (Servedio and Noor 2003).

4 Conclusions and future perspectives

The main conclusion of this PhD study is that the genus *Fucus* is now becoming a model organism for research in speciation, the evolution of life cycles and reproduction in a young XY system. The following specific conclusions can also be drawn from this PhD study:

i. The male and female sex-biased genes found in the young XY system of *Fucus* show unique evolutionary pathways that affect each sex differently. The male-biased genes evolve faster at the level of the protein sequence than females. Male-biased gene expression levels seem conserved between *Fucus* species whereas females have a higher diversity of expression levels.

ii. Male sex-biased gene expression is male limited and limited to reproductive tissues, whereas female sex-biased gene expression, is expressed in both sexes and all tissue types. These patterns of sex-biased gene expression in *Fucus* seem to arise from a down-regulation of expression of the pleiotropic female genes in male receptacles.

iii. The use of target capture sequencing in *Fucus* species is a highly effective and efficient technique to analyse genome wide SNPs to characterize genomic variation , between and within *Fucus* species.

This works also integrates into two large international research projects. Firstly, Phaeoexplorer - which aims to produce transcriptome data and annotated genome assemblies for >80 brown algal species, all of which have different phylogenetic distances from the model brown algae *Ectocarpus*, so that the data produced can be used to answer key questions about the biology and evolutionary history of brown algae. Secondly, SEXSEA - which aims to use a plethora of sexual characteristics found in brown algae to gain novel insights into the functional and evolutionary interactions between the sex chromosomes and key eukaryotic reproductive and life cycle features.

For *Fucus* this will help answer how the transitions of life cycles occurred and which sex first became hermaphroditic during its transitions.

Despite the sequencing of the genomes for *F. serratus*, *F. distichus* (Phaeoexplorer) and *F. vesiculosus* (University of Gothenburg see: https://www.ncbi.nlm.nih.gov/data-hub/genome/GCA_014849475.1/), we have yet to identify the XY chromosomes, the origins of the sex determination regions and locations of the male and female-biased genes in the genome. Additionally, the exact evolutionary age of the haploid life cycle and the XX/XY system in *Fucus* is yet to be determined. The evolutionary age of *Fucus*, haploid life cycle require additional data from closely related species which will soon be available from Phaeoexplorer.

Finally, the new method of whole genome analysis using the target capture sequencing probes developed in this PhD will help resolve the controversial number of species found in *Fucus* and help understand the genetic basis of what delimitates different species. Our method of target capture sequencing could also be used to detect the specific genes behind pre- and post- zygotic barriers and elucidate the specific genes and certain molecular mechanisms behind speciation.

The upcoming results from both projects, the methods tested in this PhD and the very large comparative genomic data sets produced will dramatically change the brown algal research landscape in the very near future.

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EDITED BY
Georgina Valentine Wood,
University of Western Australia,
Australia

REVIEWED BY
Nahlah Abdullah Alsuwaiyan,
University of Western Australia,
Australia
Kai Xu,
Jimei University, China

*CORRESPONDENCE
William J. Hatchett
william.hatchett@nord.no

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A review of reproduction in the seaweed genus *Fucus* (Ochrophyta, Fucales): Background for renewed consideration as a model organism

William J. Hatchett[1]*, James A. Coyer[2], Kjersti Sjøtun[3], Alexander Jueterbock[4] and Galice Hoarau[1]

[1]Ecology Division, Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway, [2]Shoals Marine Laboratory, University of New Hampshire, Durham, NH, United States, [3]Department of Biological Sciences, University of Bergen, Bergen, Norway, [4]Algal and Microbial Biotechnology Division, Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway

The genus *Fucus* dominates the intertidal and shallow subtidal rocky reefs of the North Atlantic and also is commonly found in the intertidal of the North Pacific. It likely diversified 12.2-2.7 mya into two genetically distinct lineages: Lineage 1 with one species in the North Pacific and two in the North Atlantic; and Lineage 2 found only in the North Atlantic (one species recently introduced into the North Pacific). With 10 accepted species, *Fucus* spp. (and the Fucales) are unique among algae in having a diplontic life cycle, whereby the only haploid stage is the single-celled gamete. Further, *Fucus* spp. produce eight eggs in each oogonium; have hermaphroditic and dioecious species in each lineage; display sperm:egg ratios differing by more than one order of magnitude; have synchronized and predictable release of gametes; are capable of self- and/or cross- fertilization and asexual (fragmentation *via* adventitious branching) reproduction; readily hybridize in culture, as well as the field; and form ecads (free-living individuals with morphological variability linked to habitat) by hybridization or polyploidy. Consequently, the genus is an excellent model for a variety of studies in reproductive biology, employing laboratory and field manipulations as well as detailed genetic studies using the molecular 'omics'. We review here the relevant literature in order to fully understand and appreciate the unique opportunities that *Fucus* spp. provide as model organisms for future studies of reproduction.

KEYWORDS

***Fucus*, reproduction, review, diplontic life cycle, selfing, hybridization, fertilization, ecads**

Introduction

The brown algal genus *Fucus* (Ochrophyta, Fucales) is an important ecosystem engineer on sheltered and moderately exposed rocky shores of the North Pacific and the North Atlantic. As providers of an important foundational habitat, the species collectively provide shelter and food for a plethora of invertebrate and fish species, as well as a substrate for attachment of epibionts (Lüning, 1990; Chapman, 1995).

Fucus also is important as a food source for large land herbivores (both wild and domestic), as well as human cultures. For example, reindeer venture into the intertidal in high Arctic regions to forage on *Fucus* when snow cover is too deep and/or rain-on-snow icing makes it difficult to obtain their normal diet of lichens (Hansen et al., 2019). Elsewhere in northwestern Europe, seaweeds (including *Fucus* spp.) have been consumed by, and fed to, sheep, cattle, and pigs, both historically and presently (see references in Blanz et al., 2020). Sheep that escaped the annual round-up in Iceland and Norway often survived the harsh winters by foraging on intertidal *Fucus* (Landsborough, 1857). The feral and protected North Ronaldsay or Orkney sheep on North Ronaldsay (northernmost island of Orkney) have modified their gut biome, as a result of confinement to the intertidal by humans, to subsist almost entirely on seaweed, including *Fucus* (Balasse et al., 2005; Ruggeri, 2015). With respect to humans, *Fucus* spp. have been a food source, processed to a form of 'soda ash' for soap and glass making, used for centuries as fertilizer in maritime areas that could not support livestock for manure (Pereira & Cotas, 2019), and had/have a variety of traditional medicinal uses, albeit of suspect effectiveness (https://medlineplus.gov/druginfo/natural/726.html; but see Catarino et al., 2018).

Students and researchers began studying coastal intertidal regions as soon as marine laboratories were established along the shores of the North Atlantic, beginning in the mid-1800s (e.g., Station Biologique de Roscoff, 1859; Woods Hole, USA, 1871; Kristineberg Marine Research Station, 1877; Laboratory of the Marine Biological Association at Plymouth, 1885; Marine Biological Laboratory (USA), 1888; University of Oslo Drøebak, 1894) and a prominent subject for these early investigations was the readily available *Fucus*. However, perhaps the first published description of *Fucus* was over 300 years ago, in a 1711 paper by René-Antoine Réaumur that included detailed illustrations of what appears to be a combination of *Fucus vesiculosus* and *F. serratus* (Figure 1).

Coyer et al. (2006a) first proposed a North Pacific origin of *Fucus* based on a phylogeny derived from both a variable and conserved region of the mitochondrion that revealed: 1) sister-taxa to *Fucus* are found only in the North Pacific, and 2) high haplotype and nucleotide diversity in the variable mt region was present in the North Pacific, whereas only a single haplotype (shared with the North Pacific) was found in the North Atlantic. A later phylogenetic study using 13 protein-coding genes also supported a North Pacific origin of Fucales dating to 19.5-7 mya with divergence of the genus *Fucus* at 12.5-2.7 mya (Cánovas et al., 2011), agreeing with an earlier estimate of *Fucus* divergence at 2.3-5.5 mya using single-strand conformation polymorphisms (SSCP) of a mtDNA spacer region (Hoarau et al., 2007). However, the most recent *Fucus* phylogeny, with more extensive sampling throughout the Arctic and Subarctic and using 21 mtDNA-IGS haplotypes, placed the ancestor of the *F. distichus* complex (likely near the ancestral member of the genus) in the low Arctic/Subarctic (Laughinghouse et al., 2015). Sequence analysis of nuDNA and mtDA also revealed two distinct lineages within the genus, Lineage 1 with two accepted species and Lineage 2 with eight accepted species (see https://www.algaebase.org/search/genus/detail/?genus_id=71; Almeida et al., 2022).

Recent studies have illustrated the dynamic nature of speciation in *Fucus*. For example, microsatellite markers suggest that *F. radicans* diverged from *F. vesiculosus* the northern Baltic within the last 400 years (Pereyra et al., 2009). On the other hand, however, is extinction; the glacial relict populations of *F. virsoides* along the Slovenian coast declined significantly by 2010 and disappeared entirely by 2016 (Battelli, 2016).

Excellent reviews on the broad subjects of phylogeny (Cánovas et al., 2011); physiology (Chapman, 1995; Colvard et al., 2014; Colvard & Helmuth, 2017); gamete release and settlement/recruitment (Chapman, 1995; Brawley et al., 1999); and ecology (Chapman & Johnson, 1990; Chapman, 1995; Wahl et al., 2011) have been written and publications on various aspects of *Fucus* have increased dramatically, closely tracking key technological advancements (Figure 2). This review will address and update aspects of reproduction in *Fucus*.

General Characteristics

Sexual dimorphism (when reproductive) and the realization of dioecy and hermaphroditism in *Fucus* species were determined in the 1800s. Reproductive individuals produce numerous receptacles at the apical tips of branches, each of which contain many conceptacles. In dioecious species, all conceptacles on an individual contain either antheridia or oogonia (Figure 3); hermaphroditic species have conceptacles containing both antheridia and oogonia (see Engel et al., 2005). There are no monoecious species of *Fucus* (e.g., one individual with receptacles containing conceptacles with either antheridia or oogonia). *Fucus* spp. exhibit a diplontic life cycle with male heterogamy (XX/XY) (Heesch et al., 2021) and gametes are the only haploid cells. Sex of dioecious species often can be determined by eye in the field when reproductive: male

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FIGURE 1
Illustration of *Fucus serratus* from Réaumur (1711). Note that one thallus appears to have receptacles and vesicles characteristic of *F. vesiculosus*, perhaps mistakenly interpreted as stemming from the same holdfast, when in fact, it may be two independent attachments, one on the other (see insert). Permission to reuse this image was confirmed by Service des Archives et du Patrimoine historique under the free use of public archive documents.

receptacles appear red/orange-colored because of carotenoids in eyespots for the negatively phototaxic sperm contained within antheridia of the conceptacles (Decaisne & Thuret, 1845), whereas female conceptacles are green or brown because of chloroplasts in the eggs (Whitaker, 1931). The egg begins dividing ~24 hrs after fertilization (Thuret, 1855).

All species of *Fucus* reproduce sexually, but sexual reproduction is difficult in brackish conditions. In *F. vesiculosus*, for example, the low salinity (<5 PSU) typical of the Baltic Sea: 1) drastically reduces the release of gametes; 2) diminishes longevity and motility of released gametes; 3) reduces fertilization rates; and 4) fosters polyspermy, which is lethal in *Fucus* and increases dramatically in brackish conditions (Brawley, 1991; Brawley, 1992; Serrão et al., 1996; Serrão et al., 1999a). Consequently, asexual reproduction (fragmentation *via* adventitious branching) is common among populations existing in marginal environments, including ecads (discussed below) and *F. radicans* in the Baltic (Tatarenkov et al., 2005; Pereyra et al., 2009; Johannesson et al., 2011). In the northern Baltic, *F. radicans* recruits both sexually and asexually, varying from complete sexual to >90% monoclonal with phenotypic variation significantly lower in monoclonal stands than in multi-clonal groups (Johannesson et al., 2012). Furthermore, a shift from sexual to asexual reproduction has occurred in a few

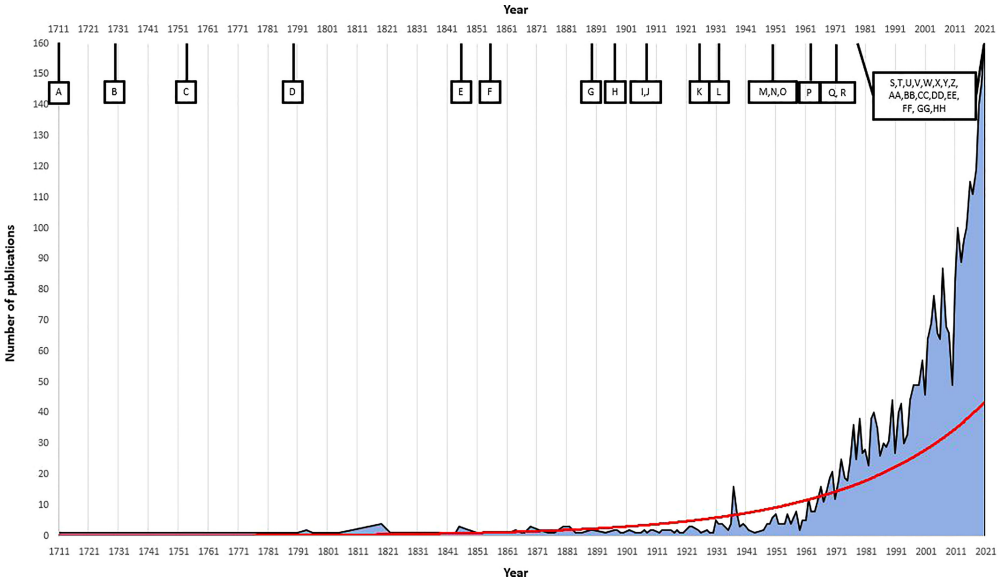

FIGURE 2
Correlation of number of *Fucus* publications and key advances in *Fucus* reproduction/research and general technological advancements over time. We used the software *Dimensions* (Digital Science; https://app.dimensions.ai/discover/publication) to search for '*Fucus*' in titles and abstracts of all journals and all dates. Red line is the exponential trendline based on the number of publications released. **(A)** 1711, first publication on *Fucus* reproduction (Réaumur 1711); **(B)** 1730, advancement in microscope technology (Tromp, 2015), **(C)** 1753, first taxonomic description of *Fucus* Linnaeus (Linneí and Salvius, 1753); **(D)** 1790-1900, early chemical, physiological and biological descriptions (Woodward, 1791; Stackhouse, 1801; Kniep, 1925); **(E)** 1845, initial research on life history, reproductive methods and taxonomy reassignment (Genus *Fucus*) (Decaisne & Thuret, 1845); **(F)** 1854, first description of fertilisation in *Fucus* and first cross-experiments (Thuret, 1855); **(G)** 1890, beginning of laboratory cultures (Campbell, 1889); **(H)** 1897, first cytological study of *Fucus* (Farmer & Williams, 1897); **(I)** 1909, first observation of fertalisation and total number of chromosomes (Yamanouchi, 1909); **(J)** 1909, early ecological studies (Baker, 1909; Baker, 1910); **(K)** 1925, first successful cross experiments (Kniep, 1925); **(L)** 1931, invention of the electron microscope (Freundlich, 1963); **(M)** 1950, first electron microscope image of *Fucus* (Manton & Clarke, 1950); **(N)** 1950, beginning of autocological research in *Fucus* (Knight & Parke, 1950; Burrows & Lodge, 1951); **(O)** 1951, natural hybridization experiment (Burrows & Lodge, 1951); **(P)** 1962, further studies on *Fucus* chromosome number (Evans, 1962); **(Q)** 1970, detailed experimentation on gamete release and gamete physiological structure and characteristics for *Fucus* (Pollock, 1970); **(R)** 1970-1995, in-depth research on individual, population and community ecology in *Fucus* (Chapman, 1995); **(S)** 1977, first generation sequencing (Heather & Chain, 2016); **(T)** 1979, detailed investigation on natural morphological variation and phenotic plasticity in *Fucus* (Scott & Hardy, 1994); **(U)** 1980, formal demographic analysis on *Fucus* species (Gunnill, 1980); **(V)** 1970-1990, development of PCR and microsatellites (Kaunitz, 2015; Saeed et al., 2016); **(W)** 1985, discovery of pheromonal gamete attraction in *Fucus* (Müller & Gassmann, 1985); **(X)** 1980-1991, comprehensive studies on egg production in *Fucus* (Vernet & Harper, 1980; Robertson, 1987; Ang, 1991); **(Y)** ~1990, detailed research in rates of reproduction, settlement, recruitment and population modelling in *Fucus* begun (Chapman, 1995); **(Z)** 1997-1999, rDNA and nrDNA sequencing of internal transcribed spacer region in *Fucus* and SSU and LSU sequences (Leclerc et al., 1998; Rousseau et al., 1997; Rousseau & de Reviers, 1999; Serrão et al., 1999a); **(AA)** 2002-2003, design of polymorphic microsatellite markers for *Fucus* (Coyer et al., 2002c; Engel et al., 2003); **(BB)** 2005, next generation sequencing begun (Heather & Chain, 2016); **(CC)** 2006, RNA extraction method designed for *Fucus* and tested with RT-PCR, RNA-labelling and Northern analysis methods (Pearson et al., 2006), **(DD)** 2006, complete mitochondrial genome for *F. vesiculosus* and mtDNA-based phylogeny showing the two *Fucus* lineages (Oudot-Le Secq et al., 2006; Coyer et al., 2006a); **(EE)** 2011, third generation sequencing (Heather and Chain, 2016); **(FF)** 2013, first investigation in sex-biased gene expression in *F. vesiculosus* (Martins et al., 2013); **(GG)** 2013-present, effects of climate change on *Fucus* distribution (Jueterbock et al., 2014; Rothäusler et al., 2018; Rugiu et al., 2018; Rothäusler et al., 2019); **(HH)** 2020-to present, complete annotated genome and transcriptome using illumina and nanopore platforms (https://phaeoexplorer.sb-roscoff.fr/home/).

absolute marginal populations of *F. vesiculosus* in the northern Baltic Sea (Tatarenkov et al., 2005).

It is important to realize that asexual reproduction does not necessarily reduce fitness, (Preston et al., 2022 and references therein). For example, *F. vesiculosus* in the Baltic Sea can exist in two forms: the most common being the epilithic form (attached, sexual and rarely asexual) and less commonly, the benthopleustophytic form (free-living and asexual) found on any substrate within the photic zone (Preston et al., 2022 and references therein). However, each form likely will respond differently to future environmental changes. Additionally, asexual reproduction may conserve existing genotypes by

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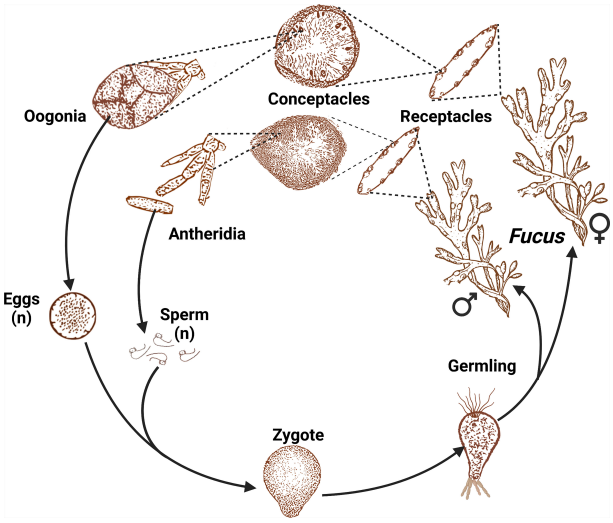

FIGURE 3
Dioecious life cycle of the genus *Fucus*. A representative illustration, not to scale, adapted from Müller (1991) footage, provided by Technische Informationsbibliothek (TIB) and created with BioRender.com. Receptacles of a hermaphroditic species contain both antheridia and oogonia, whereas in dioecious species (pictured), an individual is either male (all receptacles contain antheridia) or female (all receptacles contain oogonia).

preventing recombination of new genotypes, unlike selfing (see below) (Ardehed et al., 2015).

In *Fucus*, adventitious branches can form from both the thallus and holdfast, with regeneration most rapid from the midrib region of the thallus (Fulcher & McCully, 1969; Fulcher & McCully, 1971; McLachan et al., 1971; Van Alstyne, 1989). Branches can be induced by herbivory or in response to no obvious physical stimulus (Van Alstyne, 1989). The epidermal cells grow outward forming distinct 'embryos' instead of lateral branches, which are indistinguishable from early sexually produced embryos (Fulcher and McCully, 1969; McCook & Chapman, 1993).

Detached adventitious branches may reattach to the substratum *via* rhizoids and develop into apparently functional male and female thalli (asexual recruitment), which subsequently can form large clones with skewed sex ratios (Tatarenkov et al., 2005; Johannesson et al., 2011; Ardehed et al., 2015). For example, a large *F. radicans* female clone in the northern Baltic was distributed over 550 km and a large male clone over 100 km; both were likely to be a few thousand years old (Johannesson et al., 2011; Ardehed et al., 2015). Populations in more southerly Baltic locations, however, almost exclusively displayed sexual recruitment (Johannesson et al., 2011; Ardehed et al., 2015).

More recently, experiments with *F. radicans* demonstrated that temperature and light interactively resulted in the highest success of re-attachment of adventitious branches (Schagerström & Salo, 2019). Light level alone had no effects on success in cooler water temperature while success in high water temperature under low light levels was very low. Their results further suggested that rhizoid formation (re-attachment success) depends on the net primary production (metabolic balance) of the adventitious branches.

The notion that sexual reproduction in *Fucus* is impeded in areas of low salinity has been challenged by recent studies detailing a much more complex picture. Ardehed et al. (2016) found high levels of sexual reproduction in some *F. radicans* populations inhabiting the extreme low salinity (2-3 PSU) Gulf of Finland. Kinnby et al. (2019) showed that low salinity, temperature, and oxygen (i.e., high stress) were associated with high number of adventitious branches in *F. vesiculosus* and *F. radicans* populations within the Baltic, but outside the Baltic, high salinity, high phosphate, and low turbidity were positively correlated with adventitious branching. Kinnby et al. (2019) hypothesized that variation in patterns of adventitious branching between populations was due either to genetic differences arising from local adaptation unrelated to the physical factors that were measured or from stochastic effects

TABLE 1 Main reproductive period for *Fucus* spp. as recorded in different studies.

Lineage	Species	Mating type	Main sexual reproductive period	Location (GPS Coordinates)	Reference	Comment
1	*Fucus distichus*	H	(Summer)-Autumn	Maine, USA(42.98, -70.60)	Sideman & Mathieson (1983b)	"Dwarf form"
			Spring-Autumn-(Winter)	Maine, USA(42.98, -70.60)	Sideman & Mathieson (1983b)	subsp. *edentates*
			Spring or Autumn	Maine, USA(42.98, -70.60)	Sideman & Mathieson (1983a)	subsp. *evanescens*
			Autumn-Winter	British Columbia, Canada (49.26, -123.12)	Ang (1991)	Conceptacles fertile all year
			Spring	Nova Scotia, Canada(44.48, -66.08)	Edelstein & McLachlan, 1975	
			Autumn-Winter-(Spring)	Stavanger, Norway(58.99, 5.70)	Fredriksen (1985)	Small morphotype (subsp. *anceps*)
			Spring-(Summer)	Skagerrak, South-Norway (58.99, 9.75)	Steen & Rueness (2004)	Large morphotype (subsp. *evanescens*)
			Spring-(Summer)	Kiel Bight, Germany(54.44, 10.19)	Schueller & Peters (1994)	Large morphotype (subsp. *evanescens*)
			Winter-Spring	New Hampshire, USA(See reference)	Mathieson & Hehre (1982)	
			Winter-Spring	New Hampshire, USA(See reference)	Mathieson & Hehre (1982)	subsp. *edentatus*
			Spring-Summer	New Hampshire, USA(See reference)	Mathieson & Hehre (1982)	Large morphotype (subsp. *evanescens*)
			Spring-Summer	Southern Gulf of St. Lawrence, Canada(See reference)	Johnson et al. (2012)	subsp. *edentatus* and subsp. *evanescens*
			Spring	Orkney, UK(59.02, -2.99)	Perry & Hill (2015)	
			Spring-Summer	Avacha Bay, Russia(53.04, 158.60)	Kashutin et al. (2019)	subsp. *evanescens*
1	*Fucus serratus*	D	(Autumn)-Winter-Spring	Lofoten-Norway(68.15, 14.03)	Fredriksen (1990)	
			Autumn-Winter	Stavanger, Norway(58.99, 5.70)	Fredriksen (1990)	
			Autumn-Winter-Spring-(Summer)	Skagerrak, South-Norway (58.99, 9.75)	Steen & Rueness (2004)	
			(Autumn)-Winter	Oslofjord, South-Norway (59.30, 10.62)	Sundene (1953)	
			Autumn	Misterhult, Sweden(57.56, 16.73)	Malm et al. (2001)	
			Summer	Blekinge, Sweden(56.14, 15.39)	Malm et al. (2001)	
			Autumn-Winter	Isle of Man, UK(54.08, -4.76)	Knight & Parke (1950)	
			(Summer)-Autumn	Devon, UK(50.31, -4.089)	Knight & Parke (1950)	
			Summer	North Spain(See reference)	Arrontes (1993)	Conceptacles fertile all year
			Summer-(Autumn)	Southern Gulf of St. Lawrence, Canada(See reference)	Johnson et al. (2012)	
			Summer-Autumn	Isle of Man, UK(54.07, -4.60)	Williams (1996)	

(Continued)

TABLE 1 Continued

Lineage	Species	Mating type	Main sexual reproductive period	Location (GPS Coordinates)	Reference	Comment
			(Spring)-Summer-Autumn	UK(See reference)	d'Avack & Marshall (2015)	
2	*Fucus ceranoides*	D(H)	Spring-Summer	South Norway(See reference)	Lein (1984)	Hermaphroditic individuals probably are hybrids
			Not known	Iberian Peninsula(NA)	Gómez Garreta (2000)	Receptacles present all year, not necessarily fertile
2	*Fucus vesiculosus*	D	(Spring)-Summer-Autumn	Barsebäck, Sweden(55.77, 12.89)	Carlson (1991)	
			(Spring)-(Summer)	Hanko peninsula, Finland (59.88, 23.24)	Bäck et al. (1991)	
			(Spring)-Summer-(Autumn)	Lofoten-Norway(68.15, 14.03)	Fredriksen (1990)	
			(Spring)-Summer	Stavanger, Norway(58.99, 5.70)	Fredriksen (1990)	
			(Spring)-Summer	Skagerrak, South-Norway (58.99, 9.75)	Steen & Rueness (2004)	
			(Spring)-Summer-Autumn	Southeastern Sweden,(See reference)	Berger et al. (2001)	
			Spring-(Summer)	Isle of Man, UK(54.08, -4.76)	Knight & Parke (1950)	
			(Spring)-(Summer)	Devon, UK(50.31, -4.089)	Knight & Parke (1950)	
			Summer-Autumn-(Winter)	Ría de A Coruña, Spain(43.36, -8.35)	Viana et al. (2015)	Differences between sites
			Unknown	Iberian Peninsula(NA)	Gómez Garreta (2000)	Receptacles present all year, not necessarily fertile
			Spring	New Hampshire, USA(See paper)	Mathieson & Hehre (1982)	Conceptacles fertile all year
			Autumn	Blekinge, Sweden(56.14, 15.39)	Malm and Kautsky (2003)	
			Spring-(Summer)	Viana do Castelo, Portugal (41.67, -8.83)	Monteiro et al. (2012)	Conceptacles fertile all year
			Spring-(Summer)	Sines, Portugal(37.87, -8.82)	Ladah et al. (2003)	Conceptacles fertile all year
			Spring	Kiel Fjord, Germany(54.41, 10.19)	Wahl et al. (2010)	Conceptacles fertile all year
			Summer-Autumn	Waquoit Bay, USA(41.56, -70.52)	Yates & Peckol (1993)	
2	*Fucus radicans*	D/Veg	(Summer)	Öregrund, Sweden(60.33, 18.42)	Forslund & Kautsky (2012)	Main reproductive period first part of June
			(Summer)-(Autumn)	Saaremaa, Estonia(58.33, 23.08)	Schagerström (2013)	
			(Spring)-Summer	Gävle, Sweden(60.69, 17.24)	Schagerström & Kautsky (2016)	
2	*Fucus chalonii*	D	Summer	North Spain(NA)	Gómez Garreta (2000)	
2	*Fucus spiralis*	H	(Summer)-Autumn	Lofoten-Norway(68.15, 14.03)	Fredriksen (1990)	
			Summer (Autumn)	Stavanger, Norway(58.99, 5.70)	Fredriksen (1990)	
			Summer-(Autumn)	Skagerrak, South Norway (58.99, 9.75)	Steen & Rueness (2004)	

(Continued)

TABLE 1 Continued

Lineage	Species	Mating type	Main sexual reproductive period	Location (GPS Coordinates)	Reference	Comment
			Unknown	Iberian Peninsula(NA)	Gómez Garreta (2000)	Receptacles present all year, not necessarily fertile
			Summer-(Autumn)	New Hampshire, USA(43.05, -70.71)	Niemeck & Mathieson (1976)	
			Summer-(Autumn)	Isle of Man, UK(54.08, -4.77)	Subrahmanyan (1960)	
			Summer	Orkney, UK(59.02, -2.99)	Perry & Hill (2015)	
			Spring-(Summer)	Viana do Castelo, Portugal (41.67, -8.83)	Monteiro et al. (2012)	
			Spring-(Summer)	Sines, Portugal(37.87, -8.82)	Ladah et al. (2003)	
			(Spring)-Summer	North Wales(See reference)	Ferreira et al. (2015)	
			Autumn-Winter	Yerseke, Netherlands(51.51, 4.04)	Coelho et al. (2001)	
			Spring-Summer-Autumn-Winter	São Miguel Island, Portugal (37.73, -25.63)	Neto (2000)	Conceptacles fertile all year
2	*Fucus guiryi**	H	Spring-Summer	Tarifa, Spain(36.01, -5.57)	Sánchez de Pedro et al. (2019)	Fertile individuals all year, different between sites
			Spring-(Summer)	Viana do Castelo, Portugal (41.67, -8.83)	Monteiro et al. (2012)	
			(Spring)-Summer	Northern Portugal(See reference)	Zardi et al. (2015)	
			Autumn	Southern Portugal(See reference)	Zardi et al. (2015)	
2	*Fucus virsoides*	H	Spring-Summer-Autumn	Bay of Kotor, Montenegro (42.43, 18.64)	Mačič (2006)	
			Summer	Rovinj, Croatia(45.08, 13.62)	Zavodnik (1973)	
2	*Fucus cottonii*	Veg(D)	NA	Galway, Ireland(See reference)	Sjøtun et al. (2017)	Reproduces by fragmentation, however, some populations with receptacles have been found

D, dioecious; H, Hermaphroditic; Veg, vegetative. Spring=March-May, Summer=June-August, Autumn=September-November, Winter=December-February, '()'= covers less than half of the period, (See reference) = multiple sampling locations. *Now *F. limitaneus* and *F. macroguiryi* (see "Hybridization" in text). As the listed reproductive periods are rough estimates of optimal environmental conditions, population differences, and interactions with other environmental factors can shift and/or blur the identified ranges.

of population separation, and emphasized the need to identify additional environmental factors that may explain the predominance of asexual reproduction in the Baltic Sea. These studies clearly demonstrate that the recently formed postglacial Baltic Sea (8000 yrs; Björk, 1995), with steep gradients in physical characteristics from south to north (this and all further directional references are poleward), is a challenging and marginal environment for the closely related *F. radicans* and *F. vesiculosus* in terms of allocating resources to sexual or asexual reproduction.

Fucus spp. in Lineage 2 exhibit a general spring-summer reproduction period in most studies (Table 1), but in the low salinity Baltic Sea, *F. vesiculosus* displayed two peaks of reproduction: early summer (May-June) and late autumn (September-October) (Table 1; Berger et al., 2001). Summer-reproducing individuals initiated receptacle development and produced more, but smaller eggs in response to short-day laboratory conditions, whereas receptacle development was independent of daylight in autumn-reproducing individuals, suggesting the presence of two distinct genotypes (Berger et al., 2001). In southern Europe, mature oogonia are present all year in *F. vesiculosus* and *F. spiralis* (Table 1).

Maximum reproductive peaks for *F. serratus* on open coasts of the NE Atlantic occurred in both the spring and the autumn (March and September) and in the spring-autumn in the NW Atlantic, but in the Baltic Sea, mainland Swedish populations were reproductive in the autumn (October-November) and on the coast of Öland in the summer (June-July) (Malm et al., 2001). Fertilization success in *F. serratus* decreased markedly as salinity decreases, more so than for *F. vesiculosus*, but unlike *F. vesiculosus*, asexual reproduction was not observed in the Baltic (Malm et al., 2001). Non-overlapping reproductive periods also have been observed among *F. distichus* populations off the New England coast in the US (Sideman & Mathieson, 1983a; Sideman

& Mathieson, 1983b; Pearson & Brawley, 1996). In general, reproductive seasons vary within and among species of *Fucus* (Table 1) and clearly, continuation of non-overlapping reproductive periods within populations of a species could increase the probability of eventual speciation.

Gamete structure, release, and fertilization

Studies of reproductive structures in *Fucus* began soon after microscopes were developed. Decaisne & Thuret (1845) published one of the first microscopic descriptions of *Fucus* antheridia and 'spores', stating that the "transparent corpuscles (are) nearly pear-shaped, each one inclosing a single red globule; each one of these corpuscles is furnished with two very thin cilia, by means of which it moves with extreme vivacity". Conceptacles were described by Bower (1880), followed by descriptions of mitosis, meiosis, physiology of *Fucus* spermatozoids, conceptacle development, and egg development (e.g., Farmer & Williams, 1897; Farmer & Williams, 1898; Yamanouchi, 1909; Robbins, 1916; Roe, 1916; see also reviews by Whitaker, 1931; Fritsch, 1945). The first electron micrographs of *F. serratus* sperm revealed that the base of the anterior flagellum was enveloped by a flexible membrane of unknown function and the flagellum was covered with 'hairy' appendages; both were absent in the posterior flagellum. (Manton & Clarke, 1950). For further microscopic descriptions of gamete structure and fertilization in *Fucus*, see: Pollock, 1970; Brawley et al., 1976; Callow et al., 1978; Motomura, 1994. Recent advances in electron microscopy (high pressure freezing, microinjection of fluorescent dyes) examining ultrastructure, distribution, and *de novo* formation of plasmodesmata in *F. distichus* promise to advance studies of receptors and cell-to-cell communication (Nagasato et al., 2015).

In *Fucus*, there are eight eggs per conceptacle (Serrão et al., 1999b; Coyer et al., 2002b), however the number of eggs per receptacle can be species-specific; for example, *F. vesiculosus* produces 10x more eggs than *F. serratus* (Malm & Kautsky, 2003) and hermaphroditic species produce significantly fewer sperms/egg (40:1) than dioecious species (400:1) (Vernet & Harper, 1980). Additionally, sperm in the hermaphrodite *F. spiralis* are much smaller (0.7 1 < 0.46 µm, > 0.21) than in the dioecious *F. vesiculosus* and *F. serratus* (1.58 < 1.25 µm > 0.92), suggesting that the smaller hermaphroditic sperm have fewer energy reserves for swimming in search of eggs (Vernet & Harper, 1980). Experiments have indicated, however, that sperm numbers do not limit fertilization (Berndt et al., 2002). Egg volume varies widely among *Fucus* species, ranging from 235 x 10^3 µm^3 in *F. spiralis* to 68 x 10^3 and 181 x 10^3 µm^3 in *F. vesiculosus* and *F. serratus*, respectively (Vernet & Harper, 1980).

As soon as gametes were observed with the early microscopes, it was realized that *Fucus* provided a favorable system for the study of fertilization (Thuret, 1855); later observations revealed that *Fucus* sperm was attracted to the eggs (Robbins, 1916; see also references in Müller & Seferiadis, 1977). The extremely small amounts of attractant were below detection by instruments until the early 1970s when the pheromone fucoserratene was isolated and identified from eggs of *F. serratus* (Müller & Jaenicke, 1973). Maier and Müller (1986) reviewed extraction methods, identified sexual pheromones in several species of brown algae, including *Fucus*, and detailed how the passage of *Fucus* sperm through a critical concentration level induces a phobic return to the source of pheromone. Given the natural and complex chemical 'noise' that exists in the marine environment, it is perhaps not surprising that introduction of anthropomorphic pollution has the potential to increase the level of 'noise' and affect fertilization in *Fucus* (Steele, 1977).

To achieve maximum fertilization success in *Fucus*, gametes must be synchronously released from nearby individuals and under optimal environmental conditions. While mature oogonia were found all year in receptacles of *F. vesiculosus*, *F. spiralis* and *F. guiryi* (now *F. limitaneus*, Almeida et al., 2022) in a study from North-Portugal, release of eggs (measured as egg settlement) was mainly observed during late spring and summer, with very low settlement observed during the rest of the year (Monteiro et al., 2016). Andersson et al. (1994) measured egg release of *F. vesiculosus* from the Baltic Sea, and observed peaks that occurred in a semilunar pattern. In addition, the highest egg release took place during the evening, between 18:00 and 22:00. Serrão et al. (1996) demonstrated that photosynthesis was necessary for gamete release during calm conditions when extremely high levels of fertilization success, mostly >90%, were achieved (see also Pearson and Brawley, 1996, Pearson et al., 1998). Higher levels of fertilization success (100%) were noted for *F. vesiculosus* in calm conditions within a one-hour interval of a 6-7 hr high tide, 2-3 hrs after being covered by the rising tide (Berndt et al., 2002). Experiments demonstrated that release of gametes was correlated with depletion of dissolved inorganic carbon (DIC) in isolated tide pools during increased light conditions and that sensitivity of gamete release to high water motion was DIC dependent. Specifically, the boundary layer surrounding the receptacle becomes thicker in calm conditions and DIC becomes limiting to photosynthesis, acting as an initial signal on a pathway leading to gamete release (Pearson et al., 1998). In conclusion, gamete release in *Fucus* requires a sunny day, calm conditions, and high tide immersion (Brawley, 1990; Brawley, 1992; Pearson et al., 1998; Berndt et al., 2002; Monteiro et al., 2016).

Low-tide release of gametes, however, is possible under certain conditions. Berndt et al. (2002) documented gamete release in *F. vesiculosus* following several hours of submergence by a rising tide as photosynthesis is required to prepare receptacles for gamete release. They noted that release at low tide occurred after several days of stormy weather and

postulated that the normal high tide release was subsequently inhibited and too many mature gametangia were present in the conceptacles leading to release at low tide.

On the other hand, time of day, tidal height, and wave exposure also influenced egg release and settlement patterns, which further differed according to mating type. Ladah et al. (2008) found that dioecious *F. vesiculosus* released more eggs later in the day and at a lower tide than the hermaphroditic *F. spiralis* which released few eggs throughout the day and at all tides. Thus, the importance of low tide or high tide in gamete release is equivocal.

Dispersal of released gametes is very limited. According to a model, small propagules released one meter from the substratum in turbulent water motion will be transported to the substratum within 2-25 seconds or during 1-6 waves (Denny & Shibata, 1989) and only a few meters laterally during this time frame. Furthermore, *Fucus* sperm are too short-lived to be effective agents of dispersal and are attracted to egg-produced pheromones at only µm to mm distances. Additionally, eggs are fertilized quickly after release, are subjected to lethal polyspermy, and the negatively buoyant zygotes secrete a sticky substance for rapid adherence to the substrate (Kropf, 1992; Serrão et al., 1996; Muhlin et al., 2008). Thus, viable *Fucus* gametes and zygotes are likely to disperse only a few meters from the parent (Arrontes, 1993; Serrão et al., 1997; Engel et al., 2005; Muhlin et al., 2008). Strong genetic structuring (isolation-by-distance) revealed by microsatellites among some populations of *F. serratus* implied limited dispersal due to salinity gradients (e.g., Coyer et al., 2003; Coyer et al., 2011a); whereas the lack of genetic structuring among nearby populations of *F. vesiculosus* may be due to high gene flow, inbreeding depression, microscopic forms persisting from previous generations, and/or inappropriateness of using neutral genetic markers to detect the presence of sub-populations (Zardi et al., 2013; Teixeira et al., 2016).

Additionally, long-distance dispersal *via* rafting of fertile *Fucus* that interbreed with attached individuals may increase connectivity among populations (Muhlin et al., 2008). For example, *F. vesiculosus* possesses air bladders on the vegetative thalli allowing rafting to distant locales when detached and free-floating (Coleman & Brawley, 2005; Tatarenkov et al., 2007; Muhlin et al., 2008; Rothäusler et al., 2015) or when attached to floating objects such as buoyant seaweed, natural wood, and anthropogenic debris (Thiel et al., 2011). Rafting may be particularly important in dispersal of hermaphroditic species as only one fertile individual is necessary for successful colonization *via* a stepping-stone dispersal over longer distances. Indeed, genetic analysis strongly infers the success of rafting for hermaphroditic *Fucus* species (Coleman & Brawley, 2005; Coyer et al., 2011b). Long-distance dispersal of clones *via* detached adventitious branches (asexual reproduction) also occurs. For example, Ardehed et al. (2015) found single thalli of *F. radicans* genetically assigned to clones from distant sites rather than from the population in which they were found and reported finding single and vital thalli 18 and 50 km from the nearest population. Such dispersal is important because in a new area, a unisexual population (=clone) may evolve into a bisexual population and initiate sexual reproduction (Ardehed et al., 2015).

Selfing

Simultaneous hermaphroditism can lead to self-fertilization (selfing), an important aspect of evolutionary biology. Hermaphroditism has been reported for 10 animal phyla and ~5% of animal species, a percentage that increases substantially if the highly specious insects are excluded (Jarne & Auld, 2006). In flowering plants, the transition from outcrossing to selfing occurs in many independent lineages (10-15% of seed plants) and may be a driver of speciation (Wright et al., 2013). Within the genus *Fucus*, *in vitro* fertilization experiments have demonstrated selfing in *F. spiralis* (Pollock, 1970; Vernet & Harper, 1980; Müller & Gassmann, 1985) and genetic investigations have supported selfing in *F. spiralis, F. guiryi*, and *F. distichus* (Billard et al., 2005; Coleman & Brawley, 2005; Engel et al., 2005; Coyer et al., 2007; Perrin et al., 2007; Billard et al., 2007; Coyer et al., 2011c; Almeida et al., 2017; Whitaker et al., 2017).

Selfing usually is considered to be deleterious (see Wells, 1979, and references therein). First, it increases the probability that recessive maladaptive genes will become homozygous and subsequently decrease adaptation and fitness (e.g., 'dead end' of Stebbins, 1974). Secondly, genetic recombination is limited and further reduces genetic potential to enhance survival and reproduction in a changing environment. Thirdly, effective population size may be reduced.

However, the notion of selfing being a 'dead end' over the long term is unclear and the evolutionary and ecological mechanisms need further investigation (Wright, et al., 2013). Some advantages to selfing exist (see Wells, 1979, and references therein). In angiosperms, for example, it is possible that environmental conditions will inhibit pollen dispersal, thereby leading to extinction unless selfing is employed. Additionally, when pollinators and/or mates are rare, or when sperm is limiting (or only self-sperm is available for fusion with eggs due to phrenological incompatibility; Engel et al., 2005) and outcrossing is uncertain, selfing offers reproductive assurance (see references in Vernet & Harper, 1980; Wright, et al., 2013, and Perrin et al., 2007). Selfing also allows transmission of a whole genome through both the male and female functions to the next generation (Fisher, 1941). Furthermore, selfing is a viable means of colonization requiring only one fertile individual and can be an advantage in a mixed population of two species that produce sterile hybrids. Prolonged selfing also can lead to purging of deleterious homozygotes and reduce inbreeding

depression (Schoen, 2005; Igić et al., 2006) in unchanging environments. And finally, high-fitness selfed individuals with low rates of recombination at adaptive loci may facilitate colonization by locally-adapted genotypes (Eriksson and Rafajlović, 2021)

Selfing may be advantageous in the upper shore *F. spiralis* as the severe desiccation stress may maintain favorable co-adapted gene combinations by reducing recombination (shuffling of genetic material between male and female chromosomes during meiosis) (Stebbins, 1950; Engel et al., 2005). Additionally, selfing may be important in the closely related species *F. spiralis* and *F. macroguiryi* (formerly *F. guiryi* Almeida et al., 2022) that are sympatric along a vertical exposure gradient in the intertidal regions of northern Portugal and southern France. The two species are separated by a meter or so on the shore, and although extensive gene flow occurs between the species in sympatry (only *F. macroguiryi* is present in southern Iberia), experiments suggest that strong selection on physiological traits across the intertidal gradient maintains the distinct genetic and morphological species within their preferred vertical distribution (Zardi et al., 2011). The prevalence and importance of selfing (relative to outcrossing) in these species in sympatry remains to be determined.

The most recent common ancestor of *Fucus* probably occurred in the Atlantic/Arctic Ocean Basin, where subsequent diversification occurred after opening of the Bering Strait 5.5-5.4 mya (Cánovas et al., 2011). In this area, selfing may be an important and overlooked aspect underlying the diverse morphological forms exhibited by the hermaphroditic *F. distichus* complex throughout its range in the low- and sub-Arctic. Sequence analysis of a variable intergenic spacer and a conserved portion of the 23S subunit in the mitochondrion was unable to differentiate the several species/subspecies of *F. distichus* (e.g., *F. evanescens*, *F. gardneri*, *F. anceps*; Coyer et al., 2006a; Cánovas et al., 2011; Laughinghouse et al., 2015). Laughinghouse et al. (2015) found a distinct Arctic haplotype, clearly showing the ancestor of the *F. distichus* complex to be centered in the low Arctic/Subarctic and invoked glacial cycles in maintaining the various morphs.They postulated that during an interglacial period, the central Arctic becomes a mixing bowl, from which populations expand further into the northern regions of the Atlantic/Arctic. As ice advances southward during the following glacial period, these populations disperse south to widely separated suitable habitats and subsequently adapt to local conditions. During the next interglacial, the locally-adapted populations again expand to the central Arctic Ocean and admixture again occurs, thereby diluting the previously evolved local adaptations (Laughinghouse et al., 2015). The opportunity for differential importance of selfing may exist, with greater selfing occurring in the expanded populations during glacial advance than in the admixture during glacial retreat.

Mating system

The evolution of reproductive strategies has been extensively studied in plants as they exhibit a range of mating systems from hermaphroditism to monoecy to dioecy (Geber et al., 1999). In angiosperms, dioecy appears to be the derived state based on theoretical, empirical, and phylogenetic studies (Charlesworth, 2002; Charlesworth, 2006) and in *Fucus*, dioecy is thought to have evolved from ancestral hermaphroditism (Billard et al., 2005; Billard et al., 2007).

The hypothesis was supported by a recent study of ancestral states in the family Fucales. Using 13 protein-coding genes, Cánovas et al. (2011) established hermaphroditism as ancestral in the family (in accordance with plants), but switching from derived dioecy back to hermaphroditism in one species in each of the two *Fucus* lineages. The switch to the diploid sex-determination system from the haploid UV (*via* a hermaphroditic intermediate) occurred in several families of Fucales ~17.5 mya and the transition toward hermaphroditism within diploid lineages has occurred independently in several genera of the Fucaceae (Heesch et al., 2021).

It also may be significant that hermaphroditic species of *Fucus* frequently occupy exposed or higher shores, whereas dioecious species are found in the more frequently submerged lower or shallow subtidal regions (Vernet & Harper, 1980). Furthermore, multi-gene phylogenies of *Fucus* suggested that switching from dioecy to hermaphroditism has coincided with colonization of more extreme environments (Billard et al., 2010; Cánovas et al., 2011). Two hypotheses may explain the pattern. First, selfing may be favored among species in the higher shores because more frequent desiccation renders cross-fertilization more hazardous, and secondly, repeated exposure to the same physical conditions of the high shore does not 'penalize' the lack of recombination and genetic diversity in hermaphrodites, whereas evolving and high biological diversity/interactions in the more physically stable lower/submerged shores favors genetic diversity provided by dioecy and recombination (Vernet & Harper, 1980; Billard et al., 2010).

A recent and detailed phylogenetic analysis of the low latitude hermaphroditic clade in the Iberian Peninsula and Northern Africa (*F. macroguiryi*, *F. spiralis*, *F. limitaneus)* by Almeida et al. (2022) suggested that the strong metapopulation structure within southern *F. vesiculosus* (dioecious) in restricted habitats favored parapatric speciation of an ancestral hermaphrodite lineage. A single ancestral hermaphrodite diversification event ~0.54 mya led to the *F. macroguiryi* and *F. limitaneus/F. spiralis* clades which differ in vertical ranges on rocky shores, with the morphologically and ecologically similar *F. limitaneus* and *F. spiralis* diverging more recently (~0.34 mya). Thus, the evolution of selfing lineages from outcrossing progenitors, a feature that is common among higher plants and

some animals, has occurred several times in *Fucus* (Almeida et al., 2022).

Hybridization

Morphological variability within and between *Fucus* spp. is legendary (e.g., Sideman and Mathieson, 1985; Rice & Chapman, 1985; Rice et al., 1985; Munda & Kremer, 1997; Kalvas and Kautsky, 1998; Anderson & Scott, 1998). The ability to detect differences in DNA sequences, either indirectly or directly, has provided additional taxonomic characters with which to examine morphological differences and has proved to be especially important in unraveling some of the taxonomic confusion in *Fucus*, a genus consisting of 717 described names, of which 10 are currently accepted as species (https://www.algaebase.org/search/genus/detail/?genus_id=71).

Some species are more related than others. Using sequences of the internal transcribed region (ITS-1, 5.8S, ITS2) of nuclear ribosomal DNA, Serrão et al. (1999a) established the presence of two lineages (or clades) within the genus: Lineage 1 and Lineage 2 (see Table 1 for species within each Lineage), subsequently supported with the 23S subunit and intergenic spacer regions of mitochondria (Coyer et al., 2006a). Additionally, each Lineage contains hermaphroditic and dioecious species with reciprocal habitat specialist species: (*F. spiralis*=high intertidal; *F. serratus* low intertidal/subtidal and generalist species *F. vesiculosus* = high to low intertidal; *F. distichus* = high intertidal pools to low intertidal) (Coyer et al., 2006a). More recently, Cánovas et al. (2011) used 13 protein-coding genes to further clarify the two lineages and indicated that species in Lineage 1 are characterized by being northern in distribution, adapted to cold water and stress-receptible, whereas species in Lineage 2 are more southern in distribution and generally stress-tolerable. As discussed below, hybridization in *Fucus* occurs among species within a Lineage, rarely between, and always involves a parental species pair in which one parent is a hermaphrodite (e.g., *F. spiralis*, *F. distichus*) and the other is dioecious (e.g., *F. vesiculosus*, *F. serratus)* (Coyer et al., 2007).

A more complicated dynamic is evident in the low latitude hermaphroditic *F. spiralis* complex. Initially, two genetically and morphologically distinct entities, *F spiralis*-High and *F. spiralis*-Low, corresponded to *F. spiralis* var *spiralis* and *F. spiralis* var *platycarpus*, respectively, in addition to a third entity named *F. spiralis*-South (Billard et al., 2007; Billard et al., 2010; Coyer et al., 2011c). Using microsatellites in tandem with expressed sequence tags for partial sequencing of 14 protein-coding genes, *F. spiralis* var *platycarpus* (*F. spiralis*-Low) was synonymized with *F. spiralis*-South as *F. guiryi* (Zardi et al., 2011), a distinct entity in southern Iberia (allopatry), but as it was also distributed across *F. vesiculosus* and *F. spiralis* clades in Northern Portugal (sympatry), extensive hybridization and introgression is possible (Zardi et al., 2011).

A new study using a phylo-transcriptomic approach based on short read transcriptomic data (RNA-seq) of warm affinity Lineage 2 populations existing in sympatry (Britain, NW Iberia) and allopatry (SW Iberia, Morocco) further clarified the complex patterns of cryptic speciation (Almeida et al., 2022). Specifically, *F. spiralis*-Low diverged earlier than the others, necessitating removal of *F. spiralis*-Low from *F. guiryi* and designation as new species *F. macroguiryi* that is genetically, morphologically, and physiologically distinct from all others and is introgressed *F. guiryi* (Cánovas et al., 2011; Almeida et al., 2022). *F. guiryi* on the Canary Islands has thus reverted to the previously described *F. limitaneus* (Almeida et al., 2022).

Reports of field individuals with an intermediate morphology between two co-existing *Fucus* species have occurred several times in the past century (Gard, 1910; Gard, 1915; Powell, 1963; see also references in Scott & Hardy, 1994). Some viewed these forms as ecotypes, but others (e.g., McLachan et al., 1971; Scott & Hardy, 1994) stated strongly that hybridization most likely was the root of the considerable morphological variation in *Fucus*. At that time, verification of hybridization required difficult and labor-intensive crossing studies, although several attempts were made (Thuret, 1855; Williams, 1899; Sauvageau, 1909; Kniep, 1925; Burrows and Lodge, 1951; Burrows and Lodge, 1953; Bolwell et al., 1977; Scott & Hardy, 1994; Kim et al., 1997). Few zygotes resulting from the laboratory crosses between various species of *Fucus*, particularly between Lineage 1 and 2 species and *F. vesiculosus* x *Ascophyllum nodosum* (Kim et al., 1997), survived beyond a few days (e.g., Scott & Hardy, 1994).

The advent of genetic techniques made detection of hybridization much easier and more robust. For example, nuclear and cytoplasmic DNA reveal relatedness among individuals at the population level and above, whereas plastid DNA (mitochondria, chloroplasts) are maternally inherited. Microsatellites in particular have been invaluable for much more detailed studies of hybridization and introgression in many plants and animals, including the genus *Fucus*, as the bi-parentally inherited markers clearly delineate heterozygosity and parentage using both plastid and nuclear DNA.

The first molecular-based studies of hybridization in *Fucus* used the nuclear rDNA-ITS1 sequence, the *Rubiso* spacer in chloroplasts, and *nad*11gene in mitochondria; demonstrating that the plastids are maternally inherited in *Fucus* (Coyer et al., 2002a; Coyer et al., 2002b), although some 'paternal leakage' has been observed (Coyer et al., 2004; Hoarau et al., 2009). Results confirmed hybridization and introgression between Lineage 1 species *F. evanescens* (=*distichus*, see Coyer et al., 2007) and *F. serratus* in both field specimens and laboratory crosses, and further revealed that: 1) hybridization was asymmetrical, with the dioecious *F. serratus* contributing sperm and the hermaphroditic *F. evanescens* the eggs, and 2) hybridization was restricted to sympatric, rather than allopatric stands (Coyer et al., 2002a; Coyer et al., 2002b). Using 10 microsatellite loci of a

mixed population consisting of native *F. serratus* and introduced *F. evanescens* in Denmark that had existed for 60-100 yrs, revealed: 1) nearly 13% of the individuals were F_1 hybrids (also asymmetrical as described above), 2) ca. 1.5% of genes were introgressed into each parental species, and 3) F_1 hybrids displayed lower survivorship (Coyer et al., 2007).

Several subsequent studies have employed microsatellites to examine hybridization and introgression in Lineage 2 *Fucus* spp. Engel et al. (2005) used five loci and found a higher proportion of genetically intermediate individuals (*F. vesiculosus* x *F. spiralis* hybrids) in two sympatric populations (12.5 and 14.2%) than in parapatric populations (5.6 and 9.0%). As for *F. serratus* x *F. distichus* (Coyer et al., 2002a), *F. spiralis* (hermaphroditic) eggs were fertilized by *F. vesiculosus* (dioecious) sperm (Billard et al., 2007). Hybridization was far more common in mixed populations, although did occur in separate distributions. In some cases, *F. spiralis* x *F. vesiculosus* F_1s were fertile (Billard et al., 2007; Billard et al., 2010). More recently, microsatellite analysis was combined with network analysis to reliably determine the occurrence of present-day hybridization between *F. spiralis* and *F. vesiculosus* (Moalic et al., 2011).

Hybridization also has been demonstrated among hermaphroditic species of Lineage 2. Short read transcriptomic data (RNA-seq) consistently indicated gene flow between *F. macroguiryi* and *F. limitaneus*, exceeding that between *F. macroguiryi* and *F. spiralis*. The pattern is best explained by assuming that *F. macroguiryi* was present further south than *F. spiralis* during glacial stages and farther from the ice limits, with extensive gene flow between *F. macroguiryi* and *F. limitaneus* in the south during relatively lengthy glacial periods contributing to the observed introgression signal (Almeida et al., 2022).

Clearly, hybrid fitness will determine the fate of a hybrid zone, and three scenarios are possible: 1) if there is no selection against the hybrids and introgression is extensive, all individuals become hybrids; 2) if introgressed individuals become established and/or are adapted for new habitats, new lineages can evolve; and 3) if hybrids are less fit, pre-zygotic isolating barriers can evolve to strengthen selection against formation of hybrids (=reinforcement), as less fit hybrids can be viewed as energetically expensive 'mistakes' (summarized in Hoarau et al., 2015). Two lines of evidence suggest that in *Fucus*, scenario 3 is most likely. First, *F. serratus* x *F. evanescens* hybrids have lower survivorship and reduced fertility than either parent (Coyer et al., 2007; Hoarau et al., 2015).

Secondly, reinforcement of pre-zygotic isolation appears to have evolved in older contact zones of *F. serratus* and *F. distichus*, but not in younger contact zones. Hoarau et al. (2015) examined hybridization and introgression of the two species in contact zones: 1) near Denmark where *F. distichus* was introduced ~150 yrs ago, 2) in Iceland where *F. serratus* was introduced ~150 yrs ago, and 3) northern Norway where the two species have co-existed since the end of the Last Glacial Maximum ~10,000 yrs ago. Both Danish and Icelandic populations revealed a high proportion of hybrids (13-24%) and several F_1 individuals, whereas the Norwegian populations displayed a low proportion of hybrids (2-3%) and an absence of F_1 individuals (Hoarau et al., 2015). Additionally, the success rate of interspecific laboratory crosses to one-week old embryos was significantly lower in the older contact zones of Norway than in the younger contact zones of Denmark and Iceland, again suggesting selection against hybridization and for pre-zygotic isolation (Hoarau et al., 2015).

Similarly, the introgression signal from *F. macroguiryi* (hermaphroditic) into *F. vesiculosus* (dioecious) in secondary contact has decayed, but is still detectable (Almeida et al., 2022). The decay may be due to steady reinforcement of species boundaries in the sympatric range, as observed for *F. distichus* and *F. serratus* in Norway (Hoarau et al., 2015), or a consequence of reduced contact time during range expansion (Almeida et al., 2022).

If gamete release in *Fucus* is delayed by environmental conditions such as high-water motion, mature gametes accumulate in the conceptacles. Several studies have demonstrated that when ripe receptacles are stored in the laboratory for several days before releasing gametes, species-specific barriers are diminished and hybrids can be produced (Bolwell et al., 1977; Edwards et al., 1997; Edwards, 1999). Additionally, fertilization success was significantly reduced when eggs were retained in the receptacles for ~ 3 weeks due to unfavorable environmental conditions for release (Serrão et al., 1999a).

Despite the wide occurrence of hybridization among *Fucus* spp., pre-zygotic mechanisms, such as asynchronous release of gametes, have evolved to significantly reduce hybridization. Monteiro et al. (2012, 2016) studied gamete release among four sympatric species of *Fucus* in northern Portugal. Dioecious *F. vesiculosus* and *F. serratus* released gametes during daytime neap tides, while hermaphroditic *F. guiryi* and *F. spiralis* released gametes during night-time high tides during the same phase of the semilunar cycle, effectively reducing the potential for hybridization with the dioecious *F. vesiculosus*. As the divergence between hermaphroditic and dioecious species may be > 1 mya (Cánovas et al., 2011), the shift in periods of gamete release is remarkably rapid.

Ecads

An especially interesting aspect of Fucales is the existence of ecads and the role of hybridization and polyploidy in their existence. Ecads are free-living individuals with morphological variability linked to habitat (Clements, 1905); in the case of *Fucus*, to the low-energy muddy shorelines of estuaries and high-intertidal salt marshes, and presumably arise from attached 'parental' species. Fucoid salt marsh ecads have been known for over 100 years for *F. vesiculosus* and *F. spiralis* in the North

Atlantic (Cotton, 1912; Baker & Bohling, 1916), and more recently *F. gardneri* (part of *F. distichus* complex; see Coyer et al., 2006a; Cánovas et al., 2011) in the North Pacific (Ruiz et al., 2000; Kucera & Saunders, 2008). Ecads also are known in the closely related fucoid *Ascophyllum nodosum* (Chock and Mathieson, 1976; Chock and Mathieson, 1979; Mathieson & Dawes, 2001; Mathieson et al., 2001). All fucoid ecads are characterized by the absence of a holdfast; a dwarf morphology; asexual reproduction; and curled, proliferating thalli (see also references in Wallace et al., 2004) and may be a major source of biomass and productivity in these habitats (Tyrrell et al., 2012; Tyrrell et al., 2015).

In one of the first uses of microsatellites to examine hybridization in *Fucus*, Wallace et al. (2004) concluded that: 1) the *muscoides*-like *Fucus* ecad in Maine (USA) salt marshes consisted mainly of *F. vesiculosus* x *F. spiralis* F_1 hybrids; 2) another ecad (*F. vesiculosus* ecad *volubilis*) may have arisen through introgression between fertile hybrids and *F. vesiculosus*; and 3) introgression had likely occurred between *F. vesiculosus* and *F. spiralis*. A later study using microsatellites and mtDNA analysis showed that *muscoides*-like *Fucus* ecads in Iceland were consistent with asymmetrical hybridization between the dioecious *F. vesiculosus* sperm and hermaphroditic *F. spiralis* eggs, whereas similar ecads in Ireland were the result of polyploidy (Coyer et al., 2006b).

Sjøtun et al. (2017) examined the complexity of *Fucus* ecads at three sites in western Ireland. In one location, a morphological cline existed with small *Fucus* individuals lacking bladders in the upper intertidal salt marshes ranging to *F. vesiculosus* in mid-intertidal; nuclear DNA content ranged from 1-1.8 pg, suggesting polyploidy in some individuals. At Locality 2, microsatellite analysis revealed salt marsh individuals were derived mainly from *F. vesiculosus*, whereas at Locality 3, salt marsh individuals were *F. vesiculosus* x *F. spiralis* hybrids with greatest affiliation to *F. spiralis*. DNA content of the small individuals from Locality 2 (ca. 4 pg) suggested they were octoploids, whereas the individuals from Locality 3 formed two groups based on DNA content: one with 3.9-4.6 pg and the other with 1.5-2.8 pg. Furthermore, DNA content of individuals in Locality 3 varied between 1.1-2.8 pg in *F. vesiculosus* and 2-3.5 pg in *F. spiralis*, demonstrating a somewhat stepwise increase in both species consistent with polyploidy. The authors hypothesized that the small salt marsh *Fucus* originated from genome size changes in the parents.

Neiva et al. (2012) used microsatellite loci to examine *Fucus* ecads from Oregon (US) in the North Pacific and Ireland and concluded that they were more related to *F. gardnerii*, and *F. spiralis*, respectively. Additionally, they suggested that fucoid ecads are evolutionarily independent populations stemming from hybrid or polyploid origins that confer a fitness advantage over their parental species in a marginal and/or stressful habitat.

An interesting ecad is *F. vesiculosus* growing on intertidal mussel beds in the Wadden Sea and along the North Sea coast, first described by Nienburg (1925, 1927) and later by others (Wohlenberg, 1937; Nienhuis, 1970; van den Hoek et al., 1979). The *F. vesiculosus* ecad lacks the species' characteristic gas bladders; reproduces vegetatively; and does not have a holdfast, being attached to the muddy substratum *via* the mussel's byssal threads (Albrecht & Reise, 1994). The association is mutual: the ecad prevents mussels from sinking into the mud, whereas the mussels anchor the ecads and allows steady growth (Nienburg, 1925; Nienburg, 1927). It is unknown if *F. spiralis* co-occurs on mussels, so derivation of the ecad by hybridization or polyploidy remains unknown.

Recently, a study of attached (epilithic) and free-living (benthopleustophytic) forms of *F. vesiculosus* in the Baltic Sea revealed the presence of polyploidy (likely through autopolyploidy) throughout the majority of populations regardless of form with important implications in population structure (Preston et al., 2022).There is no direct evidence of sexual reproduction in the free-living form, which probably originated asexually *via* detached pieces of thalli aggregating in sheltered locations (Preston et al., 2022), and presumably without a functioning holdfast. Thus, the free-living form is at least 'ecad like'. Although the free-living form was less genetically diverse than the attached, genetic diversity was still within expected limits for both forms and frequent asexual reproduction in the free-living form did not reduce the overall genetic variation in *F. vesiculosus*. Gene flow within and among the forms differed at various spatial scales, but the free-living populations were judged to be more unstable and at increased risk of local extinction (Preston et al., 2022).

Evolution of diplontic life cycle and sex-biased genes

In all orders of brown algae, sex is determined in the haploid stage of the gametophyte generation except for the Fucales, where sex is determined *via* haploid gametes during the diploid state (Coelho et al., 2019). Fucales also are of interest because of the relatively recent transition from haploid to diploid sex determination (Silberfeld et al., 2014), switching from ancestral hermaphroditism to dioecy and in some species, back to hermaphroditism (Billard et al., 2005; Billard et al., 2007; Cánovas et al., 2011). The switching of reproductive method coincides with a dramatic rise in sea levels ca. 75 mya and could have opened new ecological niches for Fucales (Heesch et al., 2021). It also is important to note that hermaphroditic lineages are better colonizers of marginal habitats via increased reproductive assurance and the maintenance of locally adaptive traits (Cánovas et al. 2011).

Genes that are differentially expressed in males and females (sex-biased genes) have been well documented across a wide number of animals, plants, and brown algae (see references in

Hatchett et al., 2021). A comparative transcriptomic study of vegetative and gender-specific reproductive tissue in *F. vesiculosus* revealed striking differences (Martins et al., 2013). For example, cell cycle and meiotic pathways were over-expressed in male (not female) reproductive tissue relative to vegetative tissue, as well as genetic information processing pathways associated with sperm production. Further, the number of sex-biased genes were ~3-fold higher in male relative to female tissue and the average expression level of male-biased genes was greater than female-biased genes. Candidate sex-biased genes in females were limited to those with likely roles in cell wall/matrix modification, whereas a variety of male-biased genes were related to development; signaling and signal perception; and potential flagella-localized proteins.

Hatchett et al. (2021) examined the evolution of sex-biased genes in vegetative and reproductive tissue of male and female *F. serratus* and *F. vesiculosus* with RNAseq and *de novo* reference transcriptome assembly. While very few genes were differentially expressed between male and female vegetative tissue (8-9% in each species), thousands of genes were differentially expressed in the reproductive tissues. A similar proportion of the genome displayed tissue-biased expression between receptacle and non-receptacle tissue, demonstrating that the majority of tissue- and sex-biased expression was allocated to the reproductive structures.

The authors also found that male-biased genes were highly conserved between the two species, with clustering of male reproductive samples by sex rather than by species. Furthermore, overexpression of male-biased genes was >3-fold the number of female-biased genes and conserved male-biased genes were enriched in functions related to gamete production, sperm competition, and flagellar proteins. The increase in male biased gene expression of the transcriptome also suggested that males may experience relaxed purification selection or stronger selection than females, a trait found in many other species of eukaryotes (Hatchett et al., 2021 and references therein). Female-biased genes were uniformly and highly expressed throughout the female and male tissues, thus sexual conflict over gene expression in *Fucus* may be resolved by down-regulating expression of pleiotropic female genes in male receptacles and restricting expression of male-biased genes to the male reproductive tissue resulting in an increase of male biased gene expression.

Climate change and *Fucus* reproduction

Several studies employing ecological niche modeling or other species distribution models (SDMs) comparing present-day vs. projected future distributions have been performed for *Fucus* under contrasting IPCC (Intergovernmental Panel on Climate Change) climate change scenarios (e.g., Nicastro et al., 2013; Assis et al., 2014; Jueterbock et al., 2014; Jueterbock et al., 2016). In general, all have demonstrated a northward shift into expanding suitable habitat and decline or extinction in the southern edge populations due to rising temperatures. For example, experiments with *F. serratus* confirmed that thermal extremes will regularly reach physiologically stressful levels in Brittany (France) and further south by the end of the 22nd century (Jueterbock et al., 2014).

On the other hand, expansion into northern habitats will require adaptations to cooler water and 24 hr light/dark months. Recent studies in *Fucus* species have focused on how climate change influences average seawater temperature, salinity and pCO_2 and how these changes affect reproductive success. Predicted future changes in seawater conditions for *F. radicans* in the Northern Baltic Sea, showed a high tolerance in photosynthesis and growth, but decreased survival and cessation of sexual reproduction (Rothäusler et al., 2018; Rugiu et al., 2018). In the southwest Baltic Sea (Kiel Fjord), *F. vesiculosus* was unaffected by elevated pCO_2 and/or warming, but matured earlier with a subsequent earlier gamete release (Graiff et al., 2017). Furthermore, southern edge populations of *F. vesiculosus* are exposed to higher sea and air temperatures (a proxy for climate change) have significantly lower biomass of reproductive tissue and smaller number of receptacles per individual (Ferreira et al., 2015).

Additionally, increased glacial melting may decrease salinity in some areas. For example, *F. vesiculosus* along the Finnish coast currently tolerates 5.8 PSU, but this is projected to reach 2.5 PSU by the end of the century (Meier et al., 2012). At 2.5 PSU egg release was reduced and at 3.5 PSU, sperm cells began to swell, drastically reducing reproductive success (Rothäusler et al., 2019). As discussed above, low salinities also increase the probability of lethal polyspermy (see 'General Characteristics, above).

Future directions

The continuing and rapid development of the 'omics' (genomics, transcriptomics, proteomics, metabolomics), as well as CRISPR/Cas9 techniques, provide invaluable tools to address even more questions in *Fucus* reproduction (as well as evolutionary history), especially with the future release of full genome data for several species (Table 2), and the ease of laboratory culturing and subsequent manipulation of gene lines. There also, however, remains the need for basic investigations in reproductive ecology, particularly among the lesser studied Lineage 1 species. Taking a larger view, investigating the various modes of reproduction (e.g., hermaphroditism, dioecy, hybridization, selfing, asexual reproduction, ecads) in *Fucus* may well lead to new insights in the study of reproduction in other organisms with a diplontic life cycle, consequently, *Fucus* can be a useful model organism. We offer below a small subset of questions to stimulate thoughts of future research.

TABLE 2 Current or soon to be available (SA) genomic data for *Fucus* spp.

Species	Sequencing type	Reference	Accession number	Size
Fucus distichus	Genome	Phaeoexplorer	SA	691.15 Mbp
	Transcriptome	Hatchett et al. (2021)	GJHE00000000	22.09 Mbp
	Mitogenome	Hughey & Gabrielson (2017)	NC_034672	36.40 Kbp
Fucus serratus	Genome	Phaeoexplorer	SA	1.15 Gbp
	Transcriptome	Hatchett et al. (2021)	GJHE00000000	30.59 Mbp
Fucus vesiculosus	Genome	Unpublished	ASM1484947v1	1.51 Gbp
	Transcriptome	Hatchett et al. (2021)	GJHE00000000	24.23 Mbp
	Mitogenome	Oudot-Le-Secq et al. (2006)	NC_007683	36.39 Kbp
	Chloroplast	le Corguillé et al. (2009)	NC_016735	124.986 Kbp
Fucus spiralis	Transcriptome	Hatchett et al. (2021)	GJHE00000000	24.63 Mbp
Fucus ceranoides	Transcriptome	Unpublished	HACY00000000	47.44 Mbp
Fucus virsoides	Transcriptome	Falace et al. (2018)	PRJNA524465	N/A

Phaeoexplorer: https://phaeoexplorer.sb-roscoff.fr/home/. Size of each assembly is represented by gigabase pairs, megabase pairs, kilobase pairs (Gbp, Mbp & Kbp, respectively). N/A, not available.

1. Why is Lineage 2 much more specious than Lineage 1 and how can whole genome analysis (and genome-wide markers such as SNPs) resolve the numerous and controversial number of species in Lineage 2? Although the genus evolved in, and radiated from, the central Arctic (Cánovas et al., 2011; Laughinghouse et al., 2015), far more species have emerged in the relatively younger North Atlantic than in the older North Pacific. Is it simply that there was less competition from other species of algae in the North Atlantic or did the diplontic life cycle of reproduction favor *Fucus* spp. in a younger habitat?
2. How/why is *F. vesiculosus* so successful in such diverse habitats (e.g., sheltered to moderately exposed, rocky shores to salt marshes, degree of immersion/emersion, brackish to marine salinity)? Comparative genomics/transcriptomics may reveal the presence/absence of genes, differential levels of gene expression, and/or an important role of epigenetics (heritable and reversible changes in transgenerational phenotypes without corresponding changes in DNA sequence) that may be unique to *F. vesiculosus*.
3. The existence of ecads leads to many ecological and genomic questions. For example, what are the longevity and growth patterns of ecads resulting from hybridization relative to those arising from polyploidy and how are both influenced by the local environment? From a genomic perspective: 1) what is the genetic basis for the hybridization or polyploidy dichotomy and is it reversible; 2) is the genome size divergence (e.g., Sjøtun et al., 2017) due to non-coding elements or gene duplication, 3) how do the mechanisms of inducement differ in dioecious (*F. vesiculosus*) and hermaphroditic species (*F. distichus*); and 4) is epigenetics a key factor or are different regions/genes in the genome important? Finally, is the *F. vesiculosus* ecad on mussels in the Wadden Sea a result of hybridization or polyploidy?
4. How does rate of selfing in hermaphroditic species vary with changing environments?
5. How does frequency of hybridization vary as a function of stochastic and dynamic environmental conditions (field and laboratory cultures)?
6. Detailed field studies of gamete release and fertilization success of Lineage 1 species are needed for comparison to the much more studied *F. spiralis* and *F. vesiculosus* in Lineage 2. For example, hermaphroditic *F. distichus* shares shore position with the dioecious *F. vesiculosus* and *F. serratus* is mostly subtidal with no equivalent in Lineage 1. How do aspects of reproduction in Lineage 1 vary as a function of tidal cycle, light, and water motion? Given the different shore positions, how do selfing rates and success differ when comparing *F. spiralis* and *F. distichus*?
7. What is the genetic basis for the morphological differences (particularly receptacles) among the various morphs of *F. distichus* (*F. anceps, F. evanescens, F. edentatus*, *F. gardneri*, etc.) and *F. vesiculosus* (with and without vesicles)? Is epigenetics present in *Fucus* spp.?
8. Does the metabolome (see Parrot et al., 2019) on *Fucus* fronds differ from that on receptacles and if so, why, and how do the differences influence gamete release?
9. What is the genomic/transcriptomic basis for non-overlapping reproductive seasons of *F. vesiculosus* observed in the Baltic Sea?
10. What is the genetic basis for reproductive isolation and the connection between prezygotic barriers to fertilization and within-species sexual selection?
11. How can whole genome sequencing identify sex chromosomes and molecules involved in sperm/egg receptors?

12. How will global changes (e.g., temperature, salinity, dissolved CO_2) affect aspects of *Fucus* reproduction (development time, gamete output/size/dispersal, fertilization, dispersal of fertilized eggs, etc.)?

Author contributions

WH: Original concept, drafting and editing of the manuscript, producing/providing figures, and tables. JC: Drafting and editing of the manuscript, design of figures. KS: Table and editing of the manuscript. AJ: Editing of the manuscript. GH: Editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1051838/full#supplementary-material

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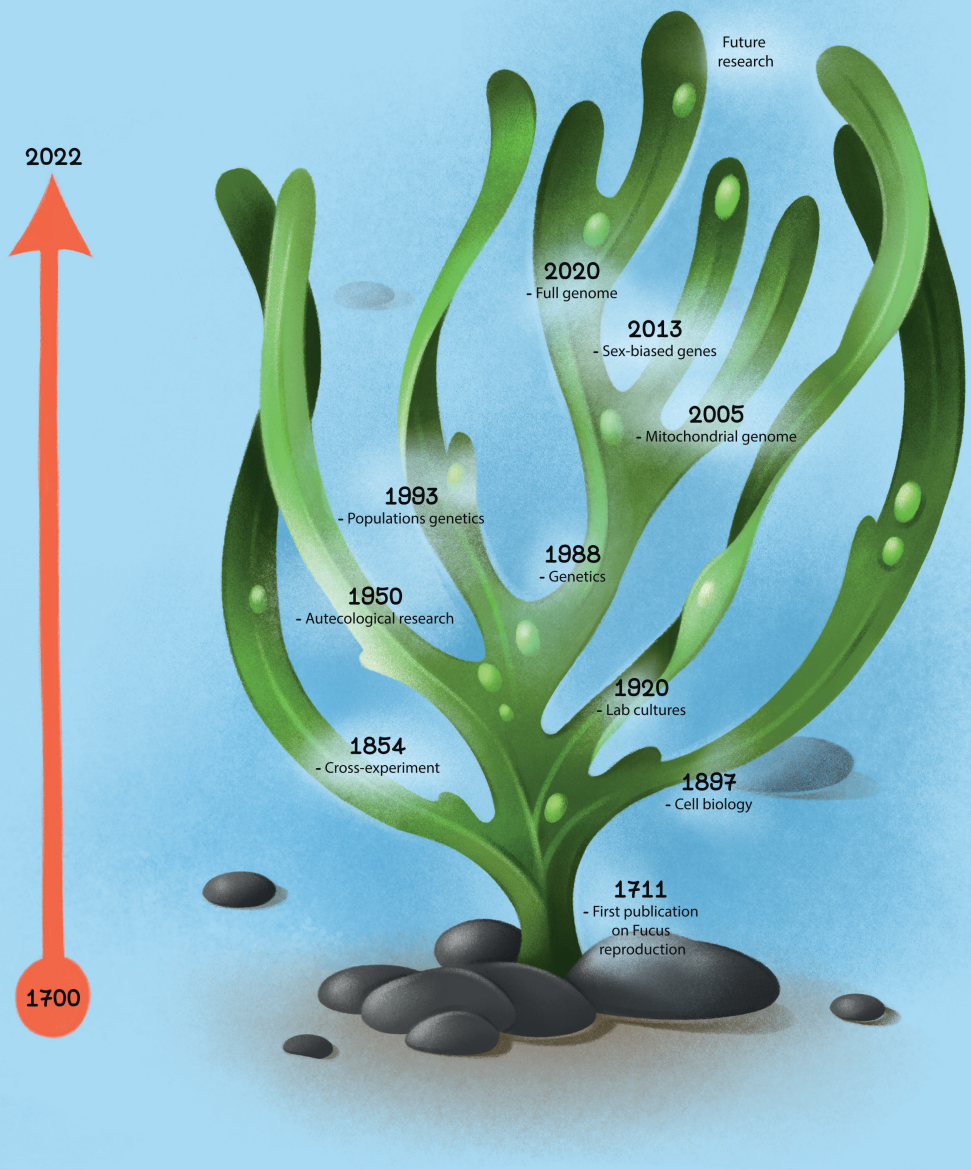
The growth and proliferation of research in *Fucus* reproduction



Paper II

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

William J. Hatchett[1], Alexander O. Jueterbock[1], Martina Kopp[1], James A. Coyer[3], Susana M. Coelho[2,4], Galice Hoarau[1], Agnieszka P. Lipinska[2,4#]

[1]Faculty of Biosciences and Aquaculture, Nord University, 8026 Bodø, Norway

[2]Sorbonne Université, CNRS, Algal Genetics Group, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, 29680 Roscoff, France

[3]Shoals Marine Laboratory, University of New Hampshire, Durham, NH 03824, USA

[4]Department of Algal Development and Evolution, Max Planck Institute for Biology, 72076 Tuebingen, Germany

#Corresponding author;

Agnieszka P. Lipinska, alipinska@tuebingen.mpg.de

Social media:

"Making sexes: genetic insights from *Fucus* algae"

@Aga_Lipi, @MPI_Bio

ORCID

Lipinska, Agnieszka P 0000-0002-1829-8293

Coelho, Susana M 0000-0002-9171-2550

Jueterbock, Alexander O 0000-0002-0659-3172

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 has 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 to 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 and Temeles 1989; Connallon and Knowles 2005; Ellegren and 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 and 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 rely largely on the regulation of sex-biased gene (SBG) expression (Ellegren and Parsch 2007; Parsch and Ellegren 2013; Grath and 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 and 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 to female-biased genes or unbiased genes (Parisi et al. 2003; Ranz et al. 2003; Yang et al. 2006; Voolstra et al. 2007; Zang et al. 2007; Martins et al. 2013; Parsch and

Ellegren 2013; Harrison et al. 2015; Yang et al. 2016). 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 have been found so far (Zemp et al. 2016; Sanderson et al. 2019; Cossard 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 to 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 & Pietrokovshi 2014; Harrison el al. 2015; Sayadi et al.2019). In contrast, female-biased genes often evolve at similar or slower rates compared with unbiased genes possibly due to larger pleiotropic constraints (Ellegren and 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 and Knowles 2005; Hayward and 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 towards 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 around 17.5 Mya (Heesch et al. 2021). While the transition to diploid sex determination from the haploid system seems to be irreversible, further transitions towards

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 and 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.

In this study, we focused on the dioecious species of two distinct lineages, *Fucus serratus* and *Fucus vesiculosus* (Fig.S1), that dominate the rocky intertidal North Atlantic shoreline. The two lineages evolved around 0.9 to 2.25 Mya 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; Wallace et al. 2004; Billard et al. 2005; Coyer et al. 2007; 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 *Fucus serratus–Fucus 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 and 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 *Fucus serratus* and *Fucus 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, 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 RNASeq 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 5mg 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, USA) and potential PCR inhibitors were removed with the OneStep-96TM 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 1µg RNA using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs)and sequenced on the Illumina NextSeq 500 (150-bp pair-end reads), using the NextSeq 500/550 High Output Kit v2.5 (300 Cycles).

RNAseq analysis and *de novo* reference transcriptome assembly

Sequencing data were demultiplexed using the Bcl2Fastq Conversion Software (v. 2.20, Illumina). 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). Prior to *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 100bp 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 log2(TPM+1)<1 were considered not expressed.

Orthofinder (v. 2.3.3) (Emms and Kelly 2019) was used to find orthologous genes between all four *Fucus* species (Table S1). We used orthogroups with single and/or mulicopy-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 (v. 3.9 bioconductor) (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. vesiculosus* - *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 (version 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 and Nielsen 2000; Yang 2007). The difference in mean dNdS 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, NSsites = 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-square distribution with the degree of freedom equal 1 and FDR correction were calculated using pchisq 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:

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

where *xij* 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 analysis was performed using RStudio (R version 3.6.3).

Gene Ontology analysis

EggNOG v5.0 (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 and 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 "Methods" section for details) after filtering out the transcripts with low expression or high similarity to other transcripts. BUSCO v3 (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, padj<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) (Table 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 (2,993 and 2,772 genes in *F. serratus* and *F. vesiculosus*, respectively) (Fig.2A). In contrast, in vegetative tissues, only 20 and 22 genes were sex-biased in *F. serratus* and *F. vesiculosus*, respectively (Table 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 (2,315 MBGs vs 678 FBGs in *F. serratus;* and 2,025 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). In 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 (log2(TPM+1)<0) of the given gene in the other sex (Fig. 2B). Interestingly, between 60-90% 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 1,416 genes in *F. serratus* and 59% or 1,201 genes in *F. vesiculosus*) which is consistent with 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 to unbiased genes in male and female vegetative and female reproductive tissues in both species (Fig.2C, p<2e-16 in all pairwise Wilcoxon tests).

Tissue-biased gene expression

We analyzed transcript abundance in the reproductive versus vegetative tissues within each sex and species to identify genes with tissue-biased expression (FC>=2, p_{adj}<0.05 (FDR-adjusted p-value)) (Table 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 to 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 non-reproductive tissue in both sexes and species (Fig.3B, Wilcoxon test, p<1.4e-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 9,401 and 8,758 one-to-one orthologs between *F. distichus – F. serratus* and *F. spiralis – F. vesiculosus* respectively, and 9,778 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 inter-specific orthologs.

Firstly, 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% to 75% of the orthogroups containing male-biased genes were common between *F. serratus* and *F. vesiculosus*. In contrast, only 20% to 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 (Chi-square test, p<2.4e-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 9,778 orthogroups with single copy genes, 21% (2,070 orthogroups) contained genes with sex-biased expression in at least one of the two species (Table S4). Again, male sex bias was strongly correlated across the two lineages and applied to roughly 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 towards 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 to 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* (7,759 orthologs); *F. vesiculosus* – *F. spiralis* (7,103 orthologs)) using the YN00 package in PAML4 (Yang 2007) (Table S6).

In both dioecious species, female-biased genes showed similar rates of non-synonymous to synonymous substitutions (dN/dS) to that of unbiased genes (Fig.6A, permutation test, $p>0.07$). In 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 (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 $p<2e-16$).

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 models 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 *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* and *F. vesiculosus*) with the average of background ω values. We also performed the same test choosing forward branches leading to: 1) all four *Fucus* species, 2) *F. serratus* - *F. distichus* lineage and 3) *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 (8 male-biased genes, 2 female-biased genes and 84 unbiased genes) in *F. serratus* and 119 genes (9 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 to unbiased genes (Chi square 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 to 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 (Fig.S2, Fig.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 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 if 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 (ca. 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. 2018). 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 to many model organisms (Grath and 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 less than 4% (658) of *Ectocarpus* genes being sex-biased during the reproductive stage in contrast to to 8% (2,993) in *F. serratus* and 9% (2,772) 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% (5,442) 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 (Zemp et al. 2016; Harkess et al. 2015), 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*; 2,315 MBGs vs 678 FBGs in *F. serratus*; 2,025 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 and 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 post-fertilization. Evidence for 'gamete-mediated mate choice' (GMMC) 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 and 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).

In 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. MBGs 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 motilityn. In contrast to MBGs, FBGs showed more variability and had species specific expression patterns indicated by significantly increased Euclidean distances, compared to 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 to 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 and Wade 2010; Gershoni and 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 *e.g.* 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 and 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 non-synonymous 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. Further, 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 and 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 *Fucus serratus* and *Fucus 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 is needed.

In summary, MBGs and FBGs in *Fucus* seem to follow different evolutionary paths and are under different selective pressures. MBGs evolve faster at the level of the protein sequence, but their expression levels remain very similar between *Fucus* species. In 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 and Knowles 2005; Wray 2007; Tirosh and Barkai 2008; Liao et al. 2009, Loehilin 2019, Martin 2013). 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; Wallace et al. 2004; Billard et al. 2005; Coyer et al. 2007; 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 pre-zygotic 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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Author contributions

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

Competing interests

None declared.

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 accessions: GJHE00000000, GJHF00000000, GJHR00000000, GJHG00000000. The versions described in this paper are the first versions.

FIGURES

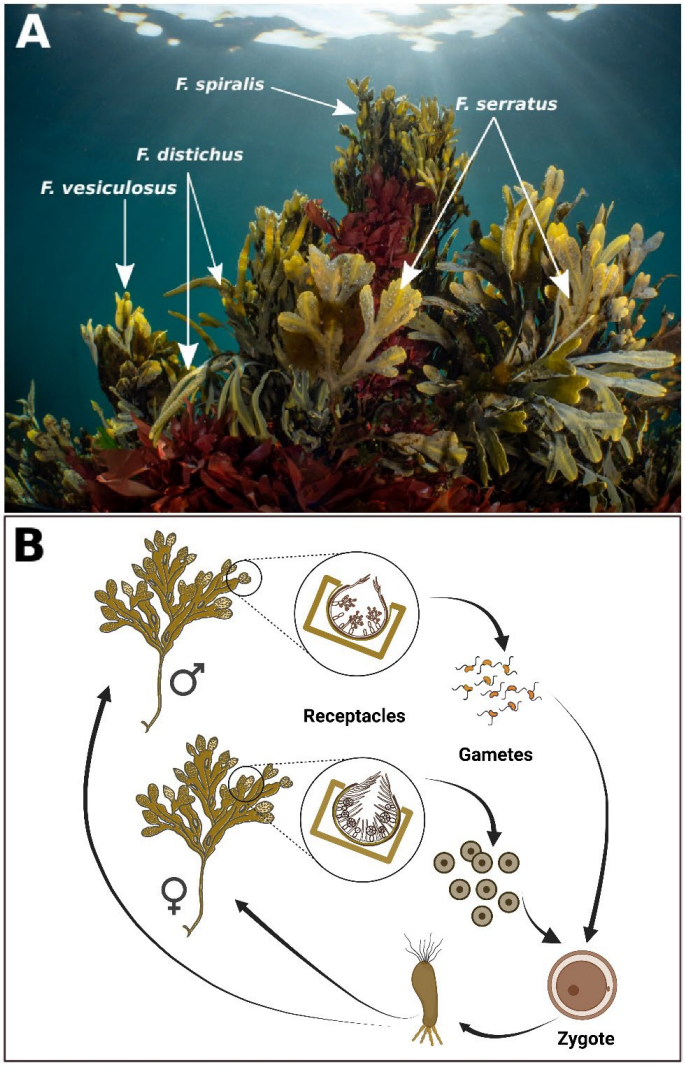

Figure 1. *Fucus* species co-ocurring in their natural habitat. A) *Fucus spiralis* (top), *Fucus distichus* (center-left), *Fucus serratus* (center-right) and *Fucus vesiculosus* (bottom-left) living in sympatry. B) Diplontic life cycle of dioecious *Fucus.* Gametes are produced in the receptacles of 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.com (B).

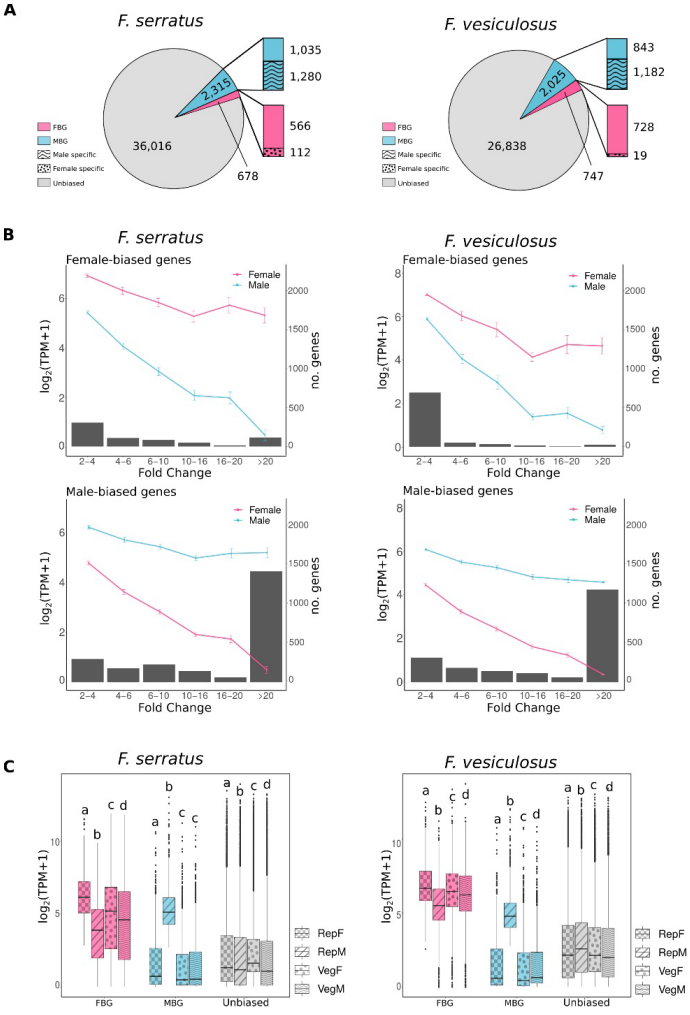

Figure 2. Sex-biased gene expression. A) Number of sex-biased genes (MBG - male-biased and FBG – female-biased) in *F. serratus* and *F. vesiculosus* reference transcriptomes. Unbiased genes were defined as p_{adj} >0.05 or showing less than 2-fold difference between the sexes. Bars represent the proportion of sex-specific genes among the sex-biased genes in each species. B) Mean expression levels (log2(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 standard errors. Bar plot indicates the number of genes in each FC category. *C*) Boxplot showing the mean expression levels across the replicates (log2(TPM+1)) of female-biased (pink), male-biased (blue) and unbiased (grey) genes in male and female reproductive and vegetative tissues. The letters above the plots indicate significant differences within each gene group (pairwise Wilcoxon test, p<0.05). RepF - female reproductive tissue; RepM - male reproductive tissue; VegF – female vegetative tissue; VegM – male vegetative tissue.

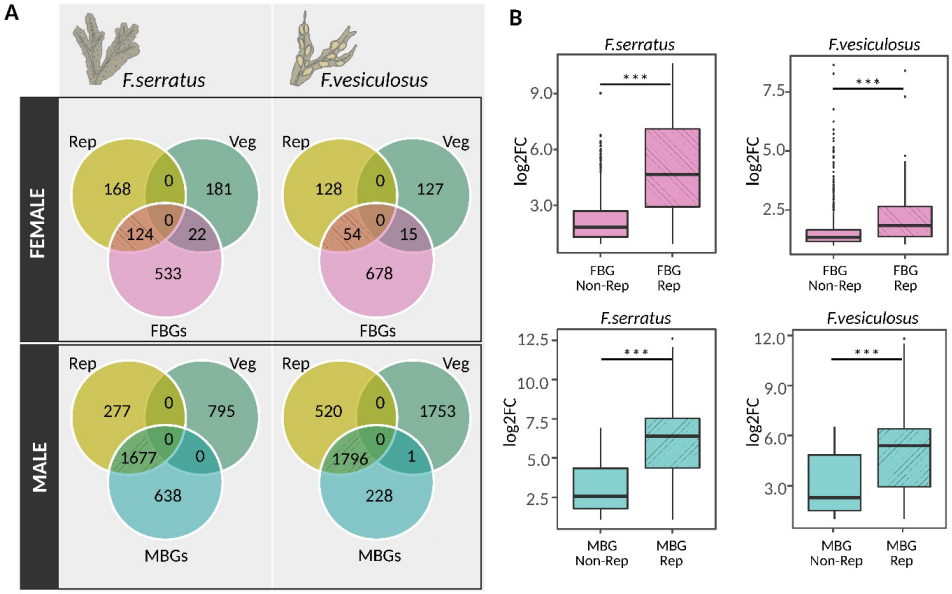

Figure 3. Sex-biased genes (SBGs) are over-expressed specifically in the reproductive tissue. A) Venn diagram shows numbers of significantly differentially expressed genes between reproductive (Rep) and vegetative (Veg) tissues of males and females from *F. serratus* and *F. vesiculosus* (FC>2, padj<0.05). The shaded overlap highlights female-biased genes (top) and male-biased genes (bottom) that were over-expressed in reproductive tissue. B) Overall levels of sex-biased expression (log2FC) of SBGs up-regulated in reproductive (Rep) or vegetative tissue (Non-Rep) (Wilcoxon test, p<1.4e-06).

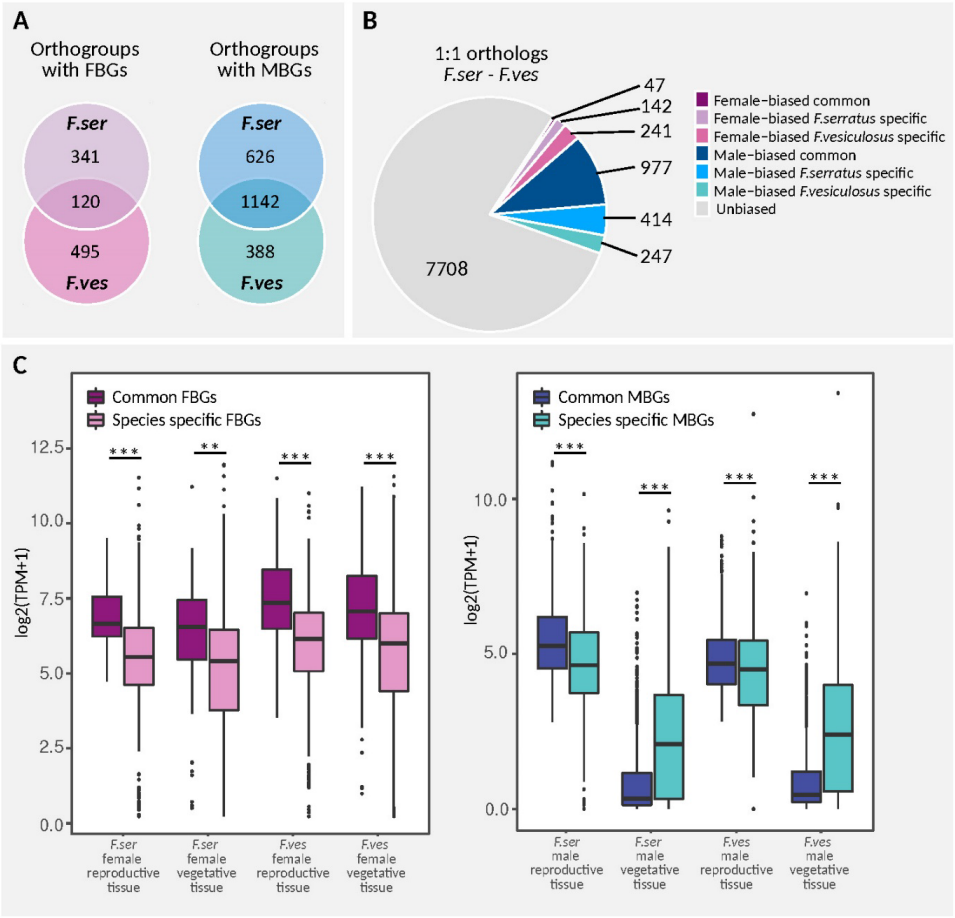

Figure 4. Conservation of sex-biased gene expression across *F. serratus* and *F. vesiculosus* species. A) Numbers of orthogroups with female (pink, FBS) and male (blue, MBS) sex-biased genes shared between dioecious species. Orthogroups with multi-copy genes of a species were included if at least one of the paralogs exhibits 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 (log2(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.*

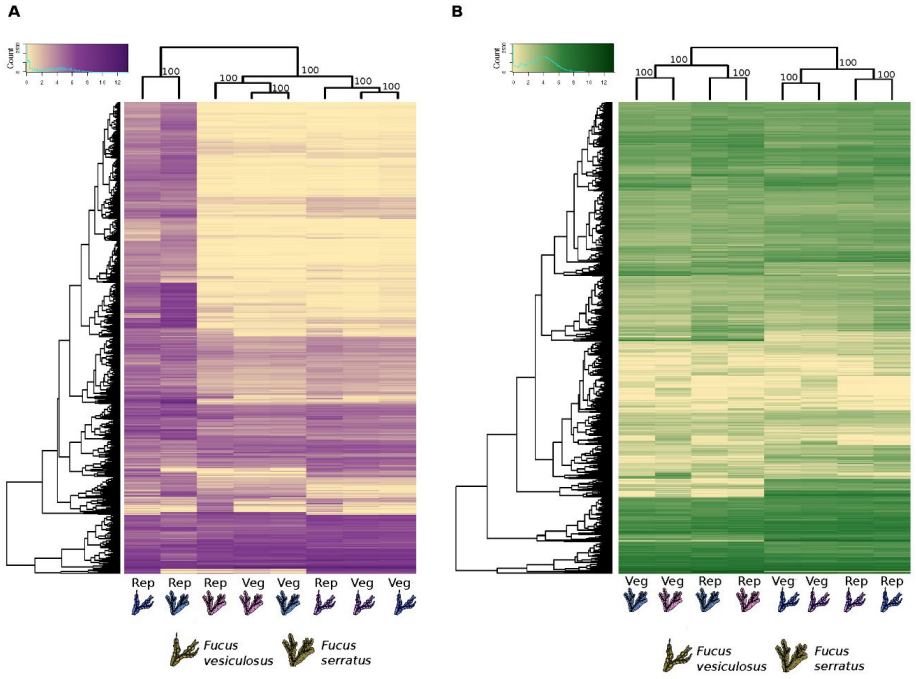

Figure 5. Heatmaps and hierarchical clustering of gene expression levels (log2(TPM+1)) for all single copy orthologs among *F. serratus* and *F. vesiculosus*. The dendrogram was generated using hierarchical clustering with 1000 bootstraps (pvclust package, R). A) Sex-biased genes (at least one sex-biased gene in one of the studied species); B) unbiased genes (none of the genes was sex-biased).

Rep – Reproductive tissue, Veg – Vegetative tissue, Pink - female, Blue – male.

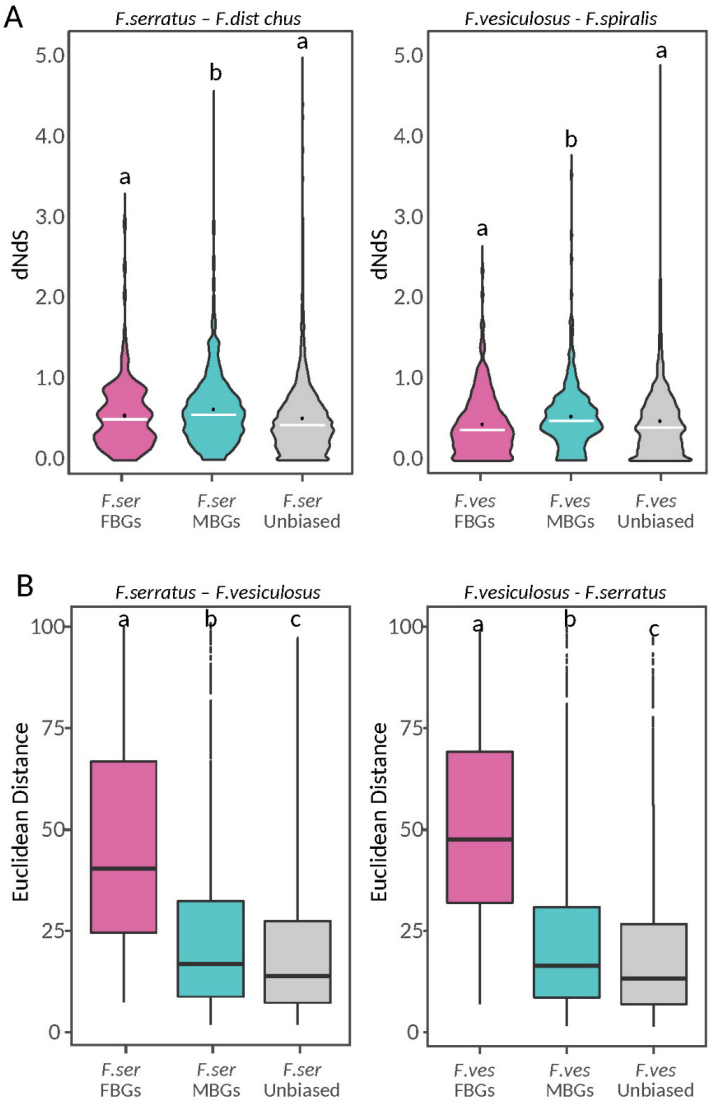

Figure 6. Evolution of sex-biased genes. A) Evolutionary rates measured as dN/dS between species pairs (*F. serratus/F. distichus* and *F. vesiculosus/F. spiralis*) for unbiased, female-biased, and male-biased genes in the two dioecious *Fucus* species. White bar indicates the median, black dot marks the 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*. Different letters above the plots indicate significant differences (pairwise Wilcoxon test; $p<4.3e-10$). FBGs – female-biased genes, MBGs – male-biased genes.

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Supplementary data

Table S1. Sequencing and assembly summary.

Table S2. Number of sex-biased and tissue-biased genes in *F. serratus* and *F. vesiculosus; DESeq2 (FC>=2, pdj<0.05).*

Table S3. Expression levels (log2(TPM+1) and fold change (logFC) of sex-biased and tissue-biased genes in *F. serratus* and *F. vesiculosus*, and tissue-biased genes in hermaphrodite *F. distichus* and *F. spiralis.*

Table S4. Gene orthology statistics.

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

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

Table S7. Evolutionary rates measured as dN/dS between species pairs (F. serratus/F. distichus and F. vesiculosus/F. 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 *F. serratus* and *F. vesiculosus*, Fisher exact test, p<0.01.

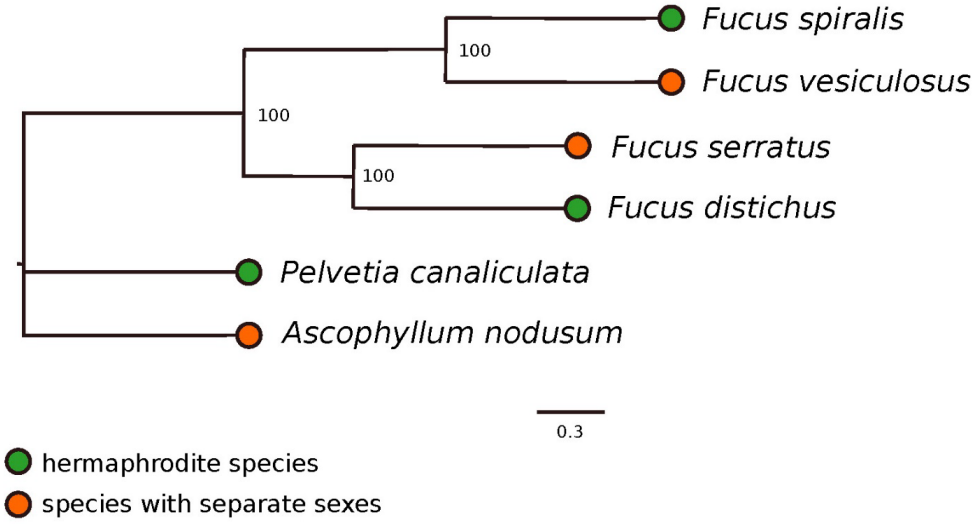

Figure S1. Phylogenetic relationships between the four *Fucus* species used in this study.

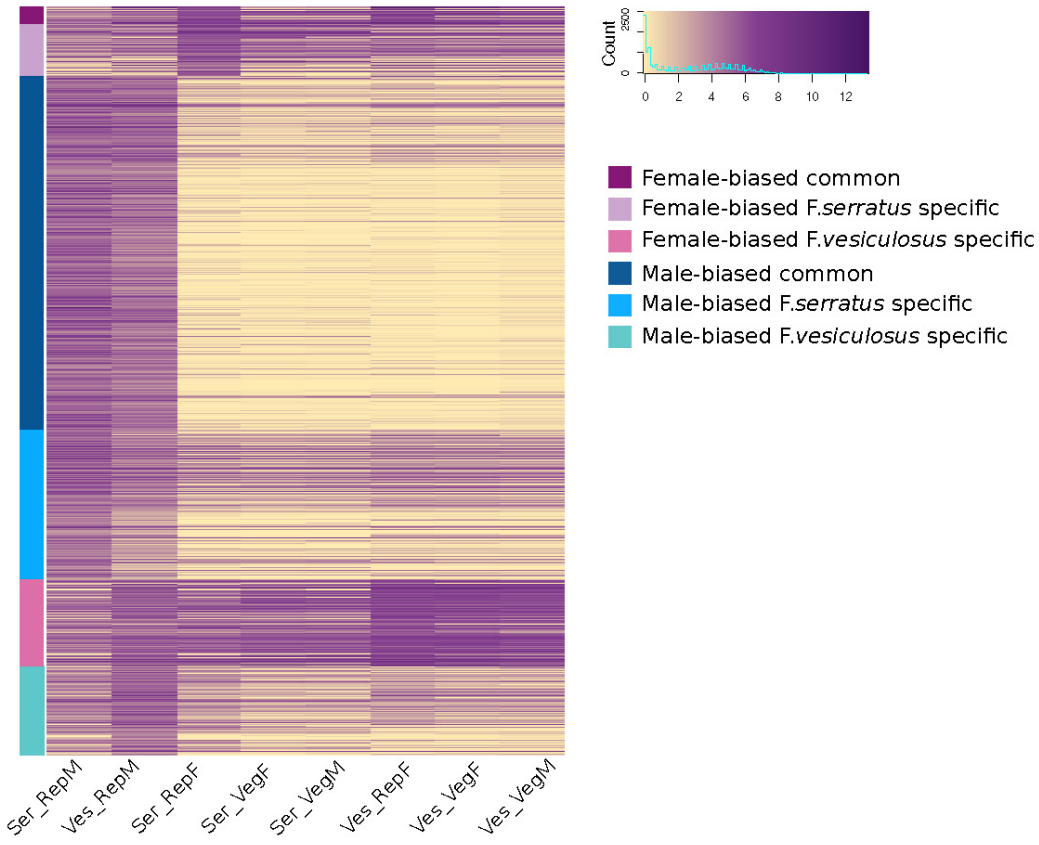

Figure S2. Sex-biased gene expression among single copy orthologs in *F. serratus* and *F. vesiculosus.*

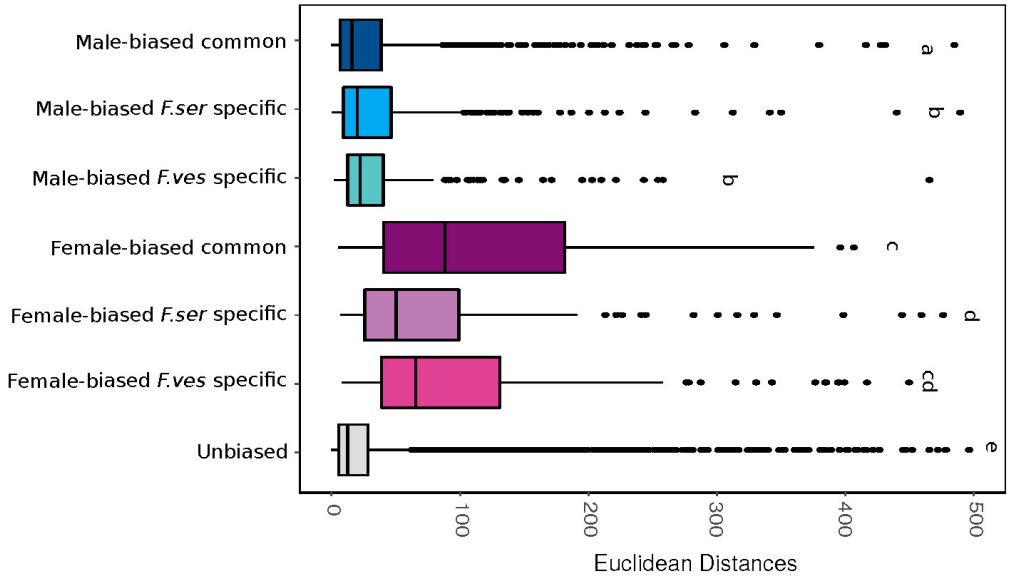

Figure S3. Expression divergence measured as Euclidean distances between single copy orthologous genes of *F. serratus* and *F. vesiculosus*.

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The brown seaweed *Fucus*, also known as rockweed, are familiar objects both on the shore and in salt marshes in the North Pacific and the North Atlantic. *Fucus* species are unique among most other brown algal species as they reproduce via sperm and egg rather than a separate life stage for reproduction. *Fucus* species come in all shapes and sizes, however determining the identity of each species has confused scientists for hundreds of years. Species can often look identical, similar or completely different due to the formation of hybrids of two species and/or species shaped to fit a different environment. Thus *Fucus* species provide us with an interesting model organism for research in the formation of species, the evolution of different reproduction methods and the evolution of the XY reproductive system similar to humans. Analysing the transcriptome of reproductive and vegetative tissues in *Fucus* species showed that male and female sex biased genes experience different evolutionary pressures and have their own evolutionary paths. Using the newly generated genomic data, we then tested a new genotyping method for *Fucus* species, known as target capture sequencing. This method was very successful, generating tens of thousands of molecular markers across the entire genome. Target capture sequencing will likely facilitate future research in this field and involve a large number of individuals, at a low cost. The future is looking bright and exciting for research on *Fucus* which now has the resources and tools to answer key questions about the formation of its many complex shapes and species, the evolution of its varied methods of reproduction and the evolution of a young XY reproductive system.

NORD University

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