HYBRIDIZATION AND MICROBIOME COMPATIBILITY OF *LAMINARIA OCHROLEUCA* AND *LAMINARIA DIGITATA*

Word count: 29697

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02013163

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Master's dissertation submitted to obtain the degree of Master of Science in Biology.
Academic year: 2024 – 2025

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ABSTRACT

DE CLERCQ, KATO. University Ghent. May 2025. **Hybridization and microbiome compatibility of *Laminaria ochroleuca* and *Laminaria digitata***.

Laminaria ochroleuca and *Laminaria digitata* are two economically and ecologically important kelp species found along European coasts. These brown macroalgae play vital roles in marine ecosystems as habitat formers and primary producers. Climate change is driving shifts in their distributions, increasing the potential for natural hybridization between these species. This study investigates whether *L. ochroleuca* and *L. digitata* can form viable and fertile hybrids and explores how hybridization may influence their associated microbiomes. Although previous studies indicated reproductive barriers, the possibility of successful hybridization under controlled conditions has not been clearly established. Using clonal gametophyte cultures, a series of intra- and interspecific crosses were performed and monitored for reproductive development, hybrid viability and morphological growth. Genetic analyses based on microsatellites confirmed successful hybridization in multiple crosses. Additionally, 16S rRNA gene sequencing showed that microbiome composition was primarily influenced by environmental origin, resulting in hybrid microbiomes largely shaped by laboratory conditions. These findings suggest that *L. ochroleuca* and *L. digitata* are reproductively compatible under specific conditions. This work contributes to kelp breeding research and positions hybridization as a potential strategy for reinforcing aquaculture sustainability in the context of climate change.

Keywords: *Laminaria digitata*, *Laminaria ochroleuca*, hybridization, microbiome, reproductive success, marine macroalgae

INTRODUCTION

Laminariales: a vital part of coastal ecosystems

Kelps are large brown algae of the order Laminariales. They create extensive underwater forests that are vital for supporting coastal ecosystems and marine biodiversity (Smale et al., 2013; Teagle et al., 2017). In aquatic environments, they represent the largest photosynthetic organisms and are known for being the most structurally complex seaweeds (Kawai et al., 2013). Found in temperate to cold seas, along rocky coastlines, kelps grow from the upper subtidal zone to depths of several meters. They provide habitat and nursery grounds for diverse marine species, including those of economic importance (Fragkopoulou et al., 2022; Pereira et al., 2019), with key ecosystem services such as fisheries production, nutrient cycling, and carbon removal largely supported by six major forest-forming kelp genera: *Ecklonia*, *Laminaria*, *Lessonia*, *Macrocystis*, *Nereocystis*, and *Saccharina* (Eger et al., 2023). Kelps influence sedimentation, reduce erosion, and support fisheries by offering shelter and food. Their canopy also fosters shade-tolerant organisms (Mauger et al., 2021; Pereira et al., 2019a). Despite their importance, kelp cultivation faces challenges due to poor environmental conditions and stressors, including anthropogenic disturbances like pollution and climate change-induced events. These stressors not only undermine their productivity, but also jeopardize the vital ecological services they provide to marine ecosystems (Gorman & Connell, 2009; Qiu et al., 2019). Gaining more understanding of these species is vital to address the challenges they face and protecting their critical contributions to coastal ecosystems.

Taxonomy and Classification

The order Laminariales belongs to the class Phaeophyceae, known as brown algae, within the phylum Ochrophyta (Guiry & Guiry, 2024). It includes large, multicellular macroalgae predominantly found in marine environments. Within Laminariales, several families are commonly recognized: Laminariaceae, which includes *Laminaria* found in temperate to polar regions; Lessoniaceae, featuring *Macrocystis* common in the temperate Southern Hemisphere; and Alariaceae, which includes *Alaria* from temperate and subarctic regions (Bolton, 2010). Classification within Laminariales is based on morphological traits, reproductive structures, and molecular data. Advances in molecular phylogenetics have enhanced understanding of relationships within this order, leading to more precise classifications and insights into evolutionary patterns (Yoon et al., 2001). For example, molecular evidence has also led to the recognition of the family Costariaceae, which includes *Agarum*, *Costaria*, *Dictyoneurum*, and *Thalassiophyllum* (Berchtenbreiter et al., 2024; Lane et al., 2006).

The genus *Laminaria* constitutes a critical macroalgal genus in temperate to polar rocky coastal ecosystems, particularly within the northern hemisphere (Bartsch et al., 2008). Its significance is evident in its species diversity, substantial biomass, ecological dominance and economic importance. Nonetheless, comprehensive understanding of *Laminaria* species remains limited (Bartsch et al., 2008). Both *Laminaria digitata* and *Laminaria ochroleuca* are perennial species, which live for more than two years and grow and reproduce over multiple seasons (Hill, 2008; Pereira et al., 2019). The North Atlantic species *L. digitata*, commonly known as oarweed, is found from the Arctic to the cold-temperate regions of southern Brittany (France) in the northeastern Atlantic and Massachusetts (USA) in the northwestern Atlantic (Martins et al., 2019). Found attached to bedrock or other hard surfaces, *L. digitata* inhabits the lower intertidal and sublittoral zones, extending down to 20 meters in clear waters. This species thrives in moderately exposed environments or in areas with strong currents (Hill, 2008).

The primary species forming kelp forests in Southern Europe is *L. ochroleuca*, often known as golden kelp. This warm-temperate species is distributed along the Portuguese and northwestern Spanish coasts, the Azores, Morocco, the strait of Messina (Italy), Brittany (France), as well as the English and Bristol Channels (Pereira et al., 2019). This species typically inhabits deep intertidal pools and high subtidal zones, extending to depths of up to 30 meters. Its ability to colonize deeper areas is directly influenced by light availability (Pereira et al., 2019). Studies indicate that *L. ochroleuca* may be spreading poleward in response to climate change and could potentially replace other *Laminaria* species in the future (Franco et al., 2018; Pereira et al., 2019; Smale et al., 2015). Seaweed biogeography is largely driven by temperature tolerance, making climate change a key driver of range shifts. Understanding the consequences is essential, as these shifts will alter interactions between coexisting species (De La Hoz et al., 2019; Hargrave et al., 2017; Straub et al., 2016).

The division of *Laminaria* into distinct clades has been supported by molecular evidence, leading to the recognition of *Saccharina* as a separate genus (Berchtenbreiter et al., 2024; Lane et al., 2006). The phylogenetic relationships among *Laminaria* species are complex and uncertain due to a lack of reliable fossil records. Re-assessing the phylogeny and using modern sequencing techniques are needed to unravel relationships within the *Laminaria* genus (Bolton, 2010). Rothman et al. (2017) conducted time-calibrated analyses to clarify the taxonomic divisions within the genus, as shown in Figure 1.

The results revealed that *Laminaria* splits into two major clades, each associated with distinct temperature regimes. The warm-adapted species *L. pallida* and *L. ochroleuca* are closely related sister species that form a clade with *L. abyssalis* from the South Atlantic and *L. rodriguezii* from the Mediterranean (Rothman et al., 2017). All four species share a common ancestor in the North Atlantic. The cold-adapted *L. digitata* and *L. hyperborea* form a second clade, see Figure 1 (Rothman et al., 2017). The division of the *Laminaria* genus into those two clades is also supported by microsatellite-based genetic analyses (Mauger et al., 2021). The phylogenies indicate that *Laminaria* originated in the North Pacific around 25 million years ago, followed by at least two migration events through the Bering Strait, which opened approximately 5.3 million years ago (Bolton, 2010; Rothman et al., 2017). This is also supported by molecular phylogenetic analyses and divergence time estimates derived from dated phylogenetic trees based on plastid, mitochondrial, and nuclear genomic data, as determined by Starko et al. (2019). One migration resulted in *L. solidungula* in the Arctic, while the other gave rise to the remaining Atlantic species and the Mediterranean endemic *L. rodriguezii* (Rothman et al., 2017). This North Atlantic colonization was succeeded by a gradual southward expansion along the west coast of Europe into the Mediterranean and two recent migrations across the equator (about 1.34 and 0.87 million years ago, respectively), leading to *L. abyssalis* in Brazil and *L. pallida* in southern Africa (Rothman et al., 2017).

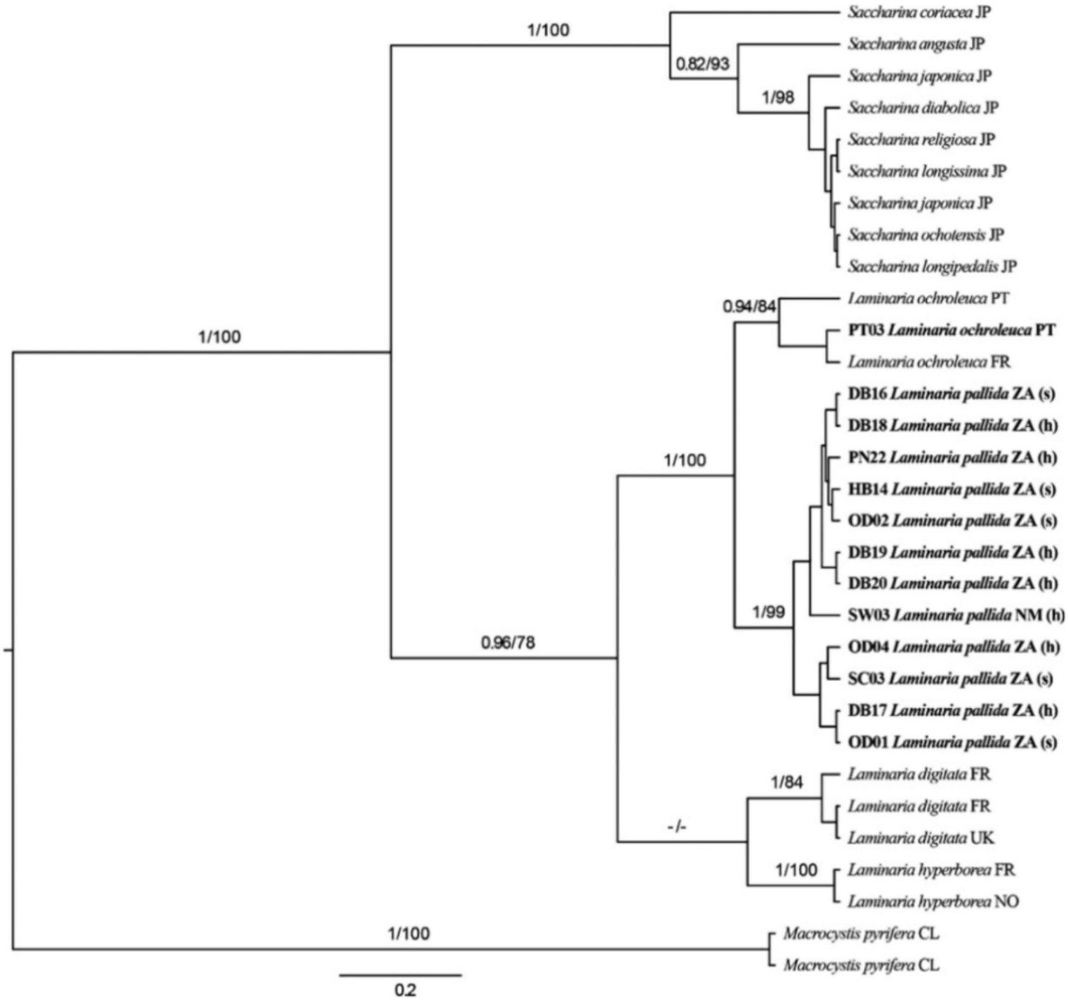

Figure 1: Time-calibrated phylogeny of the genus Laminaria based on four molecular markers, by Rothman et al. (2017).

Morphology and life cycle

Laminariales are characterized by their complex morphology, which includes a holdfast, stipe and blades, see Figure 2 (Kawai et al., 2013; Lane et al., 2006). Kelps use a holdfast to anchor themselves to rocky substrates. This root-like structure secures them and provides stability in turbulent waters (Kawai et al., 2013). The holdfast develops as individual haptera grow from the meristematic tissue at the base of the stipe (Lane et al., 2006; Tuya et al., 2011). New haptera are easily formed, allowing kelps to create new attachments as layers grow in different directions (Bartsch et al., 2008; Tuya et al., 2011). The stipe, extending from the holdfast, supports the blades and helps to stay upright for capturing sunlight. Blades play a vital role in photosynthesis and nutrient uptake. They are also essential for reproduction, especially for species in the family Laminariaceae as they contain the reproductive tissue called sori (Ihua et al., 2020; Kawai et al., 2013).

Morphology is highly influenced by hydrodynamic conditions (Vettori & Nikora, 2017). The structure of Laminariales is adapted to their aquatic environment, allowing them to thrive in various conditions from shallow coastal waters to deeper subtidal zones. Depending on exposure levels, blades may be flat or ruffled. In calmer areas with less wave action, blades tend to be ruffled, which promotes flapping, enhancing light and nutrient uptake but also increasing drag (Huang et al., 2011). A typical "stretched droplet" shape is common among blades, possibly reducing drag forces (Vettori & Nikora, 2017). The anatomy of Laminariales includes several key features such as the central vascular system for nutrient transport and specialized cells called meristems, which facilitate growth (Lüning, 1993). They contain various tissue and cell types, including a photosynthetic epidermis, cortex, medulla, sieve tubes and mucilage ducts (Hurd et al., 2014). Meristem growth in kelps, located in the basal intercalary region between the blade and stipe, is essential for blade elongation and the development of reproductive structures (Figure 2). The meristematic region supports blade expansion, which can facilitate the induction of the sori (Pang and Lüning, 2004).

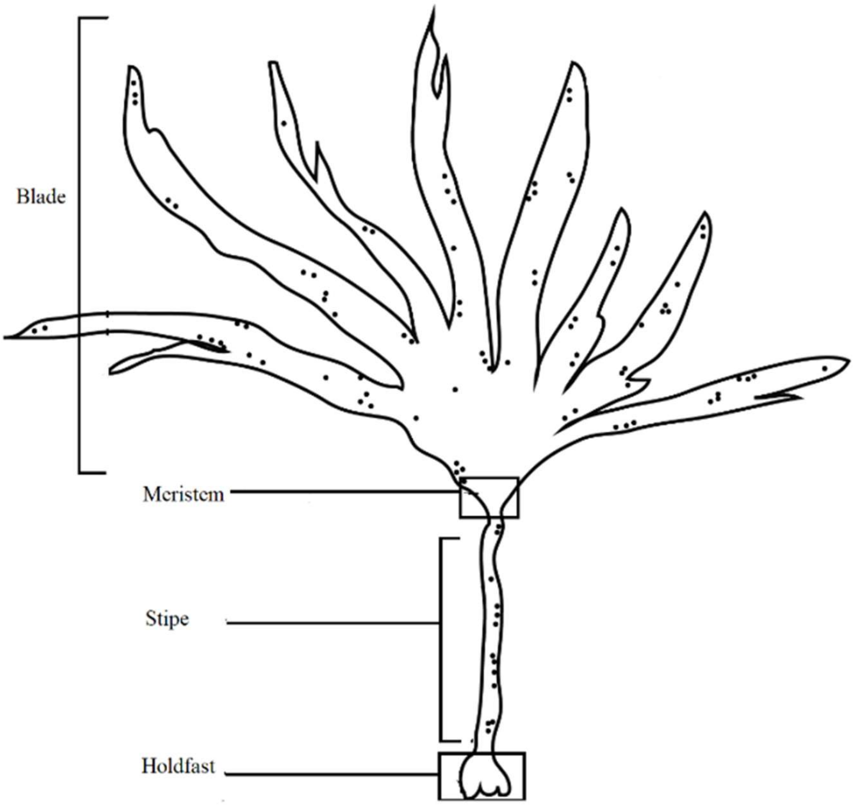

Figure 2: Schematic presentation by Ihua et al. (2020) of the four morphological components of a L. digitata thallus; blade, meristem, stipe and holdfast.

The life cycle of *Laminaria* species is similar and illustrated in Figure 3. Individuals undergo a heteromorphic life cycle comprising macroscopic sporophytes and microscopic stages following sporulation (Lüning, 1980; Pereira et al., 2019). Within the sori, unilocular sporangia produce 32 haploid zoospores, which are predominantly released at night (Baweja et al., 2016). As sporulation progresses, the paraphyses[1] swell and become mucilaginous, enveloping the basal cell. Each haploid zoospore gives rise to a dioecious heteromorphic gametophyte, with male and female gametophytes developing as filamentous structures (Baweja et al., 2016). Each haploid zoospore develops into a dioecious heteromorphic gametophyte, with male and female gametophytes being filamentous. The male gametophyte forms clusters of colorless one-celled antheridia at the branch tips, each generating a single biflagellate spermatozoid, while the female gametophyte cells transform into one-celled oogonia, each producing one egg (Baweja et al., 2016; Lüning, 1980). Following this process, the fusion of male and female gametes results in the formation of a zygote and subsequently new

[1] Paraphyses are thread-like structures located among reproductive organs or sporangia, serving to protect them or facilitate their function (Ahmadjian & Hale, 1973).

sporophytes, as shown in Figure 3 (Baweja et al., 2016; Lüning, 1980). These microscopic stages are highly sensitive to temperature fluctuations, physical disturbances and light. Studies have shown that warming temperatures can negatively impact kelp canopies, affecting their growth and health (Bishop & Spaulding, 2017; Biskup et al., 2014).

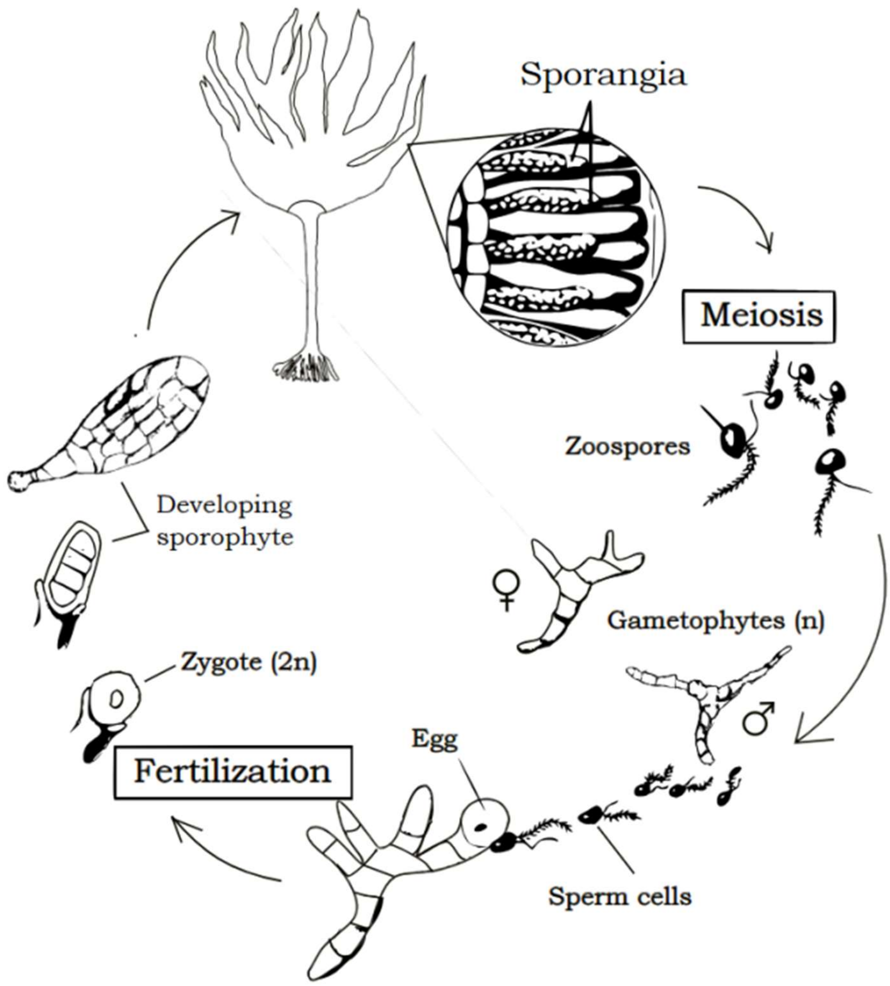

Figure 3: Life cycle of the genus Laminaria, adapted from figure by Pearson Benjamin Cummings (Campbell et al., 2008).

Sorus formation is influenced by environmental factors such as light and nutrient availability, with photoperiod being a key driver. Long daylengths typically stimulate sorus induction by synchronizing endogenous growth rhythms (Liesner et al., 2020). Experimental studies have shown that maintaining appropriate daylengths and optimal environmental conditions can trigger reproductive readiness (Liesner et al., 2020). Sorus induction can also be artificially triggered by disrupting the transport of sporulation inhibitors produced in the meristem. This can be achieved through mechanical interventions, such as removing distal blade fragments or making transverse cuts in the frond, which blocks the flow of inhibitors and allows sori to form in the distal regions. In *Laminaria saccharina*, this process was observed to occur within 10 days under controlled short-day conditions (Pang and Lüning, 2004). Short photoperiods play a role in reducing meristematic activity, which lowers the concentration of sporulation inhibitors to a critical threshold, permitting sorus formation (Liesner et al., 2020; Pang & Lüning, 2004). This process can override the natural seasonal limitations on sporogenesis, enabling year-round reproduction in controlled environments. Such methods have significant potential for aquaculture, facilitating continuous production of sporelings and accelerating kelp breeding cycles (Pang & Lüning, 2004).

Laminaria species exhibit complex reproductive strategies characterized by high mortality rates, mostly impacted by the environment. The survival rate from their microscopic stages to maturity is low, with significant mortality occurring primarily during the microscopic benthic phase (Demes & Graham, 2011). *Laminaria* species have two reproductive strategies: sexual reproduction through spores and vegetative reproduction via clonal growth. This adaptability is crucial, and the balance between these strategies is determined by, among other factors, temperature and nutrient availability (Demes & Graham, 2011; Liesner et al., 2020). Colder temperatures tend to favor sexual reproduction, while nutrient levels significantly affect vegetative growth (Demes & Graham, 2011; Liesner et al., 2020). Pheromonal signaling also plays a key role in sexual reproduction (Müller et al., 2008). Despite producing a large number of spores, the maturing process is inefficient, with environmental factors such as red algal turf and plant density significantly impacting recruitment success (Chapman, 1984). Clonality allows *Laminaria* to persist in stressful environments where sexual reproduction might be less effective. These flexible reproductive strategies enable them to thrive in diverse and changing conditions (Demes & Graham, 2011).

Kelp hybridization

Hybridization in kelp, particularly within the genus *Laminaria*, presents a complex and intriguing area of study. Hybridization refers to the reproduction between individuals from different species or genetically distinct populations (Martins et al., 2019; Miller et al., 2021). It can yield hybrid offspring with varying fitness (Martins et al., 2019; Montecinos et al., 2017). However, hybridization can be challenging, as not all species can crossbreed successfully, and the ease of hybridization varies even among closely related species (Murray, 2003). This process can be influenced by pre- and postzygotic reproductive barriers. Prezygotic barriers, such as differences in reproductive timing or gamete compatibility, restrict fertilization and preserve the genetic distinctiveness of closely related species (Montecinos et al., 2017; Monteiro et al., 2016). Postzygotic barriers, which manifest after fertilization, often result in reduced hybrid viability or fertility, impacting the survival and reproduction of hybrids (Montecinos et al., 2017). Prezygotic isolation is considered a more significant and effective barrier than postzygotic isolation (Monotilla et al., 2018). The Dobzhansky–Muller model offers a foundational framework proposing that dysfunctions, such as sterility or unviability, arise when independent mutations in different lineages lead to derived alleles that, although functional within their respective species, interact negatively in hybrids due to lack of co-evolution (Miller et al., 2021). Research on *Laminaria* hybridization involves analyzing both haploid gametophytes and diploid sporophytes. The haploid-diploid life cycle allows researchers to distinguish between the effects of reproductive barriers at different life stages (Montecinos et al., 2017).

While reproductive barriers can limit hybrid formation, successful hybridization can result in offspring with advantageous traits. One such outcome is heterosis, or hybrid vigor, where first-generation hybrids outperform their parents and offer valuable genetic variation for adapting to environmental change (Lippman & Zamir, 2007). This concept is extensively utilized in agriculture to develop high-yield crops and livestock, thereby supporting global food production (Lippman & Zamir, 2007; Martins et al., 2019). Interspecific hybridization is used to introduce desirable traits into cultivated species by crossing them with related wild species. This method addresses the lack of certain traits in cultivated plants, which may result from reduced genetic variation due to selective breeding or the absence of those traits altogether (Murray, 2003). Successful applications of heterosis, such as in kelp mariculture, have led to hybrids with enhanced thermal resilience (Martins et al., 2019). Understanding the inheritance of thermal traits is essential for selecting appropriate parent strains and guiding future genetic research. Although knowledge of thermal tolerance inheritance in kelp is limited, some studies suggest that morphological traits in hybrids may be inherited either maternally or paternally (Martins et al., 2019). For instance, hybrids of *L. digitata* and *L. pallida*, despite their separate distributions, can produce viable offspring with slight differences in thermal limits, making them suitable for studying thermal trait inheritance (Martins et al., 2019).

Previous hybridization experiments between *L. digitata* and *L. ochroleuca* have been conducted, but results remained unclear. Bartch et al. (2008) found that hybridization attempts between those species rarely resulted in the formation of viable sporophytes. Earlier studies reported the production of small F1 hybrids, but these often exhibited stunted growth and abnormal development, indicating reproductive barriers between these species. Additionally, some experiments may have confused true hybrids with partheno-sporophytes, which develop from unfertilized eggs (Bartsch et al., 2008; Martins et al., 2019). In earlier experiments, this aspect was often overlooked. Research by Tom Dieck (1992) showed that although fertilization between these species can occur, hybrid development is typically impaired, suggesting reproductive isolation. This supports the idea that while *Laminaria* species exhibit some level of cross-species fertilization, genetic differences often prevent the formation of healthy, viable hybrids.

Global warming threatens biodiversity and food production, increasing the need for heat-tolerant hybrids, a strategy successful in major crops like wheat, cotton, maize, and canola, and now applied to kelp (Sahu & Mishra, 2021; Sugumar et al., 2024). Kelp, especially brown algae, are ecologically and economically important in temperate and polar marine ecosystems (Miller et al., 2018; Smale et al., 2013), but face threats from human activities including ocean warming (Qiu et al., 2019). Unlike Asia's seaweed farming, Europe mainly relies on wild harvests (UNEP, 2023), highlighting the urgency to balance biomass demand and kelp conservation (Martins et al., 2019).

Kelp hybridization raises concerns such as genetic pollution, where cultivated kelps interbreed with wild populations and invasiveness of modified strains (Goecke et al., 2020). Some regions regulate aquaculture to limit non-native species use (Goecke et al., 2020). Future approaches may include using local cultivars or sterile varieties to prevent genetic introgression, alongside improved understanding of kelp genetics and ecological impacts (Coyer et al., 2007; Goecke et al., 2020; Murúa et al., 2020).

Polyploidy

Polyploidy, the condition of having more than two complete sets of chromosomes, is observed in seaweeds, though less frequently than in most plants (Nichols, 1980). It is often linked to complex life histories, extreme environments, and hybridization events (Hurd et al., 2014; Mortier et al., 2024). Polyploidy can result from interspecific hybridization, leading to allopolyploidy, which is facilitated by external fertilization in many seaweeds (Mortier et al., 2024; Neiva et al., 2017). Polyploid individuals often exhibit increased genetic diversity, enhancing adaptability to environmental changes. This may lead to phenotypic novelties, improving fitness traits like growth rates and stress resilience (Lavania, 2020; Mortier et al., 2024). Hybridization enhances gene expression responsiveness, enabling greater plasticity to environmental conditions, this is referred to as the polyploidy plasticity hypothesis (Mortier et al., 2024; Shimizu-Inatsugi et al., 2017). In hybrids, meiotic irregularities can also lead to triploidy, the presence of three chromosome sets, which typically results in sterility due to unbalanced gamete formation (Bradshaw & Stettler, 1993; De Storme & Geelen, 2013). Similarly, aneuploidy, the presence of an abnormal number of chromosomes, can arise from unequal segregation, though it is often nonviable (De Campos Moraes et al., 2019). While polyploidy can cause sterility in some cases (Lewis & Neushul, 1995; Meichssner et al., 2021), polyploids can still thrive through asexual reproduction which facilitates rapid colonization (Lasker & Coffroth, 1999). Consequently, polyploids may gain a short-term competitive advantage (Mortier et al., 2024).

Kelp microbiome

Microbiomes, which consist of host-associated microorganisms, are critical for the functioning of eukaryotic hosts (Hitch et al., 2022; Lazzaro & Fox, 2017; McGrath et al., 2024). A diverse microbiome benefits its host by providing various functions, especially through bacterial taxa (Hitch et al., 2022). Kelp species rely heavily on symbiotic relationships to enhance primary productivity, supplement nutrients, and aid in digestion, while the microorganisms, in turn, are protected by the host and supplied with inorganic nutrients (Gordon & Leggat, 2010; Picon et al., 2021; Stock et al., 2021).

The bacterial communities colonizing macroalgae are a subset of those found in the surrounding seawater and show both host specificity and temporal and spatial variation (Ramírez-Puebla et al., 2022). Among these bacteria, *Planctomycetes*, *Verrucomicrobia*, and *Bacteroidetes* have been identified as part of the kelp microbiome (Ramírez-Puebla et al., 2022). Research also highlights the presence of alginate-metabolizing bacteria, predominantly *Gammaproteobacteria*, with *Pseudoalteromonas* being the most abundant genus found on kelp blades (Lin et al., 2018; Picon et al., 2021; Ramírez-Puebla et al., 2022). However, since the microbiome is a dynamic "snapshot" influenced by environmental conditions, it is challenging to predict which bacteria will be present, what functions they will perform and whether their interactions will be antagonistic (Ludington, 2022; Stock et al., 2021).

The establishment of microbial communities on kelp is a complex process that includes stages of dispersal, attachment, and growth (Brislawn et al., 2018). Ongoing debates in the literature focus on the roles of selective versus neutral processes in microbiome assembly (Stock et al., 2021). The lottery model, for example, suggests a stochastic component to bacterial colonization, with only a few cells succeeding, implying selectivity (Burke et al., 2011; Lazzaro & Fox, 2017). However, studies show that bacterial communities often exhibit non-random patterns, with species-specific host associations (Lachnit et al., 2009). Factors such as bacterial-algal interactions and surface properties influence colonization, with the potential for a founder effect (Brislawn et al., 2018; Lachnit et al., 2009). Additionally, turnover processes significantly affect community structure during life stage transitions (Davis et al., 2023). Recent studies suggest that microbial distribution is not restricted to distinct patches, as would be expected if competition for space or specific binding sites was driving community structure. Instead, microbial distribution appears nearly random at the micrometer scale (Ramírez-Puebla et al., 2022). Therefore, spatial arrangement in the microbiota suggests that syntrophic[2] or metabolic interactions drive community structure, rather than competition for space (Ramírez-Puebla et al., 2022).

The microbiome of *Laminaria* species exhibits significant spatial variation, with distinct bacterial communities associated with the holdfast, stipe, meristem and blade (Ihua et al., 2020; Lemay et al., 2021). Each region forms

[2] The cooperative interaction between at least two microbial species to degrade a single substrate (Marietou, 2021).

a unique ecological niche, shaped by specific functional and nutritional needs, which reflect the non-vascular structure and localized independence of seaweed tissues (Ihua et al., 2020). The holdfast, in particular, is highly diverse, likely due to its proximity to nutrient-rich sediments and other environmental factors. Despite these insights, much of the microbiome remains poorly understood, particularly regarding the functional roles of microbial diversity across different morphological regions of kelp (Ihua et al., 2020).

L. ochroleuca, though currently underrepresented in microbiome studies, has been found to harbor Actinobacteria, which are known for their antimicrobial properties and potential anti-cancer effects (Girão et al., 2019). These Actinobacterial strains, associated with genera such as *Rhodococcus*, *Nocardiopsis*, *Microbispora*, *Microbacterium*, *Isoptericola*, and *Nonomuraeae*, have been identified on this macroalga (Girão et al., 2019). An abundance study of *L. digitata* suggests that genera like *Blastopirellula*, *Granulosicoccus*, *Psychromonas*, *Roseobacter*, *Aquimarina*, *Bacillus*, *Psychrobacter*, and *Vibrio* are likely present in these species (Izquierdo et al., 2002). These microorganisms contribute to biofilm formation (e.g., *Psychromonas*, *Planctomycetes*), polymer degradation and catalase production (*Pseudoalteromonas*), nitrogen fixation (*Azotobacter*, *Rhizobium*, *Agrobacterium*), vitamin B12 synthesis (*Ectocarpus*), auxin production (*Exiguobacterium*), morphogenesis induction (*Marinomonas*, *Bacillus*, *Cytophaga*, *Caulobacter*), antibacterial activity (*Actinobacteria*), enhancing cell division (*Roseobacter*), and promoting algal cell enlargement (*Maribacter*) (Bengtsson et al., 2012; Del Olmo et al., 2018; Holmström & Kjelleberg, 1999; Izquierdo et al., 2002). Further research is crucial to uncover the influence of these microbial populations on kelp's health, development and resilience (Ihua et al., 2020).

Societal relevance

Seaweeds, particularly kelp, are essential ecosystem engineers that play a critical role in nutrient cycling, carbon storage, sediment stabilization and supporting coastal biodiversity (Krause-Jensen et al., 2018; Miller et al., 2018). Kelp forests create habitats for marine life, contributing to ecological health and providing significant societal benefits (Smale et al., 2013; Teagle et al., 2017). Kelp is commercially valuable in industries like food production and healthcare (Baweja et al., 2009). Products such as alginates, agar, and carrageenan are used as food additives and in medical and industrial applications (Alba & Kontogiorgos, 2019; Kim & Bhatnagar, 2011). Seaweed aquaculture, a growing sector, has the potential to provide sustainable products with low carbon footprints, such as biodegradable plastics, which are more energy-efficient and easier to recycle than petroleum-based plastics (Grebe et al., 2019; Kajla et al., 2024). Seaweeds also act as natural fertilizers in agriculture, enhancing soil health and reducing the use of harmful chemicals (Nanda et al., 2022).

In addition to their commercial uses, kelp forests play a vital role in climate change mitigation by sequestering carbon dioxide and producing oxygen, through photosynthetic processes (Krause-Jensen et al., 2018). Though coastal habitats limit their carbon storage potential, expanding kelp farming to open waters could significantly enhance carbon burial (Pessarrodona et al., 2024). Kelp forests also help protect coastal communities by buffering against storm surges and erosion. They have cultural significance for local and indigenous communities, further emphasizing the need for their conservation (Kobluk et al., 2021). Citizen science initiatives like Kelpwatch engage communities in monitoring and restoring kelp forests, raising awareness and fostering a deeper connection to marine ecosystems (Bell et al., 2023; Von Gönner et al., 2023).

OBJECTIVE

This master's thesis aims to explore the following three research objectives to contribute to our understanding of interspecific kelp hybridization:

I. *To determine the hybridization potential between L. ochroleuca and L. digitata*

As climate change alters species distributions and increases spatial overlap, the likelihood of natural hybridization events may rise. Understanding hybridization potential is therefore essential. Given the close relationship between *L. ochroleuca* and *L. pallida*, which is known to hybridize with *L. digitata* (Martins et al., 2019), it is hypothesized that successful hybridization between *L. ochroleuca* and *L. digitata* can occur in laboratory settings.

II. *To examine microbiome variations among parent species and potential hybrids*

Given their fundamental role in kelp biology, microbiomes are expected to impact hybridization. Based on previous studies, it is expected that microbiome composition will differ by host species, geographic origin and tissue type (Ihua et al., 2020; Lemay et al., 2021; Weigel et al., 2022). Microbiomes in laboratory-grown specimens are anticipated to differ from those in wild-collected samples. In hybrids, microbiomes are expected to resemble those of the female parent due to developmental associations (Greiner et al., 2015).

III. *To conduct preliminary exploration of hybrid characteristics*

As a secondary focus, this study will preliminarily investigate hybrid fertility and ploidy status. It is hypothesized that the hybrids may exhibit reduced fertility or sterility, as is common in interspecific kelp hybrids (Murúa et al., 2020). Regarding ploidy, the hybrids are expected to be allopolyploid, as diploids may exhibit pairing incompatibilities, triploids are prone to meiotic failure and aneuploidy is typically deleterious (De Storme & Geelen, 2013; Mortier et al., 2024; Murúa et al., 2020).

This research seeks to contribute to a broader understanding of kelp biology, genetics and ecology. Additionally, successful hybridization may serve as a strategy to enhance resilience to climate change while contributing to more sustainable and economically viable kelp aquaculture (Lippman & Zamir, 2007; Martins et al., 2019). Advancing knowledge in this area is therefore of significant importance.

MATERIAL AND METHODS

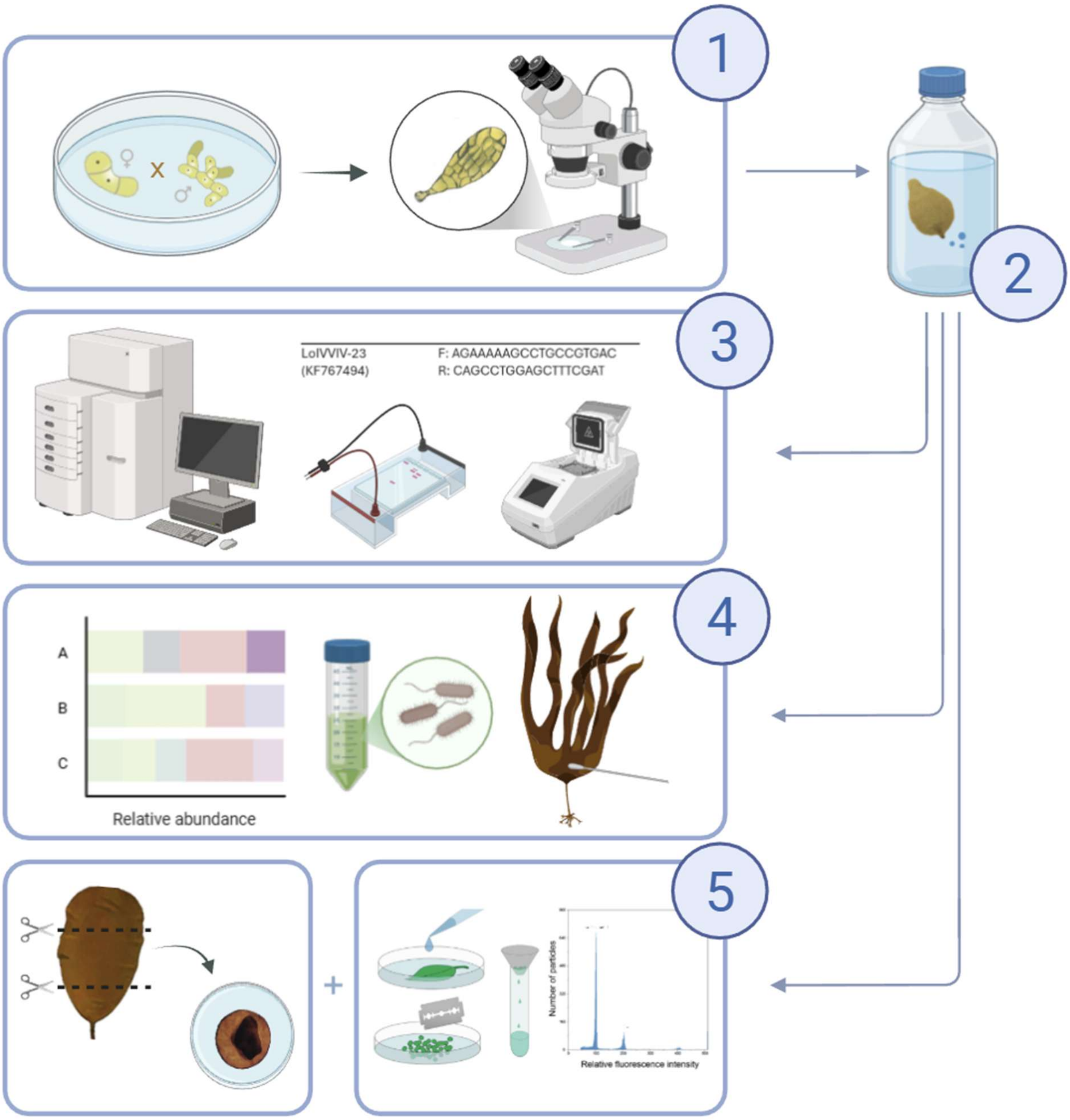

Figure 4: General overview of the experimental workflow and key research areas addressed in this thesis, illustrated using BioRender. (1) Crossing experiment conducted in Faro, (2) Cultivation of the resulting cultures in Ghent for subsequent experimentation, (3) Genetic confirmation using molecular techniques such as PCR, gel electrophoresis, and fragment analysis, (4) Microbiome comparison through blade swabs from both field-collected individuals and lab-grown specimens, and (5) Assessment of general hybrid traits, including ploidy and fertility, using flow cytometry and sorus induction, respectively.

Crossing experiment

Algal material

The algal biomass used for this research are unialgal clonal female and male gametophytes of *L. ochroleuca*, isolated from Cascais, Portugal (CCMAR Biobank), *L. digitata*, isolated from Spitzbergen, North Sea (AWI seaweed culture collection) and *L. pallida*, isolated from Swakopmund, Namibia (AWI seaweed culture collection). These were kept in a vegetative state at 12°C. They were exposed to 3 µmol photons m^{-2} s^{-1} of red light (LED Mitras daylight 150 controlled by ProfiLux 3, GHL Advanced Technology, Kaiserslautern, Germany) on a 16:8 h light:dark cycle. The samples were maintained in sterile full-strength Provasoli-enriched seawater (PES; Provasoli, 1968), with PES added at a concentration of 10 mL L^{-1}. Artificial seawater was prepared by dissolving 32 g of salt per liter of deionized water, and the resulting salinity was measured using a refractometer (Atago, Japan).

Experimental setup

The crossing experiment was carried out in collaboration with CCMAR at the Campus de Gambelas, University of Algarve in Faro. For the experiment, vegetative male and female gametophytes of *L. ochroleuca* (Och)*, L. digitata* (Dig)*,* and *L. pallida* (Pal) were gently fragmented using a pestle and mortar, sieved (diameter = 100 µm), and suspended in half-strength PES (10 mL L^{-}), to produce solutions of gametophytes with lengths of 100 µm. The gametophyte solutions were used to prepare different crosses, maintaining a density of approximately 400 multicellular gametophytes per cm^2. Every multicellular fragment was regarded as an individual gametophyte regardless of size. A total of 21 crosses were conducted, with 4 replicates of each cross. This included intraspecific (Och ♂ × Och ♀ ; Dig ♂ × Dig ♀ ; Pal ♂ × Pal ♀) and reciprocal interspecific crosses (Dig ♀ × Pal ♂; Pal ♀ × Dig ♂; Dig ♀ × Och ♂; Och ♀ × Dig ♂). For each sex of each species, three distinct strains were used (see **Appendix A**) and the strain names were adopted from the laboratory in Faro.

Table 1 outlines the crossing scheme, in which each cross is assigned a number. This numbering will be used for further discussion in the thesis. Crosses were placed into Petri dishes (5.3 cm in diameter, 1.5 cm in height) containing 14 ml of half-strength PES (10 mL L^{-}). All procedures were conducted under red light to avoid triggering reproduction. Following the treatment, all crosses underwent a recovery phase under red light (3 µmol photons m^{-2} s^{-1}) at 12 ± 0.5 °C. Crosses were randomly assigned to recovery durations of 1, 2, or 3 days. Subsequently, they were exposed to white LED light (MITRAS lightbars 150 Daylight, GHL Advanced Technology, Kaiserslautern, Germany) at an irradiance of 15 µmol photons m^{-2} s^{-1}, measured with a LI-COR LI-185B Photometer (LI-COR-Biosciences, Lincoln, NE, USA) in a 16:8 h light:dark cycle. The culture medium was refreshed every 10 days, by the replacement of 50 ml of half-strength PES per Petri dish.

Table 1: Overview of the performed crosses between female and male gametophyte strains of L. digitata (Dig), L. ochroleuca (Och), and L. pallida (Pal). Each cross was assigned a number (No.), which is used throughout the thesis for reference. Individual strain codes refer to female (F) and male (M) as designated by the Faro laboratory (Appendix A).

Cross	*No.*	*Individuals*	*Cross*	*No.*	*Individuals*
	1	Dig-F-18 x Dig-M-22		10	Dig-F-22 x Och-M-6.3
Dig ♀ x Dig ♂	2	Dig-F-9 x Dig-M-18	*Dig ♀ x Och ♂*	11	Dig-F-18 x Och-M-9.7
	3	Dig-F-22 x Dig-M-9		12	Dig-F-9 x Och-M-1.7
	4	Och-F-3.7 x Och-M-6.3		13	Och-F- 6.6 x Dig-M-22
Och ♀ x Och ♂	5	Och-F- 3.7 x Och-M-1.7	*Och ♀ x Dig ♂*	14	Och-F- 1.3 x Dig-M-9
	6	Och-F- 6.6 x Och-M-9.7		15	Och-F-3.7 x Dig-M18
	7	Pal-F-7.5 x Pal-M-1.3		16	Dig-F-9 x Pal-M-3.3
Pal ♀ x Pal ♂	8	Pal-F-3.1 x Pal-M-7.3	*Dig ♀ x Pal ♂*	17	Dig-F-18 x Pal-M-7.3
	9	Pal-F-1.2 x Pal-M-3.3		18	Dig-F-22 x Pal-M-1.3
				19	Pal-F-7.5 x Dig-M-9
			Pal ♀ x Dig ♂	20	Pal-F-1.2 x Dig-M-18
				21	Pal-F-3.1 x Dig-M-22

Quantification of ontogenetic stages

The relative abundance of four developmental stages (1: vegetative gametophytes, 2: gametophytes with oogonia, 3: gametophytes with released eggs, and 4: gametophytes with attached sporophytes) was assessed every 7 days over a 28-day period in at least 100 female gametophytes per replicate (n = 4) using an Olympus CKX41 inverted microscope (Olympus Co., Tokyo, Japan). Images of the gametophytes were taken using a Nikon D800 camera (Nikon Corporation, Japan). Structural counts were performed manually using the Simplest Manual Counter software (v1.1.1, open-source under the GNU General Public License). For each female gametophyte, the most advanced developmental stage was recorded if at least one cell within the multicellular gametophyte had reached that stage. Juvenile sporophytes were distinguished from released eggs by the presence of a first cell division, therefore zygotes were not counted separately but considered sporophytes.

Evaluation of reproductive success

After 21 days of growth, the percentage of female gametophytes containing sporophytes was assessed. After 28 days, the total number of sporophytes was counted, distinguishing between those with normal morphology and those with abnormalities. This counting was performed by examining 80 fields of view at 100× magnification.

Regular sporophytes are defined as fertilized diploid sporophytes that exhibit typical cell division patterns, have clear polar differentiation into a basal rhizoid and a proximal elongated blade, and which remain attached to the female oogonium. In contrast, abnormal sporophytes are considered unfertilized partheno-sporophytes that do not develop fully into healthy sporophytes, representing unsuccessful recruits. They are identified by two main criteria: (1) they are unattached and often lack rhizoids, and (2) they exhibit irregular morphologies (Martins et al., 2019). The parameters outlined above were used to evaluate the reproductive success of female gametophytes.

Statistical Analysis

All statistical analyses were performed using R version 4.5.0 (R Core Team, 2024), a free software environment for statistical computing and graphics. First, it was checked whether the data met the assumptions of normal distribution and homoscedasticity. The Shapiro-Wilk test and QQ-plots were used to assess normality, and Levene's Test or the Bartlett test were applied to evaluate homogeneity of variances. If both assumptions were met, a two-way ANOVA was conducted, followed by a Tukey HSD post-hoc test using the *stats* and *multcomp* packages. If assumptions were violated, the Kruskal-Wallis test and Dunn's test were used (*FSA* and *rstatix* packages). To correct for multiple comparisons in post-hoc tests, Bonferroni and Hochberg adjustment methods were applied. Statistical significance was accepted at $p < 0.05$ (R Core Team, 2024).

Cultivation in Ghent

Following an initial 4-week growth period in Faro, hybrid crosses were transferred to Ghent for continued cultivation. In the Ghent facility, cultures were maintained in 200 mL crystallizing dishes containing artificial seawater supplemented with Provasoli Enriched Seawater (PES) at a concentration of 10 mL/L. Cultures were exposed to continuous white light with an intensity ranging between 15–20 µmol photons m^{-2} s^{-1}. The culture medium was refreshed weekly to ensure optimal conditions. The largest and most vigorous sporophytes from each cross were selected and transferred to 2 L bottles, starting 8 weeks after the initial crossing. Aeration was provided to promote health and enhance growth rates, and the entire volume of medium was renewed biweekly. Growth was monitored by taking photographs of the largest sporophyte for each cross, after 3 weeks and again after 5 weeks in the 2L bottles.

After an additional 6 weeks of growth, a further selection of the most robust sporophytes were transferred to 10 L tanks equipped with aeration and maintained under the reduced light conditions of the MarBiol wet lab. From this stage onward, sterile natural seawater replaced artificial seawater in both 2L and 10L cultures, resulting in a decrease in salinity from approximately 32 ppt to 29 ppt. For the 10 L cultures, the nutrient medium was switched to F/2 (1 mL/L seawater; Cell-Hi F2P, Varicon Aqua), and environmental parameters were adjusted to a temperature of 14°C and a photoperiod of 12 hours light and 12 hours dark. Although light intensity was initially insufficient, a second TL lamp was added to improve illumination. However, light distribution remained uneven across cultures. The 10 L cultures were fully refreshed every two weeks until their final relocation to Ostend, after 18 weeks of cultivation in the 10 L tanks.

Genetic analysis of hybrid offspring

Tissue collection

Sporophytes were collected from the growing kelp crosses maintained in the lab. To minimize contamination, collection was performed using gloves and sterilized tools. After collection, the samples were gently dried with paper towels and placed in small bags filled with silica gel. As a desiccant, silica gel rapidly dehydrates the sporophyte tissue, preserving both the host material and its DNA. The silica-preserved samples were stored at room temperature until further laboratory processing.

DNA extraction, PCR and sequencing

The genomic DNA was extracted for sporophytes of all the different crosses using the OmniPrep protocol for Plant tissue by Svenja Heesch (2017), adapted for high-molecular-weight DNA extraction from brown algal tissue. The detailed protocol is provided in **Appendix B**.

Microsatellite markers, which are highly variable and species-specific, were used to confirm hybrid status and to identify the parental species. Microsatellite primers were used to select a region of the marker, which was then amplified by PCR, resulting in a fragment with length variations specific to each species. This enables the identification of two *Laminaria* genotypes in the hybrids based on the banding patterns observed on an agarose gel. Specifically, the microsatellite marker LolVVIV-23 was selected for its ability to clearly differentiate between *L. pallida*, *L. digitata* and *L. ochroleuca* (Coelho et al., 2014; Martins et al., 2019).

For the polymerase chain reaction (PCR) a TProfessional Thermocycler (Westburg, 2021) was used, by cycling samples through denaturation, annealing, and extension steps it amplified the target DNA sequences. The PCR settings outlined in the paper by Coelho et al. (2014) were applied to obtain clear patterns for all species simultaneously. The PCR products were analyzed using agarose gel electrophoresis, with the SmartLadder reference marker (200 bp–10 kb) from Eurogentec as a molecular weight standard. An image of the gel, colored with ethidium bromide, was taken with a ChemiDoc™ XR+ Imaging System (Bio Rad) and processed using Image Lab™ Software (Bio Rad). To optimize band resolution and overall amplification quality, several PCR parameters were tested: (1) halving the primer concentration and extending the gel run time, (2) increasing the annealing temperature from 55 °C to 58 °C, and (3) reducing the number of amplification cycles from 35 to 30 while maintaining an annealing temperature of 55 °C.

Finally, to confirm the hybrid status of samples and to rule out cross-contamination or parthenogenetic development, amplified DNA was analyzed on a Fragment Analyzer 5200 (Agilent Technologies). This high-resolution capillary electrophoresis system enabled precise, size-based separation of DNA fragments and generated electropherograms that clearly distinguished species-specific alleles from each parental genome.

Comparative study of the microbiome

Tissue collection

As part of a previous sampling campaign, microbiome analysis field swabs were collected from three macroalgal species: *L. digitata*, *L. ochroleuca*, and *Palmaria palmata*, as well as from a representative *Ulva* species. For the kelp species, swabs were taken from two tissue types, the blade and the meristem, due to known bacterial zonation across these regions (Ihua et al., 2020; Lemay et al., 2021). *L. digitata* samples were collected from four locations: Roscoff, France (30 May 2024); Gatteville-le-Phare, France (1 June 2024); Hoe Point, Plymouth, Cornwall (8 June 2024); and Goleen Harbour, Ireland (23 June 2024). *L. ochroleuca*, *P. palmata*, and *Ulva sp.* were collected at Hoe Point, Plymouth (8 June 2024). The decision was made to focus on these specific swabs, as they provide a base for comparing microbiome variation across geographically distinct sites, investigating tissue-specific microbial communities, and exploring interspecific differences between the main algal taxa of interest. Figure 5 provides a visualization of the sample sites, while **Appendix C** details the specific environmental conditions.

Figure 5: Geographic locations of field swab collection sites from earlier sampling campaigns.

Finally, swabs were taken from the center of the blade of each lab-grown cross, allowing for microbiome analysis of the two species, *L. ochroleuca* and *L. digitata*, along with their hybrids. Juvenile sporophytes that developed approximately eight weeks after crossing were collected, preserved in RNAlater (Thermo Fisher Scientific), and analyzed as whole individuals (referred to as 'baby sporophytes').

These samples provide an opportunity to compare lab-grown and field-derived microbiomes, as well as to investigate potential microbiome differences related to kelp age in the lab.

DNA extraction, PCR and sequencing

DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Aarhus, Denmark). Prior to extraction, the swabs were defrosted and centrifuged at 16,000 rpm for 20 minutes at 4 °C (Eppendorf Centrifuge 5425R) to concentrate microbial cells. To improve mechanical cell disruption, glass beads and lysis buffer were added to the pellet, and the samples were subjected to bead beating for 10 minutes. These modifications were implemented to ensure efficient lysis of bacterial cells commonly found in marine biofilms. Following this step, the standard QIAamp protocol was carried out as described by the manufacturer.

PCR amplification targeted the full-length 16S rRNA gene using primers 27F_BCtail-FW (TTTCTGTTGGTGCTGATATTGC_AGAGTTTGATCMTGGCTCAG) and 1492R_BCtail-RV (ACTTGCCTGTCGCTCTATCTTC_CGGTTACCTTGTTACGACTT) which included 5' extensions for barcode attachment. PCR was conducted with the Phire Tissue Direct PCR Master Mix (Thermo Fisher Scientific) under the following conditions: initial denaturation at 98°C for 3 minutes, followed by 30 cycles of 98°C for 8 seconds, 60°C for 8 seconds, and 72°C for 30 seconds. A final extension step was held at 72°C for 3 minutes. Gel electrophoresis confirmed amplification success. DNA quality was assessed, and each sample was barcoded using the Oxford Nanopore PCR Expansion 1–96 barcode primers (Oxford Nanopore Technologies, UK) following a specific PCR protocol: 95 °C for 3 minutes, 13 cycles of 95 °C for 15 seconds, 62 °C for 15 seconds and 65 °C for 40 seconds. Followed by a final extension at 65 °C for 2 minutes. DNA concentration was quantified using a Qubit fluorometer (Thermo Fisher Scientific Inc., USA). Library preparation followed the Oxford Nanopore Ligation and PCR Barcoding Kit protocol, and sequencing was performed on the MinION platform (Oxford Nanopore Technologies, UK) using high-accuracy basecalling.

Statistical analysis

Sequencing data were processed on a local server accessed via MobaXterm, connecting to the UGent Django environment. Taxonomic classification was performed using the EMU classifier (Curry et al., 2022) in combination with the SILVA 16S rRNA database. Read filtering was applied to retain sequences between 1300–1800 bp. Chloroplast and mitochondrial reads, as well as those lacking a phylum-level assignment, were filtered out after creating a phyloseq object in R (v4.5.0) using the phyloseq package (McMurdie & Holmes, 2013). Only bacterial OTUs assigned to a known genus were retained for downstream analysis. Samples with fewer than 60,000 reads, as well as mock controls, were excluded. Data were normalized through rarefaction to the sequencing depth of the least abundant sample.

Subsequent statistical analyses were performed in R. The OTU table was normalized to relative abundances, and Bray–Curtis dissimilarities were computed using the phyloseq and vegan packages. These distances were then used to visualize microbial community differences through non-metric multidimensional scaling (NMDS) with the ordinate function in phyloseq (method = "NMDS"). Samples were color-coded by variables such as Location, Tissue type, and Organism, with 95% confidence ellipses added for visual clarity.

To formally test whether microbial community compositions differed between locations and host species, permutational multivariate analysis of variance (PERMANOVA) was conducted using the adonis2 function from the vegan package. The significance was assessed using 999 permutations. To ensure the validity of the PERMANOVA results, homogeneity of multivariate dispersion was tested using the betadisper and permutest functions in vegan. This assessed whether the variation within groups (i.e., the dispersion) differed significantly among countries and organisms. If significant differences in dispersion were detected, they were considered when interpreting the PERMANOVA results. Plots were created using ggplot2 or base R functions for visualization of NMDS and dispersion analyses.

Microbial community composition was also analyzed at the genus level. Relative abundances were calculated and filtered to identify dominant genera, which were visualized with violin and ridge plots to compare lab crosses and geographic locations. Core microbiomes were defined per group (location or cross type) based on ≥80% prevalence and ≥1% mean relative abundance thresholds. Venn diagrams and tables were generated to illustrate shared and unique core taxa among groups. Statistical differences in taxon abundances across groups were assessed using Kruskal-Wallis tests and ANOVA with post-hoc Tukey comparisons.

Sorus induction

Blade tissue from sporophytes of *L. digitata*, *L. ochroleuca* and their hybrids was used to assess sorus induction as an indicator of fertility. The sporophytes had been cultivated for 32 weeks and were considered sufficiently mature for sorus induction. For each cross, the three largest individuals (≥5 cm) were selected, yielding a total of 36 samples. Tissue segments were taken from the middle third of the blade, at least 3 cm above the meristem, with the apical tip removed. This method is based on Pang & Lüning (2004), who demonstrated that exclusion of the meristem reduces hormonal inhibition of sporogenesis. Each fragment was placed in 150 mL of sterile seawater enriched with 20 mL/L of half-strength PES to ensure sufficient nutrient availability, as nutrient enrichment has been shown to promote sorus formation in kelps (Boderskov et al., 2021).

Cultures were maintained at 12 °C under a 12 h light / 12 h dark cycle, with a light intensity of approximately 30 µmol photons m^{-2} s^{-1}. Constant short-day conditions under controlled temperature and light have been shown to promote sporogenesis in kelps (Pang & Lüning, 2004). Half of the medium in the petri dishes was substituted with fresh medium on a weekly basis. Every 2 weeks, samples were monitored both visually and microscopically for the development of sori. Sorus formation was scored on a scale of 0 to 2: 0 = no sori, 1 = weak or localized sori, and 2 = strong sori across multiple areas.

Flow cytometric ploidy assessment

To assess ploidy levels, healthy sporophytes (~5 cm) resulting from the crosses involving *L.digitata* and *L. ochroleuca* were placed in 50 mL Falcon™ tubes designed to allow adequate aeration and transported to the Botanic Garden of Meise (Plantentuin Meise) to conduct flow cytometric analyses using a Partec PAS III flow cytometer (Sysmex). Blade tissue samples were processed using the CyStain™ PI Absolute P kit (Sysmex), which includes a nuclei extraction buffer and staining solution containing propidium iodide (PI). Approximately 0.5 cm² of blade tissue was chopped with a sharp razor blade in the extraction buffer, filtered through a 50 µm mesh, and incubated with PI stain according to the manufacturer's protocol. Measurements were performed using standard settings optimized for PI fluorescence (Koutecký et al., 2023) and data were recorded with FlowMax software (Sysmex). Table 2 provides an overview of the expected fluorescence peaks associated with different ploidy levels. Each of the intraspecific crosses of *L. digitata* and *L. ochroleuca*, as well as the *L. digitata* ♀ × *L. ochroleuca* ♂ hybrid, was analyzed by measuring at least 5,000 nuclei per sample. A known diploid *L. digitata* was used as an internal standard to determine relative ploidy levels.

Table 2: Predicted arbitrary peaks corresponding to various potential ploidy types. While parents are anticipated to be diploid, multiple possibilities are outlined for the hybrids.

Type	DNA content	Arbitrary peak
Diploid (L. dig & L. och)	2C	100
Diploid Hybrid	2C	100
Alloploid Hybrid	4C (2C + 2C)	200
Triploid Hybrid	3C (2C + 1C)	150
Aneuploid Hybrid	2.5–3.5C	125 - 175

RESULTS

Reproductive success

Figure 6 illustrates the reproductive structures observed in both intraspecific *L. digitata* crosses and interspecific *L. digitata × L. ochroleuca* crosses. All structures, including those in the hybrids (Figure 6b), appear to be normally developed here, with no abnormalities observed.

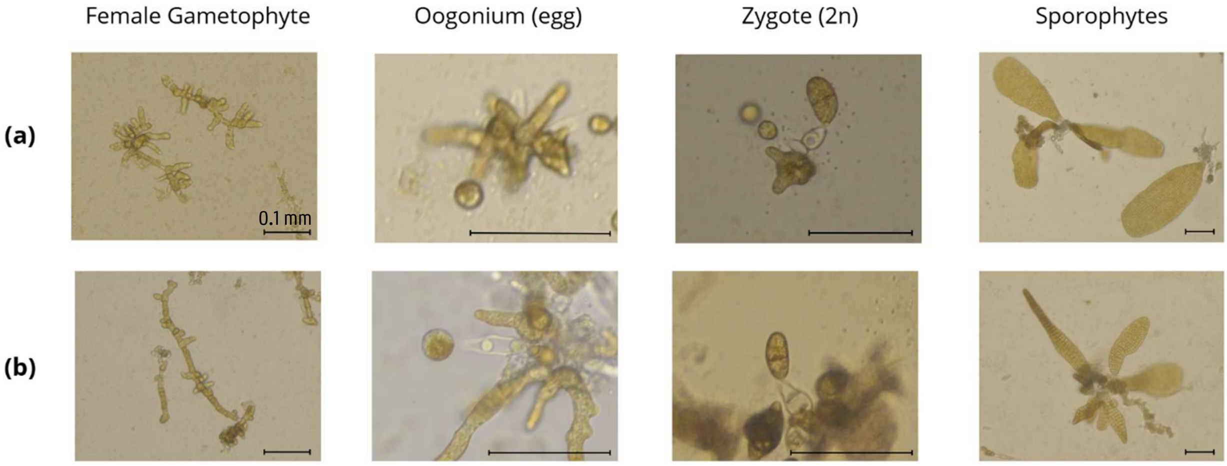

Figure 6: Microscopic images showing the different developmental stages of (a) intraspecific L. digitata crosses and (b) interspecific L. digitata × L. ochroleuca crosses. The black line indicates a scale of 0.1 mm.

The mean sporophyte percentage of the different crosses, displayed over a period of 3 weeks, is shown in Figure 7. Nearly 60% of the gametophytes in the Dig ♀ × Och ♂ cross produced sporophytes after 21 days. The crosses with the next highest percentages were the intraspecific Dig ♀ × Dig ♂ cross and the Dig ♀ × Pal ♂ cross. These results suggest that crosses involving *L. digitata* females exhibit the highest reproductive success rate. In contrast, other crosses yielded sporophyte percentages below 30%, with the intraspecific Och ♀ × Och ♂ cross having the lowest percentage (5%). Crosses with female *L. pallida* showed nearly no sporophytes after 14 days, but after 21 days this increased by 20%, indicating that these female gametophytes required more time to produce sporophytes. The crosses involving *L. ochroleuca* females exhibited a less steep slope between days 14 and 21, with the intraspecific cross showing a slight decrease in sporophyte percentage.

The proportion of reproductive gametophytes, defined as gametophytes bearing early-stage sporophytes or/and eggs, varied significantly over time. Using the Dig ♀ × Dig ♂ on Day 7 as a reference (intercept: $\beta = 0.249$, $p < 0.001$), reproductive development increased significantly over time, with positive effects observed on Day 14 ($\beta = 0.450$, $p < 0.001$) and Day 21 ($\beta = 0.523$, $p < 0.001$). However, this increase depends on the cross. For instance, the increase in reproductive gametophytes from day 7 to day 21 was greater for Dig ♀ × Dig ♂ than for Pal ♀ × Pal ♂ crosses.

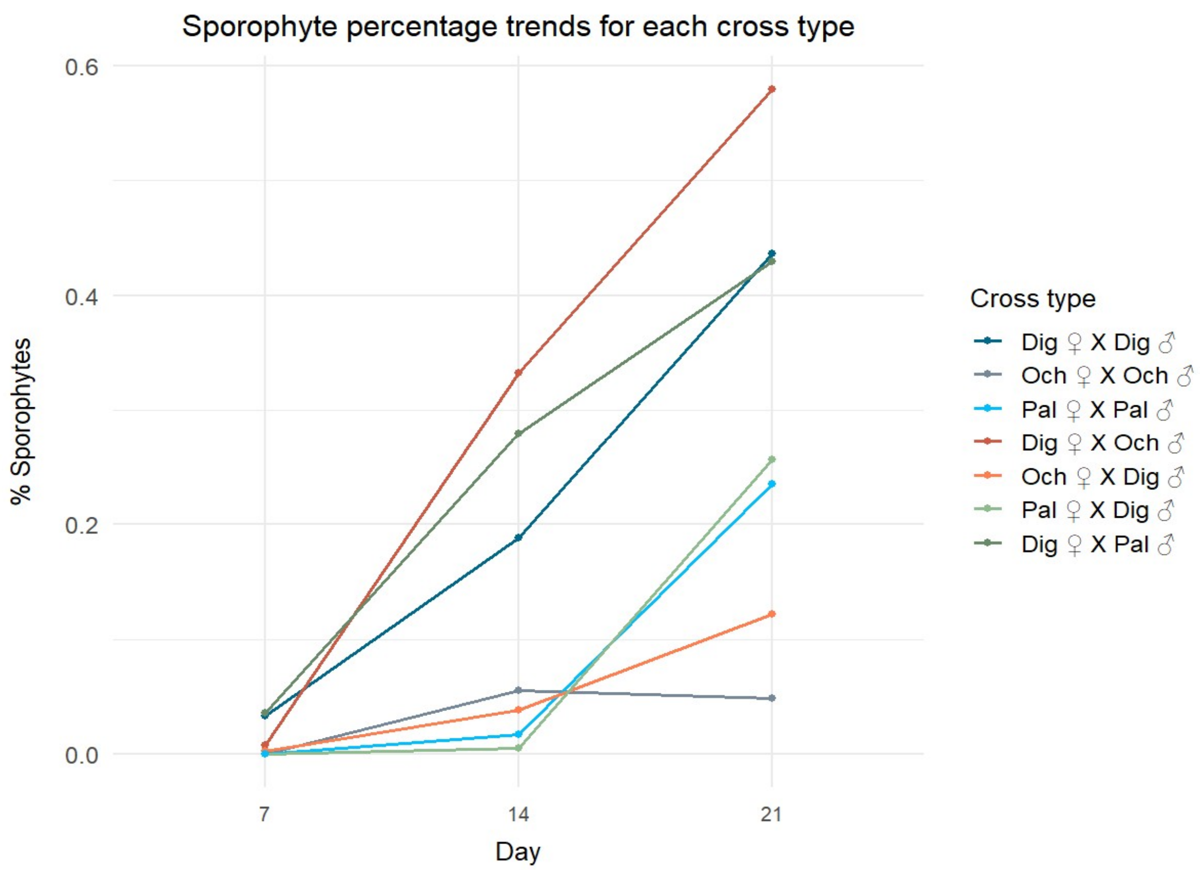

Figure 7: The average sporophyte percentage for the different cross types in function of the development. The abbreviations Och, Dig and Pal refer to L. ochroleuca, L. digitata and L. pallida, respectively. Symbols indicating the female (♀) or male (♂) used in each cross.

Figure 8 illustrates the sporophyte percentage after 21 days. Sporophyte percentage reflects reproductive efficiency by showing the proportion of gametophytes that developed sporophytes. The hybrid cross Dig ♀ × Och ♂ shows the highest mean sporophyte percentage (57.9%), followed by Dig ♀ × Dig ♂ (43.6%) and Dig ♀ × Pal ♂ (42.9%). It is worth noting again that all of these crosses involve a *L. digitata* female. Och ♀ × Och ♂ showed the lowest sporophyte development (4.9%), with a median near zero and a single outlier elevating the mean. Similarly, Och ♀ × Dig ♂ exhibited a low median, with several replicates close to zero. The Pal ♀ × Dig ♂ cross displayed substantial variability, as reflected by broad interquartile ranges. Outliers were especially notable in Och ♀ × Och ♂ (upper end) and Och ♀ × Dig ♂ (lower end), highlighting inconsistency among replicates. As seen in Figure 8, error bar lengths vary between crosses, with shorter bars indicating more uniform results and longer bars suggesting greater variability.

Boxplots showing sporophyte percentages for Day 7 and Day 14 are displayed in **Appendix D**. By Day 7, only the intraspecific Dig ♀ × Dig ♂ and the hybrid Dig ♀ × Pal ♂ crosses exhibited reproductive activity, with initial sporophyte development already evident. By Day 14, sporophyte development was observed within all crosses, though the extent varied widely.

A Kruskal-Wallis test was conducted to evaluate differences in reproductive efficiency among the seven crosses, revealing a statistically significant overall difference ($\chi^2 = 29.726$, $p = 4.432 \times 10^{-5}$). Significant differences between specific cross types, identified through pairwise comparisons using Dunn's test with Bonferroni correction, are presented in **Appendix E**. No significant differences were detected between hybrid crosses involving *L. ochroleuca* and *L. digitata*, and their respective interspecific parental combinations. A Mann-Whitney U test (Wilcoxon rank-sum) found no significant difference in reproductive efficiency between the interspecific and intraspecific groups at Day 21, suggesting similar reproductive success.

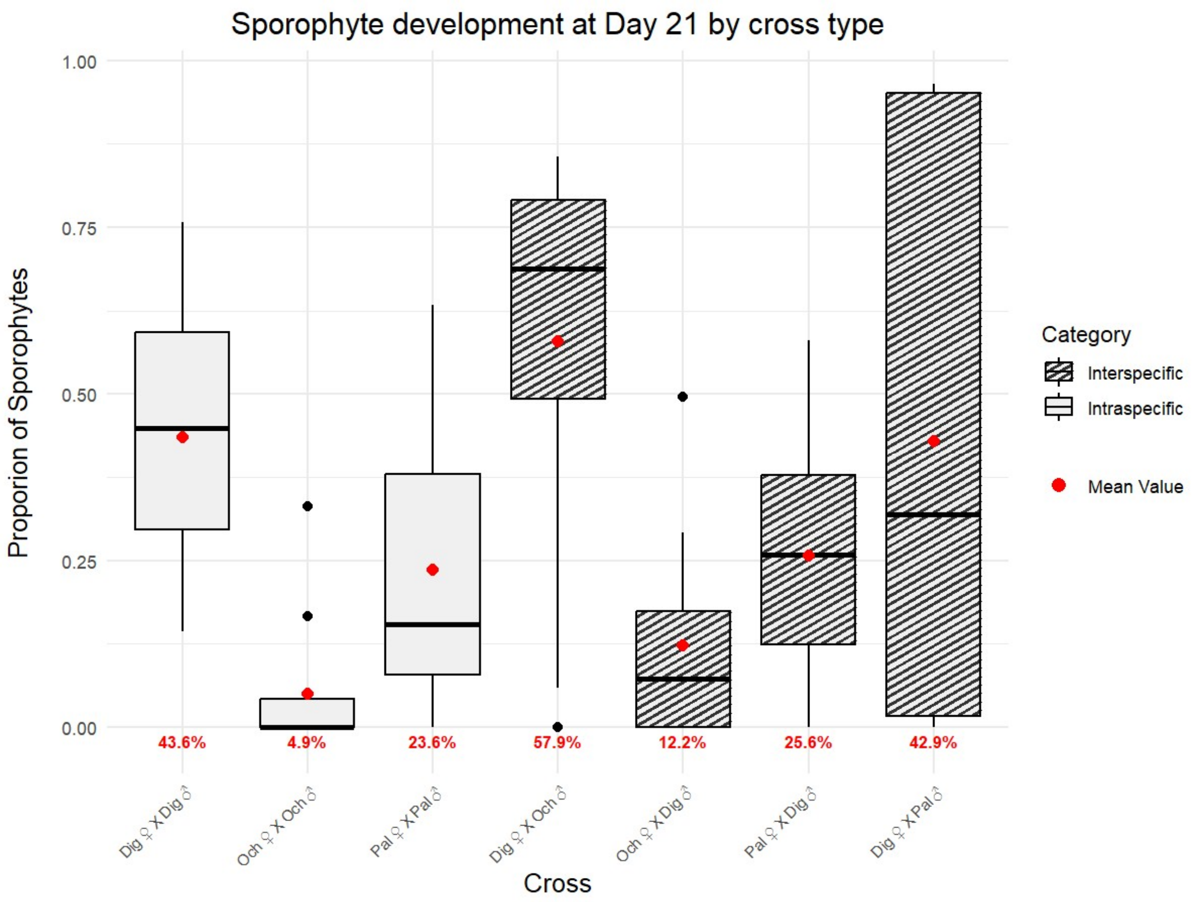

Figure 8: Proportion of sporophyte development on day 21 across different crosses. Boxplots show the median (line), interquartile range (box), and range excluding outliers (whiskers). Outliers are shown as black dots, while red dots indicate the mean. Cross types are labeled by species abbreviations (Dig, Och, Pal), with female (♀) and male (♂) symbols. Intraspecific crosses are white; hybrid crosses are striped. Error bars indicate standard deviations (SD) across replicates.

Parthenogenesis is frequently observed in kelp species (Martins et al., 2019). Therefore the number of abnormal sporophytes was quantified after 4 weeks and compared to normally developed sporophytes, see Figure 9. The mean abnormality rate over all crosses was 10.5%, significantly greater than zero ($p = 0.0045$), indicating parthenogenesis has occurred. While abnormal morphologies appeared in all crosses, it was not the dominant mechanism. Some crosses, like Dig ♀ × Pal ♂, had minimal abnormalities, while others, such as Och ♀ × Och ♂ and Dig ♀ × Dig ♂, showed ≥10% abnormal sporophytes. Intraspecific crosses had a slightly higher mean abnormality (12.2%) compared to interspecific crosses (9.3%), though this difference was not statistically significant. Crosses without sporophyte development after 28 days were excluded, leading to unequal sample sizes which limits the precision of the estimates.

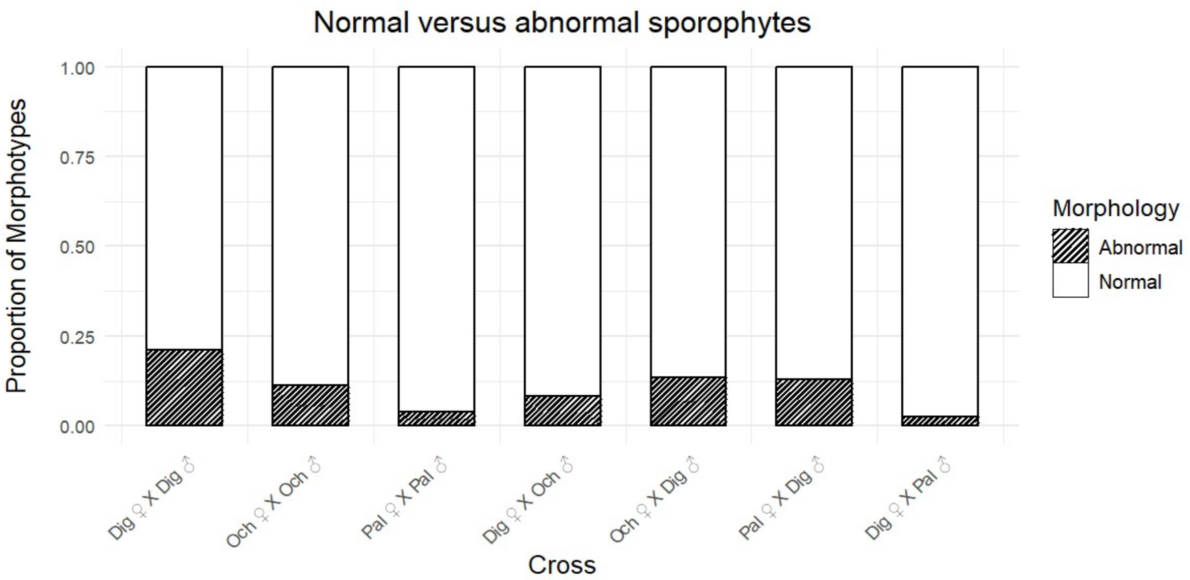

Figure 9: The proportion of normal (white) and abnormal (striped) sporophytes in different crosses, counted after 28 days of growth. The x-axis represents the various crosses, while the y-axis indicates the proportion, ranging from 0 to 1 (or 0% to 100%). The legend differentiates between normal (white) and abnormal (striped) morphology.

Reproductive efficiency was quantified by counting the number of normally developed sporophytes after 28 days of growth. This value represents the proportion of successfully developed sporophytes per gametophyte, excluding those with abnormal morphology. Mean reproductive success, expressed as a percentage, provides an estimate of the efficiency of each cross in transitioning from fertilized gametophyte to sporophyte stage. The results are presented in Figure 10. Overall, a similar trend is observed as on Day 21.

Among the intraspecific crosses, Dig ♀ × Dig ♂ exhibited the highest reproductive success, with a mean of 47%, indicating that, on average, 47 sporophytes developed per 100 gametophytes. In contrast, Och ♀ × Och ♂ demonstrated lower success, with a mean of 10.2% and a median close to zero, suggesting limited reproductive performance. Pal ♀ × Pal ♂ showed a moderate success rate of 36.9%, outperforming Och ♀ × Och ♂ but remaining below most interspecific crosses. Hybrids generally showed higher reproductive efficiency and greater variability. The Dig ♀ × Och ♂ cross shows the highest mean success (86.7%) and a broad distribution, indicating both high productivity, but also substantial variation. Pal ♀ × Dig ♂ and Och ♀ × Dig ♂ also performed well with moderate to high success. Interspecific crosses exhibited wider interquartile ranges and longer whiskers, reflecting greater variability, while intraspecific crosses were more consistent. Several outliers, especially in the Och ♀ × Och ♂ and Och ♀ × Dig ♂ cross, indicate exceptionally successful replicates. Individual replicate data is visualized in **Appendix F**.

Kruskal-Wallis and Dunn's post-hoc tests found no significant differences among the seven. In contrast, the Wilcoxon rank sum test indicated a significant difference between interspecific and intraspecific crosses ($W = 1091$, $p = 0.0398$), with interspecific crosses performing better. However, this difference disappeared when the Och ♀ × Och ♂ cross was excluded, suggesting that this cross had a disproportionate impact on the result.

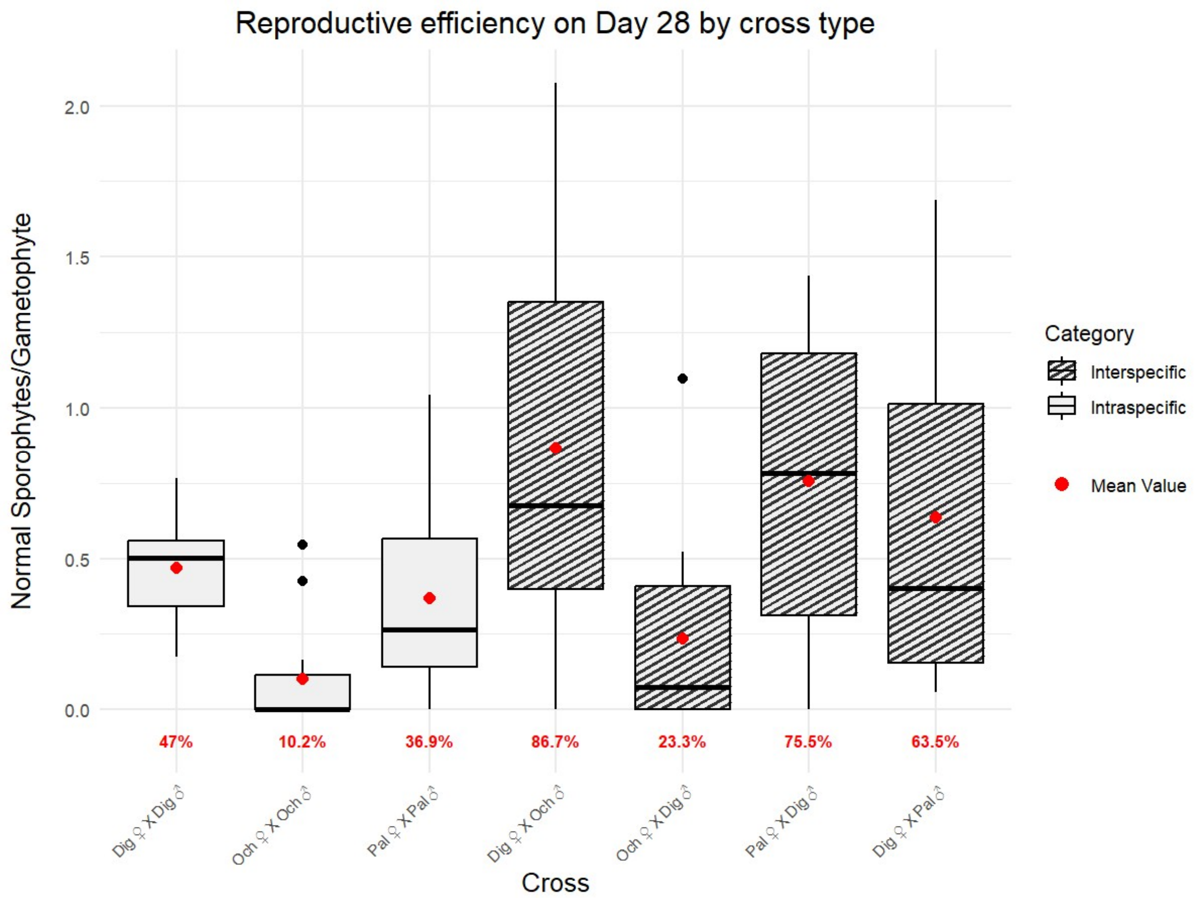

Figure 10: Sporophyte density per gametophyte after 28 days. Cross types are labeled with species abbreviations (Dig, Och, Pal), with symbols indicating the female (♀) and male (♂) parent. Each boxplot shows the median (line), interquartile range (box), and outliers (points beyond the whiskers). White boxes represent intraspecific crosses; striped boxes represent interspecific crosses.

Cultivation in Ghent

Figure 11 clearly demonstrates the variation in growth rates and morphology across the crosses. Note that all crosses eventually developed sporophytes, which was not yet the case after 4 weeks. At 8 weeks post-crossing, the largest sporophytes were transferred to aerated 2L bottles, after which a noticeable acceleration in growth was observed. The images presented in Figure 11 were captured 5 weeks following this transfer. Crosses 6, 13 and 14 appeared comparatively underdeveloped. In contrast, cross 4 looked exceptionally large, likely due to its older age of 14 weeks at the time the image was taken. Despite limited reproductive success at four weeks, the intraspecific *L. ochroleuca* crosses eventually showed noticeable growth. Figure 11 clearly shows that crosses with *L. digitata* females performed better and grew larger sporophytes, this seems to be a recurring trend.

Intraspecific and interspecific crosses involving *L.pallida* were also assessed. Intraspecific Pal ♀ × Pal ♂ crosses exhibited consistent growth, whereas hybrid crosses showed highly variable performance; crosses 17, 18 and 21 grew poorly, while crosses 16 and 19 displayed vigorous and robust growth. These results are shown in **Appendix G.**

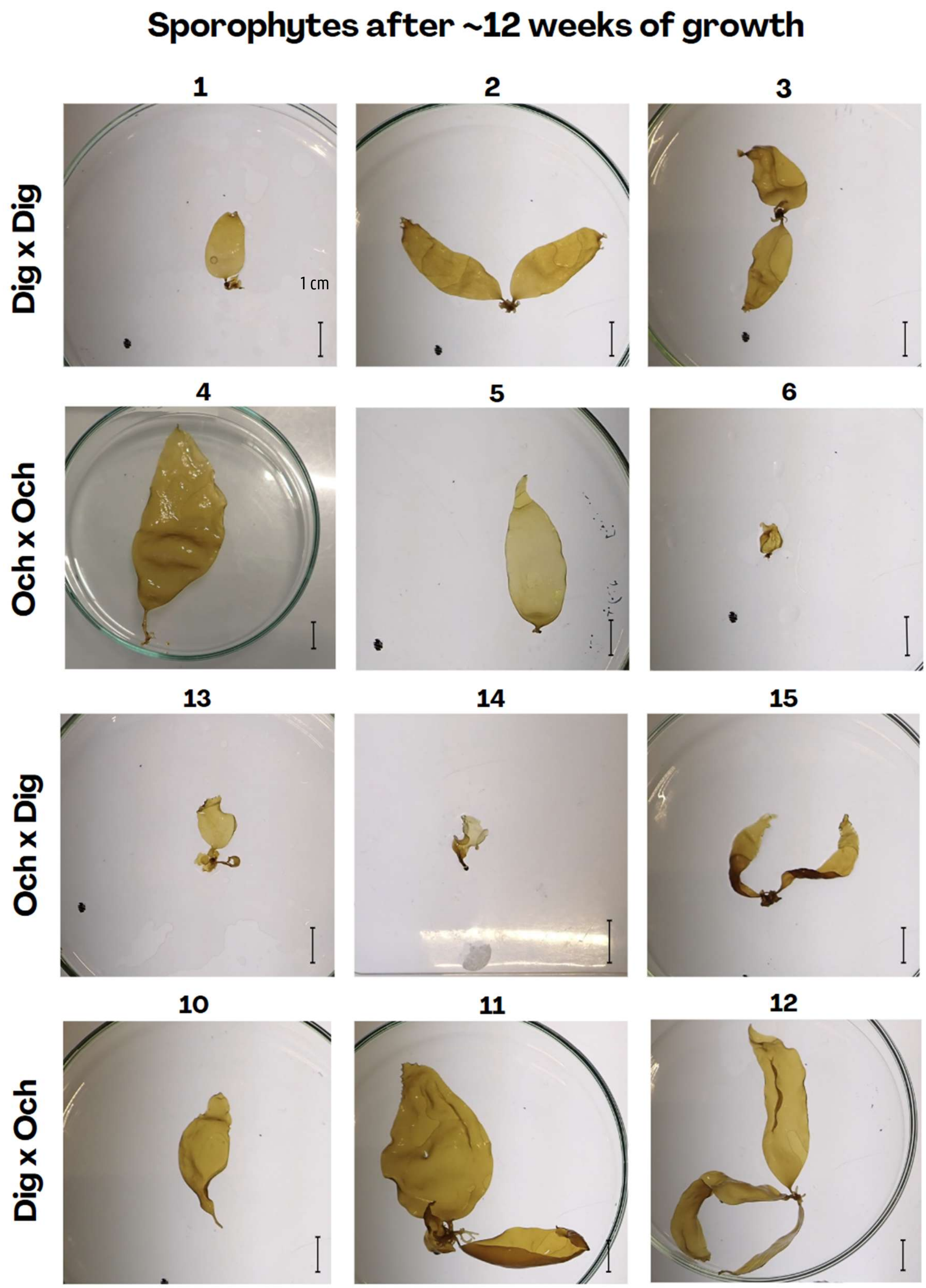

Figure 11: Sporophytes after 5 weeks of growth in aerated 2L bottles, from both intraspecific crosses (Dig × Dig; Och × Och) and reciprocal interspecific crosses (Dig × Och; Och × Dig). Och and Dig refer to L. ochroleuca and L. digitata, respectively. The total age of the sporophytes ranged from 12 weeks (no. 2, 3, 6, 10, 11, 12, 15), to 13 weeks (no. 1, 13), to 14 weeks (no. 4) and up to 15 weeks (no. 5, 14). The black line represents a 1 cm scale.

Genetic confirmation of hybrid status

Fragment analysis confirmed the hybrid status of the interspecific crosses, as individuals exhibited species-specific peaks corresponding to both parental taxa, see **Appendix H**. In the reciprocal *L. digitata* x *L. ochroleuca* hybrids, clear bands corresponding to both parental species were detected, along with some smearing and additional bands. Cross no. 14 showed only maternal *L. ochroleuca* DNA, with no *L. digitata*-specific peaks. Two sporophytes from this cross were tested and both showed the same pattern, indicating parthenogenetic development.

The microsatellite marker LolVVIV-23 failed to amplify consistently in the *L. pallida* crosses, with smearing and more bands than expected, even in intraspecific crosses. The bands of *L. digitata* and *L. pallida* also migrated very closely on the gel, making it difficult to reliably distinguish between them in the hybrid samples. Furthermore, fragment analysis was also inconclusive for *L. pallida*, as the banding patterns did not match those previously described by Martins et al. (2019).

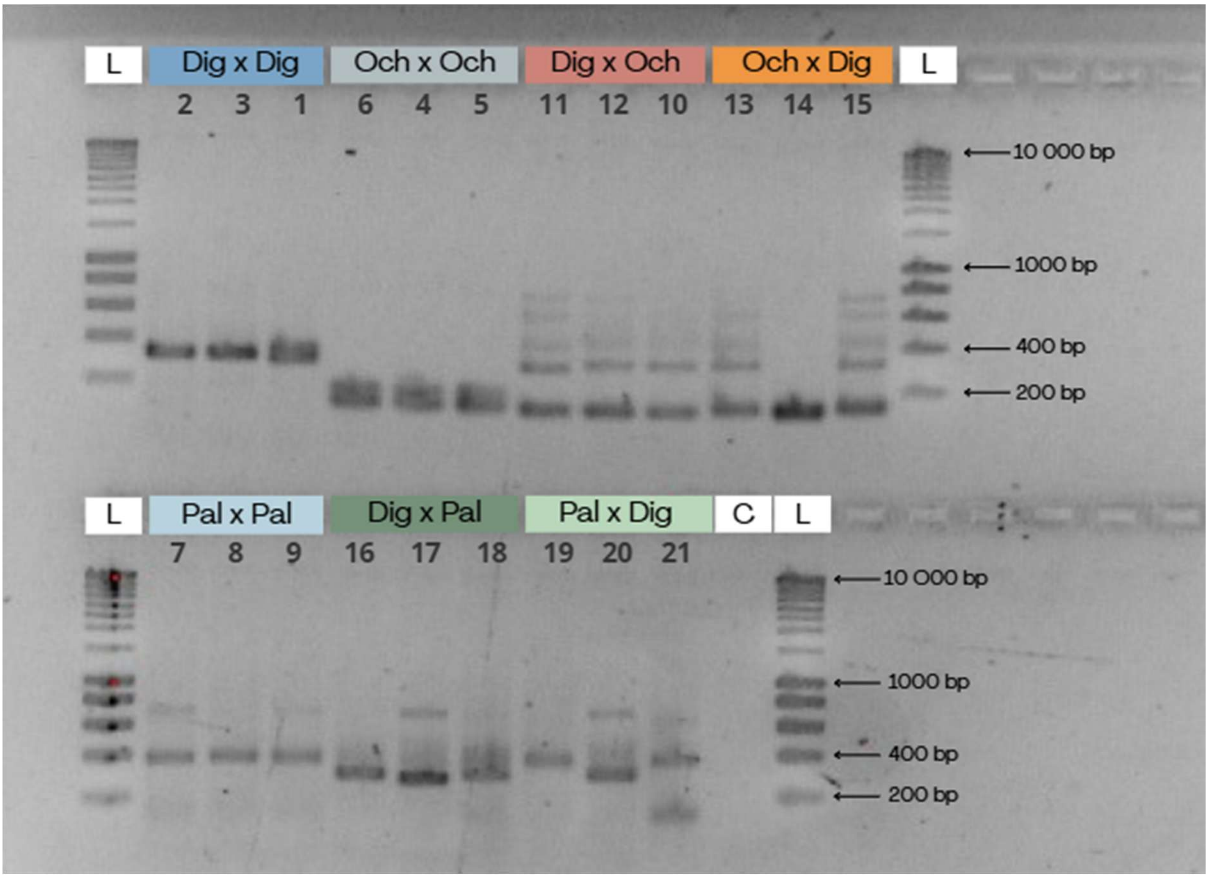

Figure 12: Electrophoresis pattern of microsatellite marker LolVVIV-23 products amplified in sporophytes derived from intraspecific (Dig x Dig; Och x Och, Pal x Pal) and reciprocal interspecific crosses (Dig x Och; Och x Dig; Pal x Dig; Dig x Pal). The numbers represent specific crosses, see ***Table 1****.*

It is important to note that the parthenogenic cross (no. 14) was included in earlier results, such as those presented in **Figure 9**, where it did not display a high percentage of abnormality. Figure 13 provides images of this cross after 28 days, illustrating that early sporophyte development appeared morphologically normal. In this cross, two replicates did not produce any sporophytes, while the remaining two replicates showed 86% normally developed sporophytes. As shown in Figure 13, this cross primarily contained normal sporophytes (A) and (B), with a smaller proportion of parthenogenic sporophytes (C).

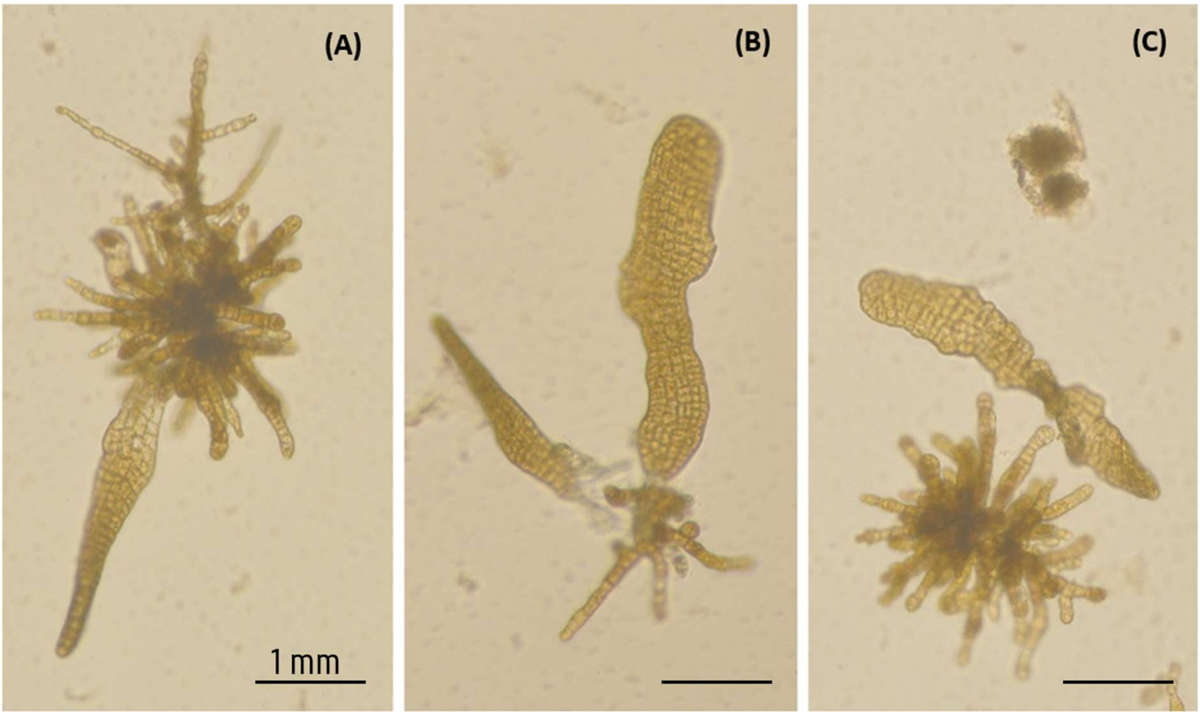

Figure 13: Microscopic images taken after 28 days, captured under 10× magnification. Panels (A) and (B) depict sporophytes classified as normal during the counting process, while panel (C) illustrates an abnormal sporophyte. The black line equals 1 mm.

Microbiome comparison

First, general patterns of microbiome composition are described, followed by an in-depth exploration of specific research questions, including variations between tissue types, geographic locations, and the main species of interest (*L. digitata* and *L. ochroleuca*). Finally, microbiomes from the laboratory crosses are examined in detail.

General microbiome patterns

To investigate the structure of microbial communities associated with *Laminaria* blades, non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities was performed. In this ordination, samples positioned closer together represent more similar microbial communities, while those farther apart reflect greater compositional dissimilarity. The NMDS axes are arbitrary but effectively summarize the main gradients in microbial community structure. Figure 14 provides an overview of microbial community variation, based solely on blade swab samples from the *Laminaria* species of interest.

As illustrated in Figure 14, samples clustered clearly by geographic origin. UK field samples (Hoe Point and Martin's Haven) formed compact clusters, suggesting consistent microbial communities across these sites. In contrast, samples from Gatteville-le-Phare (France) showed greater spatial separation, suggesting increased variability and compositional divergence relative to those from Roscoff (France) or the UK. The Ghent laboratory samples, representing cultured individuals, formed a separate and well-defined cluster on the right-hand side of the ordination, separated from the field samples along the first NMDS axis.

These spatial patterns were statistically supported by PERMANOVA analysis of field-collected samples, which revealed a significant effect of Location on microbial composition ($p = 0.001$, $R^2 = 0.388$), explaining nearly 39% of the total variation. This highlights geography as a key determinant of microbiome structure in natural *Laminaria* populations. In addition, a test for multivariate dispersion (betadisper) indicated significant differences in within-site variability ($p = 0.004$), with mainly Roscoff (France) showing higher dispersion compared to the other sites.

Differences between *Laminaria* species and hybrids are also illustrated in Figure 14. Within the Ghent cluster, there is no clear separation based on organism type. In the UK, Hoe point samples, *L. digitata* and *L. ochroleuca* (represented by diamonds and stars, respectively) exhibited some separation in the NMDS space, suggesting potential microbiome differentiation under the same environmental conditions.

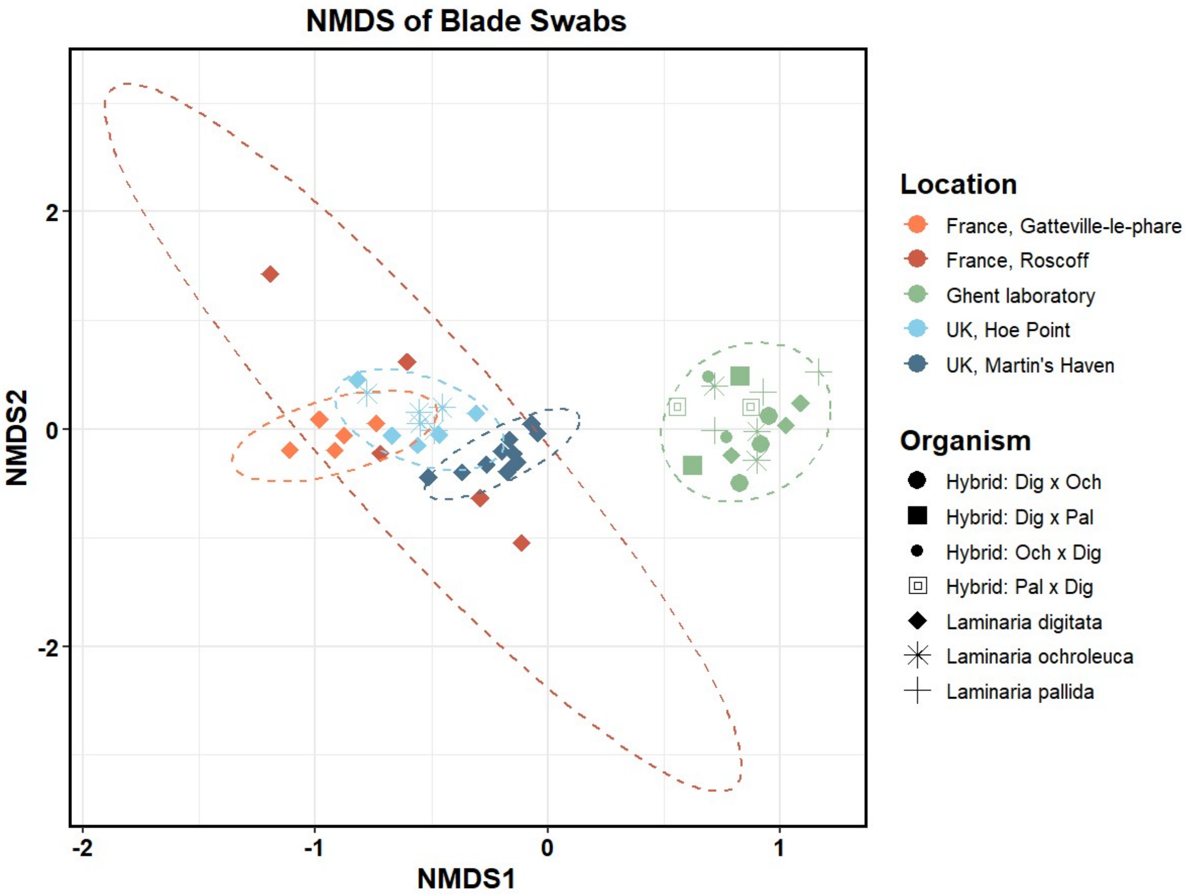

Figure 14: Beta diversity of bacterial communities associated with Laminaria blade swabs. Points represent individual samples, colored by site and shaped by organism identity (L. digitata, L. ochroleuca, L. pallida and hybrids). Ellipses indicate 95% confidence intervals by sample location. The plot highlights geographic clustering and environmental influences on microbial composition.

Appendix I presents an NMDS plot illustrating microbiome variation across different algal clades. Other species such as *Ulva* sp., *Palmaria palmata* and *Saccharina hyperborea* formed clearly separated clusters from the *Laminaria* group. *Ulva* sp., in particular, exhibited tight clustering and substantial separation from other taxa.

Thallus region and geographic location

Figure 15 depicts an NMDS ordination based on Bray-Curtis dissimilarities illustrating microbial community composition on *L. digitata*, across kelp tissues (blade vs. meristem) and sample sites. Figure 15 illustrates partial clustering of microbial communities by geographic location, although substantial overlap persists among sites. Samples from France (Roscoff) exhibit broader dispersion along the NMDS1 axis, whereas those from Ireland and the UK vary more along NMDS2. Within each location, blade and meristem tissues do not form distinct clusters, however blade samples do appear slightly shifted toward higher NMDS2 values, but this pattern is subtle.

PERMANOVA revealed that tissue type significantly influenced microbial composition at Goleen Harbour ($R^2 = 0.27$, $p = 0.01$) and Martin's Haven ($R^2 = 0.18$, $p = 0.031$). Marginal effects were found at Gatteville-le-Phare and Hoe Point ($p \approx 0.05$), with no effect at Roscoff ($p = 0.613$). A significant tissue × location interaction ($p = 0.001$) indicates that tissue effects vary by site. Conversely, location alone strongly shaped microbiomes within each tissue type, explaining 36.6% of the variation in blades and 35.8% in meristems (both $p = 0.001$). Together, these results highlight the complex interplay between host tissue and environmental context in structuring kelp-associated microbial communities.

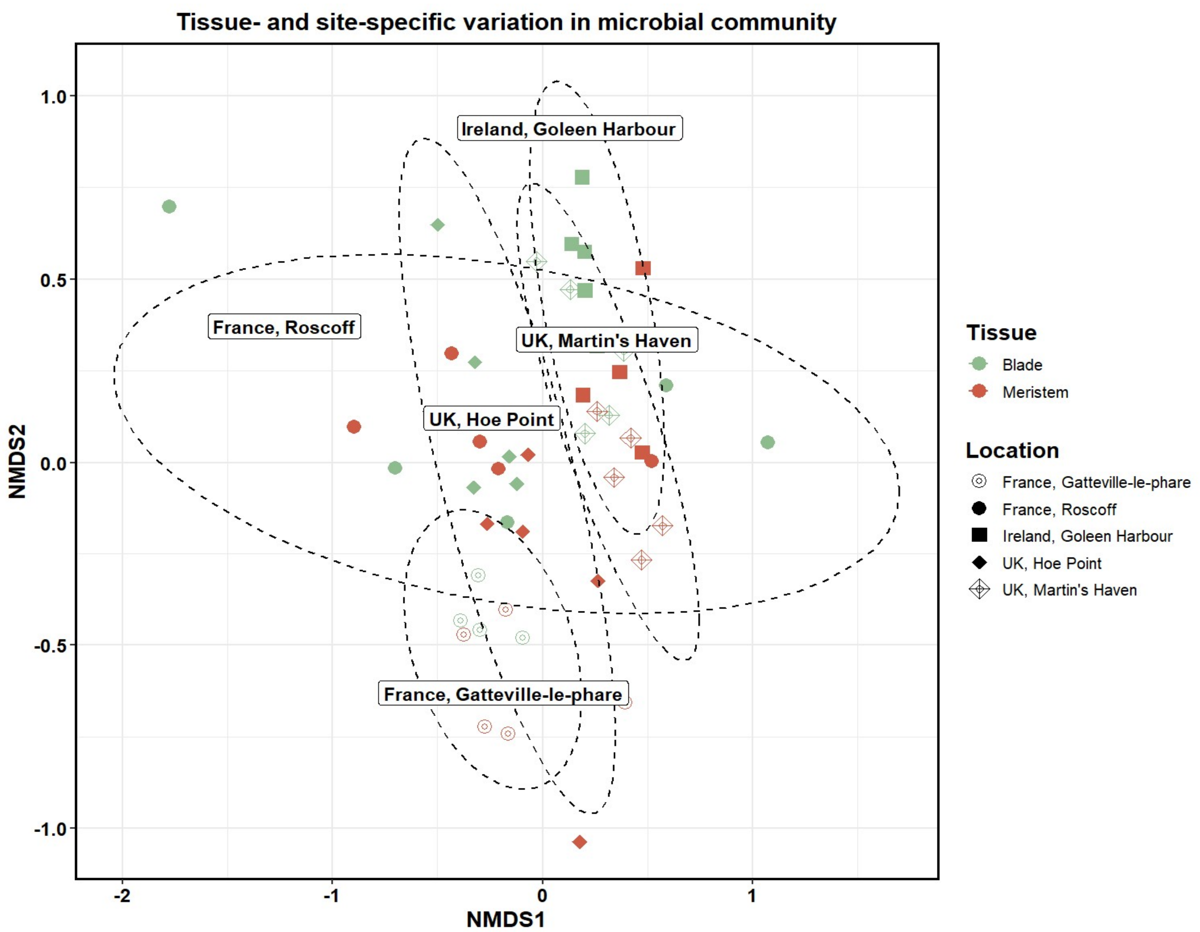

Figure 15: Microbiome composition across thallus regions of L. digitata from different field locations. Each point represents a sample, color-coded by tissue type and shaped by country of origin. Ellipses indicate 95% confidence intervals around group centroids, reflecting dispersion within sample sites.

Figure 16 highlights order-level microbial diversity and distribution across geographic locations and thallus regions, providing insights into ecological variation among microbial communities on *L. digitata*. Distinct differences in community composition are observed between sampling sites, with certain bacterial orders more prevalent at specific locations. Within the France panel, Roscoff (LDFRx) and Gatteville-le-Phare (LDFGx) exhibit notably different microbial profiles, with Enterobacterales being considerably more abundant in Gatteville-le-Phare.

The two thallus regions harbor distinct microbial communities, with certain bacterial orders preferentially associated with either blades or meristems (Figure 16). Bacterial taxa grouped as 'Other', represent the combined contribution of taxa outside the top 8 most abundant. These collectively account for a substantial portion of microbiome variation (Blade: 44.1%, Meristem: 35.6%). The abundances of Pseudomonadales, Chitinophagales, Rhodobacterales, and Flavobacteriales exhibit minimal variation, while Caulobacterales and Enterobacterales show slight enrichment in the Meristem region.

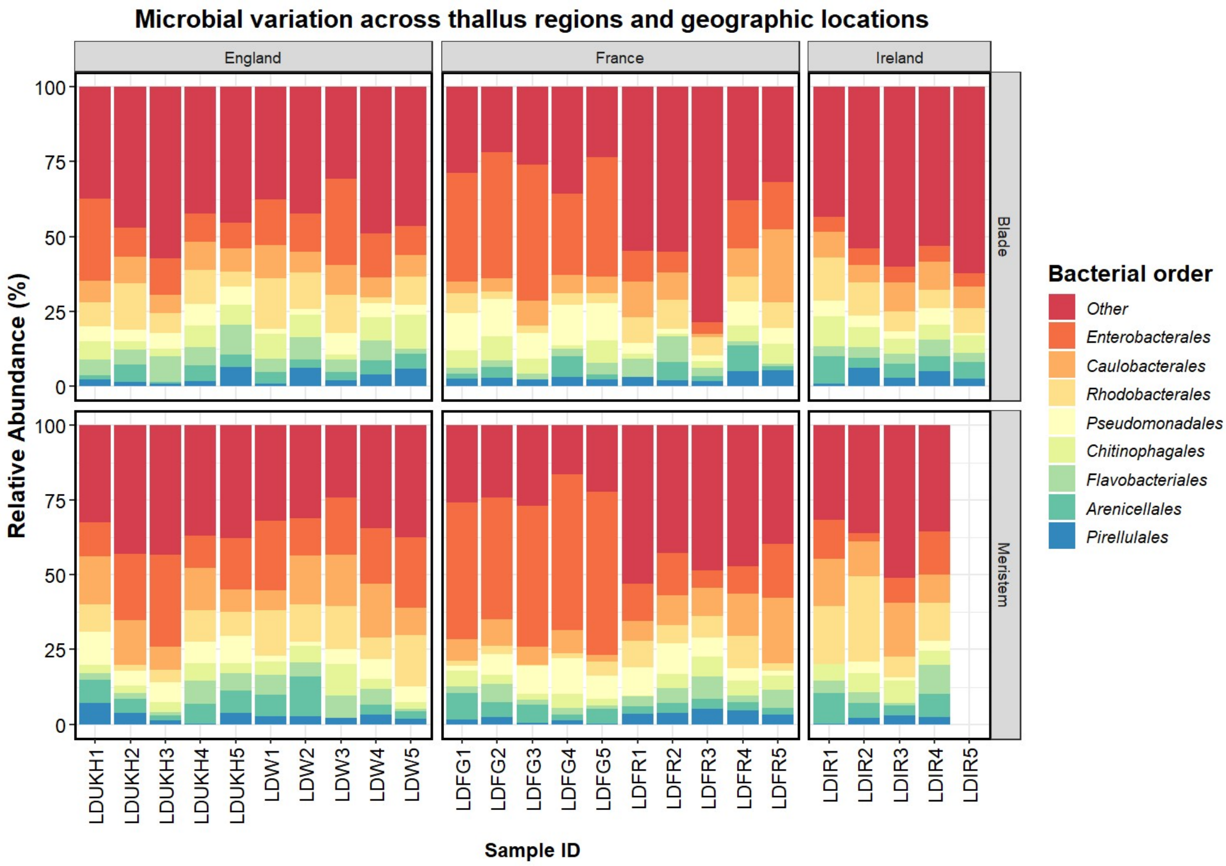

Figure 16: Microbial variation of L. digitata across thallus regions and geographic locations. The figure shows the relative abundance (%) of bacterial orders from swab samples collected in England (LDUKHx: Hoe Point, LDWx: Martin's Haven), France (LDFGx: Gatteville-le-Phare, LDFRx: Roscoff), and Ireland (LDIRx: Goleen Harbour). Samples are categorized into two thallus regions: blade and meristem. Each bar represents an individual, with colors indicating different bacterial orders.

A genus-level comparison of core microbiomes was conducted across the five coastal field sites; Roscoff (France), Gatteville-le-Phare (France), Goleen Harbour (Ireland), Hoe Point (UK) and Martin's Haven (UK). This revealed both strong location-specific microbial signatures and a conserved set of core genera. These patterns are illustrated in Figure 17 with panel A displaying the ten most abundant genera at each location and panel B highlighting the number of shared core taxa across sites, offering a comparative view of microbial overlap and divergence.

Vibrio, a genus known for its ecological flexibility, was broadly distributed across all sites, but especially abundant in some individuals from Gatteville-le-Phare, Martin's Haven and Hoe point (Figure 17a). In Roscoff and Goleen harbour, *Vibrio* exhibited narrower peaks centered at low relative abundance (0–2%), indicating a stable yet more minor role in the microbial community. In contrast, Gatteville-le-Phare showed a broader distribution ranging from 5% to 30%, reflecting higher variability between individuals. Statistical analysis confirmed significant variation in *Vibrio* abundance across sites (ANOVA, $p < 0.001$), with Gatteville-le-Phare and Hoe Point hosting significantly higher levels than Goleen Harbour and Martin's Haven (Tukey HSD, $p < 0.05$). *Psychromonas* followed a similar trend, peaking in Gatteville-le-Phare and Martin's Haven. Its abundance also varied significantly by location ($p = 0.00021$), supporting its potential as a locally responsive taxon.

Fretibacter appeared more frequently in samples from Goleen Harbour, Roscoff and Martin's Haven. While trends suggested spatial structuring, statistical support was limited (ANOVA $p = 0.047$; Kruskal-Wallis $p = 0.007$). Genera like *Rudibimonas*, *Paraglaciecola* and *Blastopirellula* were widespread and present in equal abundances across locations.

The Venn diagram (Figure 17b) highlighted eight genera shared across all sites, suggesting a resilient core microbiome. However, several locations also harbored unique taxa: Goleen Harbour (7), Gatteville-le-Phare (5) and Hoe Point (4), indicating strong local microbial signatures. In contrast, Roscoff and Martin's Haven showed no exclusive genera, implying greater microbial overlap with other sites. Detailed genus-level distributions are presented in **Appendix J.**

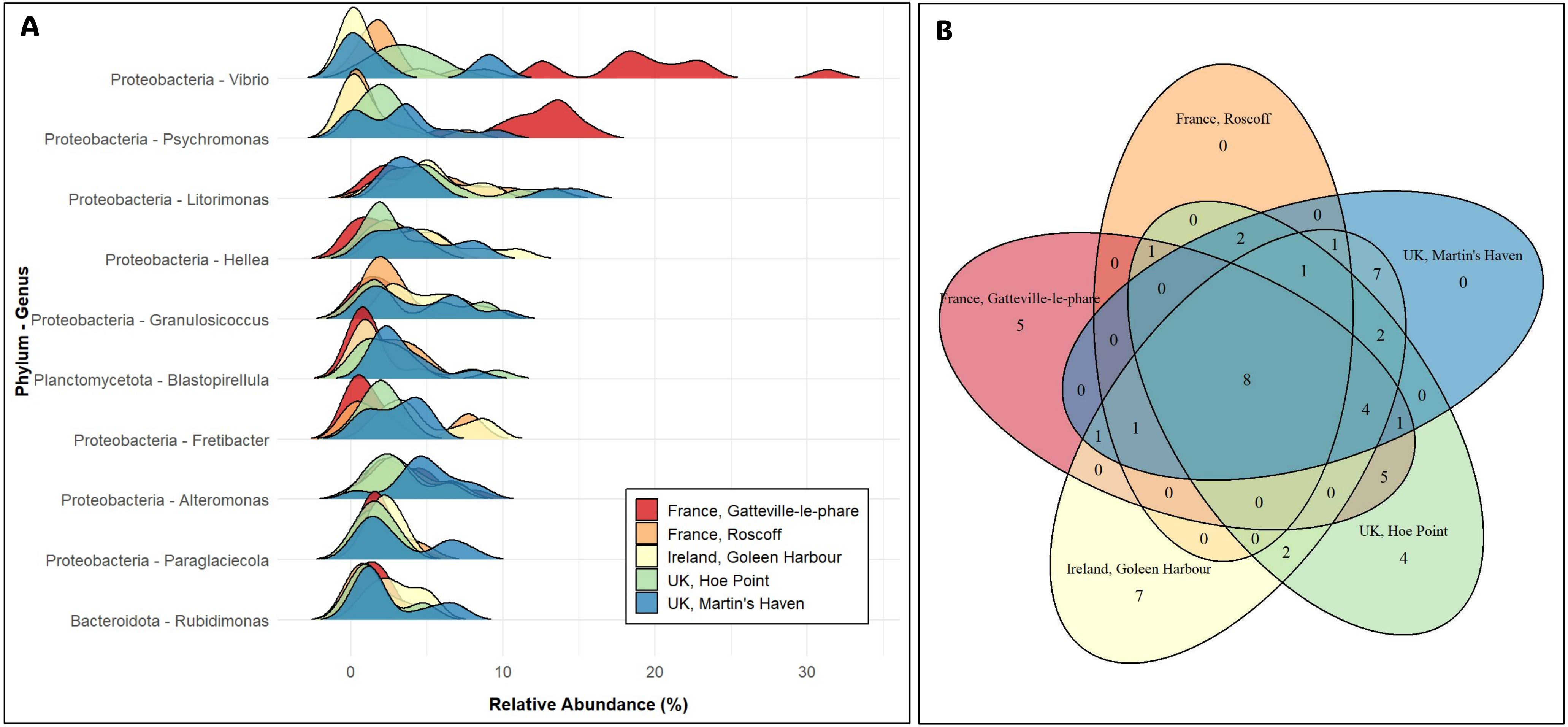

*Figure 17: Abundance and distribution of bacterial genera across five sampling locations (only blade swabs). **(A)** Ridgeplot visualizing the distribution of bacterial genera by relative abundance across sampling locations. Higher peaks represent genera that are more prevalent or consistently abundant in multiple samples. **(B)** Venn diagram displaying shared and unique genera per location, highlighting a core microbiome and site-specific taxa.*

Parent species comparison: L. ochroleuca vs. L. digitata

To assess whether the species of interest, *L. digitata* and *L. ochroleuca*, harbor significantly different microbial communities, swab samples from both the laboratory (Ghent) and field (Hoe Point, UK) environments were analyzed using NMDS based on Bray-Curtis dissimilarities, illustrated in Figure 18.

Microbial communities clustered primarily by environment rather than by host species or tissue type. Samples from the Ghent laboratory (left side of the ordination) formed a distinct and tight cluster, regardless of species identity. No pronounced differentiation between sporophyte samples and blade swabs was evident. Similarly, field samples collected from Hoe Point (right side of the ordination) exhibited no substantial separation between *L. digitata* and *L. ochroleuca*, nor a clear distinction between meristem and blade tissues within the same location (Figure 18). The ellipses illustrate the strong segregation between field and laboratory samples, particularly along the NMDS1 axis.

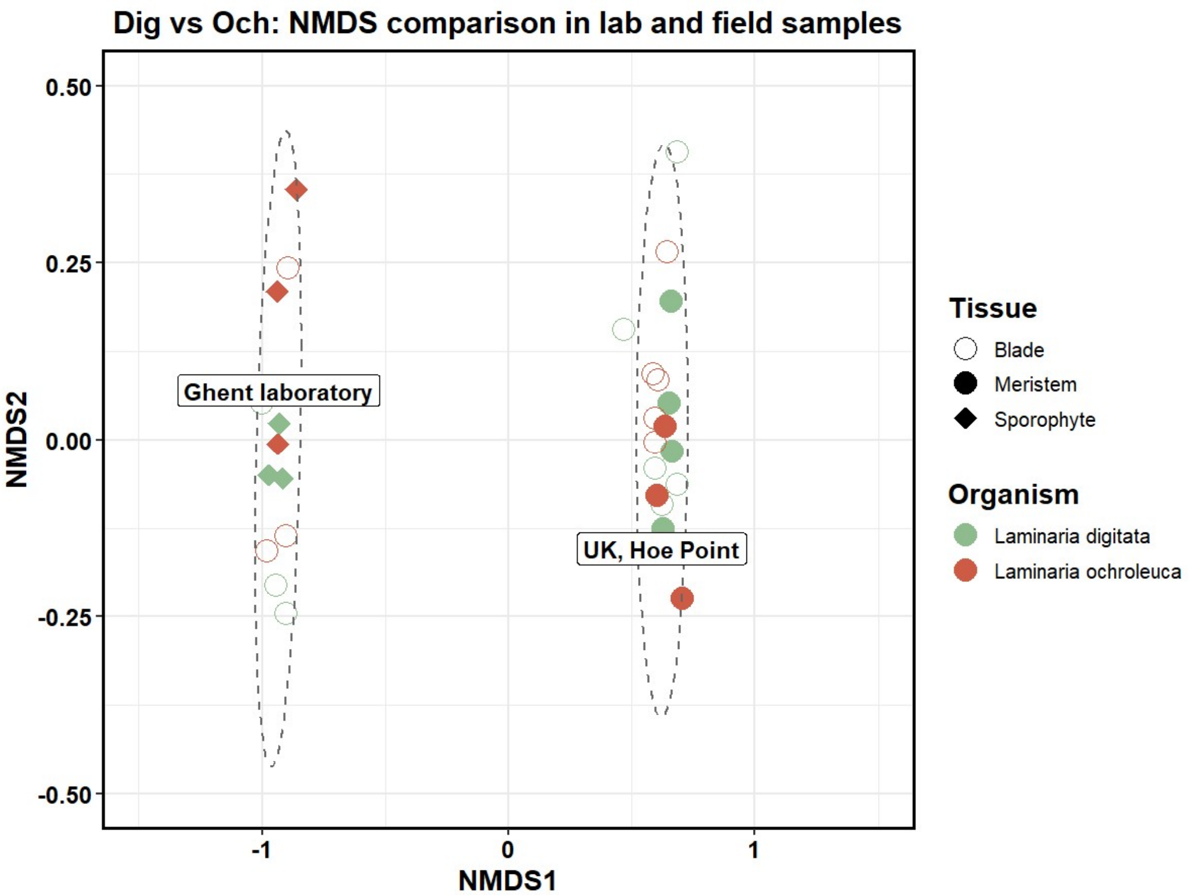

Figure 18: Clustering of samples by growth conditions (lab vs. field) for L. digitata and L. ochroleuca. Points represent individual samples, colored by species and shaped by tissue type. Ellipses indicate 95% confidence intervals around group centroids, illustrating variation and grouping related to environmental conditions.

The specific microbial community structure associated with *L. digitata* and *L. ochroleuca* varied both by host species and sampling origin, as shown by the stacked bar plot in Figure 19. Eight dominant bacterial orders were identified across all samples.

In *L. digitata*, the bacterial communities on field samples were primarily composed of taxa classified as "Other" (mean relative abundance = 38.1%), followed by Rhodobacterales (15.0%), Flavobacteriales (11.5%) and Enterobacterales (10.9%). Orders such as Pseudomonadales, Caulobacterales, Rhizobiales, Chitinophagales, and Cytophagales were also present but in lower relative abundances (all below 8%). Similarly, in *L. ochroleuca*, "Other" taxa remained the most abundant group (31.6%). However, there was a noticeable increase in the mean relative abundance of Enterobacterales (18.6%) and Rhodobacterales (16.5%), relative to *L. digitata*. Flavobacteriales and Pseudomonadales followed with mean abundances of 8.98% and 7.65%, respectively. The remaining orders exhibited similar abundance trends as observed in *L. digitata*.

Figure 19 highlights clear differences in microbiome composition between lab-grown (Ghent) and field-collected (Hoe Point, UK) samples of *L. digitata* and *L. ochroleuca*. Lab-maintained blades showed a strong dominance of Rhodobacterales, comprising on average 23% in *L. digitata* and 27% in *L. ochroleuca*. Flavobacteriales were also more abundant in lab-grown *L. digitata* (13.8%) compared to both field samples and *L. ochroleuca* lab samples (4.83%). Similar, though less pronounced, patterns were observed for Pseudomonadales and Chitinophagales. In contrast, Enterobacterales were more prevalent in field samples, which overall displayed more even and diverse microbiome profiles.

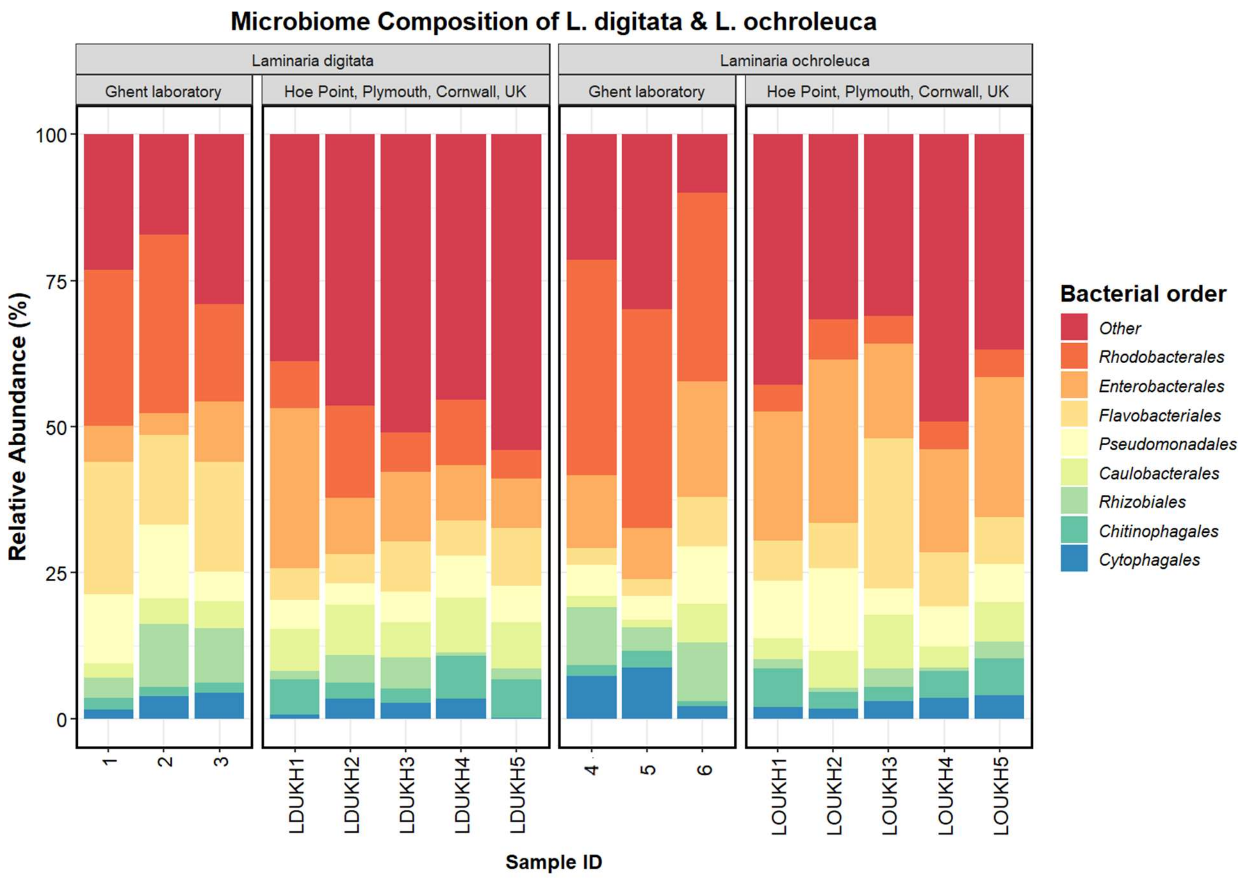

Figure 19: Microbial variation across species and environmental conditions. The figure presents the relative abundance (%) of different bacterial orders in blade swabs collected in the field (Hoe point, UK) and the lab (Ghent laboratory). Each bar represents an individual, with colors indicating different bacterial orders.

Microbiome variation in Laminaria crosses

The following analysis focused on comparing microbiomes across laboratory crosses to detect clear distinctions or recurring patterns between hybrid- and intraspecific crosses.

Figure 20 shows how microbial communities vary according to cross and sample type. The points are scattered across the plot with no clear clusters, again suggesting that the microbial communities from different crosses and sample types do not exhibit distinct visual separations. The dashed lines in Figure 20 represent the relative distances between the crosses, based on the centroids, which indicate the average positions of the communities.

The hybrid crosses (Dig ♀ x Och ♂ and Och ♀ x Dig ♂) have centroids positioned between the parental crosses (Dig ♀ x Dig ♂ and Och ♀ x Och ♂) along NMDS1 (Figure 20). The microbiomes of the two intraspecific parental crosses (Dig ♀ x Dig ♂ vs. Och ♀ x Och ♂) show the largest divergence ($R^2 = 0.267$), although this difference is not statistically significant. Overall, Dig ♀ x Dig ♂ displays the most distinct microbial profile, while hybrids and Och ♀ x Och ♂ share greater compositional similarity. The high stress value of 0.154 suggests that while this distance is informative, it may be somewhat distorted.

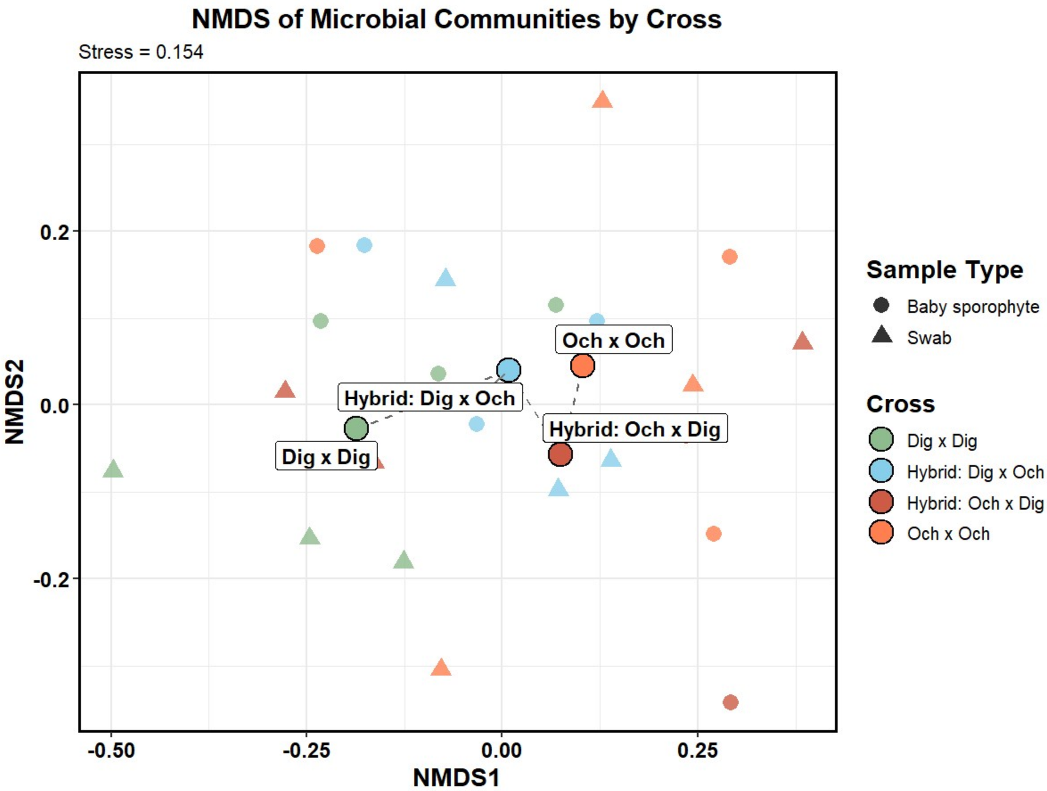

Figure 20: Microbial community structure by cross type and sample type. Points are colored by cross (Dig × Dig in green, Dig × Och hybrid in blue, Och × Dig hybrid in red, and Och × Och in orange), with shapes indicating sample type (baby sporophytes as circles, swabs as triangles). The big circles mark group centroids and dashed lines represent distances between crosses.

Appendix K shows the microbiome comparison for *L. pallida* crosses. NMDS and PERMANOVA indicate no clear clustering or significant differences between hybrid and parental groups, likely due to overall similarity in parental microbiomes. Most crosses show broadly similar microbiome composition, except for one sample (S17) being noticeably different.

The stacked bar plots in Figure 21 visually show differences in the relative abundance of dominant bacterial orders between groups. In particular, Rhodobacterales was consistently the most abundant order across all samples, but its relative abundance peaked in Och ♀ x Och ♂ sporophytes (mean: 35.9%) and was comparatively lower in Dig ♀ x Dig ♂ (24.8%). Hybrid crosses exhibited intermediate profiles, with Hybrid: Dig ♀ x Och ♂ showing higher relative abundance of Rhizobiales (15.0%) compared to Dig ♀ x Dig ♂ (8.1%) and Och ♀ x Och ♂ (9.0%). Partheno cross 14 did not display a distinct microbiome relative to other hybrids. For this cross, no swab sample was collected due to stagnated growth and underdeveloped blades.

Subtle trends included higher levels of Flavobacteriales and Pseudomonadales in *Dig ♀ × Dig ♂*, while Enterobacterales and Cytophagales were more abundant in hybrids and *Och ♀ × Och ♂* samples. Swab communities closely reflected those of the corresponding juvenile sporophytes, with only minor shifts in low-abundance taxa. Hybrids aligned with either parent depending on the individual (Figure 21).

PERMANOVA and pairwise tests showed no significant differences in community composition across lab crosses. Shannon diversity also revealed similar diversity and evenness. Sample type (swab vs. sporophyte) also had no effect, suggesting stable microbial profiles across tissue types and age.

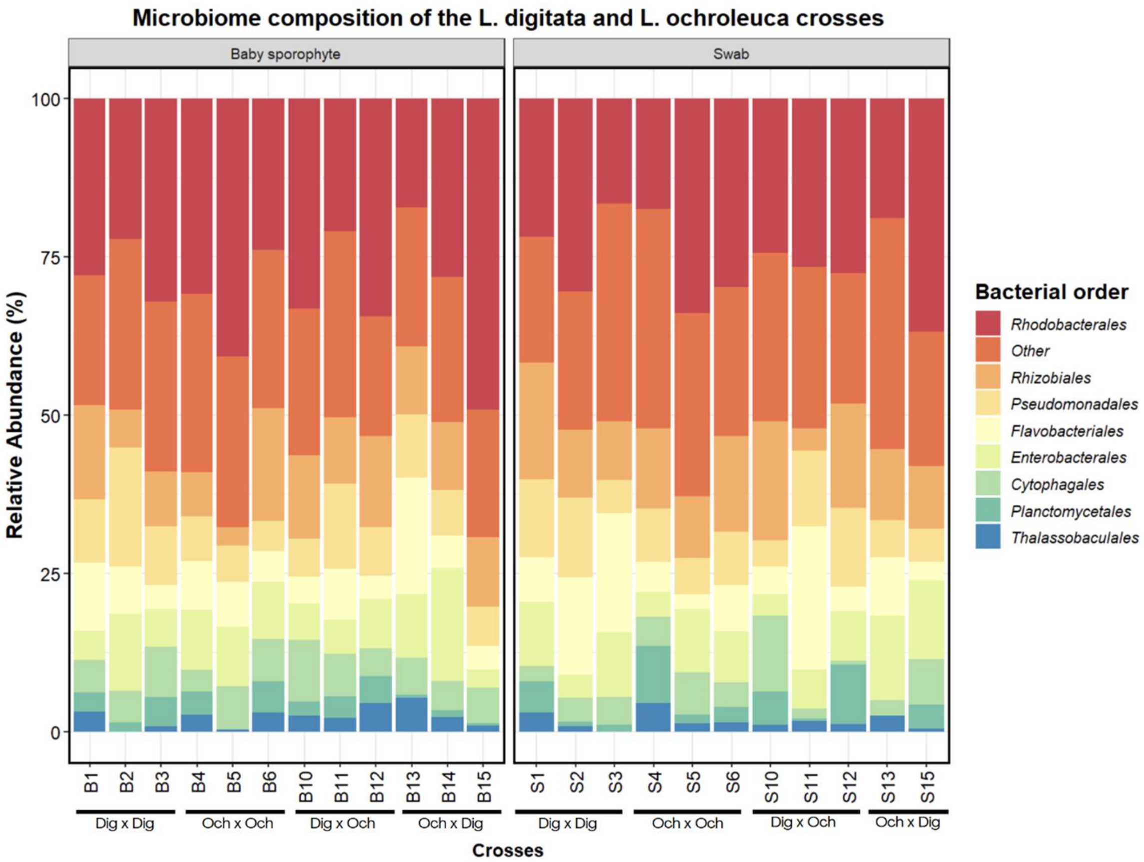

Figure 21: Abundances of the major orders in the laboratory crosses of interest. Baby sporophyte samples were collected after 8 weeks of growth, and swab samples were taken from the center of the algal blade. Each bar represents an individual, with colors indicating different bacterial orders. The bacterial orders shown represent the 8 most abundant bacterial orders across all samples. All remaining orders were grouped under the category "Other".

Analysis of microbial genera across the four cross types; Dig x Dig, Och x Och, Hybrid: Dig x Och and Hybrid: Och x Dig, revealed both a conserved core microbiome and patterns of cross-specific variation. This is again illustrated by a Ridgeplot (Figure 22a) and Venn diagram (Figure 22b).

A consistent set of genera, including *Sulfitobacter*, *Alteromonas*, *Hoeflea* and *Granulosicoccus*, was detected across all groups, indicating a core microbiome resilient to host genetic background. These taxa showed no statistically significant differences in relative abundance, highlighting their stability across different genetic contexts.

Among all examined genera, only *Paraglaciecola* displayed significant variation between groups (Kruskal-Wallis: $p = 0.015$; ANOVA: $p = 0.026$), with a specific difference between Dig x Dig and Och x Och identified by post-hoc Tukey testing (adjusted $p = 0.047$). All other genera lacked statistically significant differences in abundance.

Figure 22a presents ridgeplots of relative abundances, where taller and narrower peaks reflect consistent genus abundances across samples, and broader peaks indicate greater within-group variability. In some cases, notably in Hybrid: Dig x Och, smaller peaks were observed at higher abundances (10–20%) for genera such as *Labrenzia* and *Roseobacter*, suggesting enrichment in certain samples.

The Venn diagram in Figure 22b further illustrates taxonomic overlap and specificity. Seven genera were shared across all crosses, reinforcing the presence of a stable core microbiome. In contrast, six taxa were unique to Dig x Dig (e.g., *Methylophaga*, *Antarctobacter*) and another six were exclusive to Hybrid: Och x Dig (e.g., *Maribacter*, *Marinobacter*), pointing to cross-specific microbial communities. Notably, no unique taxa were shared exclusively between Dig x Dig and Hybrid: Dig x Och, suggesting limited microbial inheritance in this hybrid. A detailed breakdown of taxa per group is provided in Appendix L.

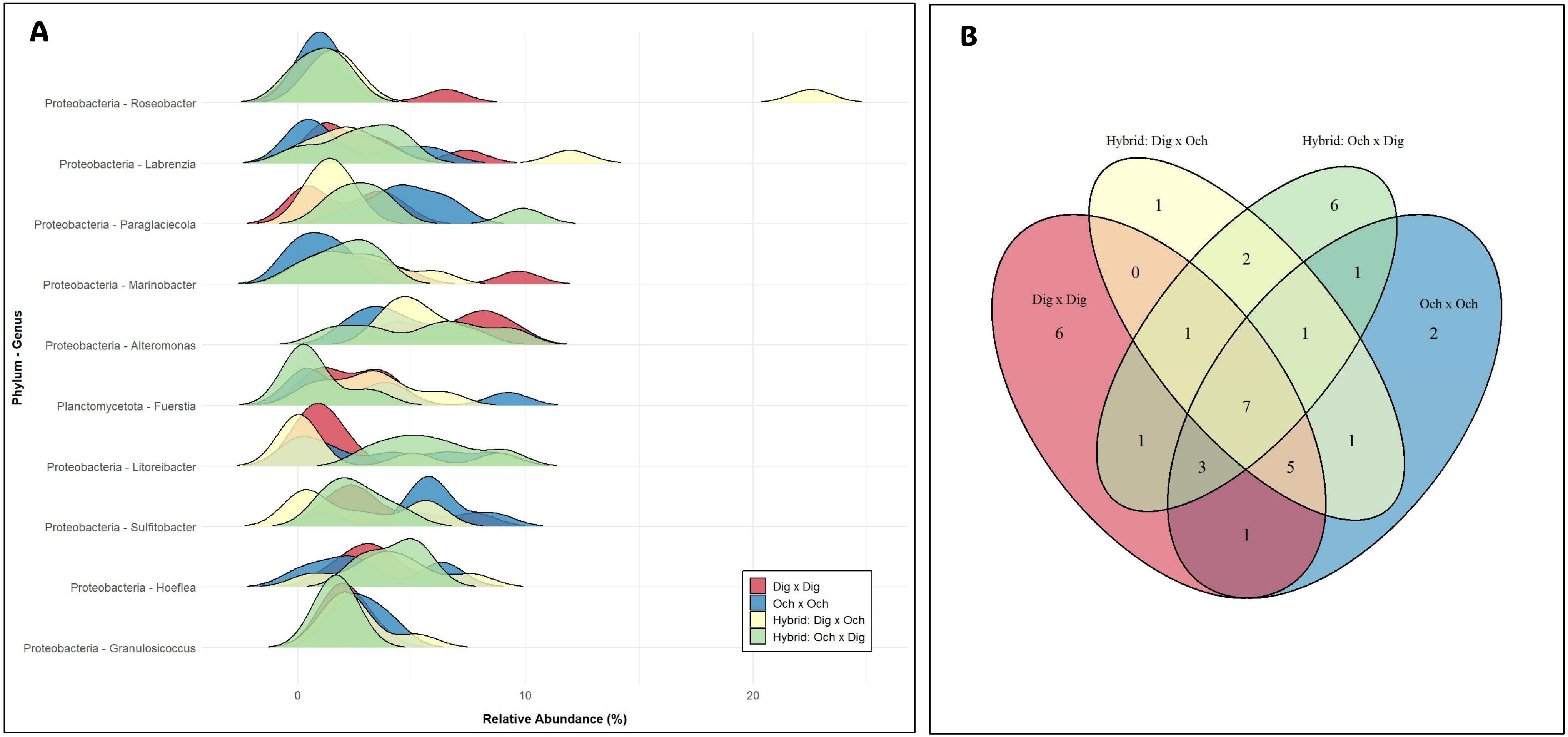

Figure 22: Distribution of bacterial genera across the laboratory cultures of interest; ***(A)*** *ridgeplot showing relative abundance patterns per genus and location, where peak height reflects prevalence within samples, and* ***(B)*** *Venn diagram displaying shared and unique genera per location, highlighting a core microbiome and site-specific taxa.*

Sorus development

During the four-week observation period, no sorus formation was observed in the kelp fragments. Typical patterns of structured sori were not evident. The tissue texture appeared diffuse and lacked the sharply defined clusters of sporangia that are characteristic of mature sori. Visual inspection revealed progressive discoloration and degradation of tissue structure in most crosses, as shown in Figure 23. Crosses involving *L. ochroleuca* females consistently appeared less healthy compared to those with *L. digitata* females across all replicates.

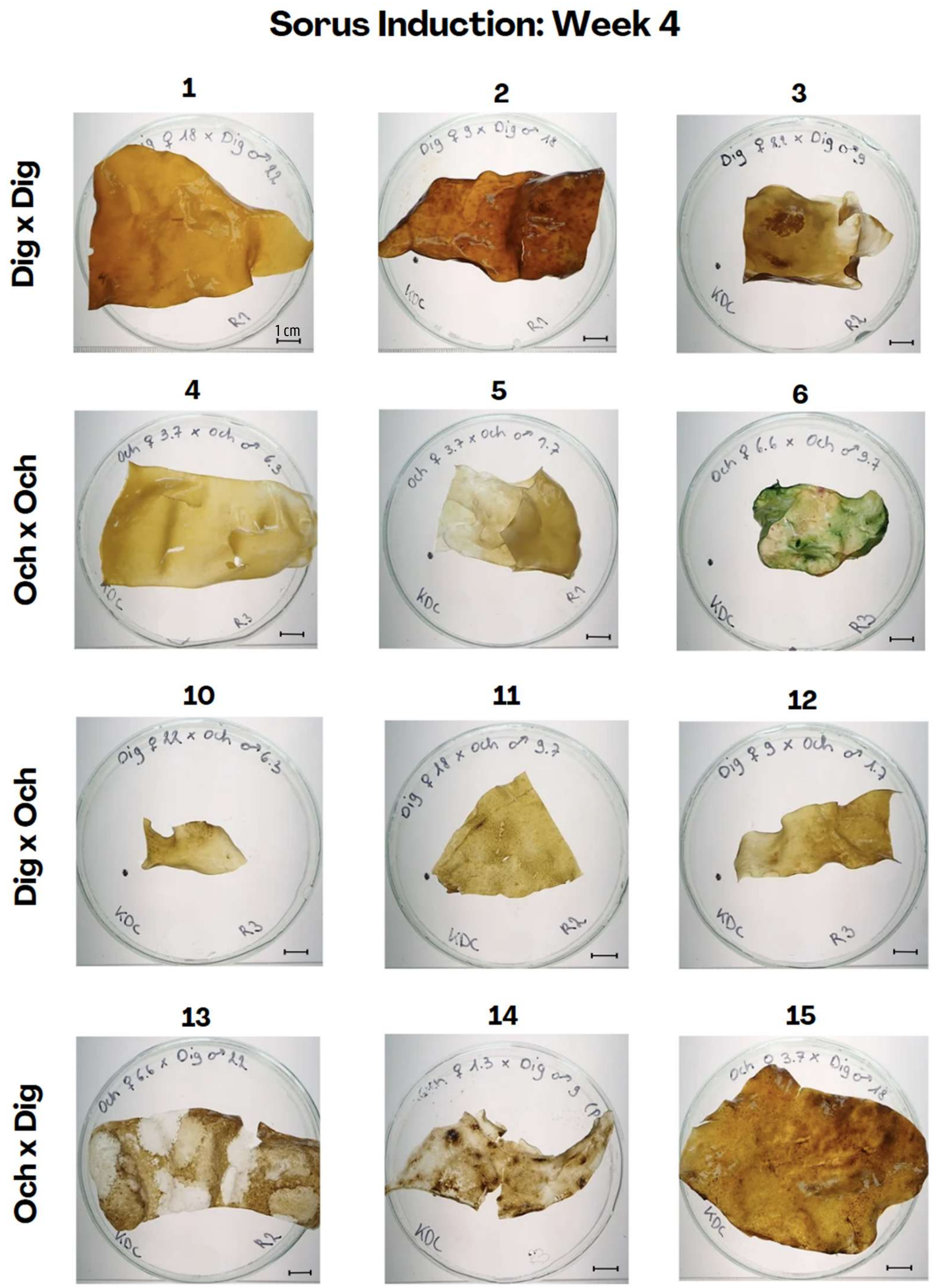

Figure 23: Sorus induction images after 4 weeks from both intraspecific crosses (Dig × Dig; Och × Och) and reciprocal interspecific crosses (Dig × Och; Och × Dig). The black line represents a 1 cm scale. Och and Dig refer to L. ochroleuca and L. digitata, respectively.

Ploidy profiles

Flow cytometry analysis was conducted to estimate the ploidy level of the different crosses and in particular the interspecific hybrids. The resulting graphs showed substantial background noise across all *Laminaria* samples. For both parental crosses (Dig × Dig and Och × Och) no clear peak could be distinguished above the background signal, with most fluorescence events clustering between 50 - 100 arbitrary units on the x-axis.

In contrast, the hybrid cross (Dig x Och) yielded a distinguishable signal that appeared after the noise, at approximately 200 arbitrary units on the x-axis. To calibrate this result and estimate genome size, the hybrid samples were co-analyzed with nuclei from *Solanum lycopersicum* (tomato), *Glycine max* (soybean), and *Zea mays* (maize). However, none of these tries yielded conclusive peaks under the same conditions. Therefore no absolute genome size could be determined, only the hybrid signal could be visualized, see **Appendix M**. These results were inconclusive and primarily informative in an experimental context rather than for characterizing hybrid ploidy.

DISCUSSION

This study aimed to assess the hybridization potential between *L. digitata* and *L. ochroleuca*, as well as potential patterns in their microbiome composition. Given the increasing overlap in species ranges due to climate change and the growing interest in kelp aquaculture, understanding reproductive barriers and hybrid traits is both ecologically and economically relevant. After presenting a comprehensive overview of the experimental results, we now discuss these findings in relation to previous literature and their broader implications.

Optimization of experimental set-up

Optimizing environmental conditions was essential to support both vegetative growth and successful gametogenesis in the hybridization experiment. Several factors, including nutrient medium, light regime, temperature and aeration, were found to influence developmental outcomes at different stages of the culture process. During the early stages, vegetative gametophytes were cultivated in sterilized seawater enriched with PES, a medium shown to support growth in brown algae (Costa et al., 2025; Ratcliff et al., 2017). Cultures remained in PES throughout the initial growth and crossing stages. After eight weeks, the most vigorous sporophytes were transferred to larger 2L bottles and eventually scaled up to 10L tanks, where f/2 medium replaced PES due to practical constraints. However, this transition coincided by a noticeable decline in growth, likely caused by both the change in nutrient composition and the lower light intensity in the Marbiol room, where the larger tanks were located. While f/2 has supported gametogenesis in *L. digitata* under blue light (Ratcliff et al., 2017), it may be less effective at sustaining vegetative growth. PES, in contrast, promotes higher biomass production and has shown better performance for maintaining gametophyte cultures in other seaweeds like *Ulva* sp. (Costa et al., 2025; Ratcliff et al., 2017).

Light quality and photoperiod critically affect both photosynthesis and life stage transitions. Vegetative gametophytes were maintained under red light at 15 °C, while exposure to white light triggered gametogenesis. The success of this transition depends on sufficient photosynthate production during the prior stage, which in turn is regulated by photoperiod (Gao et al., 2013; Ratcliff et al., 2017). Grinding and crossing of cultures occurred under red light conditions, followed by a recovery period of 1–3 days. Although the differences in recovery duration were not statistically significant, they may still partially explain the substantial variation observed between replicates. Light quality itself appears to be less critical for gametophyte growth (Lüning, 1980), but blue light (400–500 nm) is optimal for inducing gametogenesis in *S. latissima*, *L. digitata*, and *L. hyperborea* (Lüning, 1980; Lüning & Dring, 1972, 1975). The slower transition in most crosses may thus have been due to suboptimal wavelength exposure. Temperature is another key factor, often interacting with photoperiod. Some kelps initiate gametogenesis only under short-day and low-temperature conditions (Martins et al., 2017). Gas exchange also plays a role, aeration enhances gametogenesis by preventing boundary layer formation around gametophytes (Ratcliff et al., 2017). In this study, aeration was initiated after eight weeks, coinciding with the transfer to 2L bottles.

In summary, multiple interacting environmental variables, along with species-specific responses, make it challenging to define a universally optimal protocol and future research should explore alternative set-ups to optimize hybridization outcomes.

Reproductive success and growth variability

The results clearly demonstrated that interspecific hybridization is possible between *L. digitata* and *L. ochroleuca*, and to a lesser extent with *L. pallida* (**Figure 12**). Specifically, the cross Dig ♀ × L. Och ♂ yielded the highest reproductive success among all tested combinations, with a mean sporophyte percentage of 86.7% on Day 28 (**Figure 10**). This finding supports previous studies indicating hybrid vigor and the potential for reproductive success in interspecific *Laminaria* hybrids (Coelho et al., 2014; Martins et al., 2019). The variability in error bars observed in Figures 8 and 10, highlights that sporophyte development is highly dependent on the specific parental cross, with certain combinations yielding more consistent and reliable results than others.

In contrast, Och ♀ × Dig ♂ crosses displayed significantly lower sporophyte percentages and greater developmental variability. The same pattern is found with crosses involving *L. pallida*, where Dig ♀ × Pal ♂ showed moderate reproductive success while Pal ♀ × Dig ♂ performed poorly. This directional asymmetry resembles findings in the literature suggesting that maternal inheritance influences early development and hybrid success through organelle–nuclear interactions or species-specific cytoplasmic factors (Bartsch et al., 2008; Greiner et al., 2015). Photographic analysis of sporophytes after ~12 weeks of cultivation indicated the same pattern, that hybrid crosses involving *L. digitata* as the maternal parent, generally outperformed other combinations in size and morphology (**Figure 11**).

The superior performance of *L. digitata* as the maternal parent, along with the general differences observed in reciprocal crosses, may be explained by epigenetic and maternal factors (Greiner et al., 2015; X. Liu et al., 2017). In oogamous brown algae, such as species within the *Laminaria* genus, both mitochondria and plastids are typically inherited from the maternal parent. This uniparental inheritance means that the zygote receives its cytoplasmic organelles exclusively from the egg, which can significantly influence hybrid viability and development (Choi et al., 2020; Greiner et al., 2015). The sperm cell contributes mostly just its nuclear DNA. Studies have demonstrated that in interspecific hybrids, only the maternal organelles are transmitted to the offspring, underscoring the importance of the maternal cytoplasmic environment in hybrid success (Choi et al., 2020; Greiner et al., 2015). In addition to organelle inheritance, maternal factors present in the egg cytoplasm, such as mRNAs and proteins, play a pivotal role in early embryonic development (Boscq et al., 2024). These maternal cues may influence the developmental trajectory of hybrids, potentially contributing to the differences in early development observed between the crosses (**Figure 7**).

An alternative, non-mutually exclusive factor that may contribute to performance variations among crosses is pheromonal communication. Female gametophytes release volatile compounds that serve dual functions: attracting male gametes and inducing their release (Müller et al., 2008). One such compound, identified as a spermatozoid-releasing and -attracting substance, has been isolated from female gametophytes of *L. digitata* (Müller et al., 2008). This compound triggers the mass release of male gametes within seconds at very low concentrations, indicating its potency in facilitating fertilization (National Academy of Sciences, 1995). While these pheromones are generally species-specific, there is evidence suggesting that closely related species may respond to each other's pheromones to some extent. This cross-reactivity could influence the success rates of interspecific hybridization, depending on the compatibility of pheromonal signals between species (National Academy of Sciences, 1995). It is possible that *L. digitata* produces more effective or broadly responsive pheromones, enhancing its ability to attract and activate male gametes from other species, leading to higher hybridization success when *L. digitata* serves as the maternal parent. Conversely, if *L. ochroleuca* pheromones are less effective or more species-specific, this might result in lower hybridization success in reciprocal crosses.

Despite poor performance early on (**Figure 8**), *L. ochroleuca* intraspecific crosses later showed healthy growth (**Figure 11**), suggesting a potential delay in development or greater sensitivity to initial conditions. One possibility is that *L. ochroleuca* gametophytes require more time to initiate successful sporophyte development, potentially due to a slower physiological response to fertilization cues or suboptimal conditions during the early culture phase (Lüning, 1980; Martins et al., 2017). Additionally, strain-specific sensitivity to environmental factors, such as nutrient composition, light quality or the lack of aeration, may have contributed to the observed lag (Ratcliff et al., 2017; Gao et al., 2013). This delayed success underscores that reproductive performance should not only be assessed based on early-stage counts but also longer-term growth and morphological development. Additionally, it is possible that the vegetative *L. ochroleuca* gametophyte cultures used for crossing were of lower initial health or quality, which may have subsequently affected their development.

Several experimental irregularities in the Faro setup may have introduced variation or bias as well. For example, a pipetting error led to the repeated use of *Och-F 3.7* instead of the intended *Och-F 1.3* in intraspecific crosses. As a result, *Och-F 1.3* was only used in hybrid cross no. 14, the sole cross confirmed to be parthenogenetic, leaving this cross without an appropriate intraspecific control. It is therefore unclear whether *Och-F 1.3* was not sexually reproductive or simply incapable of hybridization.

Additional variation likely arose from inconsistent volumes or gametophyte densities across Petri dishes, such as reduced volume for *Dig-F 22* or lower density in *Dig-M 18*. Other possible sources of variability include pipetting errors, batch inconsistencies in medium quality and contamination by bacterial biofilms or small microbial organisms. These subtle differences in experimental conditions can impact gametophyte development and sporophyte formation. Biological variability impacts reproductive success beyond differences between species and strains, the individual-level variation may lead to differing responses under identical conditions. Ecotypic and strain-level differences play important roles, explaining that some strains consistently underperformed (Kraan et al., 2000; Liu et al., 2017).

Genetic confirmation of hybrid status

PCR amplification using the microsatellite marker LolVVIV-23 successfully confirmed the hybrid status of the interspecific crosses of interest, with distinct banding patterns indicating the presence of alleles from both parental species. However, the failure to reliably amplify *L. pallida* DNA highlights a limitation in the genetic confirmation process. Despite previous successful applications of the LolVVIV-23 microsatellite marker in *L. pallida*, amplification failures in these samples may be attributed to null alleles, which can arise from mutations in primer binding sites or the presence of PCR inhibitors such as polysaccharides and polyphenolic compounds commonly found in brown algae (Chapuis & Estoup, 2007; McDevit & Saunders, 2009). In addition, the appearance of multiple unexplained bands in **Figure 12** suggests that non-specific amplification may also have occurred (Chapuis & Estoup, 2007). This happens when non-target DNA is amplified, often leading to faint or smeared bands (Douglas, 2025). Although different PCR settings were tested to address typical causes of non-specific amplification, such as excessive primer concentrations and high cycle numbers, these factors may still have contributed to the observed results (Douglas, 2025). Smearing can also result from DNA fragmentation, excessive template DNA, degraded primers, or suboptimal annealing temperatures (Douglas, 2025). In this case, the smear appears mostly around the expected amplicon size, making it less concerning.

One possible solution could be touchdown PCR, where the annealing temperature starts 5°-10° higher and gradually decreases over multiple cycles. This method reduces mispriming and optimizes both specificity and yield (Green & Sambrook, 2018). Contamination with non-target DNA can also lead to additional bands in PCR results. Implementing strict contamination control measures and including negative controls can help address this issue. The control showed no contamination, as no bands were present. However, the crosses in Faro did show some contamination with brown biofilm or small microbes. While it is possible that PCR amplified microbial DNA in addition to kelp DNA, this is unlikely to be the primary cause of the inconclusive results.

The occurrence of parthenogenesis was confirmed through both morphological assessment and genetic analysis (**Figure 12**). In cross 14 (Och ♀ × Dig ♂), sporophytes contained only maternal DNA, and were therefore not considered true hybrids. The rate of abnormal sporophytes across all crosses was 10.5%, significantly above zero. This suggests that parthenogenesis is a background phenomenon in kelp cultures (Oppliger et al., 2007). While not dominant, it is a relevant mechanism to consider, especially when interpreting results in hybrid experiments. However, the reliability of the method used to identify abnormal morphologies, as described by Martins et al. (2019), may be limited. It is possible that all healthy sporophytes perished before genetic analysis was conducted, or that morphologies classified as normal were actually undetected parthenosporophytes (**Figure 13**). In either case, cross 14 did not exhibit clear signs of parthenogenesis during the observation period.

Thallus region shaping bacterial communities

The 16S rRNA gene ONT sequencing analysis revealed that the bacterial communities in meristem and blade swabs from adult *L. digitata* individuals are significantly different (**Figure 15**). Taxa such as Pseudomonadales, Chitinophagales, Rhodobacterales and Flavobacteriales exhibit only minor differences, with these groups remaining relatively stable across both tissue types, showing only small variations in abundance (**Figure 19**). These bacteria are typically involved in nutrient cycling and organic matter degradation, suggesting they may fulfill similar roles in both tissue types. Caulobacterales and Enterobacterales are slightly more abundant in the meristem region, potentially due to differences in nutrient availability or microbial interactions.

These findings align with a study on *L. digitata* (Ihua et al., 2020) and other research (Morrissey et al., 2019), which emphasizes the functional diversity across different niches on the host. This supports "The Competitive Lottery Hypothesis," which suggests that bacterial recruitment is influenced by functional organization rather than taxonomic composition (Burke et al., 2011; Morrissey et al., 2019). Similar observations were made in *Ulva* species, where bacterial community composition is more closely related to metabolic functions than to random processes (Burke et al., 2011). This pattern, with significant differences from apex to base, has also been found in *Caulerpa*, a siphonous algae species (Morrissey et al., 2019; Ranjan et al., 2015). Thus, the differences in bacterial composition in this study are likely driven by the functional requirements of each distinct thallus. The observed variation between morphological niches highlights the complexity of host-microbe interactions in marine macroalgae.

To better understand how bacterial community composition influences kelp-associated microbiomes, the following paragraph examines the functional roles of the dominant bacterial orders identified. Orders such as Chitinophagales and Flavobacteriales were consistently present on both the meristem and blade, highlighting their likely role in degrading complex biopolymers and maintaining aerobic microenvironments on the seaweed surface (Dang et al., 2011; Pollet et al., 2018). Notably, Chitinophagales contribute to nitrogen cycling through denitrification, encoding nitric oxide reductase, and facilitate biofilm development via production of poly-N-acetylglucosamine precursors (Fujii et al., 2022; Emma Gouwy, 2023; Izano et al., 2007). Members of Rhodobacterales, also found across both tissue types, contribute to sulfur cycling through metabolism of dimethylsulfoniopropionate (DMSP) and produce essential growth factors such as vitamin B12 (Minich et al., 2018).

Gammaproteobacteria, particularly Enterobacterales and Pseudomonadales, were detected in both tissues but showed elevated abundance in the meristem (**Figure 19**). While Enterobacterales are nitrogen fixators (Mehnaz, 2013), their high presence likely reflects anthropogenic influences such as wastewater contamination rather than a symbiotic relationship with kelp (Zhang et al., 2024). In contrast, *Pseudomonas* spp. are important biofilm formers, producing alginate to enhance structural integrity and resilience of the kelp microbiome (Dharshini et al., 2021). This support may improve kelp propagule fitness and recruitment success (Morris et al., 2016). Moreover, some *Pseudomonas* spp. produce antibiotics, potentially protecting the host from pathogens (Harmsen et al., 2010; Singh & Reddy, 2014).

Figure 21 shows the differences between baby sporophyte samples and blade swabs from the laboratory crosses. Baby sporophyte samples display a more balanced microbial composition, with lesser abundant taxa more evenly represented across the community. This may reflect higher microbial diversity due to the inclusion of multiple tissue types. However, these differences were not statistically significant.

Together, these findings highlight the functional diversity within the kelp microbiome and underscore the complex interactions that likely support kelp health, growth, and resilience across different morphological niches.

Location-specific differences in microbiomes

Microbial communities on kelp are shaped not only by thallus region but also by geographic location. This is expected, as microbial succession on kelp is strongly influenced by the surrounding seawater and local physical forces, such as wave action, water movement, and currents (Davis et al., 2023; Michelou et al., 2013; Sadeghi et al., 2024). Environmental gradients such as salinity, nutrient availability, temperature, and hydrodynamic conditions vary across locations and influence microbial composition (Weigel & Pfister, 2019; Xu et al., 2022). The results represented in **Figure 15** revealed significant differences between the sample sites, which formed distinct clusters. Roscoff (France), however, showed greater dispersion than the other sites, possibly reflecting broader ecological heterogeneity or microenvironmental variation at this location.

At the genus level, several microbial taxa exhibited clear biogeographic patterns (**Figure 17**). *Vibrio spp.* and *Psychromonas spp.* were notably more abundant in Gatteville-le-Phare (France) and Martin's Haven (UK), suggesting that these genera respond strongly to specific environmental conditions or local stressors. In contrast, genera such as *Blastopirellula* and *Paraglaciecola* were consistently present across all sampled sites, indicating broader ecological tolerance. A core microbiome of eight genera was shared among all the locations, but the presence of site-specific genera, particularly in Goleen Harbour (Ireland) and Gatteville-le-Phare, highlights the role of local filtering processes in shaping microbial assemblages.

In Gatteville-le-Phare, samples clearly showed a higher abundance of Enterobacterales, which includes the genera *Vibrio* and *Psychromonas* (**Figure 16**). This is unexpected, as Enterobacterales are typically associated with high anthropogenic pressure, which is more pronounced at sites like Roscoff and Hoe Point (UK) (Daché et al., 2024; Turner & Higgins, 2023; Y. Zhang et al., 2024). Therefore, this pattern may reflect microbial dysbiosis, a disruption in the natural microbial community balance, triggered by environmental stressors. In *Saccharina japonica*, for example, stress from increased light penetration and temperature has been shown to promote dysbiosis, characterized by a shift toward opportunistic bacteria like *Colwellia spp.* and *Pseudoalteromonas spp.* (Y. Zhang et al., 2024). Notably, the genus *Pseudoalteromonas* was a unique core taxon in Gatteville-le-Phare, suggesting that local stressors are at play (**Appendix J**). Some *Vibrio spp.* are known to act as opportunistic pathogens in macroalgae under compromised conditions and their elevated presence in Gatteville-le-Phare might reflect a pathogenic bloom, potentially driven by environmental disturbance (Egan et al., 2014). Sampling depth likely contributed to the elevated abundance of *Vibrio spp.* at Gatteville-le-Phare, which was sampled at just 1 m depth. *Vibrio spp.* are known to thrive in surface waters due to higher temperatures, light availability, and higher nutrient levels (Zhu et al., 2023). Brumfield et al. (2023) demonstrated that climate-driven factors such as warming events, altered salinity and increased storm activity further promote *Vibrio spp.* proliferation.

Lastly, the elevated abundance of *Psychromonas spp*. in both Gatteville-le-Phare and Martin's Haven likely reflects substantial inputs of macroalgal detritus in these relatively undisturbed, protected sites (**Appendix C**). This genus thrives in cold, organic-rich environments and degrades complex algal polymers (Dong et al., 2012; Lozada et al., 2023).

Beyond specific environmental patterns, both the succession of the kelp ecosystem and the microbial community could play a significant role. Studies indicate that microbial communities on kelp surfaces undergo successional changes over time (Bengtsson et al., 2012; Lemay et al., 2021). The age of kelp tissues is known to affect colonization, with older tissues typically hosting more diverse and stable microbiomes (Lemay et al., 2021). Older tissues are shown to harbor more diverse microbial communities compared to younger regions (Bengtsson et al., 2012; Weigel & Pfister, 2019). The age factor is a critical consideration when comparing field and laboratory microbiomes, as field organisms are significantly older and consequently larger in size. Larger kelp individuals, provide a greater surface area and a wider variety of microhabitats for microbial colonization (Lemay et al., 2021). Within the lab samples (baby sporophytes vs. swabs; **Figure 21**), tissue age did not significantly affect microbiome composition, likely due to the small difference and uniform growth conditions.

On a broader scale, microbial biogeography is driven by temperature, light, and chemical conditions (Gusareva et al., 2019; Rusch et al., 2007), while host-associated communities are shaped more by host-specific factors (Wood et al., 2022). Field conditions support more ecologically diverse microbial communities, highlighting the role of environmental filtering, where only taxa adapted to local abiotic conditions persist. This effect is underscored by the Ghent laboratory samples, which formed distinct clusters irrespective of species identity, indicating that uniform lab conditions strongly homogenized microbial composition (**Figure 18**). The stable and controlled conditions in the laboratory selectively favor fast-growing taxa such as Rhodobacterales, while suppressing others like Enterobacterales, as clearly illustrated in **Figure 19** (Michelou et al., 2013; Minich et al., 2018; Zhang et al., 2024). Handling procedures and filtered seawater further reduce microbial diversity in lab settings. It is essential to note that the lab specimens originated from Portugal and Norway. Therefore, some of the observed differences between lab and field samples may reflect geographic origin in addition to environmental conditions. As microbial community composition is influenced by host origin, future comparisons would benefit from using samples collected from the same location (Davis et al., 2023).

Importantly, microbial communities can show spatial structuring at fine scales. Even kelp individuals separated by just a few meters can host distinct microbiomes (King et al., 2023). This highlights the need for careful sampling design, as the choice of individuals can significantly affect observed community patterns. Seasonal changes also affect microbiome composition, and while not addressed in this thesis, they represent an important direction for future research (Kaur et al., 2023).

The microbiome composition in relation to hybridization

Understanding the influence of hybridization on host-associated microbiomes provides insight into evolutionary processes and host-microbe coadaptation (Soen, 2014). This study compared the microbiomes of *L. digitata*, *L. ochroleuca*, and their hybrids to examine the role of host genetic background in shaping microbial communities.

Comparisons between field and lab samples revealed distinct microbiome patterns, see **Figure 19**. *L. digitata* and *L. ochroleuca* share broadly similar community structures at the order level, yet exhibit species-specific differences in the relative abundance of key bacterial taxa, particularly Rhodobacterales and Enterobacterales. In the field, where environmental heterogeneity and microbial reservoirs are greater, host-specific factors appeared more influential. In contrast, the laboratory conditions promoted microbiome convergence, consistent with previous findings (Nguyen et al., 2021). Despite taxonomic overlap, field samples of *L. ochroleuca* showed higher abundances of Pseudomonadales and Flavobacteriales, groups associated with nutrient cycling and host interactions (Dharshini et al., 2021; Pollet et al., 2018). Interestingly, in the lab, *L. digitata* harbored greater levels of these taxa (**Figure 19**), suggesting that microbial associations are shaped not only by host genotype but also by environmental context.

This contrast may result from species-specific microbial competition or habitat differences. Under laboratory conditions, reduced microbial diversity and lower competitive pressures could enable Pseudomonadales and Flavobacteriales to proliferate on *L. digitata* without being displaced by competing taxa (Yawata et al., 2014). In the field, *L. ochroleuca* might offer a more stable or nutrient-rich habitat due to differences in exudates or tissue chemistry, tipping the balance toward these groups (Ghaderiardakani et al., 2020). Flavobacteriales and Pseudomonadales are often associated with organic matter degradation and stress tolerance (Bissett et al., 2008; Ramasamy et al., 2023). So another possibility is that potentially *L. ochroleuca* experiences more environmental stress in the field (e.g., higher temperatures or light exposure) which releases more dissolved organic carbon (DOC), attracting these bacteria (Bennett et al., 2024; Ghaderiardakani et al., 2020; Morrissey et al., 2019; Zhong et al., 2024). Conversely, under controlled laboratory conditions, both macroalgal species are subjected to less environmental stress and microbial colonization may be influenced more by surface compatibility, possibly favoring *L. digitata* (Ghaderiardakani et al., 2020). Furthermore, differences between Och x Och and Dig x Dig crosses may be related to the presence of green algae in the Dig x Dig 2L bottles (specifically cross 3). This algal presence could have influenced physical seawater parameters, such as dissolved oxygen levels and pH (Larkum et al., 2020; Sneed & Pohnert, 2011). Additionally, green algae may compete with bacteria for limiting nutrients, thereby influencing the microbial composition (Sneed & Pohnert, 2011). However, it is important to keep in mind that the lab-crossed individuals differ in origin from the field samples, potentially affecting the findings.

Host morphology also affects microbial colonization, with factors such as frond size, surface characteristics, and stipe flexibility playing a role in microbial settlement (Egan et al., 2013; Wood et al., 2022) (Egan et al., 2013; Wood et al., 2022). *L. digitata* has darker, more flexible blades, while *L. ochroleuca* features stiffer stipes and lighter blades (Hill, 2008; Smirthwaite, 2007). These structural differences, along with higher alginate content in *L. ochroleuca*, likely shape microhabitats and microbial filtering on algal surfaces (Kaidi et al., 2022).

Next, the results for the laboratory crosses revealed no significant effect of cross type on overall microbiome composition. This indicates that microbiome variation is primarily shaped by environment and growth conditions. These findings don't confirm previous studies showing host genotype as a major driver of microbial community structure, in both terrestrial and marine systems (Cúcio et al., 2016; Laforest-Lapointe et al., 2017).

Figure 21 illustrates differences in the relative abundances of bacterial orders between crosses, for example in the dominant order Rhodobacterales. Interestingly, hybrid crosses showed microbial profiles with centroids positioned between those of the parental types in the NMDS plot, see **Figure 20**. This suggests a potential intermediate microbiome influenced by maternal or genomic factors during microbiome assembly. However, the overall distribution of points is diffuse, with no distinct clustering, and the relatively high stress value of the NMDS indicates limited reliability of fine-scale spatial interpretation. Therefore, while the centroid positions of the hybrids may hint at a weak intermediate trend, this pattern should be interpreted with caution. It remains possible that *L. ochroleuca* traits like surface chemistry, exudates, or immune factors influence microbiome composition, but stronger evidence is needed to support this (Egan et al., 2013).

To further explore these patterns, alpha diversity metrics were examined. Interestingly, microbial richness (alpha diversity) did not differ significantly across genotypes or sample types, suggesting that while community composition may shift slightly, the overall diversity remains stable. This decoupling between taxonomic composition and diversity has also been observed in other algal-microbiome systems (Kembel et al., 2014; Morrissey et al., 2019; Zaneveld et al., 2017). For example, cross no. 14, despite its uniparental origin, exhibited a microbiome composition similar to biparental hybrids. It showed a higher relative abundance of Cytophagales and lower Enterobacterales, aligning more closely with Och × Och than Dig × Dig crosses. This may reflect subtle taxonomic shifts in the absence of paternal genetic input, even though the broader community structure remains consistent.

In contrast, *L. pallida* hybrids did not display an intermediate microbiome composition, likely due to the high similarity between the parental communities. Moreover, these crosses failed genetic confirmation and showed visible variation in growth and health, warranting caution in interpreting these results and limiting their generalizability.

At the genus level, several core taxa such as *Sulfitobacter* (Rhodobacterales) and *Hoeflea* (Rhizobiales) were consistently found across all crosses, showing stable abundances and highlighting a resilient core microbiome (**Figure 22**). Conversely, some genera like *Yoonia-loktanella* (Rhodobacterales) and *C1-B045* (Pseudomonadales) appeared exclusively in hybrids but were absent in intraspecific crosses (**Appendix L**), reflecting hybrid-driven microbiome novelty and potential creation of new microbial niches. *Yoonia-Loktanella* spp. are involved in producing terpenes, compounds that help protect macroalgae from pathogenic microbes and environmental stressors (Vigil et al., 2024). The marine bacterium *C1-B045* actively breaks down cycloalkanes, which is particularly noteworthy because the use of such bacteria shows potential for addressing oil pollution, especially in cold marine environments (Cui et al., 2024). Notably, *Paraglaciecola* demonstrated significant abundance differences between Dig × Dig and Och × Och, suggesting that host genotype influences microbial filtering.

Overall, the results suggest that hybridization can alter microbiome composition without affecting overall diversity under laboratory conditions, supporting a model of microbiome blending accompanied by functional stability (Kaur et al., 2023). The observed maternal influence on early microbial colonization highlights the role of reproductive dynamics in microbiome assembly. However, since this pattern is not reflected at the genus level, it suggests that functional traits rather than specific taxa are more important for the holobiont's stability and function, consistent with previous research (Burke et al., 2011; Vigil et al., 2024). Together, these findings highlight the need to consider both host genetics and ecological context in experimental design and contribute to a deeper understanding of host–microbe interactions in marine macroalgae.

Hybrid traits: ploidy and fertility

The exploratory flow cytometry analysis conducted, aimed to estimate nuclear DNA content in *Laminaria* species, using a protocol primarily developed for higher plants (Pellicer et al., 2021). Substantial background noise was observed across all samples, likely due to the specific biochemical characteristics of brown algae, such as high mucilage content, rigid cell walls, and the presence of polyphenols and other secondary metabolites, which are known to interfere with effective nuclear isolation and staining (Leitch & Leitch, 2013; Sliwinska et al., 2022).

In the case of the intraspecific crosses, no clear peaks were visible, possibly because the DNA signal was lost within the noise. However, the interspecific hybrid (Dig x Och) generated a reproducible peak at approximately 200 fluorescence units, which may reflect increased nuclear DNA content. This would be consistent with an allopolyploid condition (Table 2), where the combination of divergent genomes often results in higher genome size (Soltis et al., 2014). Nonetheless, this interpretation remains speculative, as no reliable internal standard was successfully applied, and genome size estimation could not be calibrated. Murúa et al. (2020) demonstrated that hybrid gametophytes of brown algae were diploid, possessing a complete set of chromosomes from both parents, suggesting that diploid hybridization cannot be ruled out.

The absence of clear peaks in parental samples may result from technical factors such as nuclear degradation, inefficient staining, or interference from cytoplasmic debris. The use of propidium iodide (PI), while standard for many plant systems, may have contributed to the poor resolution as PI is sensitive to chromatin structure and sample preparation (Doležel & Lucretti, 1995). Attempts to calibrate the hybrid's genome size using nuclei from *Solanum lycopersicum* (tomato), *Glycine max* (soybean), and *Zea mays* (maize) were unsuccessful, as these reference species did not yield clear separate peaks in combination with the hybrid. This outcome underscored the necessity of selecting appropriate internal standards that are compatible with the specific sample type and staining protocol employed. Fresh, actively growing tissue is preferred for flow cytometry, as senescent or dried material can increase background fluorescence and reduce staining quality (Pellicer et al., 2021). In this study, reference plant tissues were partially desiccated, and kelp sporophytes may have experienced stress from confinement and temperature fluctuations, potentially impacting signal quality.

While conclusive genome size measurements were not possible, this pilot analysis demonstrated the potential of flow cytometry in kelp research. Further optimization of sample preparation protocols, particularly for marine macroalgae, and the identification of suitable reference standards will be essential for reliable ploidy analysis in future studies (Sliwinska et al., 2022).

Assessing fertility in newly created hybrids is crucial to determine their ability to reproduce, persist across generations, and contribute to future breeding efforts (Liu et al., 2023). Although hybrid fertility is often limited, it is not universally absent. Coyer et al. (2007) documented a naturally occurring fertile hybrid between *Macrocystis* and *Pelagophycus*. Nonetheless, most hybrids within Laminariales and Ectocarpales are sterile due to failed sporangia development or gametophyte infertility from chromosomal mismatches (Murúa et al., 2020). The absence of sorus formation during the four-week period suggested reproductive limits in all crosses or, more likely, limitations of the experimental setup (**Figure 23**). The absence of characteristic sorus structures, along with increasing tissue discoloration and degradation across all treatments, indicates that the kelps were either not sufficiently mature or the culture conditions were inadequate for inducing reproduction.

Sorus induction in *Laminaria japonica* is highly sensitive to environmental factors, mainly temperature (optimal at 15°C), irradiance (>100 μmol photons m^{-2} s^{-1}), photoperiod and nutrient availability (Mizuta et al., 1999; Su et al., 2020). However, even under suboptimal conditions, some sori typically form, suggesting the setup here likely faced more significant limitations. Liu et al. (2023) further emphasized that successful sorus development depends on complex physiological processes, including phytohormone signaling and reactive oxygen species (ROS) regulation, which must be precisely aligned with external conditions. Inadequate control of environmental factors, along with possible tissue damage or contamination, may have hindered the reproductive capacity of both parental and hybrid individuals

First of all, the water volume of 200mL might have been too little, to compensate for this the nutrient concentration was doubled compared to the 2L bottles, also because nutrient enrichment has been shown to promote sorus formation (Boderskov et al., 2021). The nutrient-rich culture conditions could have promoted the proliferation of opportunistic bacteria and green algae, potentially exacerbating tissue deterioration (Supratya & Martone, 2024). The degradation of kelp tissues could also be attributed to the lack of aeration, which likely caused poor gas exchange, uneven nutrient distribution and microbial buildup, factors known to compromise tissue viability (Chen & Qi, 2008; Msuya & Neori, 2008). In prior experiments where sorus induction was successfully achieved, kelp discs were cultured either individually in 2-liter tanks or together in significantly larger systems, ranging from 15 to 100 liters in volume. These experiments also featured effective water mixing and aeration throughout the culture systems (Boderskov et al., 2021; Buchholz & Lüning, 1999; Gruber et al., 2011). According to the Yellow Sea Fisheries Research Institute (1989), environmental stress and microbial infections are common causes of kelp tissue degradation in aquaculture, highlighting the importance of carefully controlling culture parameters to maintain tissue health. Additionally, the kelp fragments were briefly rinsed with distilled water, which may have caused osmotic stress and was likely ineffective at removing surface contaminants (Davis et al., 2022; Wilding, 2021). Future protocols should use sterile filtered seawater and mild disinfectants to better protect tissue integrity (Edwards et al., 2016).

Furthermore, the kelps used in this study may have been too young to successfully induce sorus formation. In previous studies, the sporophytes were notably larger, ranging from 30 to 100 cm in length (Boderskov et al., 2021; Buchholz & Lüning, 1999; Pereira et al., 2019) or up to 22 months old (Gruber et al., 2011), which exceeds both the size and age of those used in this experiment. Additionally, these studies typically ran for a minimum of seven weeks, with longer durations correlating with more extensive sorus development (Boderskov et al., 2021; Buchholz & Lüning, 1999; Pang & Lüning, 2004). Gruber et al. (2011) also introduced a recovery period to allow disc tissues to heal from marginal damage, a factor worth considering in future experiments, as tissue injury may hinder sorus induction and should ideally be minimized.

Figure 23 clearly demonstrates that crosses involving *L. ochroleuca* exhibited poorer health throughout the experiment, consistent with their lower reproductive success and slower growth observed from the start in Faro. This trend is likely influenced by species-specific physiological or genetic differences, as previously discussed. It is important to note that most of the literature informing the experimental design focused on *L. digitata* populations from Helgoland (Buchholz & Lüning, 1999; Gruber et al., 2011), with only Pereira et al. (2019) addressing *L. ochroleuca*. Consequently, the experimental conditions may have been better optimized for *L. digitata*, potentially contributing to the reduced performance observed in *L. ochroleuca* crosses.

Assessing the hybrid fertility requires future studies to first establish consistent sorus induction in parental material, necessitating optimization of both environmental and methodological parameters. While the applied methodology was grounded in established research and effectively implemented, multiple trials remain essential to determine optimal conditions.

Implications of hybridization between *L. digitata* and *L. ochroleuca*

The findings of this study confirm that hybridization between *L. ochroleuca* and *L. digitata* is not only feasible under controlled laboratory conditions, but can also yield viable and morphologically normal sporophytes. This is consistent with the literature, where successful hybrids of brown algae have been created in laboratory settings and observed in the field (Kraan et al., 2000; Martins et al., 2019; Murúa et al., 2020). It underscores the potential for hybridization to affect kelp populations, especially in the context of climate-induced range shifts that may increase sympatry[3] between these species.

One significant consequence of kelp hybridization is the potential for hybrid vigor. For instance, hybrids between *L. digitata* and *L. pallida* exhibited a 2–3°C higher upper thermal tolerance compared to their parent species, suggesting that hybridization could confer resilience to warming oceans (Martins et al., 2019). Given the increasing impacts of climate change this has promising applications in mariculture and conservation, where breeding programs might leverage hybrid vigor to cultivate kelp strains better adapted to changing environments (Liesner et al., 2020). Additionally, controlled hybridization allows for the combination of desirable traits from different species while maintaining genetic diversity, which is key for long-term aquaculture sustainability (Vranken et al., 2021; Zhang et al., 2025). As shown in related macroalgal studies, hybrids may also adapt to broader environmental conditions, potentially expanding cultivation ranges (Murúa et al., 2020; Liu et al., 2023). Further investigation into the potential hybrid vigor of *L. ochroleuca* and *L. digitata* could provide valuable insights, but this was beyond the scope of the thesis.

While hybridization may offer potential benefits, it also poses significant genetic and ecological risks that must be carefully considered (Vranken et al., 2021). One such concern is outbreeding depression, where hybrids exhibit

[3] Sympatry is the term used to describe populations, varieties, or species that occur in the same place at the same time (Pham et al., 2022).

reduced fitness due to the breakdown of coadapted gene complexes, particularly when genetically divergent populations are crossed (Liesner et al., 2020; Murúa et al., 2020).

A clear distinction must be made between laboratory-induced and naturally occurring hybridization events. In controlled laboratory environments, ecological and spatial barriers are removed, which can facilitate hybridization that may not reflect what occurs in natural systems (Murúa et al., 2020). Although such experiments confirm that hybridization is biologically possible, they do not account for the full range of ecological and reproductive constraints that influence hybrid success in the wild. In natural environments, hybridization may be limited by factors such as gamete incompatibility, asynchronous reproductive timing, and environmental selection pressures (Gonzalez & Raimondi, 2024). However, interspecific hybridization within Laminariales is biologically plausible due to the conserved mating systems, the presence of homologous sex hormones, and the synchronization of phenological requirements for gametogenesis (Murúa et al., 2020)

Reproductive barriers are central to these constraints and affect both the viability and reproductive capacity of hybrids. These include prezygotic mechanisms, such as temporal or mechanical isolation, as well as postzygotic mechanisms that compromise hybrid development and fertility (Monotilla et al., 2018; Montecinos et al., 2017). For example, in studies of *Ectocarpus species*, hybrids were predominantly diploid with high levels of aneuploidy, while recombinant haploid gametophytes were rare. These findings indicate strong postzygotic barriers that reduce fertility and may limit hybrid persistence in natural populations (Montecinos et al., 2017). Similar reproductive constraints may also exist between *L. ochroleuca* and *L. digitata*, potentially restricting both hybrid viability and reproductive success. Assessing hybrid fitness requires evaluating both survival and reproductive performance. Reduced fertility and potential sterility often appear before complete hybrid unviability, as shown in comparative studies across multiple taxa (Coyer et al., 2007). A laboratory experiment such as sorus induction, can provide only limited insight into long-term reproductive outcomes, which again highlights the need for field-based research.

To further clarify these dynamics, future research should incorporate genomic tools to evaluate adaptive genetic diversity and detect genotype–environment mismatches. Such mismatches occur when inherited traits become maladaptive under new or changing environmental conditions, thereby reducing hybrid fitness (Bradshaw & McNeilly, 1991; Vranken et al., 2021). Metabolomic approaches may also offer valuable insights by identifying biochemical signatures of hybridization and novel compounds associated with hybrid traits (Murúa et al., 2020). A thorough understanding of hybridization is essential for informing conservation and management strategies, including assisted adaptation efforts aimed at increasing the resilience of kelp forests under climate change (Vranken et al., 2021).

CONCLUSION

This master's thesis investigated the hybridization potential between *Laminaria digitata* and *Laminaria ochroleuca*, aiming to evaluate the viability, fertility, ploidy characteristics, and microbiome compatibility of resulting hybrids. Given the increasing ecological and economic importance of these kelp species and the projected shifts in their distributions due to climate change, understanding the outcomes of interspecific hybridization is essential.

I. Successful Hybridization between L. ochroleuca and L. digitata

This study demonstrated that hybridization between *Laminaria digitata* and *Laminaria ochroleuca* is possible, with hybrid status confirmed through microsatellite marker analysis. The resulting hybrids developed into viable, morphologically regular sporophytes with significantly higher success rates than most controls. Many remained healthy and continued vigorous growth for over eight months, highlighting their developmental stability and potential long-term viability. These findings provide direct support for the hypothesis that these species can hybridize in controlled conditions.

II. Microbiomes are mainly shaped by environment; functional core persists in hybrids

The bacterial communities differed significantly between kelp thallus regions, reflecting functional specialization of microbial assemblages. Geographic location also shaped microbiome composition, likely driven by local environmental conditions. Laboratory-grown sporophytes had less diverse and more uniform microbiomes compared to field samples, again showing the strong influence of environment on microbial diversity. Hybrids exhibited microbial communities with intermediate profiles compared to their parent species, indicating that hybridization influences microbiome composition while maintaining overall diversity. A consistent core microbiome was observed across all crosses, supporting functional stability and resilience of key microbial roles in both hybrids and parental types. Unique microbial taxa found exclusively in hybrids point to the potential creation of novel microbial niches through hybridization. These results emphasize the complex interplay between host genetics, morphology, environment, and microbial communities, underscoring the importance of integrating ecological context in studies of kelp-microbe interactions and hybridization.

III. To conduct preliminary exploration of hybrid characteristics

Preliminary analyses highlighted flow cytometry as a promising tool for estimating genome size in *Laminaria* species, although methodological challenges, such as background interference and calibration issues, currently limit its application. Nonetheless, the detection of a distinct signal in one hybrid suggested potential for future refinement. Fertility assessments through sorus induction remained inconclusive, largely due to suboptimal experimental conditions as well as the insufficient age and size of the blades. These findings underscore the need for further protocol optimization to reliably evaluate genome size and reproductive capacity in kelp hybrids.

Overall, this research provides the first robust evidence of viable *L. digitata* × *L. ochroleuca* hybrids and offers valuable insights into their developmental stability and microbiome composition. These findings advance our understanding of interspecific kelp hybridization and hold promise for future experiments aimed at enhancing thermal resilience, eventually fostering sustainability in kelp aquaculture. Given the persistent ecological challenges affecting marine ecosystems, this area of research is becoming ever more essential.

SUMMARY

- - - - - - - - ---------- English version ---------- - - - - - - - -

Kelp forests, composed of large brown algae in the order Laminariales, are critical marine ecosystems that support biodiversity, protect coastlines, and contribute significantly to global carbon cycles (Smale et al., 2013; Teagle et al., 2017; Krause-Jensen et al., 2018). Among them, *Laminaria digitata* and *Laminaria ochroleuca* are ecologically and economically important species found in temperate North Atlantic waters (Pereira et al., 2019; Bartsch et al., 2008). *L. digitata* is characteristic of colder subtidal environments and ranges from the Arctic to the northern coasts of France and the British Isles, while *L. ochroleuca* is adapted to warmer temperate waters and has a more southerly distribution along the Iberian Peninsula and into the English Channel (Franco et al., 2018; Smale et al., 2015).

As ocean temperatures rise, the geographic ranges of these species are shifting, particularly in European coastal regions such as Brittany and southern England, where the two species increasingly co-occur (Pereira et al., 2019; Franco et al., 2018). This growing sympatry raises the possibility of interspecific hybridization, which could yield novel genotypes combining thermal tolerance and growth potential (Martins et al., 2019). However, previous studies have noted potential reproductive barriers between these species, and it remains unclear whether viable hybrids can be produced under controlled or natural conditions (Bartsch et al., 2008; Tom Dieck, 1992).

This thesis investigated three core objectives: (1) the hybridization potential between *L. digitata* and *L. ochroleuca*, (2) microbiome variation across locations, species, and between interspecific and hybrid crosses, and (3) exploratory characterization of hybrid fertility and ploidy. These objectives aimed to determine whether reproductive isolation existed between the species and whether hybrid offspring displayed unique growth patterns or microbial communities.

To address these questions, a series of controlled crosses were performed using clonal gametophytes from *L. digitata*, *L. ochroleuca*, and the closely related *L. pallida*, which has previously demonstrated compatibility with *L. digitata* (Martins et al., 2019). A total of 21 intra- and interspecific crosses were conducted, each with multiple replicates. Gametophyte development and reproductive success were monitored microscopically over 28 days, and sporophyte production was evaluated based on morphology, attachment, and cellular organization (Martins et al., 2019). Crosses involving *L. digitata* females achieved the highest reproductive success, reaching up to 86.7%, whereas crosses with *L. ochroleuca* females showed reduced reproductivity. Growth and reproductive rates varied not only between cross types but also among replicates, highlighting the influence of individual strain-specific responses. To verify hybrid status, genomic DNA was extracted and analyzed using the microsatellite marker LolVVIV-23 (Coelho et al., 2014). Fragment analysis revealed species-specific alleles in several interspecific crosses, confirming successful hybridization. Notably, in one replicate belonging to the *Och ♀ × Dig ♂* cross type, only maternal alleles were detected, suggesting a case of parthenogenesis (Martins et al., 2019).

To estimate genome size and ploidy levels, flow cytometry was conducted using propidium iodide staining on a Partec PAS III flow cytometer (Sysmex) at Meise botanic garden. In this study, flow cytometry demonstrated potential for genome size estimation in *Laminaria* species, although technical challenges such as background noise and calibration inconsistencies currently hindered accurate interpretation. Nevertheless, the observed

signal of a distinct fluorescence peak detected in a hybrid individual, indicate that methodological refinement is promising.

Reproductive capacity was assessed by cultivating central blade fragments, obtained by removing the meristem and apical tip, under short-day photoperiods and nutrient-enriched conditions for four weeks. However, no sori were observed in any of the crosses, rendering the results inconclusive. This outcome was likely influenced by suboptimal experimental conditions, such as insufficient photoperiod length, absence of aeration, and the relatively early developmental stage of the sporophyte blades (Pang & Lüning, 2004; Boderskov et al., 2021). These findings emphasize the need to refine sorus induction protocols in order to reliably assess reproductive capacity and genome size in *Laminaria* hybrids.

In addition, a comparative microbiome analysis was conducted using full-length 16S rRNA gene sequencing on the Oxford Nanopore platform. Field swabs were collected from the blade and meristem regions of mature individuals across multiple geographic locations, while laboratory samples included both juvenile sporophytes and blade swabs. Bioinformatic analysis revealed that microbiome composition was primarily influenced by sample location and tissue type (Lemay et al., 2021; Ihua et al., 2020). Field-collected samples exhibited distinct, location-specific microbial signatures, whereas lab-grown individuals, including hybrids, harbored more uniform bacterial communities. These findings suggest that environmental exposure exerts a stronger influence than host genotype in shaping microbiome composition under controlled conditions. Importantly, the field and laboratory samples differed not only in cultivation conditions, but also in age and geographic origin, which may have contributed to variation in microbial profiles, though this was not accounted for.

Within the laboratory samples, a consistent core microbiome was observed across all crosses, indicating functional stability and potential resilience of key microbial roles in both hybrids and intraspecific types. At the same time, the detection of unique bacterial taxa found exclusively in hybrids points to the potential formation of novel microbial niches as a result of hybridization. These results highlight the complex interplay between host genetics, morphology, environmental conditions, and microbial communities, emphasizing the need to integrate ecological context into studies of kelp-microbe interactions and hybrid biology.

Taken together, these findings demonstrate that *Laminaria digitata* and *L. ochroleuca* are reproductively compatible under specific laboratory conditions, with hybrids exhibiting long-term viability and the ability to reach substantial size. This study presents the first strong evidence for the viability of *L. digitata × L. ochroleuca* hybrids and offers novel insights into their development and associated microbiomes. By providing concrete proof of successful interspecific hybridization and evaluating hybrid performance in a structured framework, this work contributes meaningfully to the growing fields of kelp breeding and microbial ecology. These results advance the broader understanding of kelp hybridization and underscore its potential for enhancing thermal tolerance, with important implications for sustainable aquaculture. Future research should prioritize long-term field trials to assess hybrid fitness in natural environments and explore the functional roles of microbial taxa in promoting growth, immunity, and reproduction (Stock et al., 2021; Hitch et al., 2022). Successful ploidy assessments and sorus induction trials could offer further clarity, while strategies to prevent genetic introgression into wild populations should be considered to safeguard ecological integrity in commercial applications (Goecke et al., 2020; Murúa et al., 2020). In light of increasing ecological pressures on marine ecosystems, continued research in this area is both timely and essential.

- - - - - - - - ---------- Dutch version ---------- - - - - - - - -

Kelpwouden, gevormd door bruinwieren uit de orde Laminariales, zijn onmisbare mariene ecosystemen. Ze bevorderen biodiversiteit, beschermen kustlijnen en spelen een cruciale rol in de globale koolstofcyclus (Smale et al., 2013; Teagle et al., 2017; Krause-Jensen et al., 2018). Binnen deze groep zijn *Laminaria digitata* en *Laminaria ochroleuca* ecologisch en economisch belangrijke soorten die voorkomen in de gematigde wateren van de Noord-Atlantische Oceaan (Pereira et al., 2019; Bartsch et al., 2008). *L. digitata* is typisch voor koudere subtidale gebieden en komt voor van de Arctische regio's tot aan de noordelijke kusten van Frankrijk en de Britse Eilanden, terwijl *L. ochroleuca* beter is aangepast aan warmere gematigde wateren en een zuidelijker verspreidingsgebied kent, onder meer langs het Iberisch Schiereiland en in het Kanaal (Franco et al., 2018; Smale et al., 2015).

Door de stijgende temperaturen verschuiven de geografische verspreidingsgebieden van deze soorten, met name in Europese kustregio's zoals Bretagne en Zuid-Engeland, waar de twee soorten steeds vaker samen voorkomen (Pereira et al., 2019; Franco et al., 2018). Deze toenemende overlap creëert mogelijkheden voor interspecifieke hybridisatie, wat zou kunnen resulteren in nieuwe genotypen die thermotolerantie combineren met gunstige groeikenmerken (Martins et al., 2019). Toch wijzen eerdere studies op het bestaan van reproductieve barrières, en blijft het onduidelijk of levensvatbare hybriden zich effectief kunnen ontwikkelen onder gecontroleerde en/of natuurlijke omstandigheden (Bartsch et al., 2008; Tom Dieck, 1992).

Deze masterproef onderzocht drie centrale doelstellingen: (1) het hybridisatiepotentieel tussen *Laminaria digitata* en *Laminaria ochroleuca*, (2) variatie in microbiomen tussen locaties, soorten en tussen interspecifieke en hybride kruisingen, en (3) een verkennende karakterisering van de vruchtbaarheid en ploïdie van hybriden. Deze doelstellingen waren gericht op het vaststellen of er reproductieve isolatie bestaat tussen beide soorten en of hybride nakomelingen bepaalde groeipatronen en microbiële gemeenschappen vertonen.

Om dit te onderzoeken, werden een reeks gecontroleerde kruisingen opgezet met klonale gametofyten van *L. digitata*, *L. ochroleuca* en de nauwe verwante *L. pallida*, waarvan eerder werd aangetoond dat die compatibel is met *L. digitata* (Martins et al., 2019). In totaal werden 21 intra- en interspecifieke kruisingen uitgevoerd, verdeeld over zeven algemene kruisingstypes, elk met meerdere replicaten. Gametofytontwikkeling en reproductief succes werden gedurende 28 dagen microscopisch opgevolgd, en sporofytvorming werd beoordeeld op basis van morfologie, aanhechting en cellulaire organisatie (Martins et al., 2019). Kruisingen waarbij *L. digitata* als vrouwelijke ouder fungeerde, bereikten het hoogste voortplantingssucces, tot wel 86,7%, terwijl kruisingen met *L. ochroleuca* als vrouwelijke ouder, initieel een beperkter succes vertoonden. Groei en reproductie varieerden niet alleen tussen de verschillende kruistypes, maar ook tussen replicaten, wat wijst op de invloed van individuele responsen en de specifieke kenmerken van afzonderlijke algenstammen. Om het hybridekarakter van de nakomelingen te bevestigen, werd DNA geëxtraheerd en geanalyseerd via de microsatellietmarker LolVVIV-23 (Coelho et al., 2014). Fragmentanalyse bracht soortspecifieke allelen aan het licht in meerdere interspecifieke kruisingen, wat succesvolle hybridisatie bevestigde. Opmerkelijk was dat in één replicaat van het type Och ♀ × Dig ♂ enkel maternale allelen werden teruggevonden, wat duidt op parthenogenese (Martins et al., 2019).

Om het genoomvolume en ploidieniveau te schatten, werd flowcytometrie uitgevoerd met propidiumjodidekleuring op een Partec PAS III flowcytometer (Sysmex) in Plantentuin Meise. Flowcytometrie kwam in deze studie naar voren als een veelbelovende methode voor het inschatten van genoomgrootte bij *Laminaria*-soorten. Hoewel bij één hybride een duidelijke fluorescentiepiek werd waargenomen, bemoeilijkten technische beperkingen zoals achtergrondruis en calibratieproblemen een betrouwbare interpretatie. Hoewel hieruit geen duidelijke conclusies konden worden getrokken, duidt dit signaal wel op het potentieel voor verdere methodologische verfijning.

De voortplantingscapaciteit werd beoordeeld via sorusinductie, waarbij het meristeem weefsel van volwassen sporofyten werd afgesneden en vierkante blade fragmenten gedurende 4 weken werden blootgesteld aan korte daglichtcyclus en een voedingsrijke omgeving. In geen van de kruisingen werden sori gevormd en dus konden er geen conclusies getrokken worden uit deze resultaten. Waarschijnlijk speelden suboptimale experimentele omstandigheden een rol, net zoals de beperkte grootte van de blades (Pang & Lüning, 2004; Boderskov et al., 2021). Deze bevindingen onderstrepen het belang van verdere optimalisatie van sorusinductieprotocollen om de reproductieve capaciteit en genoomkenmerken van *Laminaria*-hybriden te onderzoeken.

Daarnaast werd een vergelijkende microbioomanalyse uitgevoerd op basis van full-length 16S rRNA-gensequencing via het Oxford Nanopore-platform. Swabs werden afgenomen van blad- en meristeemweefsel van volwassen individuen op verschillende geografische locaties. Ook de laboratoriumkruisingen werden onderzocht, hiervoor werden zowel juveniele sporofyten als blade swabs gebruikt. Statistische analyse toonde aan dat de microbiële samenstelling werd beïnvloed door de locatie van staalname en het type weefsel (Lemay et al., 2021; Ihua et al., 2020). De veldstalen vertoonden duidelijke, locatiegebonden microbiële signaturen, terwijl lab-gecultiveerde individuen, inclusief hybriden, een meer uniforme bacteriële gemeenschap vertoonden. Deze bevindingen suggereren dat omgevingsinvloeden een dominante rol spelen in het bepalen van de microbioomsamenstelling onder gecontroleerde omstandigheden. Belangrijk hierbij is wel dat de veld- en laboratoriumstalen niet alleen verschilden in groeicondities, maar ook in leeftijd en locatie van oorsprong, wat mogelijk heeft bijgedragen aan de variatie in microbiële profielen.

Voor de labo kruisingen werd een consistent kernmicrobioom waargenomen, wat wijst op functionele stabiliteit en mogelijke veerkracht van sleutelbacteriële functies bij zowel hybriden als ouderlijke types. Tegelijkertijd werden unieke bacteriële taxa aangetroffen die exclusief in hybriden voorkwamen, wat suggereert dat hybridisatie kan leiden tot het ontstaan van nieuwe microbiële niches. Deze resultaten benadrukken de complexe interactie tussen gastheer, morfologie, omgevingsinvloeden en microbiomen. Het belang van het integreren van de ecologische context in studies naar kelp-microbe-interacties en hybridisatie wordt hiermee aangetoond.

Samenvattend levert dit onderzoek robuust bewijs voor succesvolle hybridisatie tussen twee belangrijke kelpsoorten en draagt het bij aan onze kennis over mariene macroalgen, hun voortplantingsbiologie en hun interactie met microbiële gemeenschappen. Het vormt een waardevolle basis voor toekomstig onderzoek naar hybride prestaties in het veld, evenals voor de ontwikkeling van duurzame kweekstrategieën in een snel veranderende wereld (Lippman & Zamir, 2007; Sahu & Mishra, 2021).

ACKNOWLEDGEMENTS

My deepest gratitude goes to those who have supported me throughout my journey in completing this dissertation. I would like to express my sincere gratitude to Neusa Martins, Ashwin Engelen and Gareth Pearson for their valuable expertise, extensive knowledge, and guidance during my time in Faro. Additionally, the whole lab team was very supportive and always available to help. A special thanks to Axelle Defossez, my dearest friend and colleague, who stood by me in Faro while working on her thesis too. I am deeply thankful to the phycology lab in Ghent for granting me the opportunity to participate in this educational exchange.

Additionally, I am thankful to the molecular lab, especially Sofie D'hondt, for her expertise and guidance on the genetic aspects of this thesis. I would also like to express my gratitude to Sofie Vranken for her knowledge and assistance. My thanks go to Carl Van Colen and the Ugent Marine Biology research group for making the Aquarium room available for cultivation of the 10L cultures.

To Jordi Morcillo Baeza, Sofie Meeus and Frederik Leliaert, I want to express my gratitude for the guidance and advice on the flow cytometry work conducted in Meise.

Finally, I extend my heartfelt thanks to my tutor Sofie Peeters for her support and guidance from start to finish and my promotor Professor Dr. Olivier De Clerck for his guidance and constructive criticisms throughout the experimental brainstorming process and the writing of this paper.

I made use of artificial intelligence, particularly OpenAI's GPT-4, which provided assistance in various aspects of this work. The AI's capabilities in vocabulary extension, writing enhancement, and coding support contributed to the clarity and effectiveness of my dissertation.

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APPENDIX

Appendix A: Different individuals

3 Female L. ochroleuca	*3 Male L. ochroleuca*
Och-F-3.7	Och-M-1.7
Och-F-6.6	Och-M- 9.7
Och-F-1.3	Och-M-6.3
3 Female L. digitata	*3 Male L. digitata*
Dig-F-9	Dig-M-18
Dig-F-18	Dig-M-22
Dig-F-22	Dig-M-9
3 Female L. pallida	*3 Male L. pallida*
Pal-F-1.2	Pal-M-3.3
Pal-F-3.1	Pal-M-7.3
Pal-F-7.5	Pal-M-1.3

Figure A: Strain designations, adopted from Faro, for male and female individuals of the species.

Appendix B: HMW DNA extraction

OmniPrep Protocol for Plant Tissue

(adapted for extracting HMW DNA from brown algal tissue)

1. Grind samples in liquid nitrogen to a fine powder.
2. Transfer 50-100mg finely ground tissue quickly to a microcentrifuge tube containing 500µl (1000 µl for HMW, large amount of tissue) Genomic Lysis Buffer and carefully mix by inverting.
3. If lumps persist, homogenize the sample with a microfuge pestle until a homogenous suspension is acquired, approximately 30-60 strokes.
4. Add 5µl (10 µl for HMW, large amount of tissue) Proteinase K solution and incubate at 60°C for 1-2 hours. Invert the tube periodically each quarter hour.
5. Allow the sample to cool to room temperature. Do a short spin, to get rid of the aerosols on top of the lid
6. Add 200µl (400 µl for HMW, large amount of tissue) chloroform and mix by inverting the tube several times. (50x inverting)
7. Centrifuge for 10 minutes at 14,000xg and carefully remove the upper phase to a clean microcentrifuge tube.
8. Add 50µl (100 µl for HMW, large amount of tissue) DNA Stripping Solution to the sample and invert 50 times to mix. Incubate the sample for 10 minutes at 60°C.
9. Add 150µl (300 µl for HMW, large amount of tissue) Precipitation Solution and mix by inverting the tube 100 times. A white precipitate should be produced (if not, add 50µl aliquots of Precipitation Solution until a white precipitate forms).
10. Incubate sample on ice for 15 minutes.
11. Centrifuge the sample at 14,000xg for 10 minutes.
12. Transfer the supernatant to a clean tube and precipitate the genomic DNA with 500µl (1000 µl for HMW, large amount of tissue) isopropanol. Invert the tubes 50 times to precipitate the DNA.
13. Centrifuge at 14,000xg for 10 minutes to pellet genomic DNA. Remove the supernatant.
14. Add 700µl 75% ethanol to the tube and invert several times to wash the DNA pellet. Centrifuge for 10 minute at 14,000xg. (*In some samples, the pellet may be hard to see at this point and will be loosely attached to the tube.)*
15. Decant or pipette off the ethanol wash. Invert the tube on a clean absorbent surface for 5 minutes to allow any excess ethanol to drain away (do not let the pellet dry completely or it will be difficult to rehydrate).
16. Add 50µl (100 µl for HMW, large amount of tissue) TE Buffer to the pellet. Incubate the tube at 55-60°C for 30 minutes to rehydrate pellet.
17. If needed, pool samples. Add 1µl LongLife™ RNase for every 100µl TE Buffer and incubate a further 30 minutes at 55-60°C to digest RNA. (this step is only done for HMW DNA)
18. Store DNA at -20°C or -80°C.

Appendix C: Environmental conditions sample sites

Table C: Overview of abiotic and environmental conditions at sampling locations. Data include sampling depth, average sea surface temperature (SST) and salinity (PSU) during late May–early June, and general environmental context based on literature and public sources.

Location	Sampling Depth (m)	SST (°C)	Salinity (PSU)	Environmental Conditions
Martin's Haven, UK	5	~13.0	~34.5	Situated within the Skomer Marine Conservation Zone, indicating minimal anthropogenic disturbance. A remote bay with limited freshwater input (Natural Resources Wales).
Hoe Point, UK	3–4	~13.0	~34.5	Adjacent to Plymouth Sound, influenced by estuarine conditions and near a major urban and industrial area. Significant freshwater input from nearby rivers (Turner & Higgins, 2023; Wright et al., 2022).
Roscoff, France	2–4	14.2	34.78	Active port town with a marina and ferry terminal, reflecting moderate anthropogenic activity. Moderate river input; relatively sheltered from wave exposure (Carrano et al., 2021).
Gatteville-le-Phare, France	1	12.8	~35.0	Close to Roubary and Barfleur ports but designated as an ecologically protected site. Low anthropogenic pressure and limited river input (Biotope, 2014; SeaTemperature.net).
Goleen Harbour, Ireland	1–3	13.8	~34.8	Located near Crookhaven Harbour and a small inlet. Moderate freshwater input; low anthropogenic impact due to remoteness, with strong natural tidal dynamics (EOceanic.com; SeaTemperature.info).

While this table provides an overview of environmental parameters to aid the interpretation of microbial community patterns, it is based on general public data. For conclusive insights into abiotic drivers, site-specific measurements collected during the actual sampling period are required.

Appendix D: Sporophyte development on Day 7 and Day 14

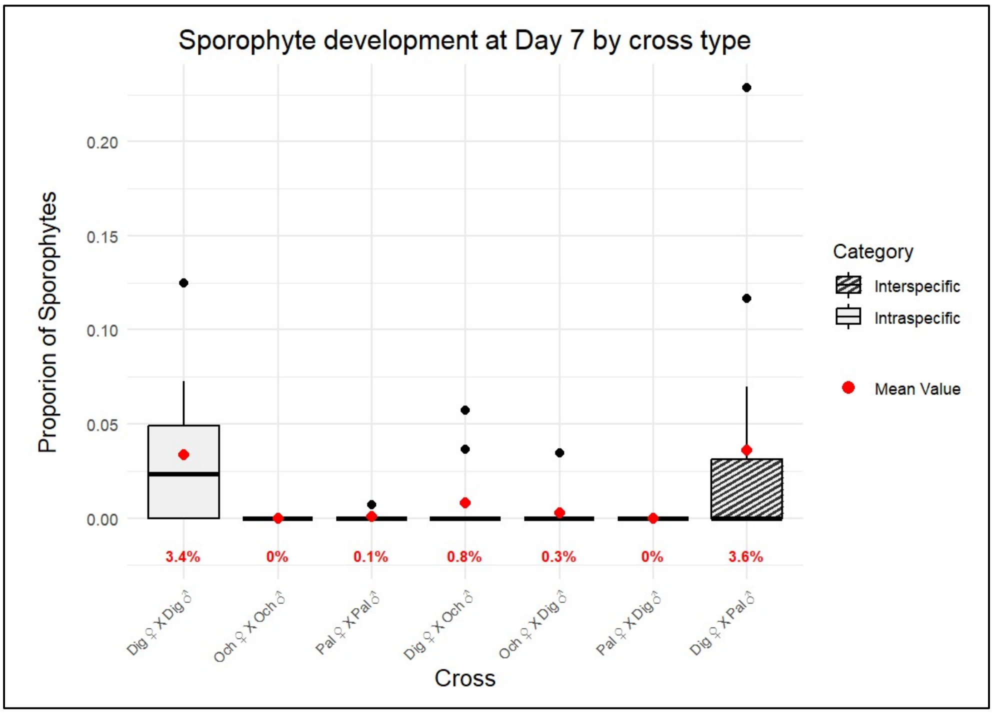


Figure D1: Sporophyte development on day 7 across crosses. Boxplots show median, interquartile range, and range (excluding outliers). Outliers = black dots; means = red dots. Cross types use species abbreviations (Dig, Och, Pal) with ♀ and ♂ symbols. Intraspecific crosses are white; hybrids are striped. Error bars show SD across replicates.

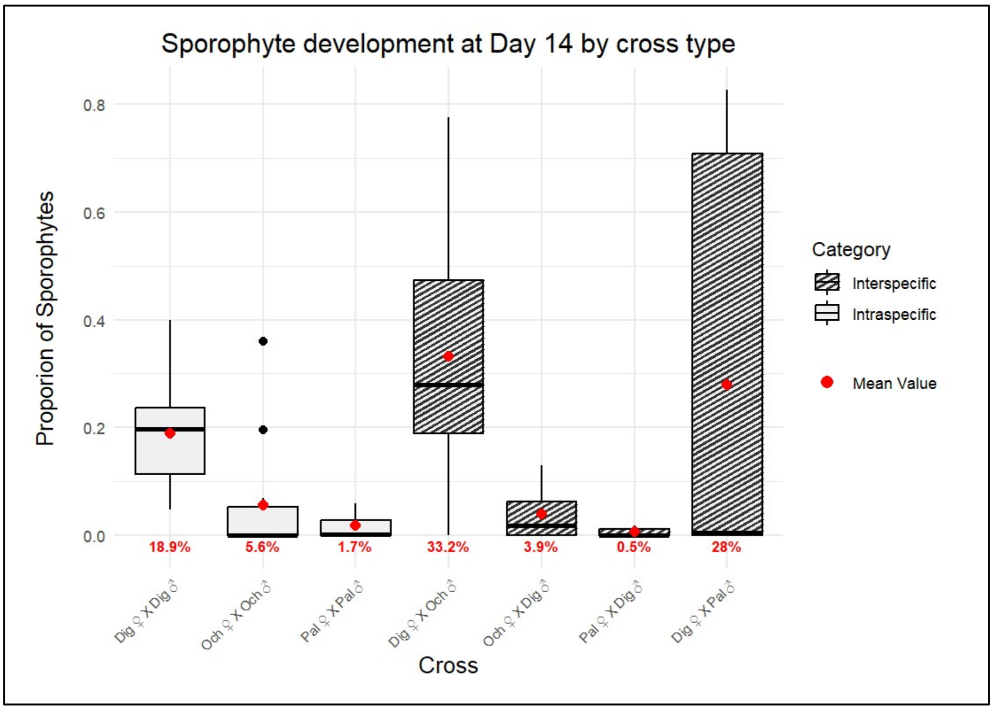


Figure D2: Sporophyte development on day 14 across crosses. Boxplots show median, interquartile range, and range (excluding outliers). Outliers = black dots; means = red dots. Cross types use species abbreviations (Dig, Och, Pal) with ♀ and ♂ symbols. Intraspecific crosses are white; hybrids are striped. Error bars show SD across replicates.

Appendix E: Significant differences in reproductive efficiency between the crosses after 21 days

Table E: Adjusted p-values from pairwise comparisons of reproductive efficiency between cross types, using Dunn's test with Bonferroni correction. Only comparisons above the significance threshold are shown.

Cross	Adjusted p-value
Dig ♀ X Dig ♂ vs. Och ♀ X Och ♂	0.0287
Dig ♀ X Dig ♂ vs. Pal ♀ X Dig ♂	0.0047
Dig ♀ X Och ♂ vs. Pal ♀ X Dig ♂	0.0032
Dig ♀ X Dig ♂ vs. Pal ♀ X Pal ♂	0.0283
Dig ♀ X Och ♂ vs. Pal ♀ X Pal ♂	0.0199

Appendix F: Facet wrap in reproductive efficiency between the crosses after 28 days

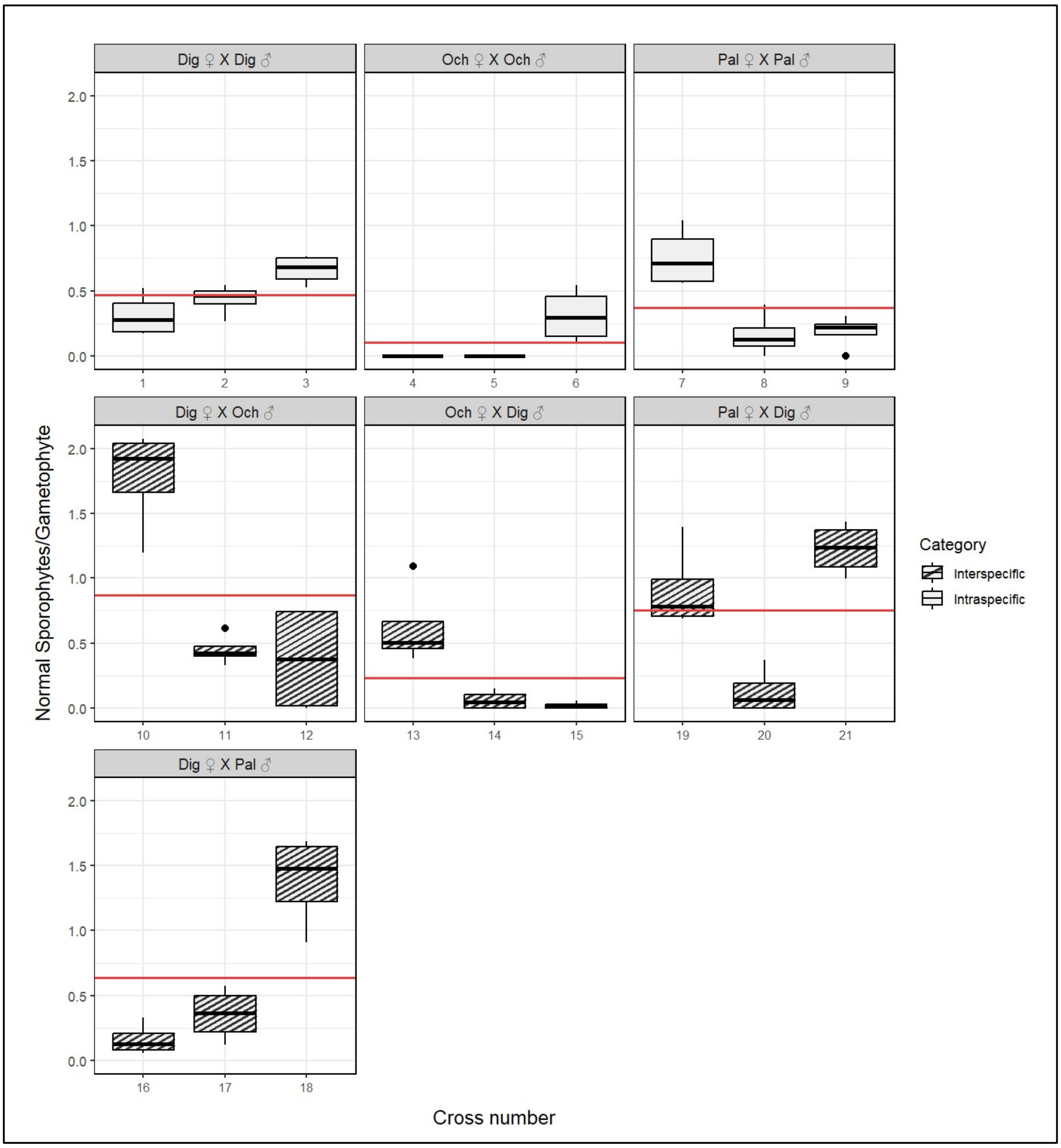


Figure F: Sporophyte density per gametophyte after 28 days as a measure of reproductive success. Cross types are displayed in separate panels using facet wrapping to visualize variation among replicates. The red line represents the mean value.

Appendix G: Sporophyte Growth for L. pallida

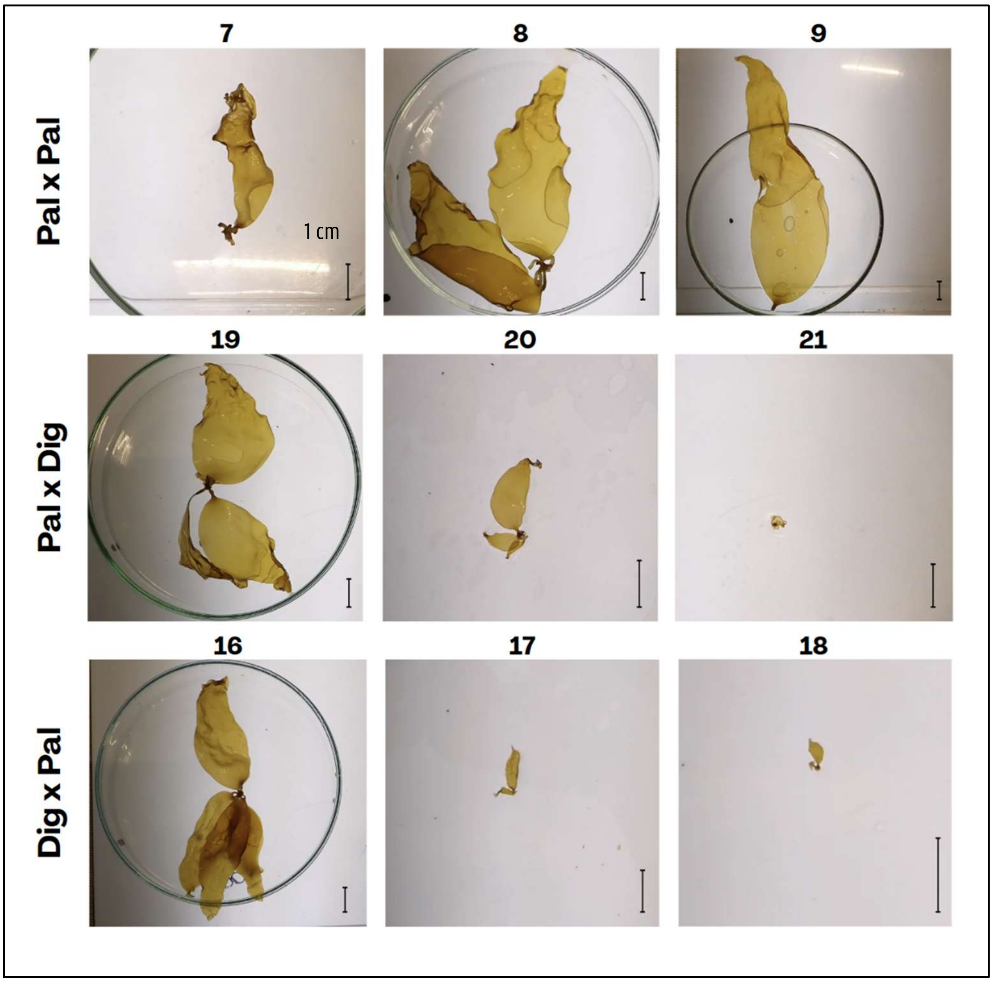
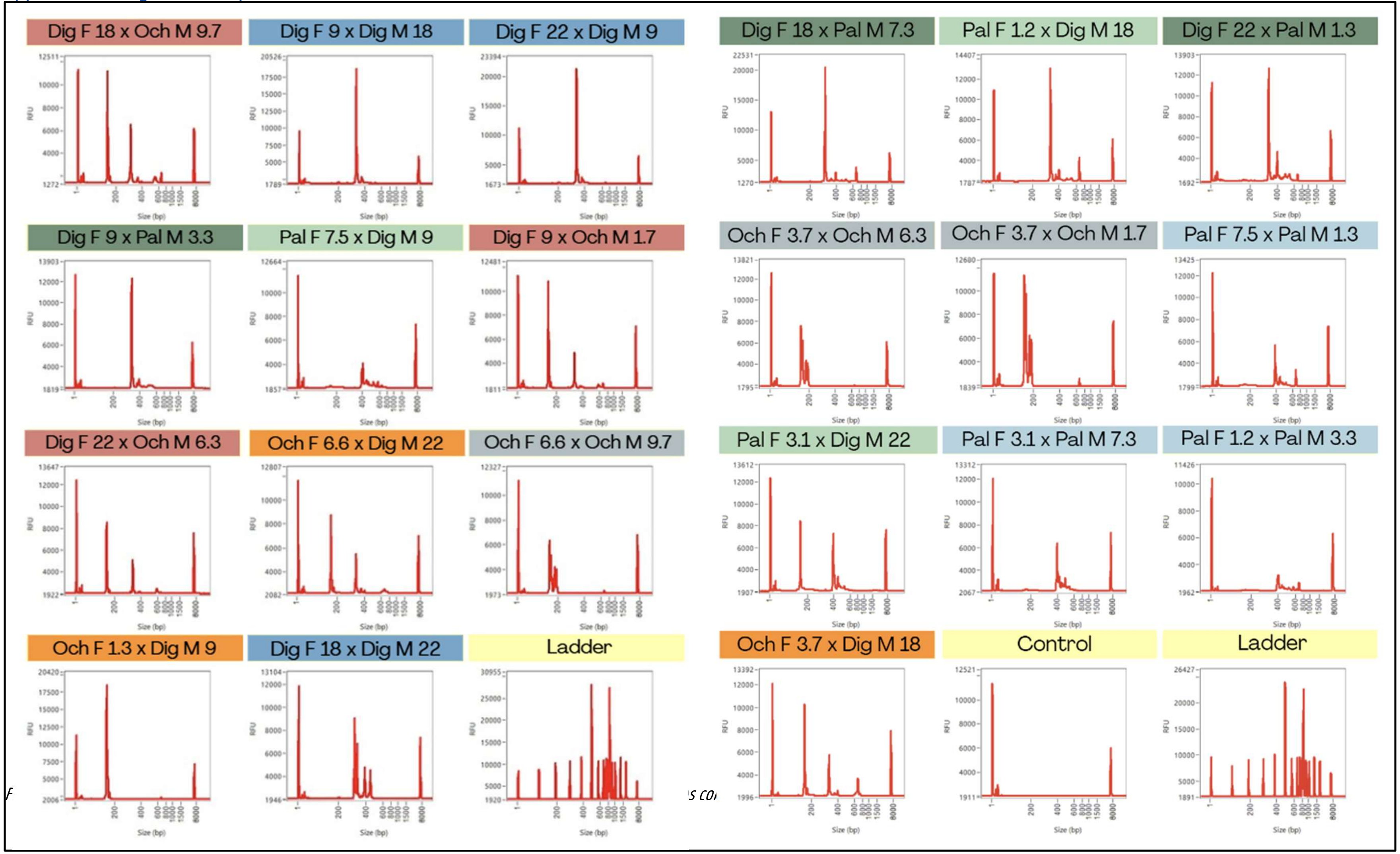


Figure G: Sporophytes after 5 weeks in 2 liter bottles (~ 12 weeks after crossing) from both intraspecific crosses (Dig × Dig; Pal × Pal) and reciprocal interspecific crosses (Dig × Pal; Pal × Dig). The black line represents a 1 cm scale.

Appendix H: Fragment analysis



Appendix I: Microbiome variation between clades

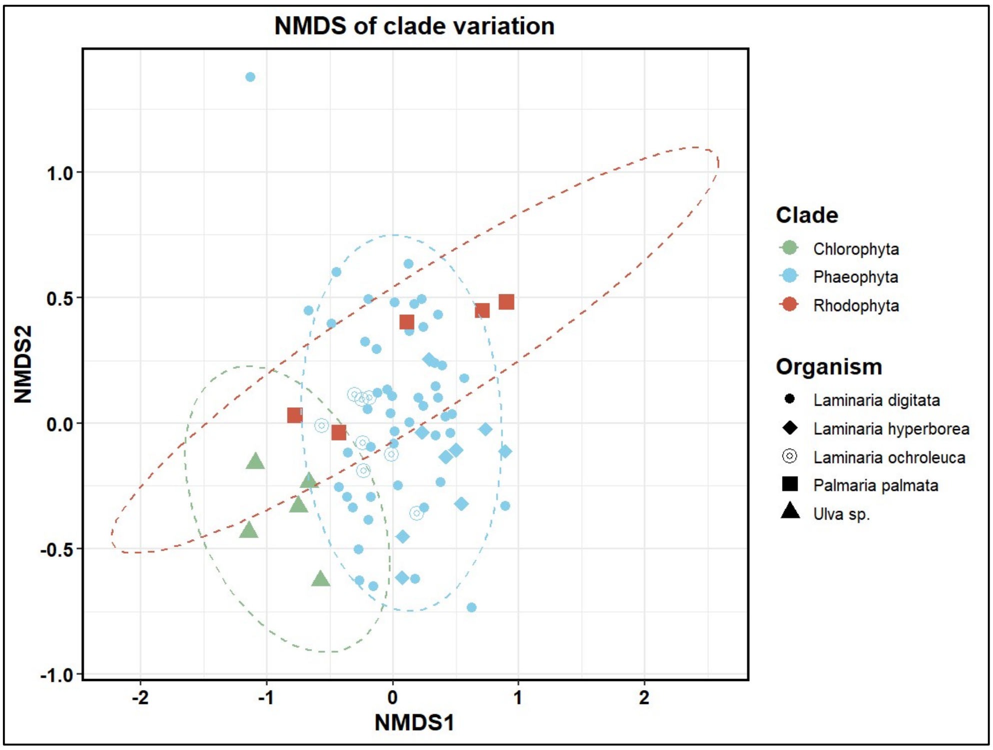


Figure I: Non-metric multidimensional scaling (NMDS) plot showing microbial community variation across different thallus regions and geographic locations. Each point represents a sample, with color indicating the clade and ellipses highlighting clustering patterns. The axes (NMDS1 and NMDS2) represent ordination scores, summarizing differences in microbial communities.

Appendix J: Core genera across sample locations

Table J: Core microbial genera per sampling location, showing overlap and uniqueness with taxonomic details.

Combination	Count	Taxa
France, Roscoff & France, Gatteville-le-phare & Ireland, Goleen Harbour & UK, Hoe Point & UK, Martin's Haven	8	Colwellia, Alteromonas, Rhodopirellula, Blastopirellula, Paraglaciecola, Litorimonas, Hellea, Arenicella
Ireland, Goleen Harbour	7	Haliangium, Tateyamaria, Candidatus Thiodiazotropha, Thiogranum, Thiolapillus, HTCC5015, Litoreibacter
Ireland, Goleen Harbour & UK, Martin's Haven	7	Stenotrophomonas, Ulvibacter, Reichenbachiella, Sphingorhabdus, Yoonia-Loktanella, Fabibacter, Fuerstia
France, Gatteville-le-phare	5	Pseudoalteromonas, Shewanella, Psychrobacter, Cobetia, Marinomonas
France, Gatteville-le-phare & UK, Hoe Point	5	Marinobacter, C1-B045, Lentimonas, Owenweeksia, Nisaea
UK, Hoe Point	4	Thalassospira, Erythrobacter, Fluviicola, Pelagibius
France, Gatteville-le-phare & Ireland, Goleen Harbour & UK, Hoe Point & UK, Martin's Haven	4	Sulfitobacter, Lewinella, Fretibacter, Rubidimonas
Ireland, Goleen Harbour & UK, Hoe Point	2	Sedimenticola, Thioalkalispira-Sulfurivermis
France, Roscoff & UK, Hoe Point & UK, Martin's Haven	2	Pleurocapsa PCC-7319, Thalassotalea
Ireland, Goleen Harbour & UK, Hoe Point & UK, Martin's Haven	2	Peredibacter, Cocleimonas
France, Roscoff & France, Gatteville-le-phare & UK, Hoe Point	1	Vibrio
France, Roscoff & Ireland, Goleen Harbour & UK, Martin's Haven	1	Leucothrix
France, Gatteville-le-phare & Ireland, Goleen Harbour & UK, Martin's Haven	1	Portibacter
France, Gatteville-le-phare & UK, Hoe Point & UK, Martin's Haven	1	Psychromonas
France, Roscoff & France, Gatteville-le-phare & Ireland, Goleen Harbour & UK, Martin's Haven	1	Sva0996 marine group
France, Roscoff & Ireland, Goleen Harbour & UK, Hoe Point & UK, Martin's Haven	1	Granulosicoccus

Appendix K: Microbiome comparison of L. pallida crosses

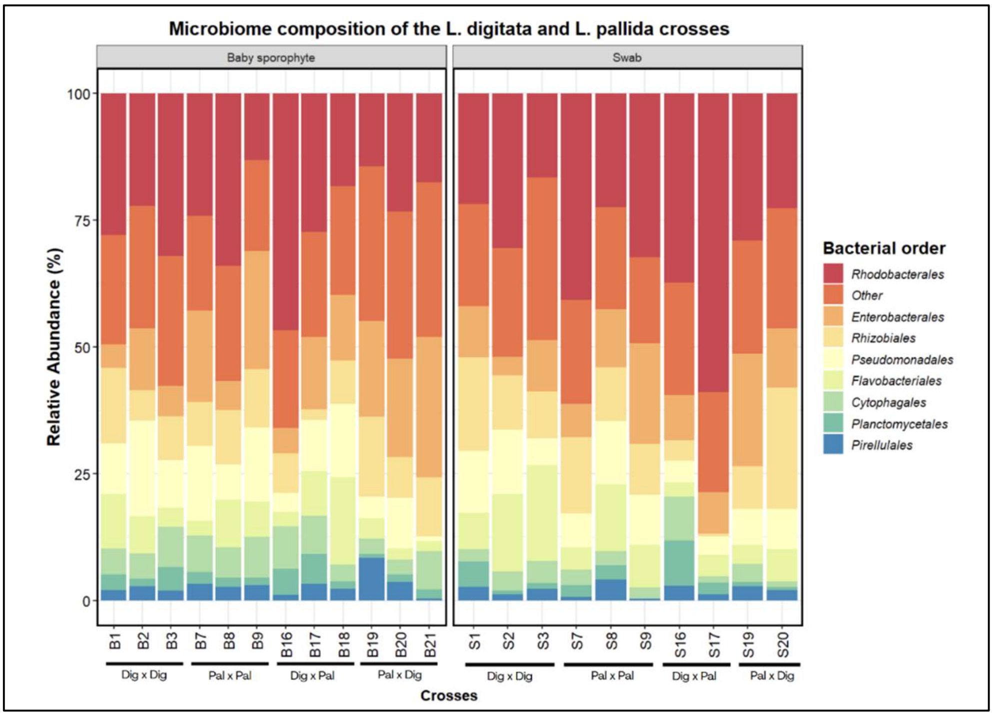


Figure K1: Microbiome composition of the crosses involving L.digitata and L. pallida. Baby sporophyte samples were collected after 8 weeks of growth, and swab samples were taken from the center of the algal blade. Each bar represents an individual, with colors indicating different bacterial orders. The bacterial orders shown represent the 8 most abundant bacterial orders across all samples. All remaining orders were grouped under the category "Other".

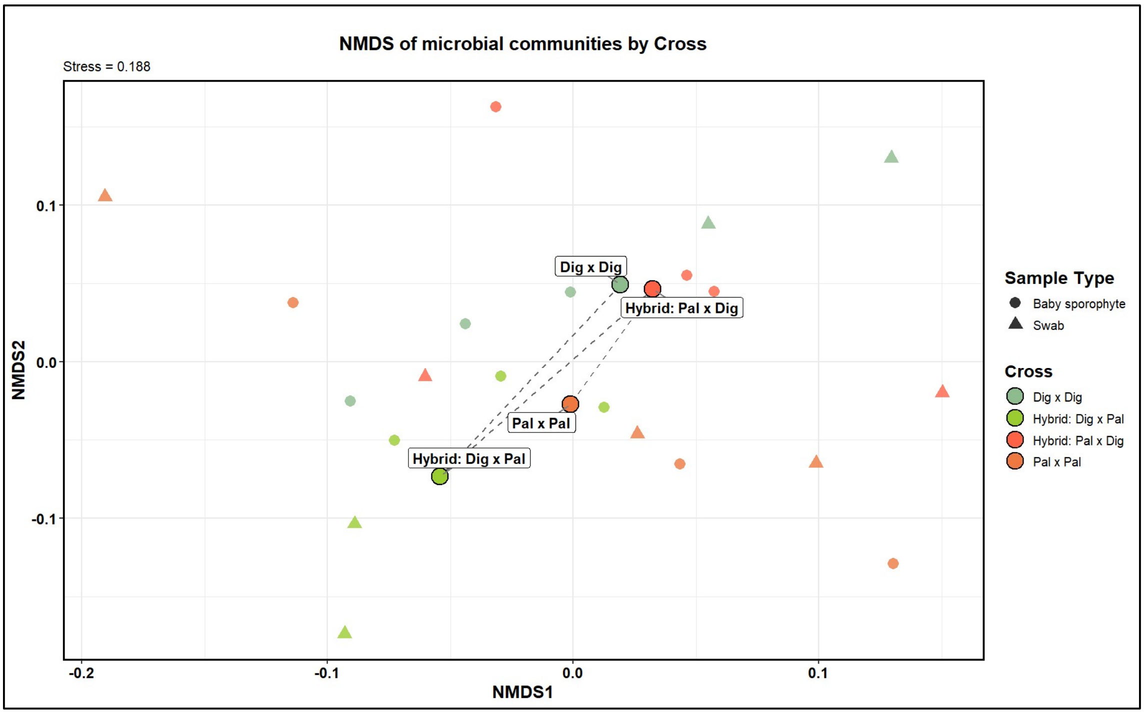


Figure K2: NMDS plot visualizing microbial community structure across different crosses and sample types. The spatial distribution of points along NMDS1 and NMDS2 axes represents microbial variability, with dashed representing distance in microbial composition between crosses.

Appendix L: Core genera across sample locations

Table L: Core microbial genera identified in the L. digitata and L. ochroleuca crosses, showing both shared and unique taxa across groups.

Combination	Count	Taxa
Dig x Dig & Och x Och & Hybrid: Dig x Och & Hybrid: Och x Dig	7	Sulfitobacter, Alteromonas, Paraglaciecola, Sphingorhabdus, Hoeflea, Fabibacter, Granulosicoccus
Dig x Dig	6	Methylophaga, Antarctobacter, Leisingera, Halioglobus, Gaetbulibacter, Haliea
Hybrid: Och x Dig	6	Maribacter, Marinobacter, Marivita, SM1A02, Phycisphaera, Litoreibacter
Dig x Dig & Och x Och & Hybrid: Dig x Och	5	Blastopirellula, Ulvibacter, Planktotalea, Lentilitoribacter, Fuerstia
Dig x Dig & Och x Och & Hybrid: Och x Dig	3	Stenotrophomonas, Pseudophaeobacter, Epibacterium
Och x Och	2	Amylibacter, OM75 clade
Hybrid: Dig x Och & Hybrid: Och x Dig	2	C1-B045, Yoonia-Loktanella
Hybrid: Dig x Och	1	Lacimonas
Dig x Dig & Och x Och	1	Labrenzia
Dig x Dig & Hybrid: Och x Dig	1	Porticoccus
Och x Och & Hybrid: Dig x Och	1	Roseobacter
Och x Och & Hybrid: Och x Dig	1	Cohaesibacter
Dig x Dig & Hybrid: Dig x Och & Hybrid: Och x Dig	1	Tateyamaria
Och x Och & Hybrid: Dig x Och & Hybrid: Och x Dig	1	Reichenbachiella

Appendix M: Flow cytometry output

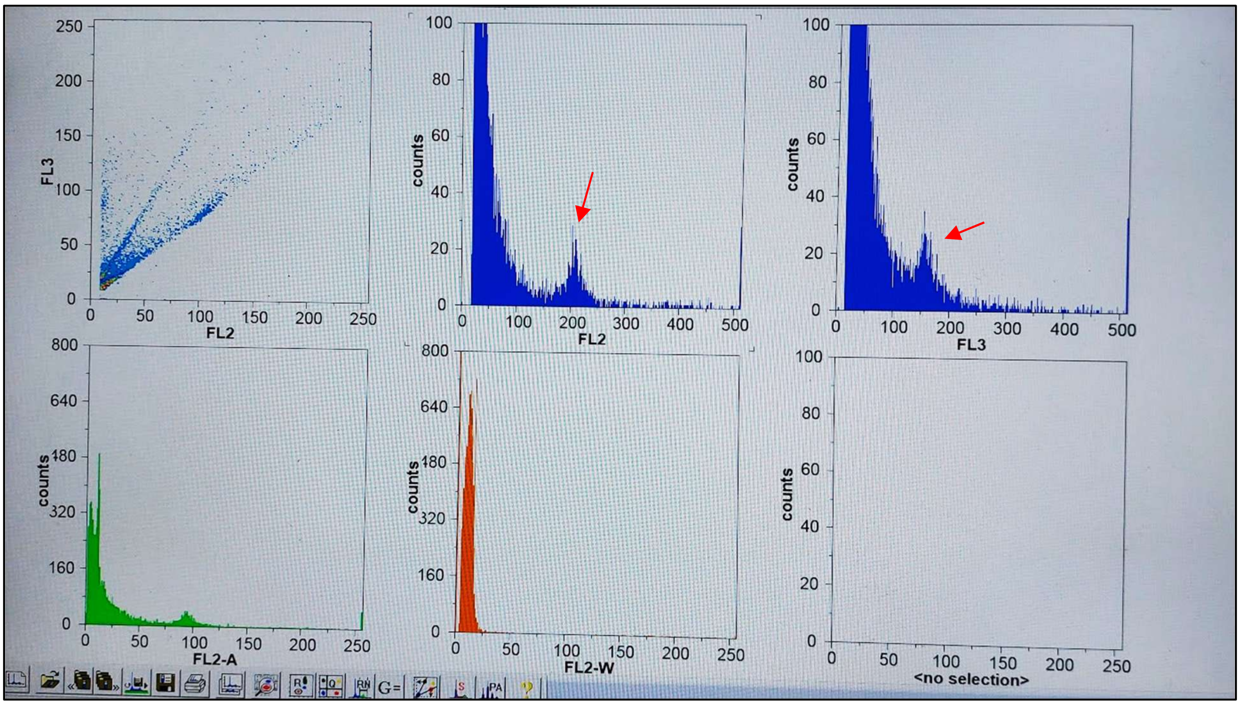


Figure M: Screenshot from the ploidy assessment of Hybrid: Dig x Och, recorded with FlowMax software (Sysmex). The red arrows point to the second peak, which was absent in individuals from interspecific crosses.