Endophytic bacteria within the green siphonous seaweed *Bryopsis:* Exploration of a partnership

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"Life did not take over the globe by combat, but by networking"

Lynn Margulis
evolutionary biologist

Life itself is contained in this one sentence.

Besides Darwin's famous 'natural selection' theory,
symbiosis, the cooperation between different organisms,
is necessary for the survival and evolution of species.

Looking at a human being, an animal, or a plant.
They all tell the same story.

Everything works according the same holistic principle of
interconnection and cooperation.

Our body contains trillions of bacterial cells, 10 times more than human cells.
Yes, you are more bacteria than human!
Without bacteria you would weigh 1.2 kg less, and yet you don't want to lose them.
Bacteria digest our food and keep us healthy.

Also seaweeds undertake close collaborations with external (ectosymbiotic) and internal (endosymbiotic) bacteria. Seaweeds are an unlimited source of oxygen and sugars which bacteria are happy to take advantage of. In exchange for these nutrients, bacteria produce growth promoting minerals and vitamins and they protect their host against environmental threats. As a result, many seaweed-bacterial associations are essential for both symbiotic partners.

This thesis focuses on the association between the feathery-like alga *Bryopsis* and bacteria inside this green seaweed. It has been known for over 40 years that *Bryopsis* houses bacteria, but nothing was known about their identity and function. The following pages take you on an exploratory trip to the hows and whys of this exciting partnership.

I hope that, while reading between the lines, I can tell you a story about the power of collaboration.
Not between two, but a lot of partners.
Each with their own talents and flaws.
Each in their own way.

"*It takes two to tango, but a whole crowd to stage dive!*"

"Life did not take over the globe by combat, but by networking"

Lynn Margulis
evolutiebiologe

Het verhaal van het leven ligt vervat in deze ene zin.

Naast Darwins gekende 'natuurlijke selectie'-theorie,
is symbiose, de samenwerking tussen verschillende organismen,
nodig voor de overleving van soorten.
Het is tevens de motor van hun evolutie.

Bekijk een mens, bekijk een dier, bekijk een plant.
Ze vertellen allen hetzelfde verhaal.

Alles functioneert volgens hetzelfde holistische principe van
onderlinge beïnvloeding en samenwerking.

In en op ons lichaam zitten biljoenen bacteriële cellen, 10x meer dan menselijke cellen.
Ja, je leest het goed, je bent meer bacterie dan mens!
Zonder bacteriën zouden wij maar liefst 1,2 kg minder wegen, geen onaangename gedachte.
En toch wil je ze niet kwijt. Bacteriën verteren ons voedsel en houden ons gezond.

Ook zeewieren gaan hechte samenwerkingsverbanden aan met uitwendige (ectosymbiontische) en inwendige (endosymbiontische) bacteriën. Zeewieren zijn een onuitputtelijke bron van zuurstof en suikers en daar maken bacteriën maar al te graag gebruik van. In ruil voor deze voedingsstoffen maken bacteriën groeibevorderende mineralen en vitamines aan en beschermen ze hun gastheer tegen bedreigingen van buitenaf. Vele zeewier-bacterie associaties zijn dan ook van levensbelang voor beide symbiose partners.

Deze thesis focust op de associatie tussen het vederwier *Bryopsis* en bacteriën aanwezig binnenin het wier. Het is al meer dan 40 jaar geweten dat het vederwier bacteriën huist, maar er was niets gekend omtrent hun identiteit en functie. De volgende bladzijden nemen je mee op een verkennende tocht naar het hoe en waarom van dit boeiend partnerschap.

Ik hoop dat ik jou, tussen de technische hoofdstukken door, een verhaal kan vertellen over de kracht van samenwerking. Niet tussen twee, maar een heleboel partners. Elk met hun eigen talenten en gebreken. Elk op hun eigen manier.

"*It takes two to tango, but a whole crowd to stage dive!*"

Tijdens mijn persoonlijke doctoraats-stage-dive heb ook ik gebruik kunnen maken van gezellige en nuttige symbioses met anderen, daarom gaat mijn dank uit naar

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en in het bijzonder naar

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voor de opdracht en het vertrouwen

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Olivier
voor het inbed- en snijwerk en de knaagdiermoppen

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om mij te leren duiken en Bryopsis herkennen in Franse wateren

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voor de hulp bij het zoeken naar ongewone dingen

Het sympathieke sequentieteam: Liesbeth, Evie en Jindrich
voor het kortwieken van de vele sequenties en om zich gewillig te laten omkopen

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voor de hulp bij die ver-draaide excelsheets

Wim
voor de symbiose tussen leek & PC en hoofd & voeten

Gwen en Anne
voor de zotheid en om samen het DGGEspook te temmen

Renata
voor de eerste opvang en de blijvende vriendschap

Sofie en Karolien
om het drie-muskutier- én symbiose-motto "Eén voor allen, allen voor één!" alle eer aan te doen

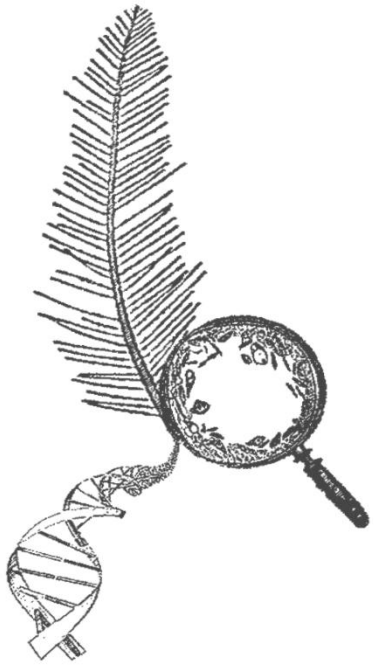
Vrienden en familie
Moeke, vake en meke
om mij te laten springen en indien nodig ook op te vangen

Dear crowd,
It was a pleasure to PhD-dive with you!

Joke

1 Chapter

Literature



What to learn from sushi: a review on seaweed-bacterial associations

Joke Hollants, Frederik Leliaert, Olivier De Clerck and Anne Willems. What to learn from sushi: a review on seaweed-bacterial associations. *Manuscript submitted as a mini-review to FEMS Microbiol Ecol.* **Author contributions:** The literature review was outlined, performed and written by Joke Hollants. Frederik Leliaert, Olivier De Clerck and Anne Willems commented on the text.

If there is one thing we can learn from sushi, it is its digestion by an alga-associated bacterium. The carbohydrate active enzyme porphyranase from the marine Bacteroidetes bacterium Zobellia galactanivorans breaks down the sulphated polysaccharide porphyran from the red alga Porphyra (nori) traditionally used to prepare sushi. Moreover, the genes coding for this porphyranase have been horizontally transferred through dietary seaweed from Z. galactanivorans to the gut microbe Bacteroides plebeius from particularly Japanese people, allowing them to digest the algae which wrap sushi rolls and other delicacies [1]. This not only indicates that the human gut microbiota may become proficient at using dietary polysaccharides by horizontal gene transfer; it also highlights the significance of macroalgal-bacterial associations.

Like sushi, algae come in many forms and flavors ranging from microscopic unicells to gigantic kelps inhabiting oceans, freshwater habitats, soils, rocks and even trees [2]. Consequently, this review needed some delimitation and is restricted to studies of bacteria associated with marine macroalgae (seaweed) belonging to the Chlorophyta (green algae), Rhodophyta (red algae) and Phaeophyceae (brown algae). Seaweed and bacteria have come a long way since algal plastids originated from endosymbiotic cyanobacteria [3 and see also Box 1]. Like their unicellular ancestors, marine macroalgae form the modern-day playground for a wide diversity of bacterial associations ranging from beneficial (mutualistic), harmful (parasitic) and neutral (commensal), over obligate and facultative, to endo- and ectophytic interactions (Box 1). This, along with applied aspects of current algal-bacterial symbioses (Box 2), makes their associations appealing for evolutionary, ecological and biochemical studies. Nevertheless, investigations of macroalgal-bacterial associations lag behind these of other marine eukaryotes [4]. Whereas the full cycle 16S rRNA approach [5] is well established to characterize the microbial associates of unicellular algae, corals and sponges [6, 7], these molecular techniques are just beginning to be applied to macroalgae [4 and references therein].

BOX 1 - Symbiosis: highlighting the beauty in biology

Symbiosis from the ancient Greek sýn 'with' and bíōsis 'living' stands for 'living together'. In 1879 the German mycologist Heinrich Anton de Bary was the first to use the term to describe the relationship between fungi and algae in the formation of lichens. In this context, he defined the term symbiosis as 'the living together of two dissimilar organisms, usually in intimate association, and usually to the benefit of at least one of them'. The last decades, the term has been used more widely to cover beneficial (mutualistic), harmful (parasitic) and neutral (commensal) interactions that can change over time for any given set of partners [8]. It has to be noted, however, that in practice the term symbiosis is often associated with mutualistic associations only [9]. Either way, symbiotic relationships encompass both long term, obligate associations in which the symbiotic partners entirely depend on each other for survival as well as transient, facultative interactions in which the partners can exist independently of one another. These symbiotic relationships can be epi- or endobiotic with one symbiotic partner (i.e. the symbiont) living on or within the other (i.e. the host), respectively. Even though symbiosis is generally assumed to involve only two partners, most hosts accommodate complex symbiont communities consisting of multiple species [8]. Accordingly, the list of symbiotic relationships is endless. Protective sea anemones that hitchhike on the back of hermit crabs, photosynthetic algae living inside coral hosts, small cleaner fish visiting larger clients, wood-digesting protozoans in termite stomachs, and tick-eating oxpeckers on the backs of zebra, elephants, hippopotamuses and other large African animals, are only some of nature's best symbiosis examples. Besides these eukaryote-eukaryote interactions, also eukaryote-prokaryote symbiotic associations are widespread in nature. Well-known examples include the human microbiome, nitrogen fixing rhizobia inside root nodules, bioluminescent *Vibrio* species within squid light organs, chemosynthetic bacteria which associate with marine invertebrates and various insect-bacterial interactions (for an overview see: http://iss.cloverpad.org/Default.aspx?pageId=552623). Compared to prokaryotes, eukaryotes are limited in their biochemical repertoire. Therefore, eukaryotic hosts associate with bacterial symbionts which expand their physiological capacities, allowing them to invade novel metabolic and ecological niches. Symbioses are thus the ultimate examples of success through collaboration and support fundamentally important processes [8]. The 'endosymbiotic theory' which claims that eukaryotic organelles like mitochondria and chloroplasts are of endosymbiotic origin [3], is a fine example of this essential significance of symbiosis throughout life's history. Symbioses have been and are to this very day anything but a marginal or rare phenomenon. In fact, according to the late evolutionary biologist Lynn Margulis (1938-2011), "*we abide in a symbiotic world*".

From kitchen secrets to sushi: a historical overview

Foundations

The first report of a seaweed-bacterium alliance – although artificial – is one that altered bacteriology forever. In 1881, Walther Hesse, a German physician, joined Robert Koch's laboratory to study the bacteria responsible for his patients' illnesses. But, like his colleagues, Hesse encountered major technical problems attaining pure bacterial cultures on solid gelatin-based media. The gelatin often liquefied due to bacterial enzymes or because of the temperature of the laboratory. When he vented his frustrations to his wife Fanny, she suggested using a seaweed extract, agar-agar, which she had used to thicken her jellies and puddings for years [10]. The practical application of this kitchen secret accelerated bacteriological research greatly, opening the way also for real life macroalgal-bacterial studies. In fact it was Walther Hesse himself who developed agar plate techniques to count bacteria

in water samples. Techniques the ship's physician Bernard Fischer (1889) used to great success in the tropical waters of the Sargasso Sea during the Plankton Expedition of the Humboldt Foundation across the Atlantic Ocean [11]. Throughout that trip Fischer noted that the greatest abundance of culturable marine bacteria was associated with planktonic organisms and seaweeds. Hans Gazert (1906) who was in charge of the bacteriological investigations of the German South Polar Expedition made similar observations in the South Atlantic and Antarctic Ocean where some of the largest bacterial populations were found in the vicinity of seaweeds [11]. Although these observations are mainly founded on a high influx of organic matter from the remains of dead seaweeds [11], also symbiotic (here defined as mutualistic) associations with living macroalgae might have contributed.

BOX 2 - Beyond sushi: the applied aspects of seaweeds and the role of bacteria therein

Seaweeds are macroscopic, photosynthetic eukaryotes which inhabit marine environments. Marine macroalgae are phylogenetically unrelated and belong to two different eukaryotic supergroups: the Archaeplastida (green and red algae) and Chromalveolata (brown algae) [12]. As key and engineering species they play critical roles in the structuring and biodiversity of marine communities [13]. Besides these significant natural functions, marine macroalgae also possess a wealth of applied aspects. First of all, seaweeds are a substantial part of the daily diet in Asian countries and are included in a great variety of dishes such as sushi, salads and soups. In the west, seaweeds are largely regarded as health food, but the last decades there is a renewed interest in the Americas and Europe in their use as sea vegetables [14-16]. In addition, algal cell wall polysaccharides such as alginate, agar and carrageenan have commercial significance as food additives with preservative, prebiotic and gelling properties [14, 17]. Because of this latter feature, seaweed sugars are also used in a variety of industrial and laboratory applications with agar-based solid culture media as one of the best examples [10, 18]. On top of that, marine macroalgae are one of nature's most rich resources of biologically active compounds. They form an important source of iodine and produce various metabolites with antimicrobial and antimacrofouling activities. As a result, seaweed-derived compounds have mayor therapeutic applications and can be used in cosmetics or antifouling paints [19-21]. Besides this, macroalgae can be used as animal feed additives, fertilizers and biofilters [22-25], and are a potential source of bioethanol [26]. For most of the applications mentioned above, the algae need to be farmed at a grand scale. Seaweed mariculture is a huge industry in Asian countries as recent cultivation figures suggest a harvest of tens of millions of tons per year (http://www.seaweed.ie/index.html). However, as this success gradually promotes monocultures, bacterial diseases have started to surface [27]. Surface associated pathogenic bacteria cause substantial financial losses and are a major threat to the mariculture industry [28]. From this point of view, there is an extensive need to characterize seaweed associated pathogenic and decomposing bacteria [4]. On the other hand, also an increasing interest in beneficial macroalgal-bacterial associations exists as many bacterial epiphytes represent a rich source of compounds with an array of biological activities [29, 30]. Moreover, it has been proven that seaweed associated bacteria are involved in secondary metabolite production originally attributed to the host [29]. Since seaweed mariculture for chemical compound production is technically challenging, epiphytic bacteria may represent a more promising and manageable source of bioactive metabolites. Therefore, it is anticipated that increasing numbers of natural product research teams will turn their focus to seaweed associated bacteria instead of their hosts [20].

Simultaneously with these initial notes of seaweed-bacterial alliances at sea, scientists in the laboratory deduced similar conclusions from their preliminary late 19th century macroalgal culture work. The German botanist Georg Klebs (1896) was aware of the presence of bacteria in his seaweed cultures and tried to set up pure, axenic cultures of filamentous and siphonous algae. While he was successful in growing the algae, he was not able to keep his cultures bacteria-free [31]. Even though Klebs was a former assistant of Anton de Bary who first introduced the term 'symbiosis' in biology, it was Johannes Reinke (1903) who was the first to suggest a true symbiotic marcoalgal-bacterial partnership. The occurrence of *Azotobacter* as an epiphyte on marine algae let him to propose that a symbiosis may exist in which the algae supply *Azotobacter* with carbohydrates and use the nitrogen fixed by the bacteria [11, 32]. Also Edgar Johnson Allen (1910), director of the Marine Biological Association of the United Kingdom, and his collaborator E.W. Nelson recognized a symbiotic aspect in xenic marcoalgal cultures [31]. As they laid the foundations for seaweed culture, they noticed good growth of algae only when small quantities of natural seawater were added to the artificial culture media. Allen remarked that these effects may be caused by products of the metabolism of bacteria [31].

First cultivation and microscopy studies

It took until after World War II for Luigi Provasoli and colleagues to establish the first bacteria-free cultures of the green foliaceous seaweed *Ulva* using newly discovered antibiotics [31]. Provasoli, however, observed that the typical foliose morphology of *Ulva lactuca* was lost in the absence of bacteria and – even more interesting – that the normal thallus morphology was restored when certain bacteria previously isolated from the algal surface were re-added to the culture medium [33, 34]. In 1955, Harold and Stanier [35] were the first to exhaustively describe the bacterium *Leucothrix mucor* which was found consistently as an algal epiphyte, showing macroalgae not only to interact with bacteria but also to represent a distinct source of new microbial taxa. With the introduction of electron microscopy to study the macroalgal ultrastructure in the '70s, an intriguing new form of seaweed-bacterial interactions was discovered. In addition to epiphytic bacteria, various siphonous seaweeds such as *Bryopsis, Caulerpa, Chlorodesmis, Halimeda, Penicillus* and *Udotea* were also shown to harbor intracellular bacteria within their cytoplasm and/or vacuolar systems (see Box 3) [36-41]. Simultaneously with these early microscopic observations, the first cultivation studies aiming to examine the total diversity of bacteria associated with macroalgae arose. Although the bacteria were initially identified only by morphological and biochemical tests, the epiphytic flora on seaweeds was

clearly very diverse, covering numerous bacterial taxa [42-51]. Not only were these macroalgal associated bacteria distinct from the surrounding seawater communities, they also appeared host-specific with clear differences in occurrence among green, red and brown seaweeds [46, 48-50]. A stable association between algal hosts and bacteria was observed [46, 49, 50], even though the bacterial flora could vary between seasons and/or between different parts of the algal thallus [42, 45, 47]. From these and other studies in the '70s and '80s, Bolinches and coworkers [52] concluded the existence of both positive and negative macroalgal-bacterial interactions based on the algal capacity to produce organic compounds and oxygen that are utilized by bacteria. In turn, bacteria produce morphogenic factors, fixed nitrogen, enzymes and vitamins which promote algal growth [34, 48, 53-56]. In addition, epiphytic bacteria as well as the seaweed hosts themselves produce antibiotic substances which prevent colonization of the algal surface by bacterial competitors and pathogens [51, 57].

Emergence of molecular techniques

Although the number of macroalgal-bacterial studies risen steadily during the last two decades, these have not significantly increased our understanding of macroalgal-bacterial interactions as postulated above. Thanks to the improvement of analysis techniques, both symbiotic partners can be characterized biochemically and phylogenetically in more detail. However, many questions remain [4]. In the following sections we review the current knowledge on the diversity and functional ecology of bacterial communities associated with green, red and brown marine macroalgae.

Chemical interactions between seaweeds and bacteria

The relationship between macroalgae and bacteria in which seaweeds provide nutrients, while the bacterial community promotes algal growth and protects the host against pathogens, has been elaborated over the last 20 years. Figure 1.1 depicts the complex, chemically mediated interplay of beneficial and detrimental relations that exists between macroalgae and bacteria. The variety and nature of these chemical interactions have been exhaustively reviewed by Goecke and coworkers [4], and are summarized in the remainder of this section.

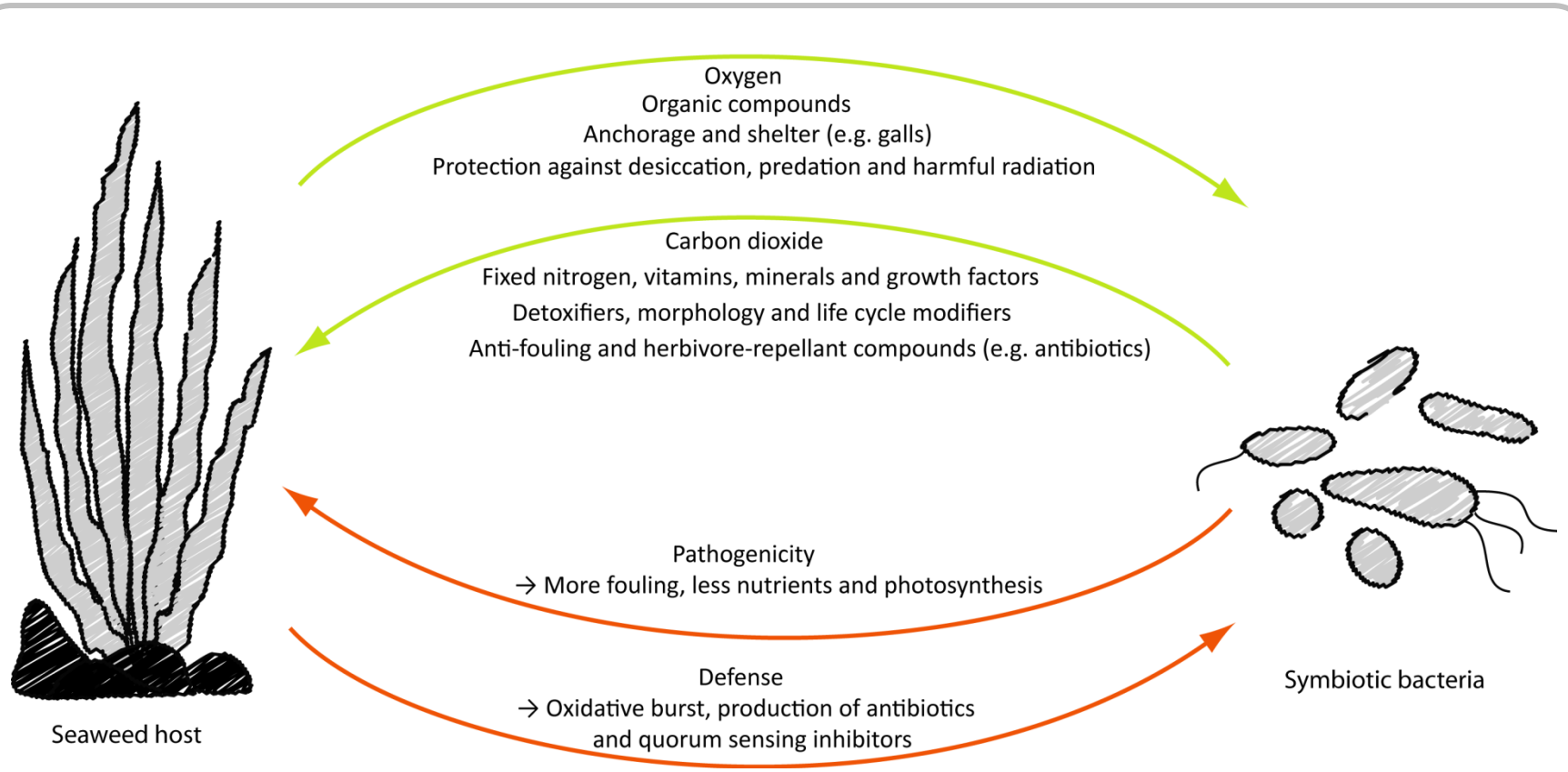


Figure 1.1: Overview of beneficial (green) and detrimental (red) interactions between macroalgae and bacteria.

Seaweed partner

From the algal host perspective, macroalgal-bacterial interactions are not unexpected. Seaweed surfaces provide a protected and nutrient rich 'hot spot' for opportunistic bacteria that are abound wherever organic material is available [30]. In most cases molecular investigations have confirmed the outcome of initial cultivation studies, i.e. that the attraction of bacteria by seaweeds turns out to be highly specific. While the composition of the bacterial flora can change over seasons, life span and different thallus-parts as a result of biotic and abiotic factors [58-60], marine macroalgae generally associate with specific bacterial communities that differ significantly from those occurring in the surrounding seawater [61, 62]. Recently, however, Burke and colleagues [13] found highly variable bacterial species compositions among local individuals of *Ulva australis* by means of in-depth 16S rRNA screening, suggesting each *U. australis* plant hosts a unique assemblage of bacterial species. Moreover, using a metagenomic approach they subsequently showed that the bacterial community composition on *U. australis* is driven by functional genes rather than the taxonomic or phylogenetic composition of its species [63]. This implies that functional groupings (or "guilds") of – not necessarily phylogenetically related – bacterial species exist of which the composition on a single algal individual is determined stochastically by recruitment from within those guilds. Even if the specificity of a seaweed-associated bacterial community may be based on functional genes rather than species, it is known that the physiological and biochemical properties of the algal host predetermine the composition of the adhering bacterial communities. For example, algal cell wall components and secondary metabolites can trigger specific interactions between seaweeds and beneficial bacteria [64, reviewed in 65]. Algal bioactive compounds also have antimicrobial properties – with interesting biomedical and industrial applications (see Box 2) – which protect the seaweed surface from bacterial pathogens, grazers and biofouling, i.e. the undesirable accumulation of micro- and macroorganisms as biofilms on the seaweed surface [4 (Table 5), 21, 28, 65-67]. Besides these bioactive compounds, macroalgae control bacterial colonization by interfering with bacterial quorum sensing (QS) systems which regulate bacterial cell-to-cell communication [4 (Table 6), 68-70]. In addition to these induced defense mechanisms, seaweeds also possess non-specific defense responses against bacterial pathogens similar to the 'oxidative burst' process of higher plants [71, 72].

Bacterial partner

Many bacteria growing on seaweed surfaces are able to enzymatically decompose algal cell walls, making them key players in biotransformation and nutrient recycling in the oceans [4 (Table 2), 18]. Also specific, beneficial bacterial-macroalgal interactions are based on the bacterial capacity to mineralize algal organic substrates and subsequently supply the seaweed host with carbon dioxide, minerals, vitamins and growth factors [30, 55, 56, 73, 74]. Several studies also revealed that seaweed associated bacteria are important sources of fixed nitrogen and detoxifying compounds [4 and references therein, 75, 76]. Besides nutritional and growth promoting effects, bacteria may shape the morphology and life cycle of their algal host. Bacterial effects on morphogenesis have been reported in foliaceous green macroalgae such as *Ulva* and *Monostroma* [34, 77-81], and have been shown to be controlled by a highly potent differentiation inducer, thallusin, isolated from well-defined associated bacteria [4 (Table 4), 82]. Thallusin and other bacterial metabolites, including QS molecules, also play a role in the host's life cycle completion as well as in algal spore release and germination [4 (Table 4), 82-87]. Furthermore, QS inhibitors and antimicrobial compounds produced by numerous epiphytic bacteria work in concert with seaweed derived metabolites (see above) to protect the seaweed surface from pathogens, herbivores and fouling organisms [4 (Table 4), 30, 88-93]. Pathogenic bacteria can cause severe degradation of algal host cells or even lead to seaweed mortality, causing major financial losses to seaweed mariculture every year (Box 2) [4 (Table 4), 94, 95]. Also biofouling forms a permanent threat to macroalgae as bacterial biofilms increase the hydrodynamic drag on their host and enhance the attachment of other fouling organisms and grazers. Biofilms may also compete for nutrients, inhibit gaseous exchange or block light, essential for photosynthesis. Thus, both bacterial and algal bioactive compounds are essential chemical mediators in macroalgal-bacterial associations which jointly control the composition and density of bacterial biofilms thereby defending the seaweed surfaces against biofouling [4 and references therein, 28]. In addition, these bacterial bioactive compounds may represent a more promising – and easier to handle – source of natural products with biotechnological applications in comparison with seaweed derived compounds (Box 2) [20, 29, 96, 97].

BOX 3 - *Bryopsis*, an underwater peacock with extraordinary features

Bryopsis (Greek, moss like) is a 1-20 cm tall feathery-like, marine green macroalga (Ulvophyceae, Bryopsidales) which inhabits temperate and tropical seas (Fig. 1.2). *Bryopsis* algae usually attach to rocks or other seaweeds and grow in the intertidal to subtidal zone, generally down to 5 m depth [2, 12, 98]. *Bryopsis* possibly originated in the late Mesozoic, about 100 my ago [99]. The genus was described in 1809 by J.V. Lamouroux, and in the past 200 years more than 200 species and intraspecific taxa have been described, of which about 55 are currently accepted [100]. Species delineation in *Bryopsis* is problematic because of rampant morphological plasticity, which is intrinsically related to its simple body plan [101]. The entire plant, built up of feathers consisting of a holdfast (rhizoids) and a central stem (axis) with branches (pinnae) on either side, has a unicellular structure without any internal cross walls (Fig. 1.2B). On that account, *Bryopsis* belongs to a unique group of marine 'giant-celled' macroalgae which are composed of a single, tubular (siphonous) shaped cell. These siphonous seaweeds exhibit a typical intracellular architecture in which the multinucleate (coenocytic) cytoplasm is restricted to a thin cell-wall associated layer that surrounds a central vacuole which occupies most of the cell volume [102]. In *Bryopsis* the peripheral cytoplasmic layer is divided into two sublayers: an outer layer adjacent to the cell wall which contains most of the organelles excluding the chloroplasts, and an inner chloroplast layer next to the vacuole (Fig. 1.2I). *Bryopsis* algae are homoplastidic, which means that they only possess one type op plastid, namely chloroplasts. These chloroplasts contain pyrenoids with starch as the principal carbohydrate storage product [36]. Another interesting phenomenon is that *Bryopsis* chloroplasts can maintain activity inside the body of some herbivorous sea slugs, rendering the animals photosynthetic [103] (Fig. 1.2H). The plastid maintenance is thought to involve lateral gene transfer from the algal food source to the slugs [104], but this has been recently questioned [105]. Although the complete *Bryopsis* plastid genome has been recently sequenced, the plastidial autonomy seems to have little to do with the size and gene content of the cpDNA itself [106]. Furthermore, the *Bryopsis* cytoplasm exhibits vigorous streaming by which organelles and nutrients are transported throughout the siphonous thallus thereby enabling optimal photosynthesis and nutrient exchange [107]. In addition, *Bryopsis* has evolved several other features to overcome the physical limitations of being unicellular [108]. *Bryopsis* produces, for example, the bioactive [also therapeutic, see reference 109] metabolite kahalalide F which protects the vulnerable alga from fish predation [110]. Cell wounding triggers a complex, multistep wound response resulting in *in loco* plug formation and subsequent synthesis of a new cell wall [37, 111, 112]. To this, *Bryopsis* algae add a surprisingly feature, i.e. the formation of protoplasts [113]. These protoplasts, which are released upon injury, are membraneless and can survive in seawater for 10–20 minutes (Fig. 1.2D). Subsequently, phospholipid membranes and a cell wall are synthesized *de novo* surrounding each protoplast, which then develops into a new *Bryopsis* plant. Protoplast formation is thus a defense as well as an effective propagation mechanism. In *Bryopsis*, this vegetative reproduction by thallus fragmentation is accompanied with sexual reproduction modes alternating between a gametophytic and sporophytic phase [2]. Despite early reports on the simplicity of the *Bryopsis* life cycle, subsequent culture observations showed a wide variety of life history patterns, even within a single *Bryopsis* species [114]. For a nice overview of life history pathways, including fragmentation, parthenogenesis and differentiation of zoospores and gametes, see Rietema [114] and Morabito *et al.* [115]. Besides these well-studied morphological, regenerative and reproductive characteristics, *Bryopsis* has long been suspected to harbor intracellular bacteria inside its cytoplasm as well as vacuolar systems [36, 37]. Endophytic bacteria have been visualized in the *Bryopsis* cytoplasm by electron microscopy at every stage of development, including the gametes [36] (Fig. 1.2E). This indicates a natural, stable relationship between the algal host and its endophytes in which both partners may provide mutualistic ecological benefits. To date, this remarkable algal-bacterial partnership has received little or no attention. However, as it has already been proven that endosymbiotic bacteria inside *Caulerpa* algae share responsibility for the successful - though sometimes highly invasive - spread of siphonous seaweeds in oligotrophic waters [75], an enlightening characterization of the bacterial partner would be welcome.

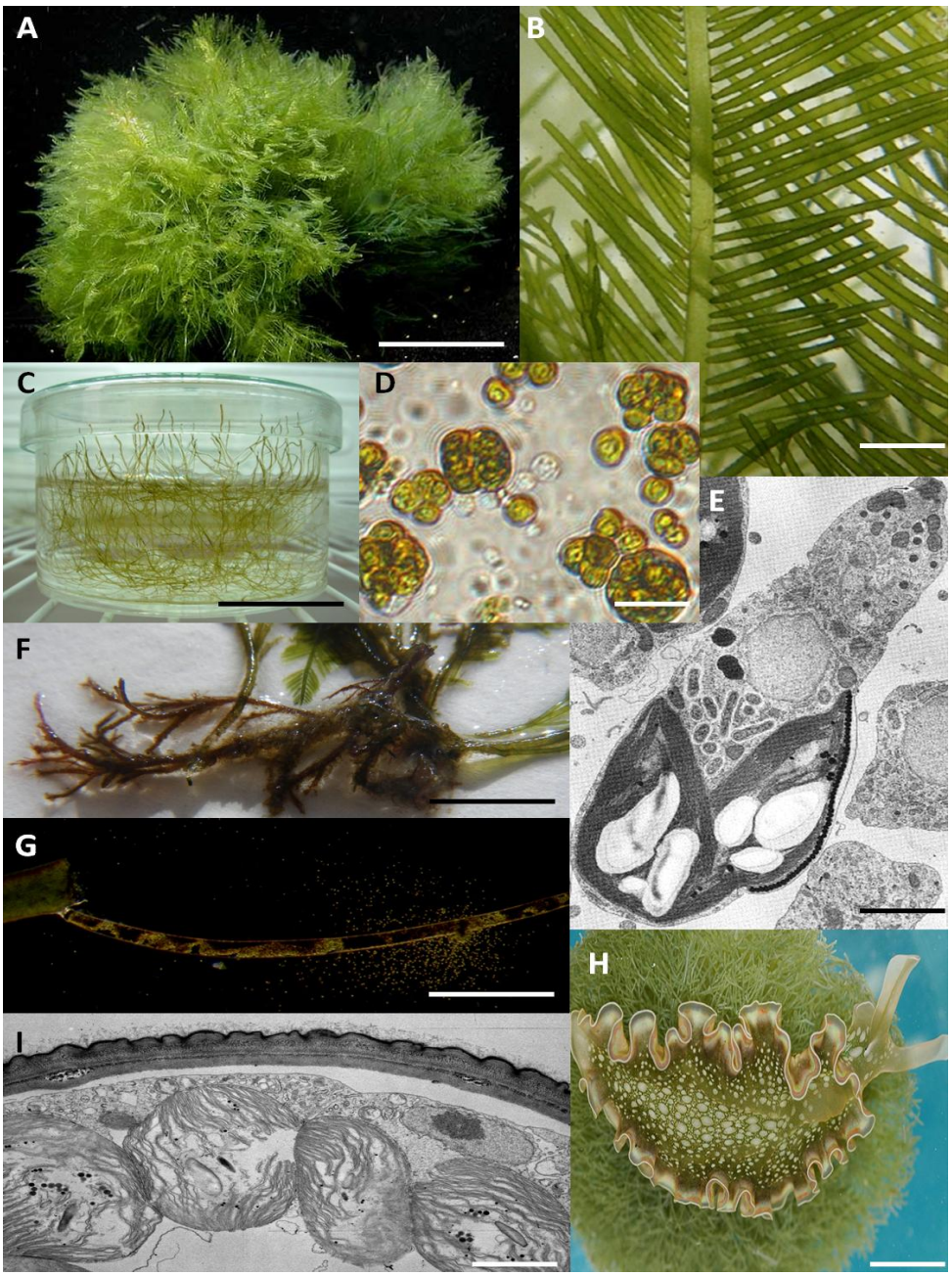


Figure 1.2: The green siphonous seaweed *Bryopsis* and its characteristic features. A: *B. pennata* forms soft, feathery clumps. Scale bar: 2.5 cm (photo by Linda Preskitt). **B:** Detail of one *Bryopsis* feather. The main axis shows no internal cross walls. Scale bar: 2.5 mm (photo: http://www.turtlejournal.com/?p=7629). **C:** *Bryopsis* loses its characteristic morphology in culture. Scale bar: 4 cm (photo by Anne Willems). **D:** Formation of protoplasts from *B. hypnoides*. Scale bar: 30 µm (photo by Lü *et al.* [106]). **E:** Electron micrograph of *Bryopsis* female gamete in longisection. Cluster of bacteria can be seen just above the chloroplast. Magnification: x20000, scale bar: 1 µm (photo by Burr & West [36]). **F:** *Bryopsis* rhizoids (holdfast). Scale bar: 5 mm (photo: http://www.turtlejournal.com/?p=7629). **G:** Release of gametes from a matured *Bryopsis* gametangium. Scale bar: 1.5 mm (photo by Joke Hollants). **H:** *Elysia clarki*, a **'**solar-powered sea slug', sequesters chloroplasts from its *Bryopsis* food. Scale bar: 1.5 cm (photo by Curtis et al. [116]). **I:** Electron micrograph of *Bryopsis* vegetative thallus in longisection. Magnification: x8000, scale bar: 5 µm (photo by Olivier Leroux and Joke Hollants).

Endophytic seaweed-bacterial relationships

Besides being epiphytic on algal surfaces, bacteria also live inside the thallus or cells. Seaweed grazers or epiphytic bacteria capable of degrading algal cell walls (see above) can damage algal thalli and provide an entrance for pathogenic and opportunistic bacteria [117-120]. These latter bacteria might become detrimental if they are able to enter the algal tissue and contribute to further disintegration of the host, finally leading to thallus rupture [4 and references therein]. In addition to these pathogenic associations, also non-detrimental seaweed-associated endophytic bacteria are described. Bacteria are present inside algal galls (i.e. abnormal tissue growths of seaweeds) reported on more than 20 species of red and brown macroalgae [reviewed in 121]. In the red seaweed *Prionitis*, endophytic bacteria are responsible for gall formation by overproduction of the phytohormone indole-3-acetic acid (IAA), thereby creating a suitable microhabitat for their own proliferation [122, 123]. Even though the benefits for the seaweed partner are not well understood, coevolution between *Prionitis* hosts and their gall-forming endobionts has been suggested [123]. Also in the red macroalga *Gracilaria dura* endophytic bacteria enhance the algal bud induction by the production of IAA and fixed nitrogen [74]. In various siphonous (single celled, multinucleate) green seaweeds, endophytic bacteria have been reported over the past 40 years (see above and Box 3). Even though these endophytic bacteria have been associated with detoxification, nitrogen fixation and photosynthetic functions [75, 124, 125], the true physiological nature of these endobiotic siphonous seaweed-bacterial symbioses remains unknown.

Bacterial diversity associated with seaweeds

Broad-spectrum seaweed-bacterial diversity studies identifying the total bacterial community are scarce. This is not surprising given that the number of seaweed associated bacteria exceeds those in the surrounding seawater by 100 to 10 000 times [42]. Total viable counts reach up to 10^7 bacterial cells per gram dry algal weight using the agar spread plate method; a number that even increases by two orders of magnitude when applying direct enumeration techniques [42, 47, 126]. Consequently, most macroalgal-bacterial studies focus on the identification and characterization of specific bacterial taxa, e.g. those with bioactive potential or pathogenic activity, rather than investigating the total bacterial diversity [79, 88, 93, 120]. Until recently, most of these investigations used traditional culture-based approaches, which are often considered insufficient since only 1% of all known bacteria are estimated to be culturable [127]. However, current molecular methods such as clone

libraries, denaturing gradient gel electrophoresis (DGGE), quantitative PCR (Q-PCR) and fluorescent *in situ* hybridization (FISH) also have their limitations for grasping the entire diversity of a microbial community, even in a single environmental sample, since they mainly reveal a snapshot in time of the dominant bacterial community members only [128].

In the following paragraphs we review 149 studies from the last 55 years which dealt with bacteria associated with a total of 159 seaweed species (36 green, 72 red and 51 brown marine macroalgae, see Table S1.1 on http://www.phycology.ugent.be/). The bacterial diversity was compared between brown, green and red seaweeds at all taxonomical levels. Wherever possible, the identity of the associated bacteria was linked to their ecological function.

Identity of bacteria associated with seaweeds: higher taxonomic ranks

Bacteria described from seaweed surfaces or within algal thalli belong to the (super)phyla Proteobacteria, Actinobacteria, Bacteroidetes (previously known as the Cytophaga-Flavobacteria-Bacteroides (CFB) group), Cyanobacteria, Firmicutes, Planctomycetes, Verrucomicrobia, Chloroflexi, Deinococcus-Thermus, Fusobacteria, Tenericutes and the candidate division OP11. In all studies reviewed, Gammaproteobacteria were the most common bacterial clade associated with seaweeds (37% relative abundance, i.e. percentage of published records), followed by the CFB group (20%), Alphaproteobacteria (13%), Firmicutes (10%) and Actinobacteria (9%) (Fig. 1.3A). On a lower taxonomic level, the orders Flavobacteriales (14% relative abundance), Alteromonadales (12%), Vibrionales (10%), Pseudomonadales (9%), Bacillales (9%), Actinomycetales (8%) and Rhodobacterales (7%) were most abundant in seaweed associated bacterial communities (Fig. 1.3B). Comparing the relative abundance of bacterial taxa on brown, green and red macroalgae, bacterial representatives of the major phylogenetic groups mentioned above were isolated from all three seaweed groups (Fig. 1.4A). Despite this similarity, green macroalgae associated more with the CFB group and Alphaproteobacteria compared to brown and red seaweeds. Brown and red macroalgae, on the other hand, harbored more Firmicutes, Actinobacteria and Planctomycetes species, respectively. Figure 1.4B shows that the discrepancy between brown, green and red seaweed associated bacteria at the order level can mainly be attributed to differences in the number of published records of Rhizobiales, Rhodobacterales, Alteromonadales, Vibrionales, Cythophagales, Flavobacteriales, Bacillales and Actinomycetales species.

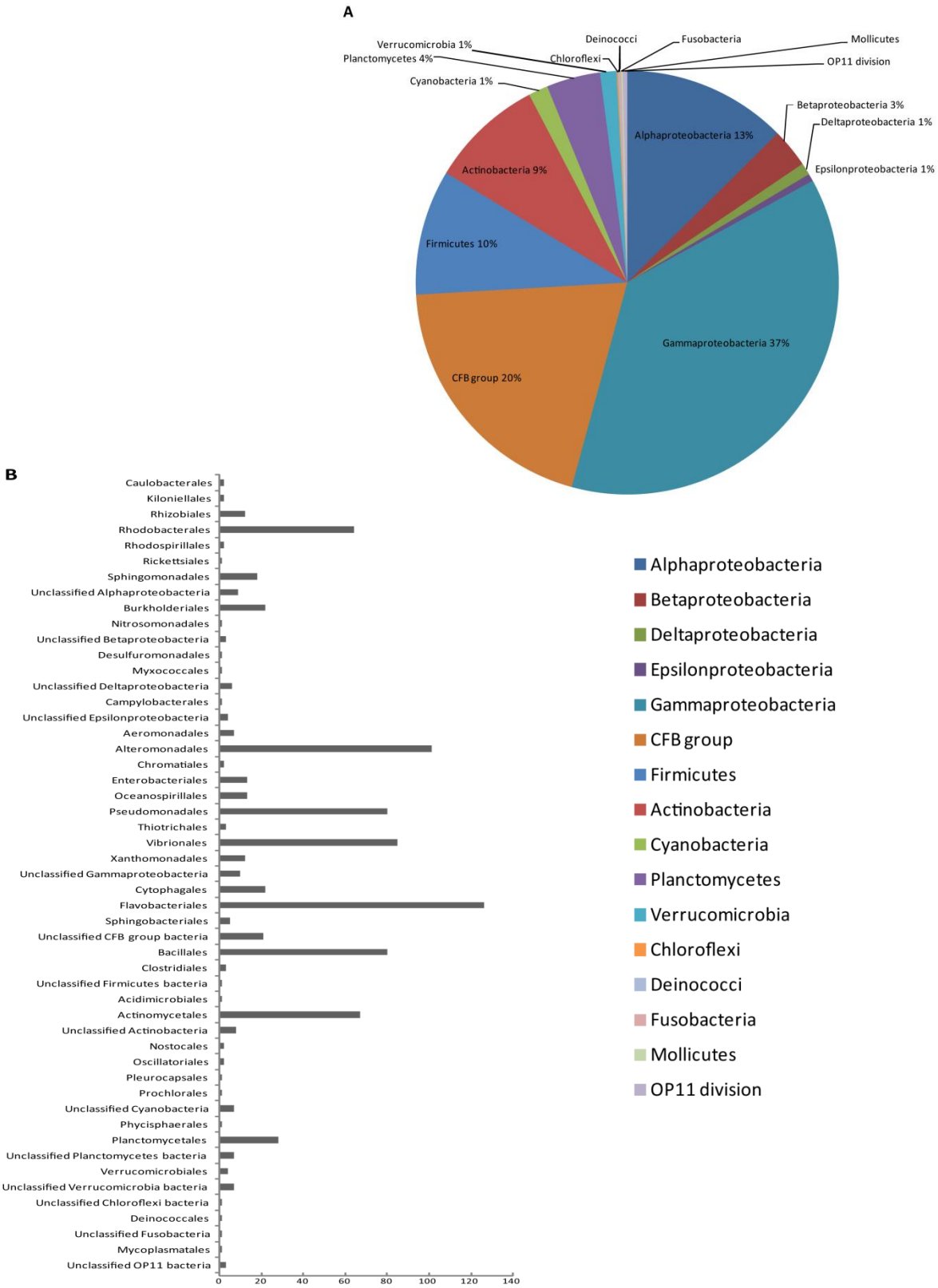


Figure 1.3: Percentage of published records of bacterial classes or phyla (A) and number of published records of bacterial orders (B) associated with seaweeds.

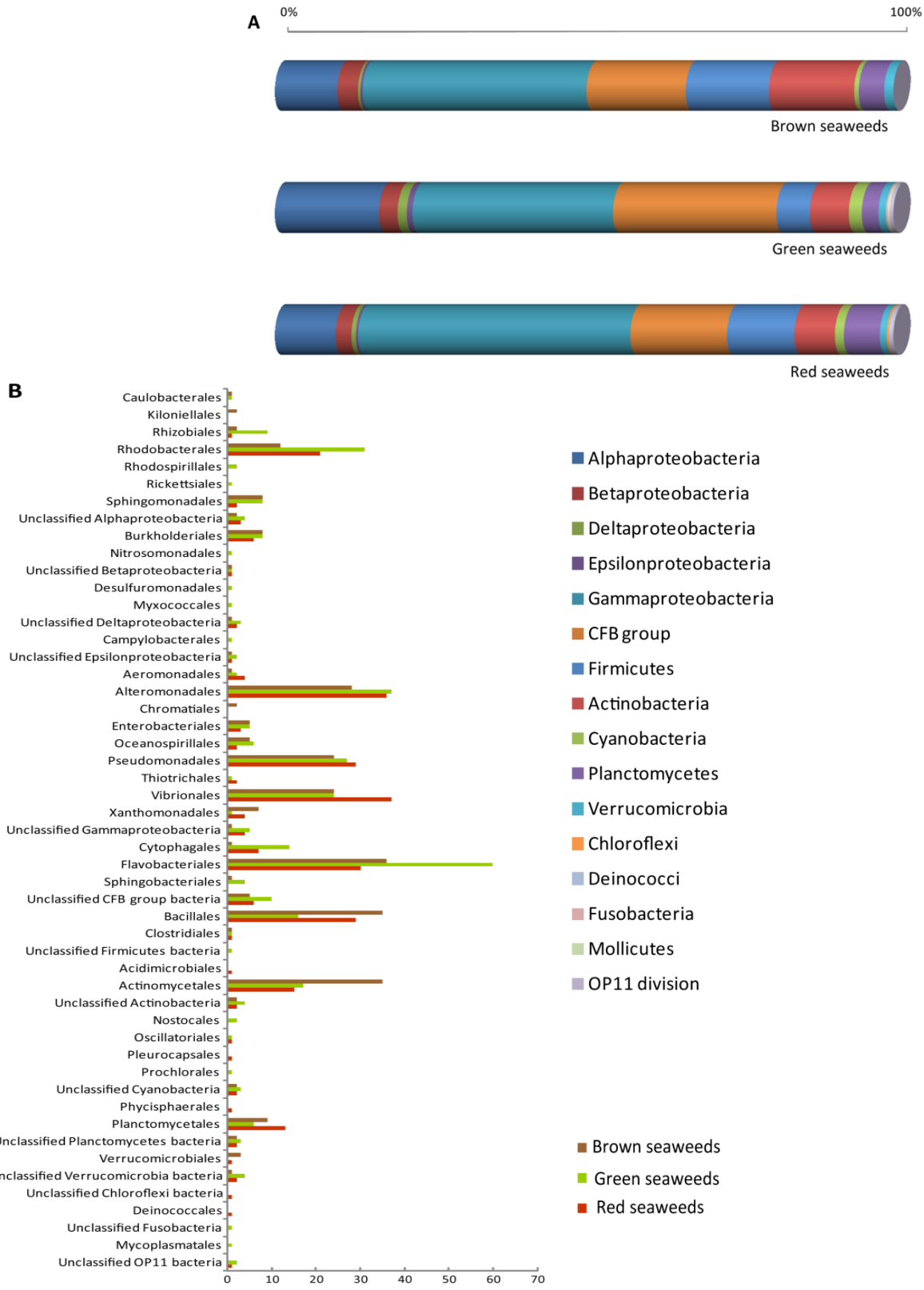


Figure 1.4: Percentage of published records of bacterial classes or phyla (A) and number of published records of bacterial orders (B) associated with brown, green and red seaweeds.

Identity of bacteria associated with seaweeds: genus/species level

The similarities observed at high taxonomic ranks appear to decrease at lower ranks of both the host and bacterial partner. Even though a consistent bacterial core community at higher taxonomic levels (i.e. Alphaproteobacteria and Bacteroidetes) was observed on different *Ulva australis* and *Saccharina latissima* samples [13, 58, 59], closely related seaweeds do not necessarily harbor the same bacterial taxa (for example different species in the genera *Fucus*, *Laminaria*, *Monostroma*, *Ulva*, *Gracilaria*, *Polysiphonia* and *Porphyra*, see Fig. S1.2 and Table S1.3 on http://www.phycology.ugent.be/). Likewise, only 33 bacterial genera including *Alteromonas*, *Bacillus*, *Flavobacterium*, *Pseudoalteromonas*, *Pseudomonas* and *Vibrio* have, to a greater or lesser extent, been described from green, red and brown seaweeds (Fig. 1.5). Genera like *Cytophaga*, *Planococcus* and *Tenacibaculum*, on the other hand, are regularly reported from green and red seaweeds, whereas they are virtually absent on brown macroalgal surfaces. Also specific bacterial species have rarely been isolated from different seaweed species, even within a single algal genus (see Table S1.3 on http://www.phycology.ugent.be/). Exceptions are outlined in Table 1.1 and include for example certain *Bacillus* and *Pseudoalteromonas* species that are present on or within a variety of brown, green and red seaweeds. This table also illustrates that several of these bacterial species (*Cellulophaga fucicola*, *Leucothrix mucor*, *Pseudoalteromonas elyakovii*, *Tenacibaculum amylolyticum* and *Zobellia galactanovorans*) were newly described from their algal host, indicating marine macroalgae represent an important habitat for the discovery of novel bacterial diversity. To date, more than 50 new bacterial species initially isolated from seaweeds have been validly published [for an overview see reference 4, Table 1]. In contrast to the similarities in bacterial communities at higher taxonomic levels, almost no individual species was consistently found on the surface of different *Ulva australis* and *Saccharina latissima* samples [13, 58]. Consequently, there does not appear to be a consistent core community of macroalgal associated bacterial species, suggesting that a large number of bacterial species are able to colonize seaweed surfaces. This variability at the species level appears to be an emerging feature of host-associated microbial communities in general [13]. Endobiotic associations, on the other hand, seem to be more uniform at lower taxonomic ranks compared to epiphytic bacteria. For example, different *Prionitis* species host similar bacteria of the *Roseobacter* group inside their galls [123]. Also different species and geographical diverse algal samples of the siphonous seaweed *Caulerpa* harbor one and the same *Herbaspirillum* species [125].

Table 1.1: Overview of bacterial species isolated from two or more host species/samples in independent macroalgal-bacterial studies. Type: EP = endophyte, FI = faecal indicator bacteria and SN = new bacterial species (sp. nov.) originally described from the algal host. Function: AB = antibacterial activity, AF = antifouling activity, AM = antimicrobial activity, AS = antisettlement of invertebrate larvae, D = disease, GF = growth enhancing activity, MG = morphogenesis activity, NF = nitrogen fixation, SZ = settlement of zoospores and QSI = quorum sensing inhibitory activity.

Bacterial species	Host (bacterial type/bacterial function)	References
Bacillus licheniformis	*Colpomenia sinuosa* (QSI), *Fucus serratus* (AB), *Palmaria palmate* (AM) and *Gracilaria dura* (EP/GF, NF)	[74, 129-131]
Bacillus pumilus	*Ecklonia cava* (AM), *Sargassum fusiforme* (AM), *Porphyra yezoensis* (AM), *Lomentaria catenata* (AM), *Chondrus oncellatus* (AM), *Colpomenia sinuosa* (AM), *Gracilaria dura* (EP/GF, NF) and *Delisea pulchra* (AM)	[29, 129, 132-134]
Cellulophaga fucicola	*Ulva australis* and *Fucus serratus* (SN)	[90, 135-137]
Cobetia marina	*Antithamnion plumula*, *Cladophora rupestris*, *Ulva linza* (GF, MG), *Ulva compressa* (GF, MG) and *Ulva lactuca* (GF, MG)	[80, 138]
Escherichia coli	*Monostroma undulatum* (FI), *Cladophora* mats (FI), *Kappaphycus alvarezii* (FI), *Laminaria religiosa* (FI) and *Ulva reticulate* (FI)	[27, 94, 139-141]
Leucothrix mucor	*Ulva lactuca* (SN), *Clathromorphum* and *Sporolithon* sp.	[35, 142, 143]
Phaeobacter gallaeciensis	*Ulva australis* (AF) and *Delisea pulchra* (AM)	[29, 90, 135, 136]
Pseudoalteromonas citrea	*Ulva* spp. (GF, MG)	[80, 86]
Pseudoalteromonas elyakovii	"*Enteromorpha*" sp. (SZ) and *Laminaria japonica* (SN/D)	[86, 144, 145]
Pseudoalteromonas gracilis	*Ulva australis* and *Gracilaria gracilis* (D)	[90, 135, 136, 146]
Pseudoalteromonas tunicata	*Ulva australis* (AF, AM) and *Ulva lactuca* (AF, AM, AS, SZ)	[29, 89, 90, 135, 136]
Shewanella japonica	*Ulva australis* (AM)	[29, 147]
Tenacibaculum amylolyticum	*Ulva* sp. (GF, MG), *Monostroma* sp. (GF, MG) and *Avrainvillea riukiuensis* (SN)	[81, 82, 148]
Vibrio tasmaniensis	*Laminaria japonica*, *Polysiphonia urceolata* and *Plocamium telfairiae* (AM)	[134, 149]
Zobellia galactanovorans	*Ulva* sp. (GF, MG), *Monostroma* sp. (GF, MG), *Delesseria sanguine* (SN) and "*Enteromorpha*" sp. (SZ)	[81, 82, 86, 150]

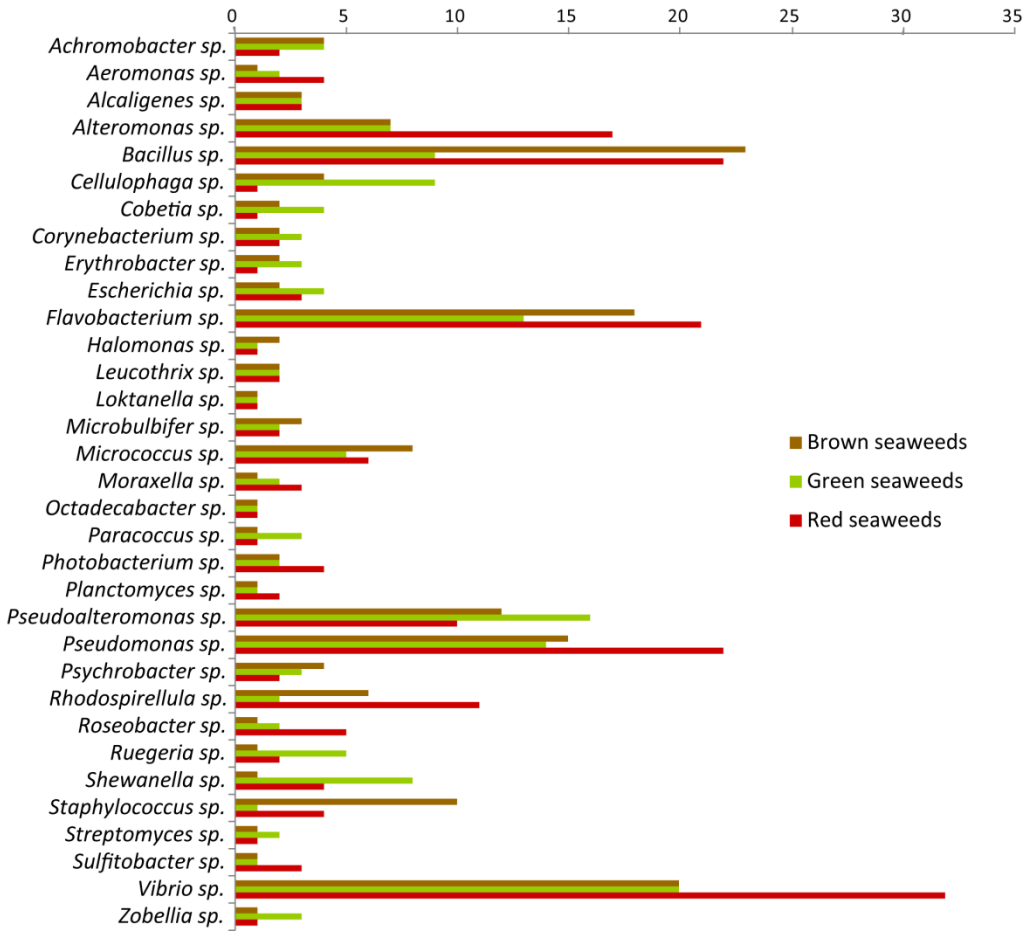


Figure 1.5: Number of published records of bacterial genera isolated from all three macroalgal groups.

Linking identity to function

Although the ecological relevance of most bacterial associates on or within macroalgae remains unclear, a number of beneficial and detrimental functions have been postulated for particular bacterial species. For example, Alpha- and Gammaproteobacteria, Cyanobacteria, Actinobacteria and CFB group species have been identified as the causative agent of various macroalgal diseases [for an overview of macroalgal diseases caused by bacteria see reference 4, Table 3]. The sushi-alga nori (*Porphyra*), for example, may be infected by species of *Flavobacterium* [Anaaki disease, 151], *Pseudomonas* [green spot rotting, 152, 153] and *Vibrio* [green spot rotting and white rot disease, 152, 153-155]. In addition, a wide variety of bacterial species isolated from seaweeds are capable of assimilating algal cell wall sugars. Besides key players in nutrient recycling processes, they are thus also potential pathogens as they can damage algal tissues and provide an entrance for opportunistic bacteria (see

above). These algal cell wall degrading bacteria mainly belong to the Alphaproteobacteria, Gammaproteobacteria and the CFB group. Especially *Alteromonas*, *Flavobacterium*, *Pseudoalteromonas*, *Pseudomonas*, *Vibrio* and *Zobellia* species possess sugar-degrading enzymes like agarases, carrageenases and aliginases [for an overview of macroalgal cell wall degrading bacteria see reference 4, Table 2]. Also antimicrobial, including antisettlement and QS inhibiting, functions which protect the algal surface from pathogens, herbivores and fouling organisms have been assigned to a broad range of seaweed associated bacterial species. Not unexpectedly, nutrient-rich seaweed surfaces attract many opportunistic micro- and macroorganisms, thereby creating a highly competitive environment in which the production of defensive compounds can serve as a powerful tool for bacteria to outcompete other surface colonizers [29, 30, 96]. As a result, the production of these antimicrobial compounds is not restricted to a certain bacterial group but appears to be widespread across alphaproteobacterial, betaproteobacterial, gammaproteobacterial, flavobacterial, actinobacterial and bacilli clades (Fig. 1.6). In particular, *Micrococcus*, *Phaeobacter*, *Pseudoalteromonas*, *Shewanella*, *Vibrio* and various *Bacillus* species are efficient producers of compounds with antimicrobial, antifouling and QS inhibiting features, making them highly successful colonizers of seaweed surfaces [4, 133]. Besides these defense functions, bacteria also sustain the normal morphology and life cycle of their algal hosts. Morphogenesis and germination of foliaceous green macroalgae was linked to the production of thallusin (see above) by an epiphytic *Cytophaga* species isolated from *Monostroma* [82]. But also other bacterial species from the CFB group and members of the Alphaproteobacteria, Gammaproteobacteria, Actinomycetales and Bacillales have been described as inducing morphogenic effects [78-81, 156]. Likewise, *Cytophaga*, *Polaribacter*, *Pseudoalteromonas*, *Pseudomonas*, *Psychroserpens*, *Shewanella*, *Vibrio* and *Zobellia* species have been shown to either stimulate or inhibit the zoospore settlement of *Ulva s*eaweeds (Fig. 1.6) [86, 157]. Growth promoting and nutritional effects, on the other hand, have been attributed to endophytic *Bacillus pumilus* and *B. licheniformis* as well as to epiphytic *Exiguobacterium homiense*, *Pseudoalteromonas porphyrae, Azotobacter* and various cyanobacterial species (Fig. 1.6) [53, 54, 73]. These latter two bacterial taxa fix nitrogen and subsequently supply it to their *Codium* host. In *Caulerpa*, another green siphonous seaweed, this nitrogen supply is provided by an endosymbiotic *Rhodopseudomonas* species [75]. In addition, *Caulerpa* also hosts photosynthetic Alphaproteobacteria in its cytoplasm [124]. These endosymbiotic associations may provide a physiological explanation for the successful – and sometimes invasive – spread of siphonous green algae in oligotrophic environments [75].

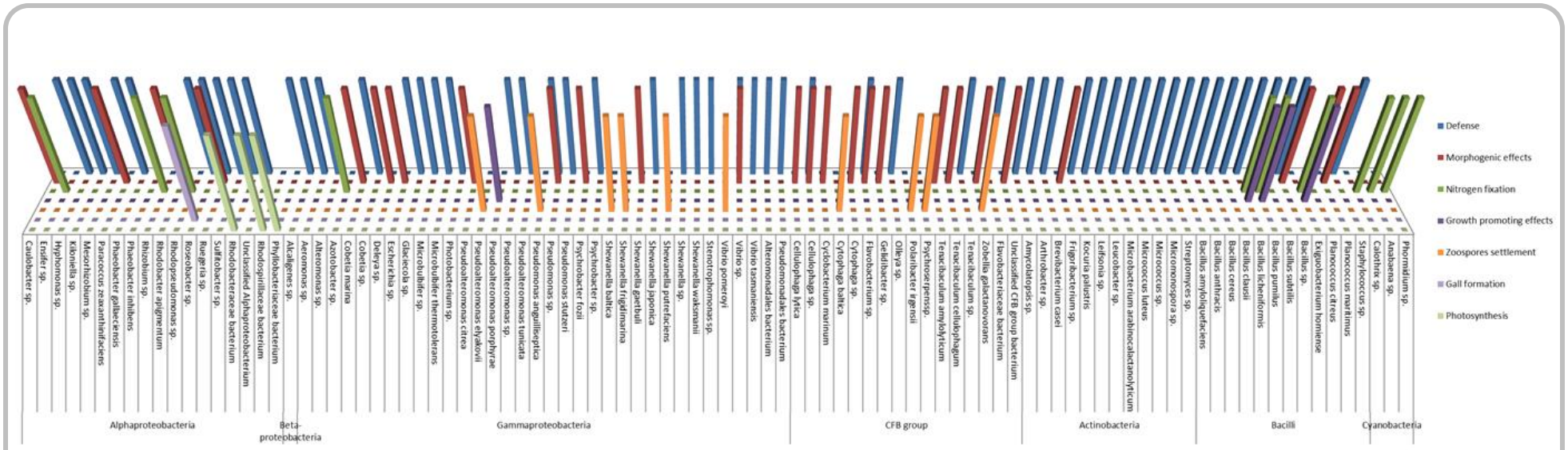


Figure 1.6: Potential host beneficial functions associated with certain bacterial genera.

Conclusion

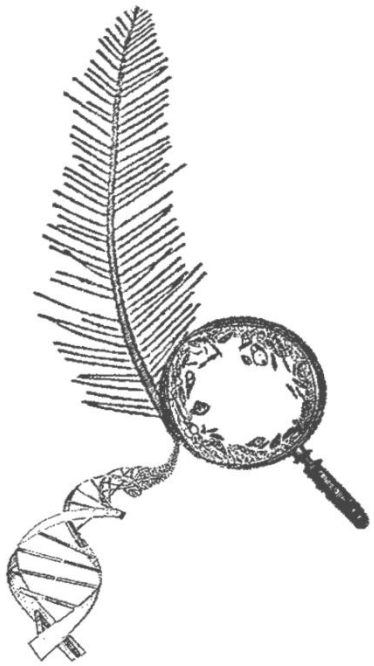
Seaweed-bacterial associations have been studied from the end of the 19th century onwards and were shown to be highly diverse, covering a wide range of beneficial and detrimental interactions between various macroalgal and bacterial partners. A rather complex – chemically mediated – interplay exists among seaweeds and bacteria based on the exchange of nutrients and minerals (Figure 1.1). Notwithstanding this diversity, all studies conducted so far have shown that seaweed associated bacterial communities are highly specific as they differ significantly from those occurring in the surrounding seawater. This specificity is predetermined by physiological and biochemical properties of both the seaweed and bacterial partner, however, the taxonomic level at which to address this specificity remains unknown. Lower levels seem not the answer as similar bacterial taxa are present on different algal hosts and, on the other hand, samples from the same seaweed species harbor distinct bacterial communities. Hence, it has been proposed that functional genes, rather than taxonomic characteristics may be the appropriate perspective from which to understand these specificity patterns [63]. Macroalgal associated bacterial communities appear to contain a consistent functional profile with features related to an algal host-associated lifestyle. Most of these functions can be performed by phylogenetically distinct bacterial taxa (Figure 1.6). Nevertheless, a definite bacterial core community at higher taxonomic levels, mainly consisting of Gammaproteobacteria, CFB group, Alphaproteobacteria, Firmicutes and Actinobacteria species, seems to exist which is specifically (functionally) adapted to life on brown, green and/or red seaweed surfaces (Figure 1.3). These three macroalgal groups, however, show some quantitative, rather than qualitative, differences as they harbor the same higher bacterial taxa at dissimilar (relative) abundances (Figure 1.4). While such an ecological coherence at high bacterial taxonomic ranks has also been observed in other aquatic systems, intra- and intercellular bacterial communities generally show more specificity at lower taxonomic levels [128]. Likewise, endobiotic macroalgal-bacterial relationships seem to be highly species-specific.

Since both epi- and endobiotic seaweed-bacterial associations are appealing from evolutionary and applied perspectives (Box 1 and 2), ecological and biochemical studies should be scaled up. Advances in molecular techniques have, however, revealed that obtaining an accurate picture of the composition of symbiotic bacterial communities presents an unusually difficult challenge [9]. Therefore, summarizing the immense bacterial diversity at the species level by integrating it into higher levels of organization (both phylogenetic and functional) would provide a framework to study

(epiphytic) macroalgal associated bacterial communities in a more practical way [128]. Nevertheless, macroalgal-bacterial studies will always remain a difficult balancing act between examining the seaweed and bacterial partner on their own or studying them as a whole (i.e. as a holobiont). Either way, there is a strong need to integrate aspects of different biological disciplines such as microbiology, phycology, ecology and chemistry in future macroalgal-bacterial studies.

2 Chapter

Objectives



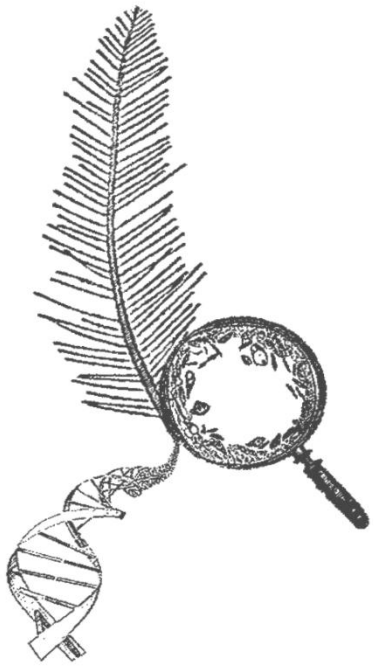
Siphonous seaweeds are common in tropical and warm-temperate marine habitats where they form a significant component of the marine flora and are among the major primary producers in coral reefs, lagoons and seagrass beds [99]. Besides these constructive aspects, several siphonous taxa are also vigorous invasive species (e.g. *Caulerpa taxifolia* and *Codium fragile*) which are known to profoundly affect the ecology and native biota in their areas of introduction [158, 159]. While the cause of this spread of siphonous green algae in a range of marine habitats is not known with certainty, unique cellular innovations alongside interactions with intracellular bacteria may provide an explanation [75]. Indeed, many siphonous seaweeds have long been known to harbor endosymbiotic bacteria [36-41] which may be associated with various metabolic functions including nitrogen fixation and photosynthesis (Chapter 1). This dissertation aims to explore the association among siphonous seaweeds and their intracellular bacterial communities, focusing on the green alga *Bryopsis* as host organism. In contrast with other siphonous seaweed hosts, *Bryopsis* can be easily cultured in the laboratory on account of its vegetative reproduction traits such as thallus regeneration and the formation of protoplasts (Chapter 1, Box 3). Moreover, only in *Bryopsis,* intracellular bacteria have been detected in both the vegetative thalli and gametes, suggesting an ancient, stable association between the algal host and its bacterial endophytes [36]. This combination of features, combined with the large collection of available cultures, makes the genus an ideal case study to address the following specific objectives:

- Phylogenetic identification of the intracellular bacterial diversity within *Bryopsis* algae
- Examination of the symbiotic nature (i.e. facultative versus obligate) of the bacterial endophytes
- Characterization of the distinctiveness of the endobiotic bacterial communities from those present in the surrounding seawater
- Characterization of the temporal and spatial stability of the intracellular communities
- Identification of the factors (i.e. ecology, geography and/or host phylogeny) shaping the endobiotic bacterial communities
- Examination of the host specificity of the *Bryopsis* endophytes
- Investigation of the degree of interdependency between the algal host and the bacterial partners
- Exploration of the function of the endophytic bacteria

The methodology used to answer these research questions and the results of this study are outlined in the following sections. Part 1 of Chapter 3 deals with the optimization of the experimental design. As this thesis is the first to explore the *Bryopsis*-bacterial partnership, all methods had to be optimized before the main objectives could be addressed. A surface sterilization protocol was designed to free the *Bryopsis* surface from epiphytic bacteria and also the subsequent full-cycle 16S rRNA gene approach was modified to meet the research questions. Part 2 presents the experimental work performed on living *Bryopsis* samples which were kept in culture throughout this study. The identity, diversity, uniqueness, stability, symbiotic nature and transmission modes of the endophytic bacterial communities within *Bryopsis* cultures were examined by means of clone libraries, denaturing gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization (FISH). Statistical analyses were performed to identify the factors responsible for the variation in endobiotic bacterial community composition. In addition, attempts to culture both the *Bryopsis* host and its endophytes separate from each other are reported at the end of Part 2. The last part of Chapter 3 describes the amplification of species specific bacterial 16S rRNA genes form natural *Bryopsis* samples and addresses the host specificity and evolution of *Bryopsis* Flavobacteriaceae endosymbionts. Also preliminary *in situ* hybridization results of *Bryopsis* sections with group- and species-specific probes are reported. Finally, the main results and future perspectives of this thesis study are discussed in Chapter 4.

3 Chapter

Experimental work



Part 3.1 Optimization of the experimental design

3.1.1. Overview

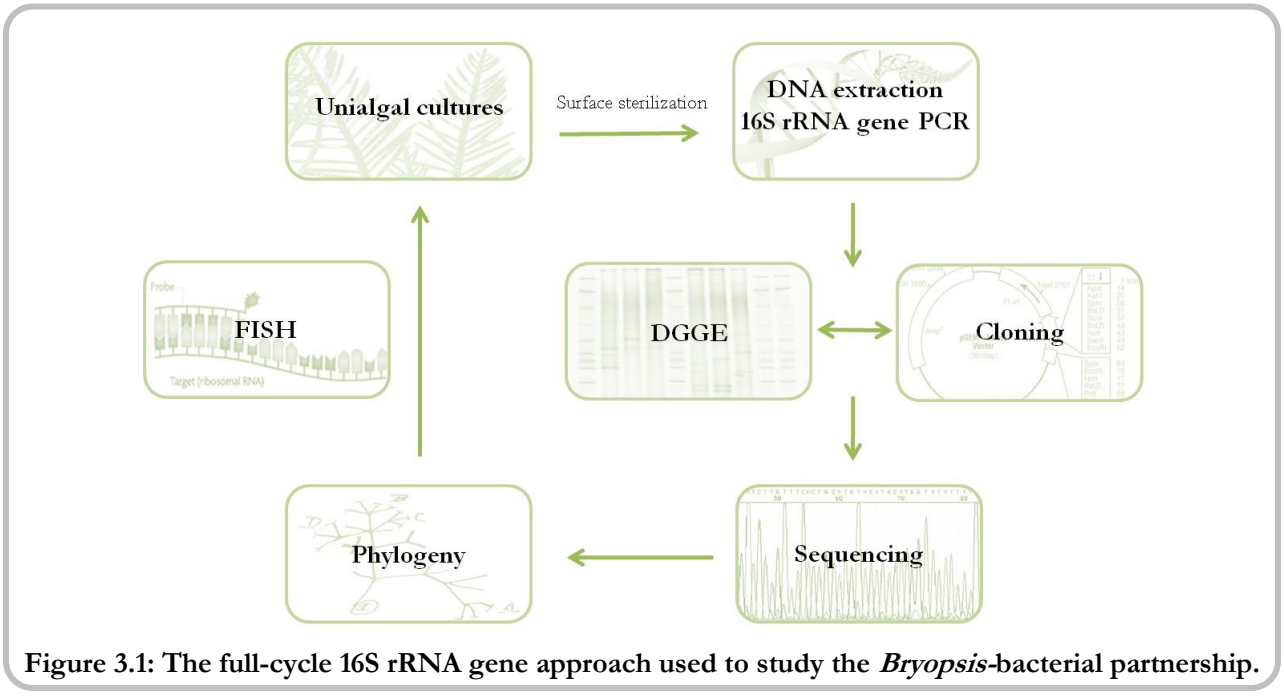


Figure 3.1: The full-cycle 16S rRNA gene approach used to study the *Bryopsis*-bacterial partnership.

Surface sterilization

Besides intracellular bacteria, marine macroalgae also harbor numerous epiphytic bacteria on their surfaces (see Chapter 1). Elimination of these epiphytes is essential to study the bacterial endophytes. Therefore, different mechanical (pipetting, sonication, vortexing, the use of beads, cytoplasm isolation by centrifugation and the formation of protoplasts), enzymatic (different enzymes) and chemical (several disinfectants and lysis buffers) surface sterilization procedures were compared to successfully free the *Bryopsis* surface from epiphytic contamination. Only a combination of CTAB buffer (cetyltrimethylammonium bromide), proteinase K and the bactericidal cleanser Umonium Master proved to be highly effective. A full description of the surface sterilization protocol and its evaluation can be found in section 3.1.2.

Molecular work: full-cycle 16S rRNA gene approach

In addition to the surface sterilization step, several other protocols from the 16S rRNA gene approach were optimized to address the objectives of this thesis. Different DNA extraction techniques, 16S rDNA PCR protocols and dereplication methods were screened. The use of different DNA extraction protocols (CTAB [160] versus Muyzer [161] protocol) had no significant effect on the denaturing gradient gel electrophoresis (DGGE) and cloning efficiency, whereas a nested rather than a direct PCR approach improved the identification rate of – especially – the low abundant community members. Attempts to avoid the interference of chloroplast 16S rDNA amplification by means of a specific primer pair (i.e. F799-R1492, [162, 163]) rather than universal bacterial 16S rRNA gene primers, were unsuccessful. The intented non-amplification of chloroplast 16S rRNA genes was accompagnied by a failure to detect all bacteria present. Furthermore, short fragment sequencing appeared a much more cost-effective technique over the RFLP method to dereplicate the clone libraries. Consequently, CTAB DNA extraction [160], 16S rRNA gene amplification with the universal primer pair F27-R1492 [164], short fragment sequencing dereplication and the nested DGGE-PCR protocol were implemented in the 16S rRNA gene approach applied on a total of 20 *Bryopsis* cultures (see Chapter 3, Part 2). To 'close' the 16S rRNA cycle, the occurrence of bacterial 16S rRNA gene sequences in their respective samples needs to be verified *in situ* [5, 165, 166]. Fluorescence *in situ* hybridization (FISH) with oligonucleotide probes targeting rRNA molecules has been widely used to identify, quantify and localize bacteria in their natural environment [127, 167]. Due to the high intrinsic autofluorescence of algal cells, however, FISH applications on macroalgae are not straightforward [168]. During this thesis several FISH attempts were undertaken on both whole mount and resin-embedded *Bryopsis* vegetative thalli and gametes. A FISH protocol with the universal bacterial EUB338 probe mix [169] was optimized on LR White sections of vegetative thalli and showed the presence of bacterial rRNA inside the *Bryopsis* cytoplasm (see section 3.2.1). Preliminary results of fluorescent hybridizations with group-specific 16S rRNA probes and a newly designed Flavobacteriaceae endosymbiont species-specific probe are reported in section 3.3.2.

Cultivation work

To examine the interdependency of the *Bryopsis* host and the endophytic bacterial partners, attempts were made to culture them separately. Exploratory antibiotic experiments were performed to 'cure' the algae of endophytic bacteria. The antibiotic mixture and/or concentrations applied seemed not sufficient to completely eliminate the bacterial epi- and endophytes without affecting the algal host (see section 3.2.4). Also several attempts were made to culture the endophytic bacteria on media mimicking the algal host. Tryouts by which *Bryopsis* cytoplasm was plated on solid agar media with and without algal extract, were unsuccessful. Cultivation attempts in liquid media supplement with *Bryopsis* extract and inhibitors for gram-positive bacteria, on the other hand, showed the growth of *Labrenzia* and Phyllobacteriaceae bacteria. The cultivation methodology and results are described in section 3.2.4. This section also reports *Bryopsis* epiphytes which were cultured during the cultivation experiments.

Functional gene analysis

Preliminary attempts were made to amplify bacterial functional genes with the *nif*H protocol described by De Meyer *et al.* [170]. Only from a small number of *Bryopsis* samples Rhizobiaceae nitrogenase reductase and Phyllobacteriaceae nitrogenase-like light-independent protochlorophyllide reductase genes (see section 3.2.1 and 3.2.3) could be successfully amplified. Most of the potential amplicons, however, showed high sequence similarities with *Bryopsis* chloroplast genes.

3.1.2. Surface sterilization of *Bryopsis* samples

Modified from: Joke Hollants, Frederik Leliaert, Olivier De Clerck and Anne Willems. (2010) How endo- is endo-? Surface sterilization of delicate samples: A *Bryopsis* (Bryopsidales, Chlorophyta) case study. *Symbiosis* **51**(1): 131-138. **Author contributions:** JH designed and performed the experiments, analyzed the data and wrote the paper. FL maintained the algal cultures. ODC collected the *Bryopsis* (BR) specimen. FL, ODC and AW commented on the manuscript.

Abstract

In the search for endosymbiotic bacteria, elimination of ectosymbionts is a key point of attention. Commonly, the surface of the host itself or the symbiotic structures are sterilized with aggressive substances such as chlorine or mercury derivatives. Although these disinfectants are adequate to treat many species, they are not suitable for surface sterilization of delicate samples. In order to study the bacterial endosymbionts in the marine green alga *Bryopsis*, the cell wall of the host plant was mechanically, chemically and enzymatically cleaned. Only a chemical and enzymatic approach proved to be highly effective. *Bryopsis* thalli treated with cetyltrimethylammonium bromide (CTAB) lysis buffer, proteinase K and bactericidal cleanser Umonium Master showed no bacterial growth on agar plates or bacterial fluorescence when stained with a DNA fluorochrome. Moreover, the algal cells were intact after sterilization, suggesting endophytic DNA is still present within these algae. This new surface sterilization procedure opens the way to explore endosymbiotic microbial communities of other, even difficult to handle, samples.

Introduction

Numerous eukaryotes maintain close associations with bacteria, either on their surface or within their tissues or cells. To examine the latter alliance it is essential to remove the bacteria which inhabit the host's surface and form a main source of contamination. However, the ubiquity of bacterial biofilms prevents the straightforward study of these endosymbionts [171]. In well established symbiosis models the surface sterilization used is in general quite aggressive. Insect eggs, larvae and adults are treated with hydrogen peroxide, formaldehyde, radiation, antibiotics or highly toxic mercury derivatives like mercuric chloride and thiomersal [172, 173]. Land plants or their symbiotic structures (e.g. root nodules) are mostly surface disinfected by means of beads, ethanol or sodium hypochlorite [174]. Despite the use of these vigorous techniques, an effective surface sterilization remains a balancing exercise. Few surface disinfection protocols result in complete removal of ectosymbionts without penetrating interior tissues and thereby neutralizing internal bacteria; while an ineffective sterilization may result in outer surface bacteria being mistaken for endosymbionts [174]. When the host is delicate, as is the case for the siphonous green alga *Bryopsis*, finding the right balance becomes even more challenging. Siphonous seaweeds are essentially single giant multinucleate cells surrounded by a xylan-cellulose cell wall, a thin parietal layer of cytoplasm and a huge central vacuole [2]. Like various other macroalgae [38, 40, 123], *Bryopsis* has long been suspected to harbor endogenous bacteria in the cytoplasm [36]. The identity of these endosymbionts, however, remains unknown. Further exploration of this algal-bacterial partnership requires an efficient surface sterilization of the *Bryopsis* host. After all, many seaweeds live in close association with numerous epiphytic bacteria, which control morphological development [34, 78, 80, 175] or are linked with various metabolic functions [53, 55, 58, 75, 83], and *Bryopsis* seems no exception [176]. Whereas the usage of axenic cultures is quite common for microalgae, for the study of marine macroalgae this is limited. In general, axenic seaweed cultures are obtained by the addition of antibiotics to the growth medium or a combination of antibiotic use and isolation of reproductive cells [31, 177, 178]. Reported attempts to efficiently remove epiphytes mechanically, chemically or enzymatically from macroalgae are even scarcer. Only a few protocols have been published for the selective extraction and subsequent application of epiphytic DNA from bacteria associated with seaweeds [171, 179]. Siphonous macroalgae, such as *Bryopsis*, offer some extra options for the elimination of epiphytes due to their giant-cell morphology and regeneration mechanisms: the cytoplasm of these algae can be isolated by centrifugation [180] and the formation of protoplasts can be easily attained through wounding [113]. However, the objective of all techniques listed above was never to study the

endophytic bacteria within these seaweeds, leaving the effect of these methods on the endophytes unaddressed.

In this study, different mechanical, enzymatic and chemical procedures for the complete elimination of epiphytes from *Bryopsis* plants, in order to study the internal bacterial communities, were compared and evaluated. The aim was to develop a new, highly effective surface sterilization technique which neither lyses the algal cells nor eliminates endophytic DNA, allowing further molecular processing of the endosymbionts.

Materials and methods

Sampling and culturing

A *Bryopsis hypnoides* strain (BR) was collected from the lower intertidal zone in Roscoff, Brittany, France in July 2008. The plant was grown in sterile 1x modified Provasoli enriched seawater [181] at 23°C under a 12:12 hours Light:Dark cycle with a photon flux rate of 25-30 μmol m^{-2} s^{-1}. Unialgal cultures were achieved by isolating apical fragments of the vegetative thalli under a binocular dissecting microscope. The selected apical fragments were maintained under the same growth conditions as described above. To obtain more material for further applications, unialgal cultures were transferred to sterile 250 ml Erlenmeyer flasks with constant aeration.

Sterilization

Unialgal *Bryopsis* samples were submitted to a single or a combination of several mechanical, enzymatic and chemical sterilization protocols listed in Table 3.1. Each protocol was followed by ten washing and vortexing steps in sterile artificial seawater (ASW). Effective removal of epiphytes was tested by incubation of the washing water and sterilized algal thalli on Marine Agar plates (Becton Dickinson) for five days at 20°C. Because many bacteria are difficult to culture, the cleaned samples were stained for 15 min with 5 μg.ml^{-1} 4',6-diamidino-2-phenylindole (DAPI) and subsequently viewed under a confocal and epifluorescence microscope (Zeiss) to determine whether the outer surface bacteria were effectively eliminated by the sterilization protocol applied. Also the intactness of the algal cells was microscopically verified.

Table 3.1: List of protocols applied for the surface sterilization of *Bryopsis* plants.

	Sterilization technique	Extended protocol
Mechanical	**Vortexing**	Repeatedly vortex the plants in 0.2 µm filtered ASW with five changes of washing water
	Ultrasonic probe sonication	Ultrasonic probe sonication of the samples in sterile ASW for 15 seconds at 30 kHz
	Ultrasonic bath sonication	Ultrasonic bath sonication of the samples in sterile ASW for 15 min at 47 kHz
	Use of beads	Add glass beads (0.5 mm, BioSpec Products) to the algal tissue and bead beat the mixture at 30 kHz for 3 x 85 seconds
Enzymatic	**Lysozyme**	Add 10 µl lysozyme (1 $mg.ml^{-1}$ in 10 mM Tris-HCl) and 190 µl sterile ASW to the specimens and incubate for 5 min at room temperature
	Proteinase K	Incubate the algal thalli in a mixture of 1 µl 20 $mg.ml^{-1}$ proteinase K and 99 µl ASW for 30 min at 60°C
Chemical	**Ethanol**	Rinse plants in 80% ethanol for 5 min
	Bleaching	Sterilize algae in 3% sodium hypochlorite for 30 seconds
	Alkaline lysis buffer	Place thalli in 80 µl sterile ASW with 20 µl alkaline lysis buffer (1 M NaOH and 10% sodium dodecyl sulfate) for 15 min at 95°C
	CTAB buffer	Put plants directly into 100 µl CTAB buffer (2 g CTAB, 1 g PEG 8000, 1.5 M NaCl, 0.02 M EDTA and 0.1 M Tris-HCl) for 30 min at 60°C
	UNSET buffer	Place samples in 100 µl UNSET Lysis Buffer (8 M urea, 2% sodium dodecyl sulfate, 0.15 M NaCl, 0.001 M EDTA, 0.1 M Tris pH 7.5) for 15 min at 55°C [179]
	Bactericidal cleanser	Sterilize plants overnight in a 1:1 mixture of 0.2 µm filtered Umonium Master (Huckert's International) and sterile ASW
	Combined approach	1. Place unialgal *Bryopsis* plants directly into CTAB buffer with 20 $mg.ml^{-1}$ proteinase K for 30 min at 60°C 2. Wash the *Bryopsis* thalli with sterile ASW 3. Repeat step 2 two times 4. Incubate overnight the washed thalli in a 1:1 mixture of 0.2 µm filtered Umonium Master and sterile ASW 5. Wash thalli in sterile ASW 6. Repeat step 5 ten times with vigorous vortexing in between the washing steps

Denaturing Gradient Gel Electrophoresis

To compare the effectiveness of the different sterilization procedures, the remaining bacterial diversity was examined by Denaturing Gradient Gel Electrophoresis (DGGE). Therefore the cleaned *Bryopsis* plants were placed in liquid nitrogen and ground with a sterile pestle prior to a total DNA extraction following a CTAB protocol modified from Doyle and Doyle [160]. The V3 region of the 16S rRNA gene was amplified by a PCR with the universal bacterial primers F357 (5'-CCTACGGGAGGCAGCAG-3') and R518 (5'-ATTACCGCGGCTGCTGG-3') [182, 183]. A GC-clamp was coupled to the forward primer to improve DGGE separation. Amplifications were performed in volumes of 50 µl containing 1 µl of target DNA, 1x PCR buffer (GeneAmp, Applied Biosystems), 100 µM dNTPs, 0.05x BSA, 0.2 µM of both primers, and 1.25 units AmpliTaq DNA polymerase (Applied Biosystems). After an initial denaturing step at 95°C for 5 min, 30 cycles of denaturation (95°C, 30 seconds), annealing (55°C, 45 seconds) and extension (72°C, 1 min) were completed, followed by a final amplification step at 72°C for 7 min. Successful amplification of the V3 region was verified through agarose gel electrophoresis. DGGE analysis of PCR amplicons was performed using the DCode Universal Mutation Detection System device (Bio-Rad) as described previously [182]. Optimal electrophoretic separation was obtained using 35-70% denaturing gradient polyacrylamide gels, running for 990 min at 70 V in 1x TAE buffer at a constant temperature of 60°C. The gels were stained with SYBR gold (Molecular Probes, Invitrogen) for 30 min followed by visualization and digital capturing of the profiles via the Molecular Imager Gel Doc XR System (Bio-Rad). Digital images were processed by means of the BioNumerics software (version 5.1, Applied Maths). On each DGGE gel, a reference marker consisting of V3 16S rRNA gene amplicons of 12 different bacterial species was included for normalization to allow comparison between gels [184].

Results

Sterilization

Attempts to efficiently eliminate the epiphytes by means of vortexing, ultrasonic bath sonication, beads, lysozyme, proteinase K or ethanol were unsuccessful. Incubation of the washing water on Marine Agar plates indicated that the former techniques were able to reduce the amount of epiphytes (data not shown), but bacteria were still visible when the sterilized algae themselves were plated or stained with DAPI (Fig. 3.2b and c, Fig. 3.3d to i). In addition, the application of ultrasonic probe sonication appeared to be too rough, the *Bryopsis* thalli were totally fragmented in a fraction of a second. Also the use of sodium hypochlorite was too aggressive, causing elimination of the

endosymbionts due to instant bleaching of the algae. When *Bryopsis* thalli treated with different lysis buffers or the bactericidal cleanser Umonium Master were cultivated on agar plates, no bacterial growth was noticeable (Fig. 3.2d and e); although, some bacterial fluorescence remained visible on the plants after DNA staining (Fig. 3.3j to o). The results mentioned above show that no single sterilization procedure was able to completely remove the epiphytes. Consequently, several combinations of two or more protocols were tested and evaluated (data not shown). Only a combination of CTAB buffer, proteinase K and the bactericidal cleanser Umonium Master proved to be highly effective. Unialgal *Bryopsis* plants were directly placed into CTAB buffer (2 g CTAB, 1 g PEG 8000, 1.5 M NaCl, 0.02 M EDTA and 0.1 M Tris-HCl) with 20 $mg.ml^{-1}$ proteinase K for 30 min at 60°C. Subsequently, the thalli were washed three times with sterile ASW and incubated overnight in a 1:1 mixture of 0.2 µm filtered Umonium Master and sterile ASW. After lysis of the epiphytes, *Bryopsis* samples were washed ten times in sterile ASW with vigorous vortexing in between the washing steps to remove the lysed bacterial DNA (step-by-step protocol of the combined approach, Table 3.1). The absence of cultivable epiphytes and bacterial DNA on the sterilized samples was verified as described above. Plating of these sterilized *Bryopsis* thalli on Marine agar showed no bacterial growth (Fig. 3.2f). More significant, however, was the staining of the sterilized *Bryopsis* samples with the DNA fluorochrome DAPI, revealing the absence of bacterial fluorescence on the surface of the algae (Fig. 3.3p to r). The algal cells themselves, on the other hand, were not lysed by the sterilization procedure as confirmed by light, epifluorescence and confocal microscopy, suggesting endophytic DNA is still present within the algae after the used chemical and enzymatic surface sterilization.

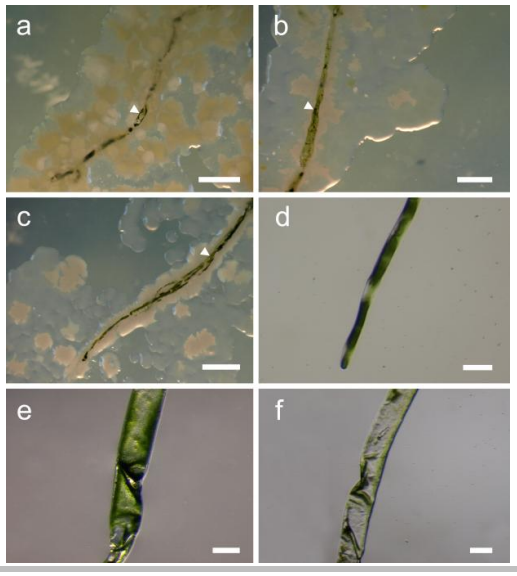


Figure 3.2: Incubation of untreated (a) and sterilized (b-f) *Bryopsis* thalli on Marine Agar plates. Like the untreated sample (a), the ethanol (b) or enzymatically (c) cleaned samples still show growth of epiphytic bacteria after five days incubation, indicating an unsuccessful surface sterilization. In contrast, *Bryopsis* plants treated with lysis buffers (d), Umonium Master (e) or the new combined approach (CTAB buffer, proteinase K and Umonium Master; f) showed no bacterial growth after plating, suggesting that (culturable) epiphytes are effectively eliminated. Scale bars: 5 mm (a, b, and c), 2.5 mm (d), and 1 mm (e and f). Arrowheads: *Bryopsis* thalli.

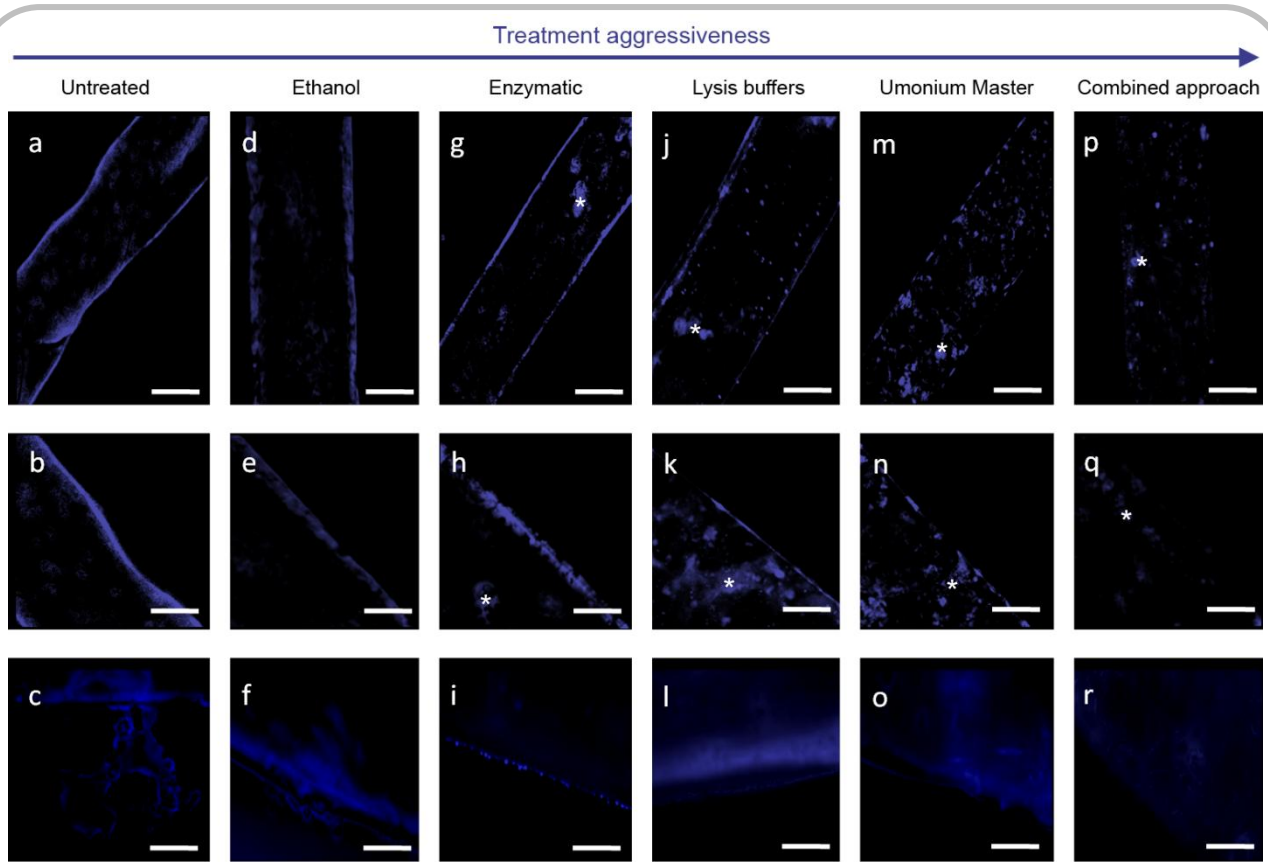


Figure 3.3: Fluorescence microscopy images of untreated (a-c) and sterilized (d-r) *Bryopsis* thalli stained with DAPI. Rows display from left to right the result of progressively more aggressive treatments, columns show increasing magnification from top to bottom. When surface sterilization is more aggressive and consequently more effective (left-right), DAPI staining of the outer surface bacteria becomes less profuse. Images of the untreated sample (a-c), the ethanol (d-f) and enzymatically (g-i) sterilized samples show an intense staining of epiphytic DNA on their cell walls. This DAPI staining becomes gradually weaker on the images of plants treated with lysis buffer (j-l) or Umonium Master (m-o), and is missing on fluorescence pictures of *Bryopsis* thalli sterilized with the new combined approach (CTAB buffer, proteinase K and Umonium Master; p-r). The latter signifies an effective surface sterilization. In addition, algal cell walls become more permeable for the DAPI stain (e.g. more fluorescent foci from nuclei and chloroplasts at the inside of the algal cells, see asterisks) as surface sterilization is more aggressive, but they were never fully lysed. Scale bars: 100 μm (a, d, g, j, m, and p; confocal microscopy images), 50 μm (b, e, h, k, n, and q; confocal microscopy images), and 10 μm (c, f, i, l, o, and r; epifluorescence microscopy images).

Denaturing Gradient Gel Electrophoresis

Total DNA, of both algal and bacterial origin, was extracted from *Bryopsis* thalli using the CTAB approach. This DNA mixture appeared to contain an excess of plant enzymes which interfere with PCR amplification. In order to decrease the algal inhibitors in the DGGE PCR, a 1:10 dilution of template DNA was used. Following electrophoresis, all samples, except the one treated with bleach, displayed an expected band of approximately 200 bp on the agarose gel. Each band on the agarose gel represents a mixture of fragments of 16S rRNA genes from potential remaining epiphytes,

endophytes and chloroplasts. Hence, DGGE was used to separate these fragments and examine the bacterial diversity surviving the various sterilization protocols applied. Figure 3.4 depicts a decrease in bacterial diversity in proportion to the vigorousness of the used sterilization. Mechanically cleaned samples show more individual DGGE bands, indicating an unsuccessful removal of epiphytes, compared to enzymatically and chemically sterilized plants. The newly presented combined sterilization protocol displayed the strongest reduction in bands and thus the most effective elimination of outer surface bacteria. Taking together evidence from the fluorescence imaging along with these molecular results strongly suggests that the remaining 16S rRNA gene diversity, including the chloroplast 16S rDNA, is of endophytic origin.

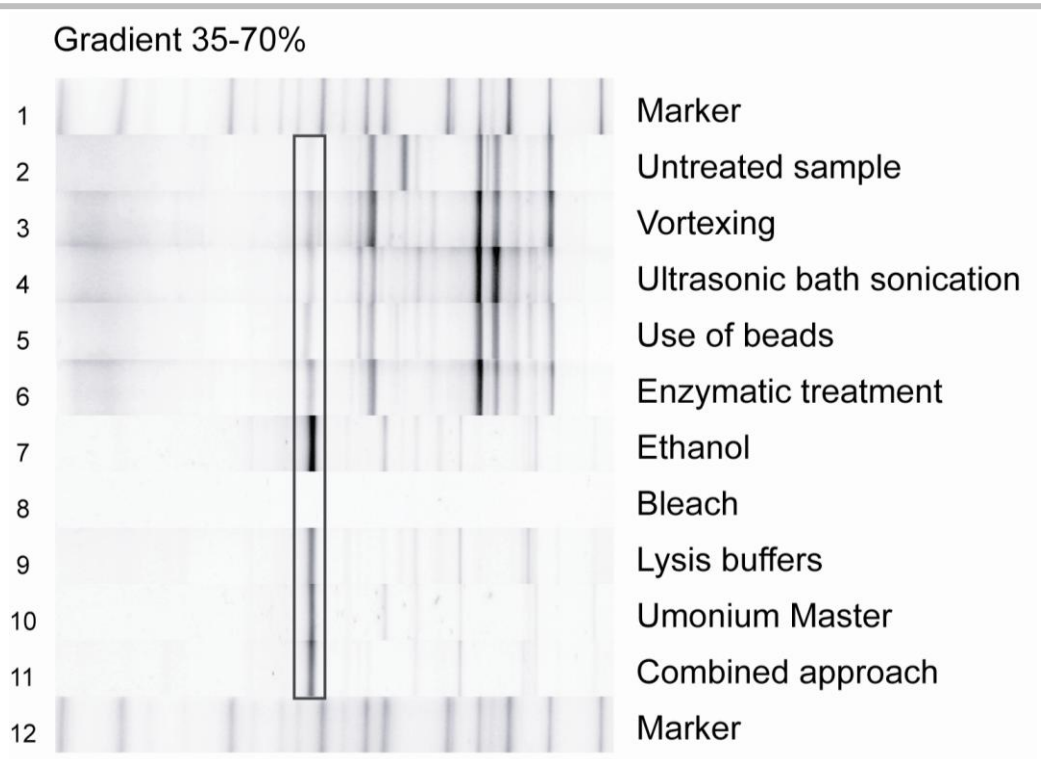


Figure 3.4: Normalized DGGE profiles of 16S rDNA fragments amplified from untreated and sterilized *Bryopsis* plants. DGGE profiles represent the bacterial diversity of the untreated (lane 2) and treated (lane 3 till 11) samples. As the disinfection protocols applied are more effective (up-down), DGGE profiles become less complex, reflecting a more successful surface sterilization. The DGGE profile of the bleach-sterilized sample (lane 8) shows no remaining bacterial diversity. Furthermore, *Bryopsis* plants treated with the new combined approach (CTAB buffer, proteinase K and Umonium Master; lane 11) still show a clear occurrence of bands, indicating endophytic DNA is still present within these sterilized plants and suitable for further molecular processing. Lane 1 and 12 contain a molecular marker used for normalization. The black box indicates chloroplasts 16S rDNA fragments as verified by DNA sequencing.

Discussion

Obtaining axenic macroalgal cultures while maintaining endophytic bacteria is challenging. Established culture techniques relying on the usage of antibiotics are inadequate as antibiotics may penetrate through the algal cell wall and eliminate the endophytes. Observations that antibiotics clearly affect the growth of algae or even kill them indicate such diffusions [31]. Also the special features of siphonous seaweeds, e.g. the formation of protoplasts and the ability to isolate their cytoplasm, are insufficient to generate epiphytic-free algal material. Since these extraction techniques both depend on cutting or squeezing the algae, the outer xenic membranes become damaged and give rise to contaminations (personal observations). It has to be mentioned that the development of protoplasts in some larger and therefore easier to manipulate siphonocladous algae, like for example *Boergesenia* and *Ventricaria*, shows potential for the formation of epiphytic-free algal material [personal observations, 102]. Hence, traditional algal cultivation and manipulation methods appear inadequate for the removal of bacterial epiphytes from *Bryopsis* plants without affecting the endophytes. Consequently, different mechanical, enzymatic and chemical surface sterilization protocols were tested and compared. None of these techniques seems on its own able to effectively eliminate the outer surface bacteria. The mechanical and enzymatic methods are highly insufficient, in accordance with observations by Burke and coworkers [171]. Also the use of various lysis buffers and disinfectants appears to be ineffective, in contrast with previous published studies [171, 179]. In these studies, Fisher and coworkers [179] successfully sterilized filamentous green algae by placing them directly in UNSET buffer (Table 3.1), and Burke and colleagues [171] fruitfully treated the green and red seaweeds, *Ulva australis* and *Delisea pulchra*, with calcium- and magnesium-free artificial seawater (CMFSW) supplemented with EDTA and a rapid multi-enzyme cleaner. These protocols, which were designed for the selective extraction of epiphytic DNA from algae associated bacteria, seem inefficient to completely sterilize *Bryopsis* externally with the aim of studying the bacterial endophytes. This objective is achieved by a combined chemical and enzymatic approach as presented here. *Bryopsis* thalli treated with CTAB lysis buffer, proteinase K and the bactericidal cleanser Umonium Master are highly effectively sterilized. They show no bacterial growth on agar plates (Fig. 3.2f) and no bacterial fluorescence on their cell wall when observed with confocal and epifluorescence microscopy after DAPI staining (Fig. 3.3p to r). Although often neglected by conventional surface sterilization protocols, the latter verification is essential since only 1% of all known bacteria are suspected to be culturable [127]. For example, the untreated sample and the ethanol and enzymatically cleaned plants all show growth when plated (Fig. 3.2a to c). On the other

hand, fluorescence images allow a more detailed assessment of the outer surface community: the untreated, ethanol-sterilized and enzymatically cleaned samples show, respectively, bacterial biofilms (Fig. 3.3a to c), reduction of surface biofilms (Fig. 3.3d to f) and destruction of the biofilms into unattached bacteria (Fig. 3.3g to i). Much less expected were the results of the DAPI staining of *Bryopsis* plants sterilized with lysis buffer or Umonium Master. While both samples indicate no bacterial existence on agar (Fig. 3.2d and e), fluorescence images prove the presence of DNA on the surface of the algae (Fig. 3.3j to o). Even after vigorously vortexing during the several washing steps, bacterial DNA remains trapped in the degraded algal cell wall which is still clearly outlined by the blue DAPI stain. Despite the fact that these cell walls are gradually more damaged as surface sterilization becomes more effective, they were never fully lysed after the different disinfectant treatments. As shown in Figure 3.3 (internal fluorescent foci from nuclei and chloroplasts, a to r) the weakened algal cell walls become permeable for the DAPI fluorochrome after chemical and enzymatic surface sterilization. This does not only indicate that internal algal and bacterial DNA is still present, this DNA also seems suitable for additional molecular processing like PCR amplification and DGGE (Fig.3.4). This molecular approach is of great value for further research on the identity and functionality of the – possibly unculturable – endosymbiotic bacteria in *Bryopsis* algae. Future investigations will probably reveal that these bacteria have significant functions within their host. Moreover, some of the compounds produced by these bacterial symbionts may have important applications like for example the production of the anticancer drug kahalalide as suggested by Kan et al. [176].

Conclusion

Although surface sterilization is a critical step in endosymbiosis research, it remains challenging, especially in delicate organisms such as algae. Certainly in new symbiosis systems, it is worthwhile to test and evaluate conventional sterilization techniques. This study demonstrates that small alterations or combinations of established disinfection protocols permit an efficient sterilization. The protocol presented here will likely be useful in studies of new and difficult to handle hosts, allowing exploration of novel symbiosis systems.

Acknowledgments

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Part 3.2 Endophytic bacterial communities of *Bryopsis* cultures

3.2.1. Endophytic bacterial diversity within Mexican *Bryopsis* samples

Modified from: Joke Hollants, Olivier Leroux, Frederik Leliaert, Helen Decleyre, Olivier De Clerck and Anne Willems. (2011) Who is in there? Exploration of endophytic bacteria within the siphonous green seaweed *Bryopsis* (Bryopsidales, Chlorophyta). *PLoS ONE* **6**(10): e26458. **Author contributions:** JH designed the experiments and wrote the paper. OL helped to design the FISH protocol. JH, OL and HD performed the experiments. JH and FL analyzed the data. FL maintained the algal cultures and helped to draft the figures. OL, FL, ODC and AW commented on the manuscript.

Abstract

Associations between marine seaweeds and bacteria are widespread, with endobiotic bacterial-algal interactions being described for over 40 years. Also within the siphonous marine green alga *Bryopsis*, intracellular bacteria have been visualized by electron microscopy in the early '70s, but were up to now never molecularly analyzed. To study this partnership, we examined the presence and phylogenetic diversity of microbial communities within the cytoplasm of two *Bryopsis* species by combining fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone libraries. Sequencing results revealed the presence of *Arcobacter*, Bacteroidetes, Flavobacteriaceae, *Mycoplasma*, *Labrenzia*, Phyllobacteriaceae and Xanthomonadaceae species. Although the total diversity of the endobiotic communities was unique to each *Bryopsis* culture, Bacteroidetes, *Mycoplasma,* Phyllobacteriaceae, and in particular Flavobacteriaceae bacteria, were detected in several *Bryopsis* samples collected hundreds of kilometres apart. This suggests that *Bryopsis* closely associates with well-defined endophytic bacterial communities of which some members possibly maintain an endosymbiotic relationship with the algal host.

Introduction

Marine macroalgal-bacterial associations range from beneficial, harmful or neutral, over obligate or facultative, to ecto- or endophytic interactions [4]. Elaborating the latter, endobiotic associations between marine macroalgal hosts and bacteria have been reported over the past 40 years. Besides reports of bacterial endosymbionts associated with red algal galls [95, 123, 185], endophytic bacteria have been microscopically observed in the vacuolar as well as cytoplasmatic regions of various bryopsidalean green algae, including *Bryopsis*, *Penicillus*, *Halimeda*, *Udotea* and *Caulerpa* [36, 38-40, 75, 124]. These seaweeds are composed of a single, giant tubular cell and form an interesting biotic environment for bacterial communities. The giant cell contains millions of nuclei and chloroplasts in a thin cytoplasmic layer surrounding a large central vacuole. The cytoplasm typically exhibits vigorous streaming, enabling transport of nutrients, organelles and various biomolecules across the plant [107]. In *Bryopsis* 'bacteria-like particles' have been visualized in the cytoplasm by means of transmission electron microscopy in vegetative thalli as well as in the gametes, the latter suggesting vertical transmission of the endophytic bacteria [36]. This implies a stable and specific relationship between the algal host and its endobionts in which both partners may provide mutualistic ecological benefits. To date, the diversity of the intracellular microbial communities associated with *Bryopsis* remains unidentified. Up till now investigations of the bacterial endophytic diversity of siphonous macroalgae have been limited to *Caulerpa* species and revealed endosymbiotic Alphaproteobacteria with the potential to photosynthesize, detoxify and/or fix nitrogen [75, 124]. The endophytic bacteria in *Bryopsis* may similarly possess ecologically significant functions and bioactive potential since *Bryopsis* is a substantial source of bioactive compounds such as therapeutic kahalalides which may be of bacterial origin [176, 186].

In order to explore these algal-endophytic bacterial interactions, we previously developed a surface sterilization protocol for the complete elimination of bacterial epiphytes from the *Bryopsis* surface (see section 3.1.2 [187]). We showed that *Bryopsis* samples treated with a combined chemical and enzymatic approach (i.e. a mixture of cetyltrimethylammonium bromide (CTAB) lysis buffer, proteinase K and the bactericidal cleanser Umonium Master) remained intact after sterilization and showed no remaining bacterial fluorescence on their surface when stained with a DNA fluorochrome. Successful 16S rRNA gene DGGE analysis following this surface sterilization treatment showed that endophytic DNA was still present within the sterilized *Bryopsis* samples, allowing specific molecular processing of the endophytes (section 3.1.2 [187]). In this study, we verified the presence of bacteria inside two *Bryopsis* species from the Mexican west coast by a

combination of fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE) and clone libraries.

Materials and methods

Ethics Statement

No specific permits were required for the described field studies, i.e. the collection of algal samples from the Mexican west coast, because marine algae are not included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, http://www.cites.org/eng/disc/species.shtml). The authors confirm that the location is not privately-owned or protected in any way and that the field studies did not involve endangered or protected species.

Algal material

Five *Bryopsis* specimens were collected in February 2009 along the Pacific Mexican coast at different sites located between Mazunte Beach (Oaxaca, southwest Mexico) and Playa Careyero (Nayarit, central Mexico) (Figure 3.5). These five samples were classified in two different species with samples MX19 and MX263 representing *Bryopsis hypnoides* J.V. Lamouroux and MX90, MX164, and MX344 representing *Bryopsis pennata* J.V. Lamouroux var. *leprieurii* (Kützing) Collins and Hervey individuals. After sampling, living specimens were rinsed with sterile seawater and transferred to the laboratory in plastic vessels containing a small amount of sterile seawater. In the laboratory, clean apical fragments of the *Bryopsis* specimens were isolated and cultured in sterile 1x modified Provasoli enriched seawater [181] at 23°C under 12:12 hours Light:Dark conditions with a photon flux rate of 25-30 μmol m^{-2} s^{-1}. This isolation procedure was repeated for several months until the *Bryopsis* cultures were free of eukaryotic contamination. Thus, the *Bryopsis* isolates were kept in culture for eight months prior to molecular analyses in October 2009. After isolation, all five unialgal *Bryopsis* cultures were maintained in the laboratory under the culture conditions described above.

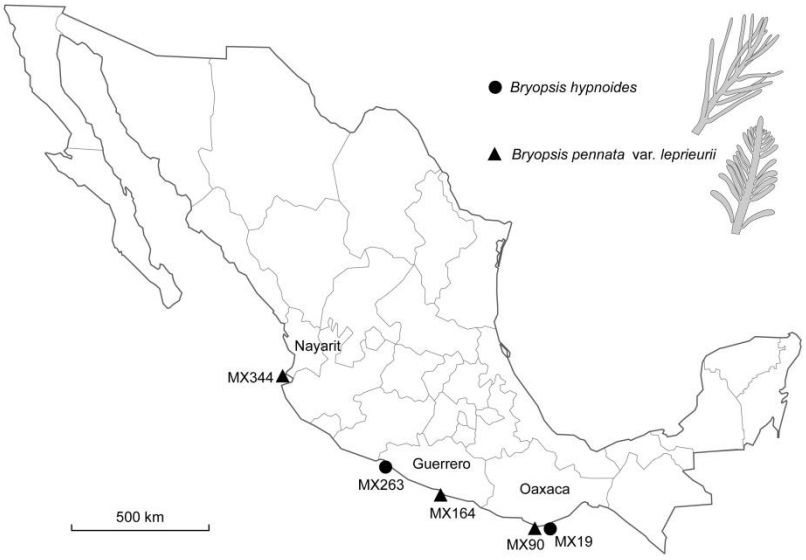


Figure 3.5: *Bryopsis* sampling sites along the Pacific Mexican coast. *Bryopsis hypnoides* (●) and *Bryopsis pennata* var. *leprieurii* (▲) samples were collected from following sites: Playa el Pantheon (MX19), Mazunte Beach (MX90), Acapulco (MX164), Playa las Gatas (MX263) and Playa Careyero (MX344).

Fluorescence in situ hybridization

Unialgal *Bryopsis* thalli were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)) buffer, pH 7.2 for 2 hours. After dehydration through a graded ethanol series from 30% to 80%, ethanol was subsequently replaced by LR white embedding medium (London Resin, UK). Samples were loaded in gelatine capsules and allowed to polymerize at 37°C for 3 days. Semithin sections were cut using glass knives on a Microm HM360 microtome (Microm International GmbH, Germany) and collected on Vectabond-coated (Vector Laboratories, USA) slides. *In situ* hybridization was performed as described by Daims *et al.* [188] with 200 µl formamide per ml hybridization buffer, an incubation of 90 min at 46°C, and the universal bacterial Cy3-labelled EUB338 probe mix [169]. Algal DNA and cell wall counterstaining was performed by adding a mix of 4',6-diamidino-2-phenylindole (DAPI) and calcofluor to the sections for 7 min in the dark at room temperature. Sections were mounted in AF-1 antifadent (Citifluor, UK) and viewed with an Olympus BX51 epifluorescence microscope fitted with a DAPI/FITC/TRITC triple band filter. The *Bryopsis* specimens were not surface-sterilized prior to hybridization due to potential morphological losses.

Surface sterilization, DNA extraction and PCR

To identify the endophytic bacterial diversity, approximately 2 grams (ww) of each unialgal *Bryopsis* sample was surface-sterilized as described in section 3.1.2 [187] prior to a total DNA extraction using a CTAB protocol modified from Doyle and Doyle [160]. These extracts, containing both algal and bacterial DNA, were subjected to *rbc*L and 16S rRNA gene PCR amplifications following protocols outlined in Hanyuda *et al.* [189] and Lane [164] with, respectively, primer pairs 7F/R1391 and 27F/1492R. All obtained PCR amplicons were purified using a Nucleofast 96 PCR clean up membrane system (Machery-Nagel, Germany) according to the manufacturer's instructions.

Cloning and DGGE

To determine the bacterial diversity, purified 16S rRNA gene amplicons from the algal extracts were cloned using the pGEM®-T Vector System (Promega Benelux, The Netherlands). For each *Bryopsis* sample a clone library of 150 clones was prepared, the diversity of which was examined via short fragment sequencing (see below). For dereplication, the short sequences of the clones were grouped into the same operational taxonomic unit (OTU) when having ≥ 97% similarity. From each OTU, representative clones were selected for full length (± 1450 bp) 16S rRNA gene sequencing (see below). Coverage of the clone libraries was verified by DGGE analysis of each *Bryopsis* DNA extract and its representative clones. A V3 PCR with primers F357-GC/R518 and subsequent DGGE analysis were carried out as described in section 3.1.2 [187], with a denaturing gradient of 45-65%. DGGE banding patterns were normalized and processed as outlined in section 3.1.2 [187]. DGGE bands from the algal extracts which showed no correspondence with OTU band positions were excised from the polyacrylamide gel following Van Hoorde *et al.* [190] and sequenced (± 150 bp) as described below.

Sequencing

*Rbc*L genes, DGGE bands as well as short and full length 16S rRNA genes were sequenced on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA) by means of the BigDye® xTerminator™ v.3.1 Cycle Sequencing and Purification Kit (Applied Biosystems, USA) according the protocol of the supplier. Primers used were, respectively, 7F/R1391 [189], F357/R518 [190], BKL1 [191] and T7/SP6 (Promega Benelux, The Netherlands). Sequences obtained were assembled in BioNumerics, compared with nucleotide databases via BLAST and chimera-checked using

Bellerophon [192]. The bacterial 16S rRNA gene and *Bryopsis* chloroplast 16S rRNA gene and *rbc*L sequences were submitted to GenBank under accession numbers JF521593-JF521615 (Table 3.2).

Phylogenetic analyses

Two sets of alignments, made using MUSCLE [193], were considered for phylogenetic analyses. The first one, consisting of a concatenated chloroplast 16S rRNA gene and *rbc*L dataset, was used for the creation of a *Bryopsis* phylogram. A second set of alignments was assembled to assess 16S rRNA gene phylogenetic relationships between the *Bryopsis*-associated bacterial endophytes and known bacterial species, including BLAST hits and algae-associated bacteria described in literature. The most suitable model for phylogenetic analysis was selected using the AIC criterion in jModelTest [194]. Subsequently, the *Bryopsis* host and bacterial datasets were analyzed by means of the maximum likelihood (ML) algorithm in PhyML v3.0 [195] under a HKY + G4 model via the University of Oslo Bioportal website [196]. Reliability of ML trees was evaluated based on 100 bootstrap replicates. Output ML trees were subsequently visualized in Mega 4.0 [197] and edited with Adobe® Illustrator® CS5.

Results

Fluorescence in situ hybridization

To confirm the observation of endogenous bacteria in *Bryopsis* made by Burr and West [36], *Bryopsis* sections were hybridized with the universal bacterial EUB338 probe mix labelled with Cy3. Figures 3.6A-C depict clear binding of the red fluorescent probe mix to bacterial rRNA present throughout the cytoplasm; both in the outer layer next to the cell wall, which contains most of the organelles except the chloroplasts (Figures 3.6A-C), as well as in the inner chloroplast layer immediately adjacent to the vacuole (Figures 3.6B-C). These hybridization results demonstrate the presence of metabolically active bacteria within the *Bryopsis* cytoplasm. Since the *Bryopsis* thalli were not surface sterilized before fixation, the EUB338 probe mix also hybridized with epiphytic bacterial rRNA on the cell wall (Figures 3.6B-C).

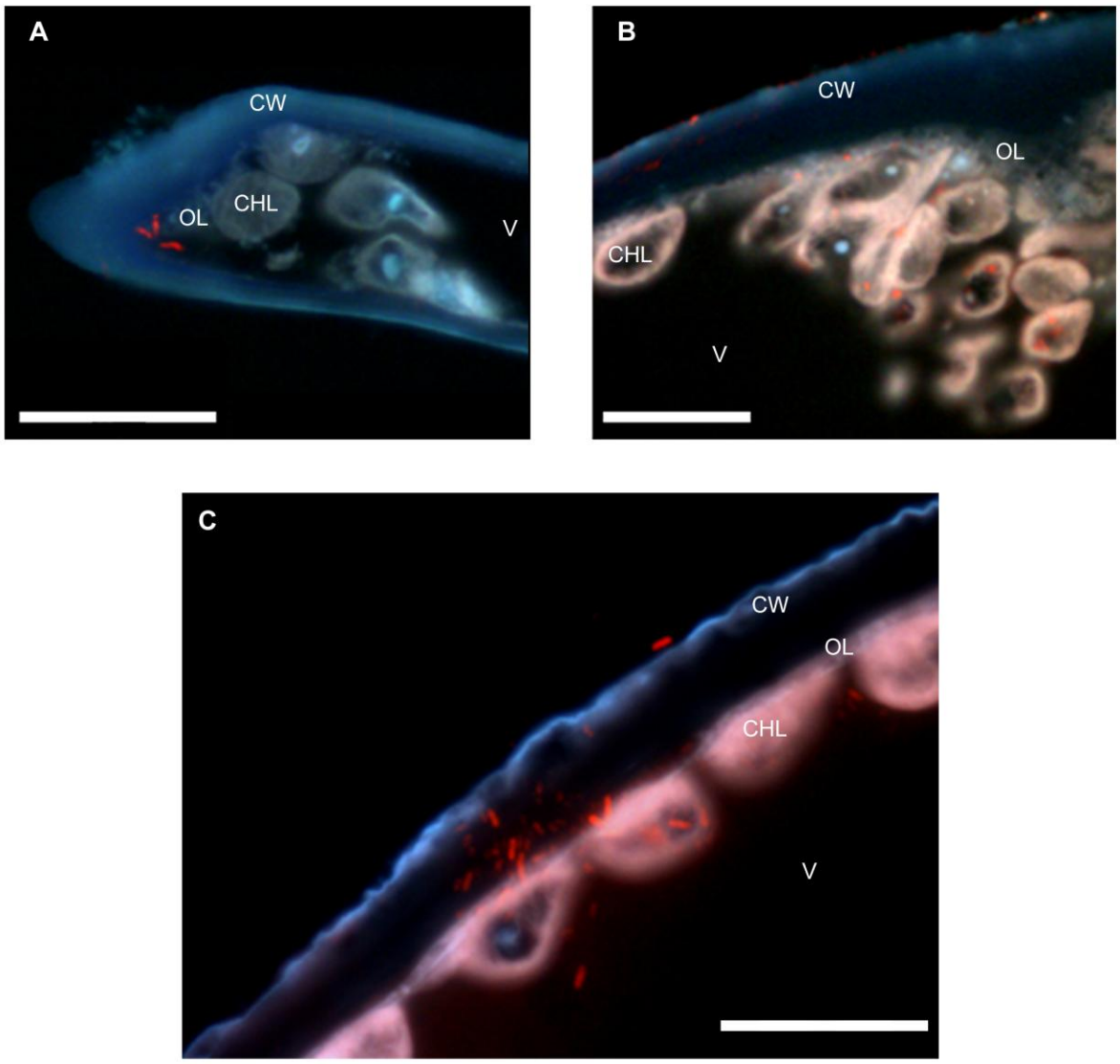


Figure 3.6: Epifluorescence microscopy images of *Bryopsis* sections hybridized with the universal bacterial Cy3-EUB338 probe mix (red). DAPI (light blue) and calcofluor (dark blue) were used as counter stains to visualize algal DNA in nuclei and chloroplasts and the algal cell wall, respectively. Metabolically active bacteria (red) are present throughout the *Bryopsis* cytoplasm: in the outer layer (OL) next to the cell wall (CW) which contains most of the organelles like mitochondria, endoplasmic reticulum, and nuclei (A-C), and in the inner chloroplast layer (CHL) immediately adjacent to the vacuole (V) (B-C). Since the *Bryopsis* thalli were not surface sterilized before fixation, the red probe also hybridized with epiphytic bacteria on the calcofluor stained cell wall (B-C). The scale bar on all images is 20 μm.

Bacterial diversity within Bryopsis algae: *Cloning*

Five clone libraries were created using the amplified 16S rRNA gene fragments from samples MX19, MX90, MX164, MX263 and MX344. After clone dereplication, 16S rRNA gene sequences from all five clone libraries covered no more than seven unique OTUs. By far the most common OTU, representing 72% of the total clones screened, showed ≥ 96% sequence similarity with the *B. hypnoides* chloroplast 16S ribosomal RNA gene (AY221722). The six remaining OTUs, on the other hand, contained bacterial sequences belonging to the phyla Bacteroidetes, Proteobacteria or Tenericutes (Table 3.2). OTU-1 was detected in all five *Bryopsis* cultures and had 96% sequence similarity with an uncultured Flavobacteriales bacterium (FJ203530) associated with the coral *Montastraea faveolata*. OTU-2 and 3 were only present in the *B. hypnoides* samples. OTU-2 is related to Mycoplasmataceae sequences amplified from the intestine of the small abalone *Haliotis diversicolor* (GU070687, HQ393440). OTU-3 is allied to unclassified Bacteroidetes bacteria associated with corals (GU118164, FJ202831) or *Acanthamoeba* species (EF140637). OTU-4 sequences were detected in cultures MX19 and MX164, and showed high similarity (≥ 97%) with Phyllobacteriaceae bacteria isolated from seawater (HM799061, FJ517108), dinoflagellates (AY258089), stromatolites (EU75366) or corals (GU118131). OTU-5 and 6 were only present in *B. pennata* var. *leprieurii* sample MX164 and are distantly related (93-94%) to, respectively, *Luteibacter* sp. (Xanthomonadaceae) present in soil (EF612351, AM930508, FJ848571) and *Arcobacter* strains (Campylobacteraceae) recovered from mussels (FR675874) and seawater surrounding seaweeds and starfish (EU512920).

Table 3.2: Taxonomic affiliation of the clones representing the bacterial OTUs, sorted per *Bryopsis* sample.

Host		16S rRNA gene sequence analysis of bacterial clones						
***Bryopsis* sample**	**Chloroplast 16S rRNA gene[1] and *rbc*L gene**	**OTU no.[2]**	**OTU representative clone name**	**Accession no.**	**OTU library %/sample[3]**	**Higher taxonomic ranks**	**Three closest NCBI matches**	**Accession no. (Query coverage/Maximum identity)**
MX19	JF521612 JF521594	OTU-3	MX19.8	JF521598	0.8%	Bacteroidetes; unclassified Bacteroidetes	Uncultured bacterium clone Dstr_N15	GU118164 (99/94)
							Uncultured bacterium clone SGUS845	FJ202831 (100/92)
							Endosymbiont of *Acanthamoeba* sp. KA/E21	EF140637 (100/91)
		OTU-2	MX19.9	JF521606	14.2%	Tenericutes, Mollicutes, Mycoplasmatales, Mycoplasmataceae	Uncultured bacterium clone GB96	GU070687 (100/97)
							Uncultured bacterium clone frc89	HQ393440 (100/93)
							Uncultured bacterium isolate SRODG064	FM995178 (100/90)
		OTU-4	MX19.12	JF521607	3%	Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	Uncultured Rhizobiales bacterium clone PRTBB8661	HM799061 (99/99)
							Uncultured Rhizobiaceae bacterium clone TDNP_Wbc97_42_3_189	FJ517108 (100/97)
							Uncultured alpha proteobacterium clone D2F10	EU753666 (100/97)
		OTU-1	MX19.14	JF521603	2.3%	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
							Uncultured Bacteroidetes bacterium clone CN77	AM259925 (100/94)
							Uncultured bacterium clone SINP825	HM127741 (99/89)
MX90	JF521615 JF521597	OTU-1	MX90.40	JF521602	6.5%	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
							Uncultured Bacteroidetes bacterium clone CN77	AM259925 (100/94)
							Uncultured bacterium clone SINP825	HM127741 (99/88)
MX164	JF521611 JF521593	OTU-5	MX164.9	JF521609	63.6%	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae	Gamma proteobacterium strain OS-28	EF612351 (100/94)
							Uncultured *Luteibacter* sp. clone SMa210	AM930508 (100/94)
							"*Luteibacter jiangsuensis*" JW-64-1	FJ848571 (100/93)
		OTU-1	MX164.14	JF521600	7.1%	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
							Uncultured Bacteroidetes bacterium clone CN77	AM259925 (100/94)
							Uncultured bacterium clone SINP825	HM127741 (99/89)

Host		16S rRNA gene sequence analysis of bacterial clones						
Bryopsis **sample**	**Chloroplast 16S rRNA gene[1] and *rbc*L gene**	**OTU no.[2]**	**OTU representative clone name**	**Accession no.**	**OTU library %/sample[3]**	**Higher taxonomic ranks**	**Three closest NCBI matches**	**Accession no. (Query coverage/Maximum identity)**
		OTU-6	MX164.20	JF521610	3.6%	Proteobacteria; Epsilonproteobacteria; Campylobacterales; Campylobacteraceae	*Arcobacter marinus* type strain CL-S1T	EU512920 (96/93)
							"*Arcobacter molluscorum*" type strain CECT7696T	FR675874 (94/94)
							Uncultured *Arcobacter* sp. clone bo13C09	AY862492 (96/93)
		OTU-4	MX164.59	JF521608	5%	Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	Phylobacteriaceae bacterium strain DG943	AY258089 (97/99)
							Uncultured bacterium clone Apal_F11	GU118131 (99/98)
							Uncultured bacterium clone MSB-2G6	EF125460 (100/97)
MX263	JF521613 JF521595	OTU-2	MX263.1	JF521605	22.6%	Tenericutes, Mollicutes, Mycoplasmatales, Mycoplasmataceae	Uncultured bacterium clone GB96	GU070687 (100/97)
							Uncultured bacterium clone frc89	HQ393440 (100/93)
							Uncultured bacterium isolate SRODG064	FM995178 (100/90)
		OTU-1	MX263.61	JF521604	4%	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
							Uncultured Bacteroidetes bacterium clone CN77	AM259925 (100/94)
							Uncultured bacterium clone SINP825	HM127741 (99/89)
		OTU-3	MX263.73	JF521599	1.4%	Bacteroidetes; unclassified Bacteroidetes	Uncultured bacterium clone Dstr_N15	GU118164 (99/94)
							Uncultured bacterium clone SGUS845	FJ202831 (100/92)
							Endosymbiont of *Acanthamoeba* sp. KA/E21	EF140637 (100/91)
MX344	JF521614 JF521596	OTU-1	MX344.2	JF521601	2.2%	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
							Uncultured Bacteroidetes bacterium clone CN77	AM259925 (100/94)
							Uncultured bacterium clone SINP825	HM127741 (99/89)

[1] Chloroplast 16S rRNA gene sequences were derived from clones MX19.1, MX90.9, MX164.1, MX263.48 and MX344.10 with an OTU library percentage of, respectively, 79.7, 93.5, 20.7, 68 and 97.8 percent per sample.

[2] All bacterial OTUs containing clones derived from different *Bryopsis* strains had minimal intra-OTU sequence similarities of ≥97% ranging from exactly 97% in OTU-4, over 99.3% and 99.7% in, respectively, OTU-2 and OTU-1, to no less than 99.9% pairwise similarity in OTU-3.

[3] Especially noteworthy is the abundance of OTU-5 in the clone library of sample MX164. While the bacterial OTUs 1, 3, 4 and 6 have a low occurrence of 0.8-7.1% and OTU-2 a considerable presence of 14.2-22.6% in their respective clone libraries, OTU-5 amounts to a substantial percentage (63.6%) of the clones of sample MX164. In addition, only *Bryopsis* sample MX263 comprised chimeric Flavobacteriaceae-*Bryopsis* chloroplast 16S rRNA gene sequences which made up 4% of the clone library of the sample.

Bacterial diversity within Bryopsis algae: *DGGE*

Coverage of the clone libraries was verified by comparing DGGE community profiles of the different *Bryopsis* DNA extracts with the banding pattern of clones from their respective OTUs, including representative clones with 16S rRNA gene chloroplast and chimeric sequences. As shown in Figure 3.7 the OTUs DGGE bands overlap well with the individual bands of the DGGE profiles of the MX extracts, indicating adequate clone library coverage. MX samples 19, 164 and 344, however, all showed one band in their DGGE profile not represented by an OTU band. Consequently, these three DGGE bands (A, B and C, respectively) were excised and sequenced. The sequence of DGGE band A showed 100% similarity with the chimeric sequences detected in MX sample 263, not unexpected given its corresponding band position with clone MX263.66. DGGE band B was identified as forming part of the OTU-2 cluster with 100% sequence similarity with clone MX19.9, whereas DGGE band C showed no correspondence with any bacterial OTU detected. Hence, the latter DGGE band was assigned to a new OTU, i.e. OTU-7. BLAST searches revealed that this OTU-7 is closely related to *Labrenzia* species isolated from the green seaweed *Ulva rigida* (FN811315), crustose coralline red algae (HM178529) and the dinoflagellate *Karlodinium micrum* (HM584720).

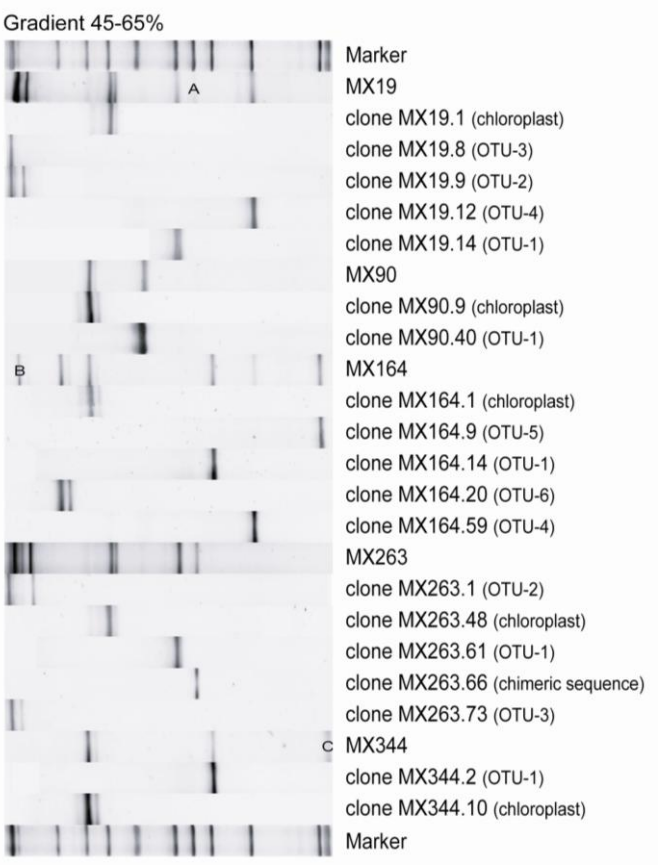


Figure 3.7: Normalized DGGE profiles of MX DNA extracts and their representative OTUs. DGGE bands marked with letters A, B and C, which did not match any of the individual OTU bands, were excised from the polyacrylamide gel and sequenced. The first and last lanes contain a known molecular marker [184] used for normalization.

Figure 3.8 depicts the endophytic diversity results from the clone libraries and DGGE analyses plotted on a phylogram representing the relations between the five *Bryopsis* samples. From Figure 3.8 we can deduce that Flavobacteriaceae (OTU-1), *Mycoplasma* (OTU-2), Bacteroidetes (OTU-3) and Phyllobacteriaceae (OTU-4) species were present in more than one *Bryopsis* sample examined. Even though the endobiotic community members were to a certain extent similar, the total diversity of the endophytic community was unique to each *Bryopsis* sample. None of the *Bryopsis* samples harbored the same number or range of bacterial endophytes.

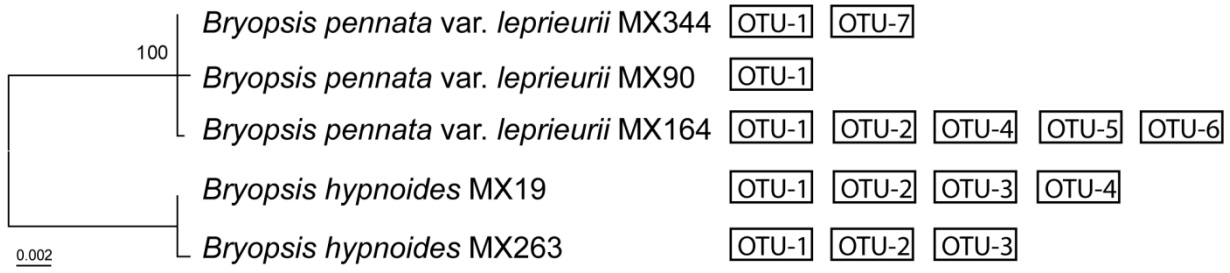
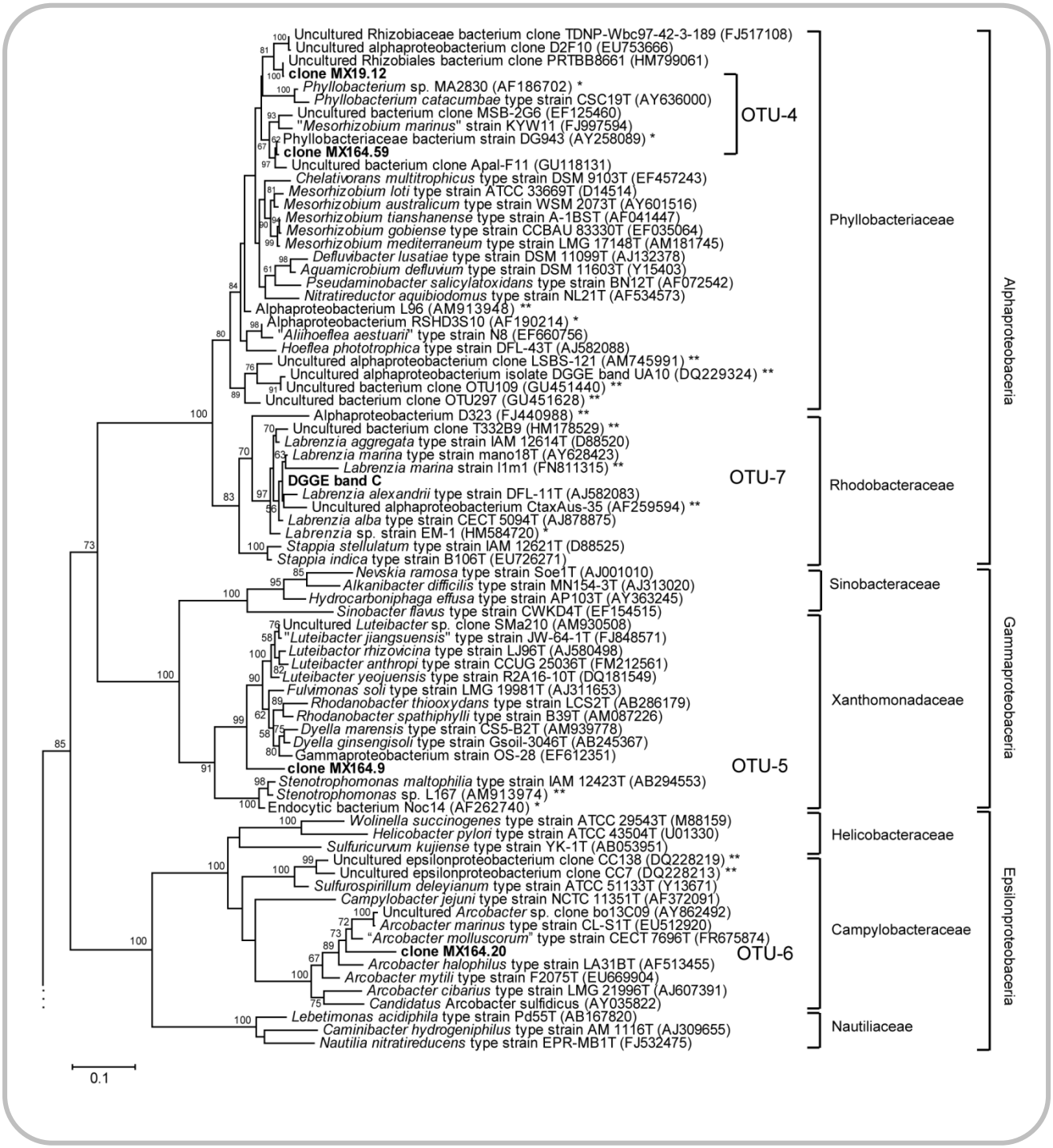


Figure 3.8: Endophytic diversity results (right) plotted against the *Bryopsis* host phylogeny (left). The OTU diversity (1-7) displayed on the right summarizes the diversity results from the clone libraries and DGGE analyses. The concatenated chloroplast 16S rRNA gene - *rbc*L maximum likelihood tree on the left classifies the *Bryopsis* MX samples in two distinct species clades with 100% bootstrap support. The scale bar indicates 0.002 nucleotide changes per nucleotide position.

Bacterial diversity within Bryopsis algae: *Phylogenetic analysis*

A wide-range phylogenetic tree (Figure 3.9) was created, which includes bacterial OTUs determined in this study (clones and DGGE bands), significant BLAST hits (Table 3.2), type strains from the Bacteroidetes, Proteobacteria and Tenericutes division, and algae-associated bacteria described in the literature (Supplementary Table S3.1, p. 65). As could be predicted from the BLAST maximum identity scores (Table 3.2), none of the endobiotic bacterial sequences clustered tightly with cultivated bacterial type strains. Consequently, all endophytic bacterial OTUs derived from *Bryopsis* represent new species or genera which in some cases match previously sequenced unclassified bacteria. These OTU sequences, however, all showed at least 93% sequence similarity with their best BLAST hit which generally resulted in phylogenetic placements with good bootstrap support. Accordingly, all OTU-1 sequences formed a distinct and well-supported (98%) clade within the Flavobacteriaceae family and most likely represent a new genus given their low sequence similarities (87% at most) with Flavobacteriaceae type strains. The similarity among the five OTU-1 sequences,

however, was 99.7%, suggesting all sequences belong to the same new Flavobacteriaceae genus even though they were derived from different *Bryopsis* samples collected several hundred kilometres apart. Likewise, the Bacteroidetes OTU-3 clones were virtually identical displaying 99.9% pairwise similarity. These OTU-3 clones, found in *B. hypnoides* samples MX19 and MX263, belong to a single clade (100% bootstrap support) of unclassified Bacteroidetes, but are distantly related to other unclassified Bacteroidetes symbionts. The OTU-2 clade, consisting of clones MX19.9 and MX263.1 and DGGE band B, fell into the genus *Mycoplasma* with 100% bootstrap support although these clones showed low levels of similarity (≤ 90%) with *Mycoplasma* type strains. All OTU-2 sequences presumably belong to one and the same new *Mycoplasma* species (99.7% intra-OTU sequence similarity). The majority of the endophytic bacterial OTUs, however, were affiliated with the Proteobacteria phylum and belonged to the Alpha-, Gamma- and Epsilonproteobacteria. Particularly, OTU-5 and 6, both consisting of clones exclusively obtained from *B. pennata* var. *leprieurii* sample MX164, most probably represent a new genus of Xanthomonadaceae and a new *Arcobacter* species, respectively. OTU-4 and 7 are robustly affiliated (100% bootstrap support) with the Alphaproteobacteria class and belong to the Rhizobiales and Rhodobacterales, respectively. Despite the high sequence similarity of OTU-7 with algal-associated *Labrenzia* species, relatedness of DGGE band C with the *Labrenzia alexandrii* type strain (AJ582083) and an uncultured *Labrenzia* bacterium isolated from *Caulerpa taxifolia* (AF259594) lacks bootstrap support. The shortness of the DGGE band C sequence (± 150 bp) and, consequently, the poor resolution within this clade, made it difficult to conclude whether OTU-7 represents a new *Labrenzia* species. Finally, OTU-4 is the only OTU containing clones derived from different *Bryopsis* samples in which the representative clones, i.e. clone MX19.12 and MX164.59, did not cluster together. This is in agreement with the 97% intra-OTU sequence similarity. Hence, both clones belong to the Phyllobacteriaceae clade with good bootstrap support (80%), but most likely represent two different new species or genera because of their low sequence similarities (96% at most) with Phyllobacteriaceae type strains.


Uncultured Rhizobiaceae bacterium clone TDNP-Wbc97-42-3-189 (FJ517108)
Uncultured alphaproteobacterium clone D2F10 (EU753666)
Uncultured Rhizobiales bacterium clone PRTBB8661 (HM799061)
clone MX19.12
Phyllobacterium sp. MA2830 (AF186702) *
Phyllobacterium catacumbae type strain CSC19T (AY636000)
Uncultured bacterium clone MSB-2G6 (EF125460)
"Mesorhizobium marinus" strain KYW11 (FJ997594)
Phyllobacteriaceae bacterium strain DG943 (AY258089) *
clone MX164.59
Uncultured bacterium clone Apal-F11 (GU118131)
Chelativorans multitrophicus type strain DSM 9103T (EF457243)
Mesorhizobium loti type strain ATCC 33669T (D14514)
Mesorhizobium australicum type strain WSM 2073T (AY601516)
Mesorhizobium tianshanense type strain A-1BST (AF041447)
Mesorhizobium gobiense type strain CCBAU 83330T (EF035064)
Mesorhizobium mediterraneum type strain LMG 17148T (AM181745)
Defluvibacter lusatiae type strain DSM 11099T (AJ132378)
Aquamicrobium defluvium type strain DSM 11603T (Y15403)
Pseudaminobacter salicylatoxidans type strain BN12T (AF072542)
Nitratireductor aquibiodomus type strain NL21T (AF534573)
Alphaproteobacterium L96 (AM913948) **
Alphaproteobacterium RSHD3S10 (AF190214) *
"Aliihoeflea aestuarii" type strain N8 (EF660756)
Hoeflea phototrophica type strain DFL-43T (AJ582088)
Uncultured alphaproteobacterium clone LSBS-121 (AM745991) **
Uncultured alphaproteobacterium isolate DGGE band UA10 (DQ229324) **
Uncultured bacterium clone OTU109 (GU451440) **
Uncultured bacterium clone OTU297 (GU451628) **
Alphaproteobacterium D323 (FJ440988) **
Uncultured bacterium clone T332B9 (HM178529) **
Labrenzia aggregata type strain IAM 12614T (D88520)
Labrenzia marina type strain mano18T (AY628423)
Labrenzia marina strain l1m1 (FN811315) **
DGGE band C
Labrenzia alexandrii type strain DFL-11T (AJ582083)
Uncultured alphaproteobacterium CtaxAus-35 (AF259594) **
Labrenzia alba type strain CECT 5094T (AJ878875)
Labrenzia sp. strain EM-1 (HM584720) *
Stappia stellulatum type strain IAM 12621T (D88525)
Stappia indica type strain B106T (EU726271)
Nevskia ramosa type strain Soe1T (AJ001010)
Alkanibacter difficilis type strain MN154-3T (AJ313020)
Hydrocarboniphaga effusa type strain AP103T (AY363245)
Sinobacter flavus type strain CWKD4T (EF154515)
Uncultured Luteibacter sp. clone SMa210 (AM930508)
"Luteibacter jiangsuensis" type strain JW-64-1T (FJ848571)
Luteibactor rhizovicina type strain LJ96T (AJ580498)
Luteibacter anthropi type strain CCUG 25036T (FM212561)
Luteibacter yeojuensis type strain R2A16-10T (DQ181549)
Fulvimonas soli type strain LMG 19981T (AJ311653)
Rhodanobacter thiooxydans type strain LCS2T (AB286179)
Rhodanobacter spathiphylli type strain B39T (AM087226)
Dyella marensis type strain CS5-B2T (AM939778)
Dyella ginsengisoli type strain Gsoil-3046T (AB245367)
Gammaproteobacterium strain OS-28 (EF612351)
clone MX164.9
Stenotrophomonas maltophilia type strain IAM 12423T (AB294553)
Stenotrophomonas sp. L167 (AM913974) **
Endocytic bacterium Noc14 (AF262740) *
Wolinella succinogenes type strain ATCC 29543T (M88159)
Helicobacter pylori type strain ATCC 43504T (U01330)
Sulfuricurvum kujiense type strain YK-1T (AB053951)
Uncultured epsilonproteobacterium clone CC138 (DQ228219) **
Uncultured epsilonproteobacterium clone CC7 (DQ228213) **
Sulfurospirillum deleyianum type strain ATCC 51133T (Y13671)
Campylobacter jejuni type strain NCTC 11351T (AF372091)
Uncultured Arcobacter sp. clone bo13C09 (AY862492)
Arcobacter marinus type strain CL-S1T (EU512920)
"Arcobacter molluscorum" type strain CECT 7696T (FR675874)
clone MX164.20
Arcobacter halophilus type strain LA31BT (AF513455)
Arcobacter mytili type strain F2075T (EU669904)
Arcobacter cibarius type strain LMG 21996T (AJ607391)
Candidatus Arcobacter sulfidicus (AY035822)
Lebetimonas acidiphila type strain Pd55T (AB167820)
Caminibacter hydrogeniphilus type strain AM 1116T (AJ309655)
Nautilia nitratireducens type strain EPR-MB1T (FJ532475)
OTU-4
OTU-7
OTU-5
OTU-6
Phyllobacteriaceae
Rhodobacteraceae
Sinobacteraceae
Xanthomonadaceae
Helicobacteraceae
Campylobacteraceae
Nautiliaceae
Alphaproteobacteria
Gammaproteobacteria
Epsilonproteobacteria
0.1

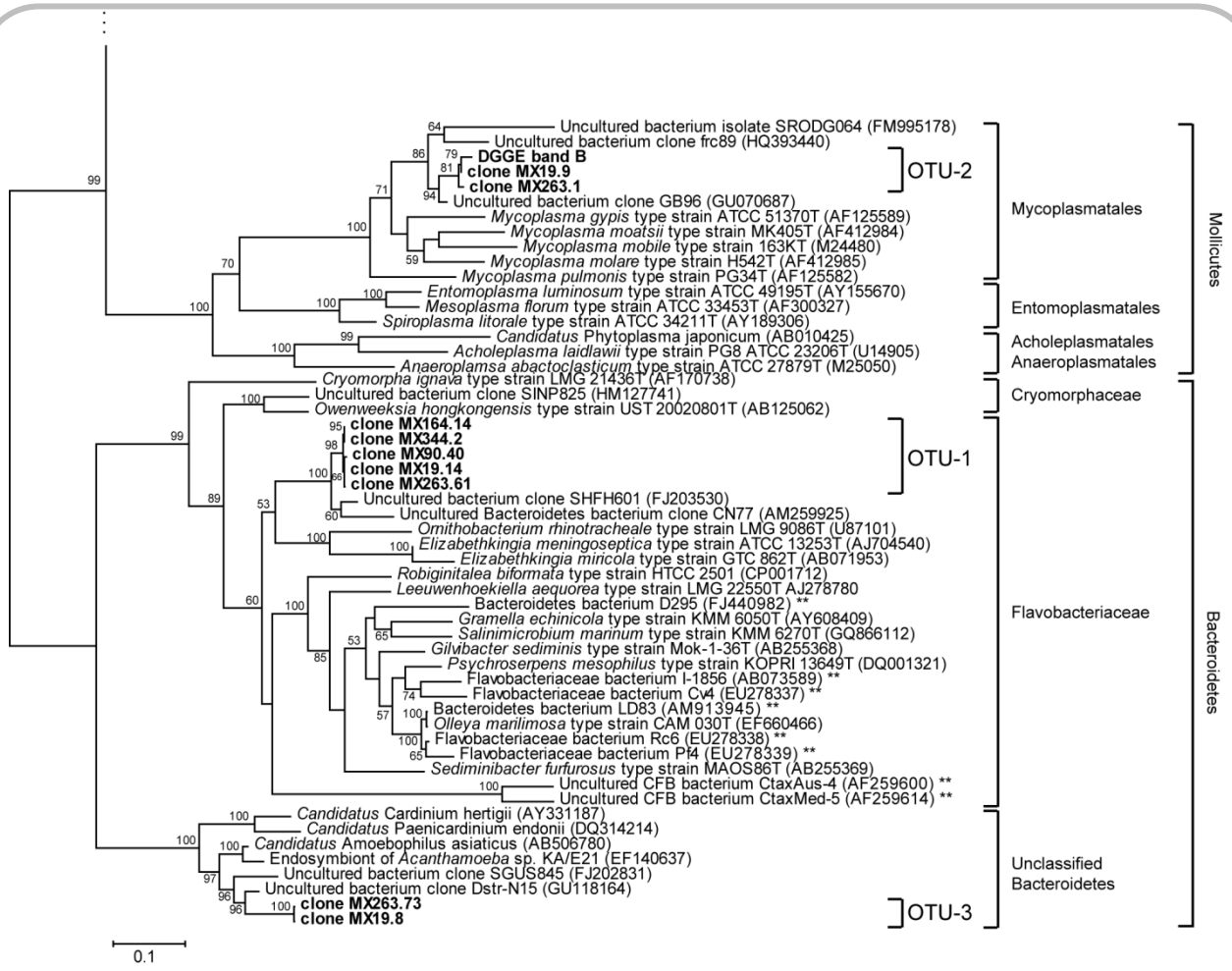


Figure 3.9: A wide-range maximum likelihood tree showing the phylogenetic positions of endophytic clones and DGGE bands. Phylogenies were inferred from 16S rRNA gene sequences determined in this study (in bold), BLAST hits (see Table 3.2), Bacteroidetes, Proteobacteria and Mollicutes type strains, and algae-associated bacteria described in the literature (see Supplementary Table S3.1, p. 65). The tree was generated in PhyML according the HKY + G4 algorithmic model. Bootstrap values above 50% are indicated at the branch nodes and the scale bar shows 10 nucleotide substitutions per 100 nucleotides. Asterisks denote sequences previously isolated from micro* and macroalgae**.

Discussion

Forty years after Burr and West [36] observed endogenous 'bacteria-like particles' in *Bryopsis hypnoides*, this is the first study to verify the presence of metabolically active endophytic bacteria inside the *Bryopsis* cytoplasm by means of the FISH technique. Mainly due to the intense background autofluorescence of algal cells, reports of successful FISH applications on macroalgae are limited to analyses of macroalgal surface-associated bacteria [168] and algal gall endosymbionts [123]. The use in this study of semi thin algal sections and a triple band filter, however, made it possible to discriminate bacterial FISH signals from autofluorescence of algal pigments using standard FISH protocols in combination with epifluorescence microscopy. Even though *Bryopsis* samples were not surface-sterilized prior to hybridization to avoid potential morphological losses, the solid embedding at the start of the FISH protocol proved successful in immobilizing the epiphytes on the *Bryopsis* surface (data not shown). This prevented the detachment and potential spread of surface bacteria during sectioning. Consequently, our FISH results strongly suggest the presence of bacteria within *Bryopsis* cells.

In this study, the first insights are provided into the identity and phylogenetic diversity of endobiotic bacterial communities within *Bryopsis*. Despite the limited number of samples studied, our results indicate that *Bryopsis* harbors endophytic bacterial communities which are not very complex (i.e. only 7 bacterial OTUs detected), but taxonomically diverse including *Arcobacter*, Bacteroidetes, Flavobacteriaceae, *Mycoplasma*, *Labrenzia*, Phyllobacteriaceae and Xanthomonadaceae members. Although the composition of the total endophytic community seems unique to each *Bryopsis* culture, Bacteroidetes, Flavobacteriaceae, *Mycoplasma* and Phyllobacteriaceae species were detected in two or more *Bryopsis* samples. In particular OTU-1 Flavobacteriaceae species are present in all five *Bryopsis* cultures, which were collected from diverse sites along the Mexican west coast. Delbridge and colleagues [124] made similar observations when comparing the endosymbiotic communities within four different *Caulerpa* species. While the endosymbiotic communities seemed unique to each *Caulerpa* individual, all community members were photosynthetic Alphaproteobacteria.

Also within *Bryopsis*, Alphaproteobacteria appear well represented. This is not unexpected, since Alphaproteobacteria are frequently associated with macroalgae [4, 125, 198], an alliance which may be linked to dimethylsulfoniopropionate (DMSP) exchange [59]. Particularly OTU-7, belonging to the marine phototrophic and CO-oxidizing *Labrenzia* genus [199, 200], is closely related to an uncultured bacterium reported by Meusnier *et al.* [125] in their study on the total bacterial community associated with *Caulerpa taxifolia*. Although *Labrenzia* species have not been reported as endophytes, the presence of Rhizobiales-specific proteins in *L. aggregata* [201] may hint at potential endosymbiotic

features. The Rhizobiales order contains various well-known nitrogen fixing plant symbionts, mainly in terrestrial habitats but also in marine environments [202]. Moreover, Rhizobiales bacteria are common epiphytes on green [59, 203], brown [58, 93] and red [203] macroalgae; and a *Rhodopseudomonas* species with the potential to fix nitrogen was isolated from the inside of *C. taxifolia* [75]. Also within *Bryopsis*, Rhizobiales species seem to be well established as clones MX19.12 and MX164.59 (OTU-4) likely represent two different new Phyllobacteriaceae species or genera clustering together with, respectively, a free-living marine Phyllobacteriaceae bacterium [204] and a dinoflagellate-associated anoxygenic photosynthetic bacterial strain [205]. In addition, we amplified a Phyllobacteriaceae nitrogenase-like light-independent protochlorophyllide reductase gene (submitted to GenBank under accession number JN048464) from *Bryopsis* sample MX164 by the *nif*H protocol described by De Meyer *et al.* [170], supporting the above suggested relatedness of OTU-4 to photosynthetic bacteria.

Besides the presence of Alphaproteobacteria in three of the five *Bryopsis* cultures studied, endophytes from the Gamma- and Epsilonproteobacteria order seem restricted to a single *Bryopsis* sample. The latter endophytes (OTU-6) most likely belong to a new *Arcobacter* species within the Campylobacteraceae family. *Arcobacter* species are mainly known as potential human and animal pathogens, but have also been isolated from diverse marine environments including seawater surrounding seaweeds [206, 207]. Despite their ecologically significant functions like nitrogen fixation, denitrification, sulfide oxidation and manganese reduction [206, 208], they are not frequently reported as endobionts [209, 210]. On the other hand, members of the Xanthomonadaceae family to which OTU-5 belongs, are well-known plant endophytes [211] and have previously been isolated from marine algae [93, 212]. Since many Xanthomonadaceae species cause plant diseases, the high number of Xanthomonadaceae endophytes within *Bryopsis* MX164 could be a sign of infection. The alga, however, showed no visible disease symptoms (e.g. bleaching), indicating a neutral or beneficial relationship.

In the Bacteroidetes group, we found two distinct clusters (i.e. OTU-1 and OTU-3) of endophytic bacteria, one within the Flavobacteriaceae family and one belonging to unclassified Bacteroidetes. The Flavobacteriaceae endophytes (OTU-1) show an especially strong association with *Bryopsis* as evidenced by their occurrence in all five samples. The phylum Bacteroidetes, and in particular the family Flavobacteriaceae, forms one of the major components of marine bacterioplankton and mediates a substantial proportion of the carbon flow and nutrient turnover in the sea during and following algal blooms [213]. Moreover, many novel Bacteroidetes members, some of which were

characterized as morphogenesis inducers [81], have been isolated from the surfaces of marine macroalgae [4]. Whereas Bacteroidetes bacteria are obviously common epiphytes on macroalgae, Meusnier and co-workers [125] suggested the existence of an endophytic Cytophaga-Flavobacteria-Bacteroidetes (CFB) bacterium within *Caulerpa taxifolia*. In addition, Bacteroidetes bacteria are well-known endosymbionts of amoebae, plant-parasitic nematodes and insects [214-216]. Phylogenetic analysis, however, revealed that the Bacteroidetes endophytes of *Bryopsis* are more closely related to bacteria tightly associated with corals and sponges [217-219] than to CFB sequences isolated from green [81, 125], brown [93] and red [29, 220] macroalgae.

Finally, three *Bryopsis* samples (i.e. MX19, 164 and 263) contained *Mycoplasma* sequences (OTU-2). Mycoplasmas are well-known human and animal parasites, but are also common members of the intestinal bacterial flora of fishes and abalones where they may provide nutrients to their hosts [210, 221, 222]. Moreover, the close affiliation of *Mycoplasma* sequences isolated from *Bryopsis* and abalone species is perhaps not at all surprising as the latter generally feeds on a broad selection of algae [223]. Also Huang and colleagues [221] postulated that the presence of *Mycoplasma* species in the intestinal microflora of the abalone *Haliotis diversicolor* could be algal-food related. Additionally, this bacterial link between *Bryopsis* and abalone species might be extrapolated to other marine gastropod mollusks, supporting the hypothesis of Rao *et al.* [186] that the production of therapeutic kahalalides by the sea slug *Elysia rufescens* as well as by its *Bryopsis* food could actually be performed through an associated microorganism. Indeed, it has been shown that several metabolites initially assigned to eukaryotes are in fact of microbial origin [4].

In summary, molecular analysis revealed, for the first time, that *Bryopsis* harbors relatively restricted but taxonomically diverse communities of endophytic bacteria. The presence of Phyllobacteriaceae, Bacteroidetes, *Mycoplasma*, and in particular Flavobacteriaceae endophytes in several *Bryopsis* samples collected hundreds of kilometres apart indicates a close association between these endophytes and *Bryopsis* plants. Even though these endophytic bacterial communities within *Bryopsis* cultures might not fully represent those that are present within the alga in its natural environment, the bacteria identified in this study are at least part of the natural *Bryopsis* endobiotic flora. Future investigations of *Bryopsis* algae in natural environments, however, are necessary to complete the *Bryopsis*-bacterial endobiosis picture.

Acknowledgments

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Supplementary Table S3.1: Bacterial 16S rRNA gene sequences isolated from algae (excluding BLAST hits) included in the phylogenetic analysis.

	Taxon label	Accession no.	Isolation source	Reference
Bacteroides	Bacteroidetes bacterium D295	FJ440982	*Delisea pulchra*	[29]
	Bacteroidetes bacterium LD83	AM913945	*Saccharina latissima*	[93]
	Flavobacteriaceae bacterium Cv4	EU278337	*Ceramium virgatum*	[220]
	Flavobacteriaceae bacterium I-1856	AB073589	Green macroalga	[81]
	Flavobacteriaceae bacterium Pf4	EU278339	*Polysiphonia fucoides*	[220]
	Flavobacteriaceae bacterium Rc6	EU278338	*Rhodomela confervoides*	[220]
	Uncultured CFB bacterium CtaxAus-4	AF259600	*Caulerpa taxifolia*	[125]
	Uncultured CFB bacterium CtaxMed-5	AF259614	*Caulerpa taxifolia*	[125]
Proteobacteria	Alphaproteobacterium D323	FJ440988	*Delisea pulchra*	[29]
	Alphaproteobacterium L96	AM913948	*Saccharina latissima*	[93]
	Alphaproteobacterium RSHD3S10	AF190214	*Pfiesteria*-like dinoflagellate	[224]
	Phyllobacterium sp. MA2830	AF186702	*Pfiesteria*-like dinoflagellate	[224]
	Uncultured Alphaproteobacterium clone LSBS121	AM745991	*Saccharina latissima*	[58]
	Uncultured Alphaproteobacterium isolate DGGE band UA10	DQ229324	*Ulva australis*	[59]
	Uncultured bacterium clone OTU109	GU451440	*Gracilaria vermiculophylla*	[203]
	Uncultured bacterium clone OTU297	GU451628	*Ulva intestinalis*	[203]
	Uncultured Alphaproteobacterium CtaxAus-35	AF259594	*Caulerpa taxifolia*	[125]
	Endocytic bacterium Noc14	AF262740	*Noctiluca scintillans*	[212]
	Stenotrophomonas sp. L167	AM913974	*Saccharina latissima*	[93]
	Uncultured Epsilonproteobacterium clone CC7	DQ228213	*Cladophora* mats	[139]
	Uncultured Epsilonproteobacterium clone CC38	DQ228219	*Cladophora* mats	[139]

3.2.2. Uniqueness, temporal stability and symbiotic nature of *Bryopsis* endophytic bacterial communities

Modified from: Joke Hollants, Helen Decleyre, Frederik Leliaert, Olivier De Clerck and Anne Willems. (2011) Life without a cell membrane: challenging the specificity of bacterial endophytes within *Bryopsis* (Bryopsidales, Chlorophyta). *BMC Microbiology* **11**: e255. **Author contributions**: JH designed the experiments, analyzed the data and wrote the paper. JH and HD performed the experiments. FL maintained the algal cultures. FL, ODC and AW commented on the manuscript.

Abstract

The siphonous green macroalga *Bryopsis* has some remarkable characteristics. Besides hosting a rich endophytic bacterial flora, *Bryopsis* also displays extraordinary wound repair and propagation mechanisms. This latter feature includes the formation of protoplasts which can survive in the absence of a cell membrane for several minutes before regenerating into new individuals. This transient 'life without a membrane' state, however, challenges the specificity of the endophytic bacterial communities present and raises the question whether these bacteria are generalists, which are repeatedly acquired from the environment, or if there is some specificity towards the *Bryopsis* host. To answer this question, we examined the temporal stability and the uniqueness of endobiotic bacterial communities within *Bryopsis* samples from the Mexican west coast after prolonged cultivation. DGGE analysis revealed that *Bryopsis* endophytic bacterial communities are rather stable and clearly distinct from the epiphytic and surrounding cultivation water bacterial communities. Although these endogenous communities consist of both facultative and obligate bacteria, results suggest that *Bryopsis* owns some intrinsic mechanisms to selectively maintain and/or attract specific bacteria after repeated wounding events in culture. This suggests that *Bryopsis* algae seem to master transient stages of life without a cell membrane well as they harbor specific – and possibly ecological significant – endophytic bacteria.

Introduction

The marine green alga *Bryopsis* has long been suspected to harbor endogenous bacteria. These intracellular bacteria have been repeatedly observed in the cytoplasm as well as vacuolar regions of algal thalli and gametes by electron microscopy [36, 37] (and personal observations see Supplementary Figure S3.1, p. 81), suggesting the presence of bacterial endophytes within *Bryopsis* is a natural phenomenon. Recently, the first insights were provided into the identity and diversity of these bacterial endophytes within two *Bryopsis* species from the Pacific Mexican coast (see section 3.2.1 [225]). Full length 16S rRNA gene analysis showed that the *Bryopsis* endophytic bacterial communities are quite low in diversity (i.e. only 7 bacterial OTUs detected) but taxonomically wide-ranging with the presence of *Arcobacter*, Bacteroidetes, Flavobacteriaceae, *Mycoplasma*, *Labrenzia*, Phyllobacteriaceae and Xanthomonadaceae species. Moreover, the same Bacteroidetes, *Mycoplasma,* Phyllobacteriaceae, and in particular Flavobacteriaceae bacteria, were detected in several *Bryopsis* samples collected hundreds of kilometres apart. This apparent spatial stability of the *Bryopsis*-bacterial endobiosis, however, raises the question whether these endophytes are a subset of the free-living bacterial community or whether there is some specificity towards the *Bryopsis* host. Although the distinctiveness between free-living and macroalgal-associated bacterial communities is well established [4, 13, 125, 203, 226], the extraordinary morphological and physiological characteristics of the *Bryopsis* host must have implications for the specificity of its bacterial endophytes. *Bryopsis* is a marine siphonous macroalga composed of a single, tubular shaped cell which contains multiple nuclei and chloroplasts in a thin cytoplasmic layer surrounding a large central vacuole [102]. While an organism composed of a giant, single cell would be prone to damage, siphonous macroalgae possess an intricate defense network that operates at various levels [4, 111]. In *Bryopsis*, for example, the metabolite kahalalide F, which shows *in vitro* therapeutic activities, protects the alga from fish predation [110]. Even if damage does occur, a complex, multistep wound response is triggered [111, 112] to which *Bryopsis* algae add a surprisingly feature, i.e. the formation of protoplasts [113]. These protoplasts are membraneless structures that can survive in seawater for 10–20 minutes. Subsequently, membranes and a cell wall are synthesized *de novo* surrounding each protoplast, which then develop into new *Bryopsis* plants. This not only suggests *Bryopsis* can exist – at least transiently – without a cell membrane, it also questions the nature of the association between the algal host and the endophytic bacterial communities present. Are these bacteria *Bryopsis*-specific, obligate endophytes (specialists) or are they rather generalists (facultative endogenous bacteria) which are repeatedly acquired from the local environment (epiphytic communities and/or surrounding sea water)?

To address this issue, we evaluated the temporal stability of the endobiotic bacterial communities after prolonged cultivation of *Bryopsis* isolates. We also examined the diversity of the epiphytic and surrounding water bacterial communities of five *Bryopsis* isolates in culture using the DGGE technique and subsequently compared these DGGE profiles with previously obtained DGGE banding patterns of *Bryopsis* endophytic bacterial communities (see section 3.2.1 [225]).

Materials and methods

Sample collection and DNA extraction

Bryopsis hypnoides (MX19 and MX263) and *Bryopsis pennata* var. *leprieurii* individuals (MX90, MX164 and MX344) were collected in February 2009 at five different sites along the Mexican west coast (see Fig. 3.5, section 3.2.1 [225]). Living algal samples were transferred to the laboratory and unialgal *Bryopsis* cultures were formed by repeatedly isolating clean apical fragments. To preserve these unialgal cultures, apical fragments were monthly transferred to fresh sterile 1x modified Provasoli enriched seawater [181]. All unialgal *Bryopsis* cultures were maintained in the laboratory at 23°C under a 12:12 hours Light:Dark cycle with light intensities of 25-30 μmol m^{-2} s^{-1}.

One year after the first endophytic community screening (see section 3.2.1 [225]), all five *Bryopsis* MX samples were resubmitted to a total surface sterilization (section 3.1.2 [187]) and DNA extraction [160] in October 2010 to evaluate the temporal stability of the endophytic bacterial communities after prolonged cultivation. To address the specificity of the *Bryopsis*-bacterial endobiosis in culture, 50 ml of 30 day old cultivation water was collected from each *Bryopsis* MX culture that had been cultivated for two years (i.e. in February 2011). These cultivation water samples were serially filtered over a syringe filter holder with sterile 11 μm and 0.2 μm cellulose acetate filters (Sartorius Stedim Biotech GmbH, Germany) to remove small *Bryopsis* fragments and to retain the planktonic microbial fraction, respectively. Bacterial DNA was extracted from the 0.2 μm filters using the bead-beating method followed by phenol extraction and ethanol precipitation as described by Zwart *et al.* [161]. Parallel with these cultivation water samples, washing water samples were obtained from all five MX isolates by repeatedly vortexing the algae in 50 ml sterile artificial seawater (ASW). These washing water samples, containing the loosely *Bryopsis*-associated bacterial fraction, were processed as described above. Subsequently, approximately 1 gram of each washed *Bryopsis* MX sample was placed in 500 μl cetyltrimethylammonium bromide (CTAB) lysis buffer supplemented with 20 $mg.ml^{-1}$ proteinase K and 2.5 μl filter-sterilized Umonium Master (Huckert's International,

Belgium) to eliminate the epiphytic bacterial fraction from the *Bryopsis* surface (see section 3.1.2 [187]). Samples were incubated for 30 min at 60°C and subsequently vortexed in 500 µl sterile ASW for 2 min. Algal material was removed by centrifugation and the DNA of the supernatants originated from the epiphytic bacterial fraction was extracted using a CTAB protocol modified from Doyle and Doyle [160].

DGGE and sequence analysis

The endophytic (EN-2010), epiphytic (EP), washing water (WW) and cultivation water (CW) bacterial community extracts were subjected to a nested-PCR DGGE approach. First, full length 16S rRNA gene amplification was carried out with the universal bacterial primers 27F/1492R following the protocol outlined in Lane [164]. PCR amplicons were purified using a Nucleofast 96 PCR clean up membrane system (Machery-Nagel, Germany) according to the manufacturer's instructions and subsequently submitted to a second PCR with primer pair F357-GC/R518 targeting the V3 region of the 16S rRNA gene. The latter amplification reaction and subsequent DGGE analysis were carried out as described in section 3.1.2 [187], with a denaturing gradient of 45-65%. DGGE banding patterns were normalized using BioNumerics 5.1 software (Applied Maths, Belgium). As standard, a marker containing the V3 16S rRNA gene fragments of all bacterial endophyte and chloroplast OTUs formerly obtained from the five *Bryopsis* MX samples (see section 3.2.1 [225]) was used (Supplementary Figure S3.2, p. 81). The temporal stability of the endophytic communities was explored by visually comparing the normalized endophytic community profiles of the DNA extracts of the MX samples made in October 2009 (EN-2009) versus October 2010 (EN-2010). To study the specificity of the *Bryopsis*-bacterial endobiosis, normalized EP, WW and CW bacterial community profiles of each *Bryopsis* sample were comparatively clustered with previously obtained endophytic (EN-2009) DGGE banding patterns (see section 3.2.1 [225]) using Dice similarity coefficients. A dendrogram was composed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm in BioNumerics to determine the similarity between the EP, WW, CW and EN-2009 samples. The similarity matrix generated was also used for constructing a multi-dimensional scaling (MDS) diagram in BioNumerics. MDS is a powerful data reducing method which reduces each complex DGGE fingerprint into one point in a 3D space in a way that more similar samples are plotted closer together [227]. Additionally, EP, WW and CW DGGE bands at positions of endophytic (including chloroplast) marker bands were excised, sequenced and identified as described in section 3.2.1 [225]. To verify their true correspondence with *Bryopsis* endophytes, the sequences of the excised bands were aligned and clustered with previously obtained (see section 3.2.1

[225]) endophytic bacterial sequences using BioNumerics. The V3 16S rRNA gene sequences of the excised DGGE bands were submitted to EMBL under accession numbers HE599189-HE599213.

Results

Temporal stability of endophytic bacterial communities after prolonged cultivation

The endophytic bacterial communities showed little time variability after prolonged cultivation when visually comparing the normalized EN-2009 and EN-2010 DGGE fingerprints (Fig. 3.10). The band patterns of the different MX90, MX263 and MX344 endophytic extracts were highly similar, whereas *Bryopsis* samples MX19 and 164 showed visible differences between the community profiles of their EN-2009 and EN-2010 DNA extracts. Both the MX19 and MX164 sample had lost the DGGE band representing the Phyllobacteriaceae endophytes (black boxes in Fig. 3.10) after one year of cultivation.

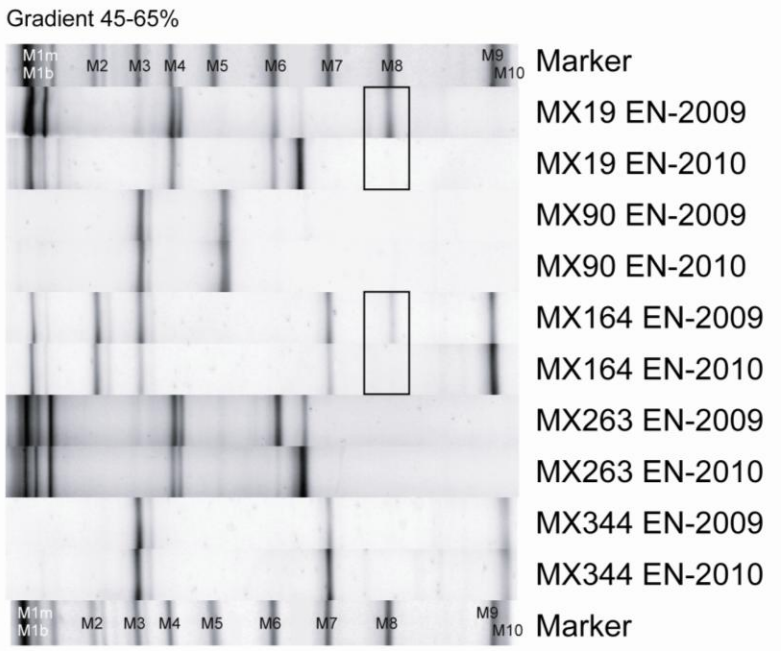


Figure 3.10: **Visual comparison of normalized endophytic DGGE fingerprints obtained from surface sterilized *Bryopsis* DNA extracts made in October 2009 (EN-2009) versus October 2010 (EN-2010).** Differences are indicated with black boxes. The first and last lanes contain a molecular marker of which the bands correspond to known *Bryopsis* endophyte or chloroplast sequences (Supplementary Figure S3.1, p. 81). This marker was used as a normalization and identification tool.

DGGE fingerprint cluster analysis: inside ≠ outside

DGGE cluster analysis showed that the endophytic (EN) banding patterns were significantly different from the epiphytic (EP), washing water (WW) and cultivation water (CW) community profiles of all five MX *Bryopsis* cultures studied. In the dendrogram (Fig. 3.11), the cluster containing the EP, WW and CW community profiles is clearly separated from the endophytic banding patterns (indicated in bold, Fig. 3.11). Also the multidimensional scaling (MDS) plot (Fig. 3.12A), which reduces the complex DGGE patterns to one point per sample, shows that the EN samples (right) are clearly apart from the epiphytic and surrounding water samples (left). Besides this, the MDS diagram showed that the EN samples did not cluster together and are distributed over the y-axis of the three-dimensional plot (Fig. 3.12A), while the EP, WW and CW samples were more or less grouped per *Bryopsis* MX sample (Fig. 3.12B). Within one *Bryopsis* sample EP-WW-CW cluster (clusters 1-5, Fig. 3.12B), however, no general grouping mode can be observed. Whereas the epiphytic community samples within clusters 2, 3 and 4 (representing *Bryopsis* samples MX90, MX164 and MX263) were more apart from their corresponding WW and CW samples, this was not the case for clusters 1 and 5 (i.e. *Bryopsis* cultures MX19 and MX344). These observations corresponded to the results of the cluster analysis of all DGGE patterns (Fig. 3.11). In addition, Figure 3.11 also shows a much larger diversity of DGGE bands in all epiphytic and surrounding water samples in comparison with the endophytic DGGE profiles.

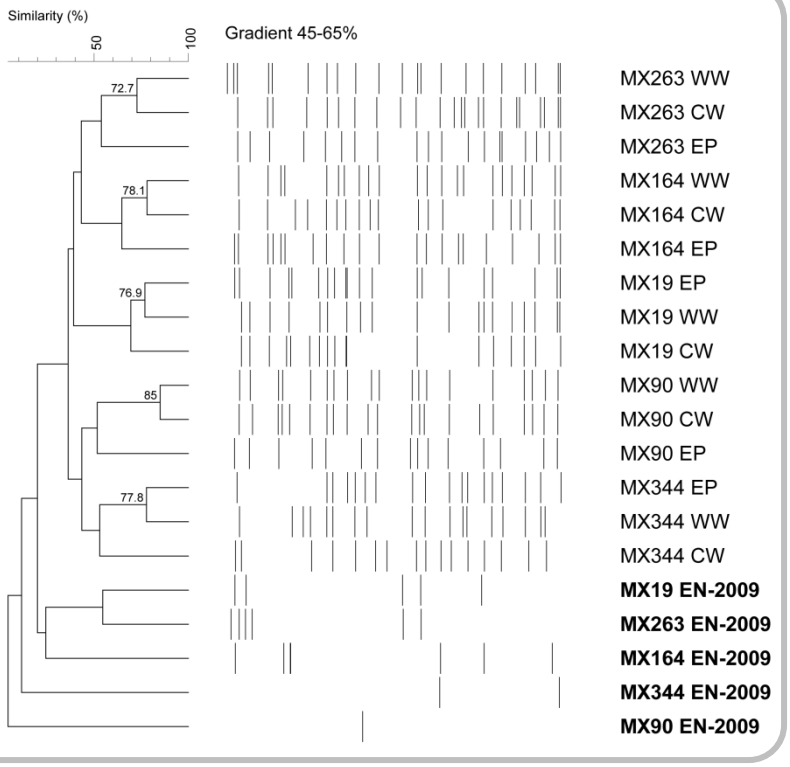


Figure 3.11: **UPGMA dendrogam showing the similarities (≥ 70%) among the endophytic (EN-2009), epiphytic (EP), washing water (WW) and cultivation water (CW) normalized DGGE fingerprints.** Cluster analysis was performed in BioNumerics using the band based Dice similarity coefficient with an optimization of 0.84% and a position tolerance of 0.48%. DGGE bands in the EN-2009 profiles identified as algal chloroplasts were excluded from the analysis. DGGE band patterns are graphically represented and similarity values above 70% are indicated above the branches.

Figure 3.12: **Three-dimensional MDS plot seen from dimension X and Y (A) and Y and Z (B) visualizing the similarities among the endophytic (EN-2009), epiphytic (EP), washing water (WW) and cultivation water (CW) DGGE fingerprints.** The MDS plot was derived from the similarity matrix generated during the DGGE cluster analysis (Fig. 3.11). Clusters 1 till 5 (B) surround the EP, WW and CW fingerprints (reduced into one point in the plot) of *Bryopsis* samples MX19, MX90, MX164, MX263 and MX344, respectively.

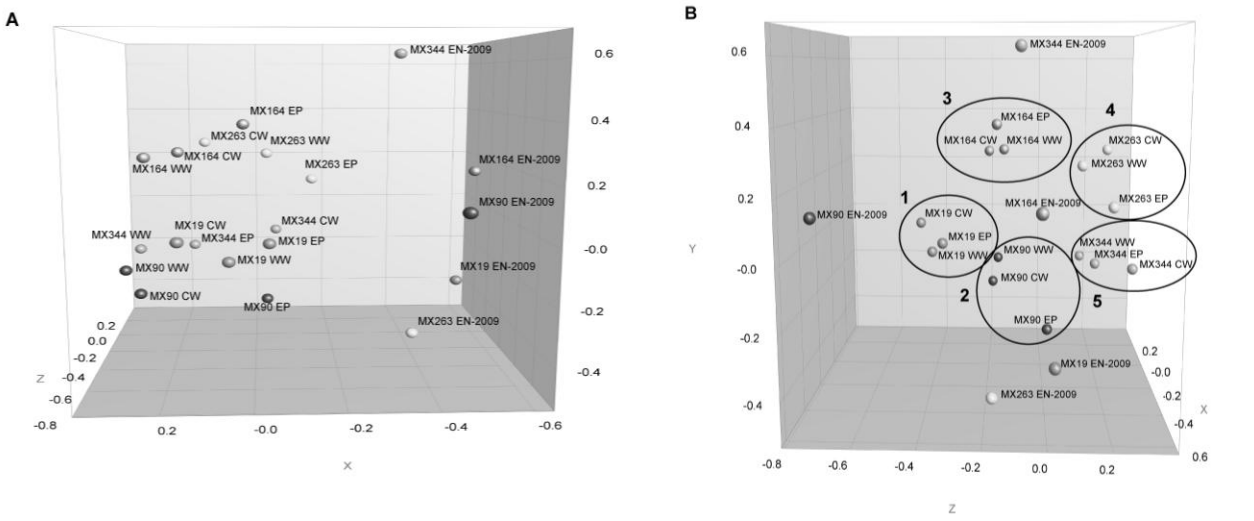
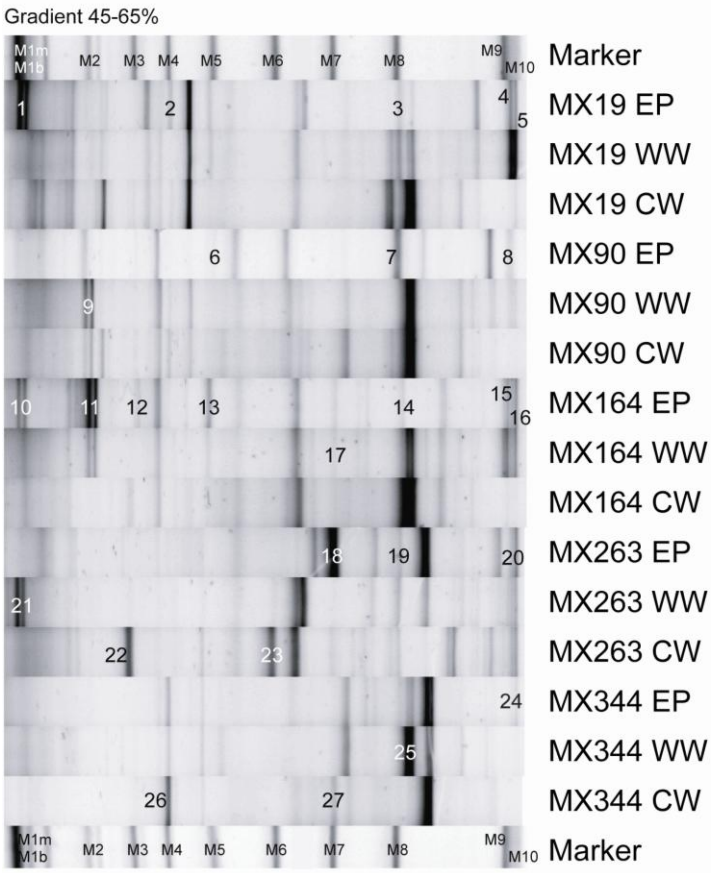


Figure 3.13: **Normalized epiphytic (EP), washing water (WW) and cultivation water (CW) DGGE fingerprints obtained from *Bryopsis* samples MX19, MX90, MX164, MX263 and MX344.** Numbers (1-27) indicate which bands were sequenced, and correspond to band numbers in Table 3.3 and Fig. 3.14. The first and last lanes contain a molecular marker of which each band (M1m, M1b, M2-M10) corresponds to a known *Bryopsis* endophyte or chloroplast sequence (see Supplementary Figure S3.2, p. 81). This marker was used as a normalization and identification tool.



DGGE band cluster analysis: inside ≈ outside

Although the community fingerprints of all EP, WW and CW samples were distinct from the EN community profiles, some overlap was noticeable between individual bands from the EP, WW and CW DGGE profiles and the EN (including chloroplast) marker bands. To examine this potential overlap, EP, WW and CW DGGE bands at positions of marker bands (Fig. 3.13, bands 1-27) were excised from the polyacrylamide gels and sequenced. Table 3.3 outlines the taxonomic identification and phylogenetic affiliation of the excised bands. The last column in Table 3.3 shows the correlation (positive$^+$ or negative$^-$) between the position of a certain EP, WW or CW DGGE band towards the marker bands and its sequence identification. From this column we can deduce that most bands at positions of marker bands M1m, M2, M8 and M10 showed sequences that matched those of the marker bands and were thus identified as *Mycoplasma*, *Arcobacter*, Phyllobacteriaceae and *Labrenzia* species, respectively. All EP, WW or CW bands at the height of Bacteroidetes (M1b), chloroplast (M3 and M4), Flavobacteriaceae (M5-7) and Xanthomonadaceae (M9) marker bands, however, showed a mismatch. Instead of being related to *Bryopsis* endophytic bacterial sequences, these latter band sequences were affiliated with Alphaproteobacterial (Caulobacterales, Rhizobiales and Sneathiellales), Gammaproteobacterial (Alteromonadales and Oceanospirillales) and Acanthopleuribacterales sequences (see Table 3.3). To validate the true correspondence of excised EP, WW and CW bands with endophytic sequences, band sequences were clustered with previously obtained endophytic bacterial full length 16S rRNA gene sequences (see section 3.2.1 [225]). The UPGMA dendrogram (Fig. 3.14) confirms that every one of the positively related bands (indicated with $^+$) was highly similar (≥ 99.2%) to endogenous sequences (indicated in bold). This dendrogram illustrates that *Arcobacter*, *Labrenzia, Mycoplasma* and Phyllobacteriaceae endogenous sequences are also present in the epiphytic, washing water and/or cultivation water bacterial communities of *Bryopsis* cultures, whereas Bacteroidetes, Flavobacteriaceae and Xanthomonadaceae sequences were strictly endogenous. In addition, *Arcobacter* and *Mycoplasma* sequences were only present in the EP, WW and/or CW bacterial communities of those *Bryopsis* MX samples in which they were also endogenously present. *Labrenzia* and Phyllobacteriaceae sequences, on the other hand, were also found in the EP, WW and/or CW bacterial communities of algal samples in which these species were not identified as being endophytic.

Table 3.3: **Taxonomic identification and phylogenetic affiliation of the excised and sequenced epiphytic (EP), washing water (WW) and cultivation water (CW) DGGE bands.**

DGGE band number	Closest matching strain in BLAST (accession number) Query coverage/Maximum identity	Phylogenetic affiliation	Correlation
MX19 EP 1	Uncultured *Mycoplasma* sp. clone MX19.9 (JF521606) 100/100	Tenericutes; Mollicutes; Mycoplasmatales; Mycoplasmataceae	M1m + M1b -
MX19 EP 2	Uncultured bacterium clone Del10081H12 (JF262029) 100/100	Proteobacteria; Alphaproteobacteria; Caulobacterales; Hyphomonadaceae	M4 -
MX19 EP 3	Uncultured Phyllobacteriaceae bacterium clone MX19.12 (JF521607) 100/100	Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	M8 +
MX19 EP 4	Uncultured bacterium isolate TTGE gel band N68 (JN185170) 100/100	Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae	M9 -
MX19 EP 5	Uncultured *Labrenzia* sp. clone DGGE band C (HE599215) 100/100	Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	M10 +
MX90 EP 6	Uncultured bacterium clone CD02003D03 (HM768522) 100/96	Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae	M5 -
MX90 EP 7	Uncultured Phyllobacteriaceae bacterium clone MX19.12 (JF521607) 100/100	Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	M8 +
MX90 EP 8	Uncultured alphaproteobacterium clone TH_d327 (EU272970) 100/98	Proteobacteria; Alphaproteobacteria; Rhizobiales, Hyphomicrobiaceae	M9 -
MX90 WW 9	Uncultured bacterium clone OTU017 (GU174663) 100/100	Proteobacteria; Alphaproteobacteria; Rhizobiales; Bartonellaceae	M2 -
MX164 EP 10	Uncultured *Mycoplasma* sp. clone MX19.9 (JF521606) 100/96	Tenericutes; Mollicutes; Mycoplasmatales; Mycoplasmataceae	M1m + M1b -
MX164 EP 11	Uncultured *Arcobacter* sp. clone MX164.20 (JF521610) 100/100	Proteobacteria; Epsilonproteobacteria; Campylobacterales; Campylobacteraceae	M2 +
MX164 EP 12	Uncultured proteobacterium clone Marsh_0_33 (JF980756) 100/100	Proteobacteria; Alphaproteobacteria; Caulobacterales; Hyphomonadaceae	M3 -
MX164 EP 13	*Acanthopleuribacter pedis* type strain NBRC 101209 (AB303221) 100/93	Acidobacteria; Holophagae; Acanthopleuribacterales	M5 -
MX164 EP 14	Hyphomicrobiaceae bacterium WPS10 (HQ638980) 100/98	Proteobacteria; Alphaproteobacteria; Rhizobiales; Bartonellaceae	M8 -
MX164 EP 15	Uncultured bacterium clone I3A_12H (EU352599) 100/98	Proteobacteria; Alphaproteobacteria; Rhizobiales; Methylobacteriaceae	M9 -

DGGE band number	Closest matching strain in BLAST (accession number) Query coverage/Maximum identity	Phylogenetic affiliation	Correlation
MX164 EP 16	*Stappia* sp. enrichment culture clone NKiNSO2 (EU983274) 100/95	Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	M10 -
MX164 WW 17	Uncultured *Sneathiella* sp. clone w-G7 (HQ727092) 100/97	Proteobacteria; Alphaproteobacteria; Sneathiellales; Sneathiellaceae	M7 -
MX263 EP 18	*Thalassomonas* sp. UST061013-012 (EF587959) 100/100	Proteobacteria; Gammaproteobacteria; Alteromonadales; Colwelliaceae	M7 -
MX263 EP 19	Uncultured Phyllobacteriaceae bacterium clone MX19.12 (JF521607) 100/100	Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	M8 +
MX263 EP 20	Uncultured *Labrenzia* sp. clone DGGE band C (HE599215) 100/100	Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	M10 +
MX263 WW 21	Uncultured *Mycoplasma* sp. clone MX263.1 (JF521605) 100/100	Tenericutes; Mollicutes; Mycoplasmatales; Mycoplasmataceae	M1m + M1b -
MX263 CW 22	Uncultured bacterium isolate DGGE gel band B12 (HQ875697) 100/93	Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae	M3 -
MX263 CW 23	*Alcanivorax dieselolei* strain PM07 (HM596594) 100/100	Proteobacteria; Gammaproteobacteria; Oceanospirillales; Alcanivoracaceae	M6 -
MX344 EP 24	Uncultured *Labrenzia* sp. clone DGGE band C (HE599215) 100/100	Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	M10 +
MX344 WW 25	*Ruegeria mobilis* strain F4122 (HQ338148) 100/99	Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	M8 -
MX344 CW 26	Uncultured bacterium clone EMar8 (FR667032) 100/94	Proteobacteria; Gammaproteobacteria; Alteromonadales	M4 -
MX344 CW 27	Uncultured bacterium clone W2-97 (HQ322761) 100/90	Proteobacteria; Alphaproteobacteria	M7 -

The band numbers correspond to the numbers (1-27) in Fig 3.13. The last column shows the correlation (positive $^{+}$ or negative $^{-}$) between the identification of a band and the sequence information of the marker band (M1m, M1b, M2-M10) at the same position.

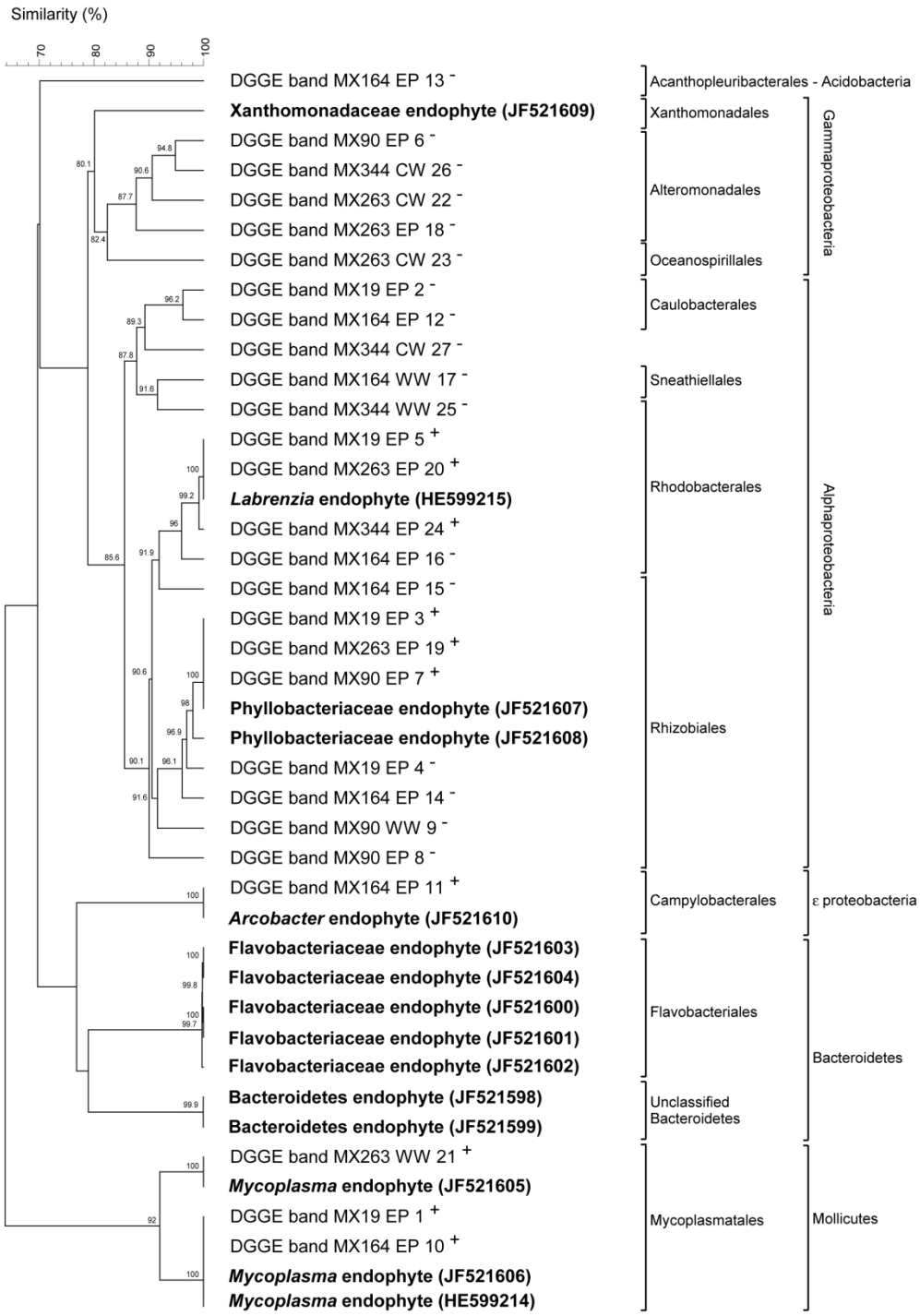


Figure 3.14: **UPGMA dendrogram showing the sequence similarities among the excised DGGE bands (numbers 1-27 in Fig. 3.13) V3 16S rRNA gene sequences and previously obtained (see section 3.2.1 [225]) endophytic bacterial full length 16S rRNA gene sequences (indicated in bold).** Cluster analysis was performed in BioNumerics. Similarity values above 80% are given above the branches. The positive or negative correlation between the sequence identification of a certain excised DGGE band and its position towards the marker bands (see Table 3.3), is indicated with $^{+}$ or $^{-}$, respectively.

Discussion

The existence of highly specific macroalgal-bacterial associations is no longer doubted [4]. Various studies revealed that bacterial communities living on macroalgae clearly differ from those occurring in the surrounding seawater [13, 58, 203, 226]. These studies, however, focused on the distinctiveness of the epiphytic bacterial communities from the free-living environmental communities and never studied the specificity of the endophytic bacteria associated with macroalgae. To our knowledge, this is the first study to address the temporal variability of the endogenous (EN) bacterial communities of *Bryopsis* isolates and their distinctiveness from the epiphytic (EP) and surrounding water (WW and CW) bacterial communities after prolonged cultivation using the DGGE technique. Taken the inherent limitations of the DGGE technique into account [228], we observed that the endophytic bacterial community profiles were notably different from the fingerprints of bacterial communities on and surrounding *Bryopsis* cultures. DGGE fingerprint cluster analysis (Fig. 3.11) and MDS (Fig. 3.12) clearly indicate that the epiphytic and surrounding water samples in all *Bryopsis* cultures were more similar to each other than to their corresponding endophytic community profile. This suggests the existence of specialized endophytic bacterial communities within *Bryopsis* algae which are clearly distinct from the outer surface and environmental bacterial communities. This apparent specificity is supported by the observation that *Bryopsis* harbors rather stable endophytic bacterial communities, which showed little time variability after one year cultivation of the algal samples (Fig. 3.10). However, examination of individual DGGE bands did reveal some similarities between intra- and extracellular bacteria. While Bacteroidetes, Flavobacteriaceae and Xanthomonadaceae species seemed exclusively endobiotic, sequence cluster analysis confirmed that *Arcobacter*, *Labrenzia, Mycoplasma* and Phyllobacteriaceae endophytes were also present in the epiphytic, washing water and/or cultivation water extracts. This latter observation is consistent with the outcome of a study conducted by Maki *et al.* [229] which revealed similar intracellular and extracellular bacterial populations in and on the harmful marine microalga *Heterocapsa circularisquama* in culture.

Although the *Bryopsis* cultures used in this study have been kept in the laboratory for almost three years due to experimental restrictions (see section 3.2.1 [225]), our data allow us to put forward some hypotheses regarding the nature of the endophytic communities within natural *Bryopsis* populations. Whereas we cannot rule out selection by artificial laboratory growth conditions, *Arcobacter*, *Labrenzia, Mycoplasma* and Phyllobacteriaceae endophytes can at least survive without the *Bryopsis* host, suggesting they might be facultative endogenous bacteria which are acquired from the local environment. This is consistent with the general perception that most plant endophytes originate

from the surrounding environment and the outer plant surface [230, 231]. Bacteroidetes, Flavobacteriaceae and Xanthomonadaceae endophytes, on the other hand, appear well adapted to an endobiotic lifestyle as they persist within the *Bryopsis* interior after prolonged cultivation. Especially Flavobacteriaceae endophytes, which are present in all five MX samples collected hundreds of kilometres apart, might be obligate endophytes which are strictly dependent on the *Bryopsis* host for their growth and survival. This co-occurrence of multiple facultative and obligate bacterial endophytes is also well documented in many land plant and insect hosts [230, 232].

Furthermore, the *Bryopsis* endophytic communities seem also rather specific as the EP, WW and CW extracts contained numerous Alphaproteobacterial, Gammaproteobacterial and Acanthopleuribacterales species which are not present in the EN samples. This apparent specificity is confirmed by our observations that EP, WW, CW (data not shown) and EN (see Fig. 3.10) extracts made at different time points revealed largely consistent banding patterns even after the algal specimens were repeatedly wounded and transferred to fresh, sterile cultivation medium (see Material and methods section). Consequently, the *Bryopsis* host seems able to selectively maintain its endophytic flora and/or to attract specific facultative endophytes after wounding. Although this may be the result of more general physiological and biochemical processes [4], the characteristic properties of *Bryopsis* might also contribute to this selectiveness. An interesting characteristic of *Bryopsis* is that following cell wounding, the protoplasm can aggregate and regenerate into a mature individual. This process involves a transient state of membrane-free protoplasts in seawater [113]. Although this transient 'life without a membrane' state might seem anything but selective, Klotchkova and coworkers [233] showed that an incompatibility barrier is present during protoplast formation to exclude foreign inorganic particles or alien cell components. Only some chosen cells or particles could be incorporated into *Bryopsis* protoplasts. Moreover, the lectins which play a key role in the aggregation process during protoplast formation [234-237] might actually be 'specificity mediators'. The description of the *Bryopsis* specific lectin Bryohealin by Kim *et al.* [236], which contains an antibiotic domain that protects the newly generated protoplasts from bacterial contamination [237], supports this hypothesis. Lectins are known symbiosis mediators in, for example, legume-rhizobia and sponge-bacterial symbioses [238, 239].

Besides the endophytic bacterial communities, also the epiphytic and the surrounding cultivation water bacterial communities seemed unique to each *Bryopsis* culture as the EP, WW and CW fingerprints of a given *Bryopsis* sample clearly clustered together. This is consistent with the general perception of highly specific macroalgal-bacterial interactions as discussed above [4]. Additionally,

since all five *Bryopsis* cultures were maintained under similar laboratory conditions, the above observation suggests that factors other than cultivation conditions contributed to the observed specificity (see Material and methods section).

Conclusion

Our results indicate that *Bryopsis* samples harbor specific and rather stable endophytic bacterial communities after prolonged cultivation which are clearly distinct from the epiphytic and surrounding cultivation water bacterial communities. Even though *Bryopsis* algae are repeatedly being exposed to a mix of marine bacteria, they seem to selectively maintain and/or attract their endophytes after repeated wounding events in culture. Despite the limitations of the experimental design, this indicates that *Bryopsis* has some intrinsic mechanisms to favour the entry of certain bacteria of possible ecological importance within its cell, suggesting macroalgal-bacterial endobioses might be as or even more specific than macroalgal-epiphytic bacterial associations. The use of species-specific primers and probes may open the way to investigate the specificity, both spatially and temporally, of the endophytic communities in natural *Bryopsis* populations.

Acknowledgments

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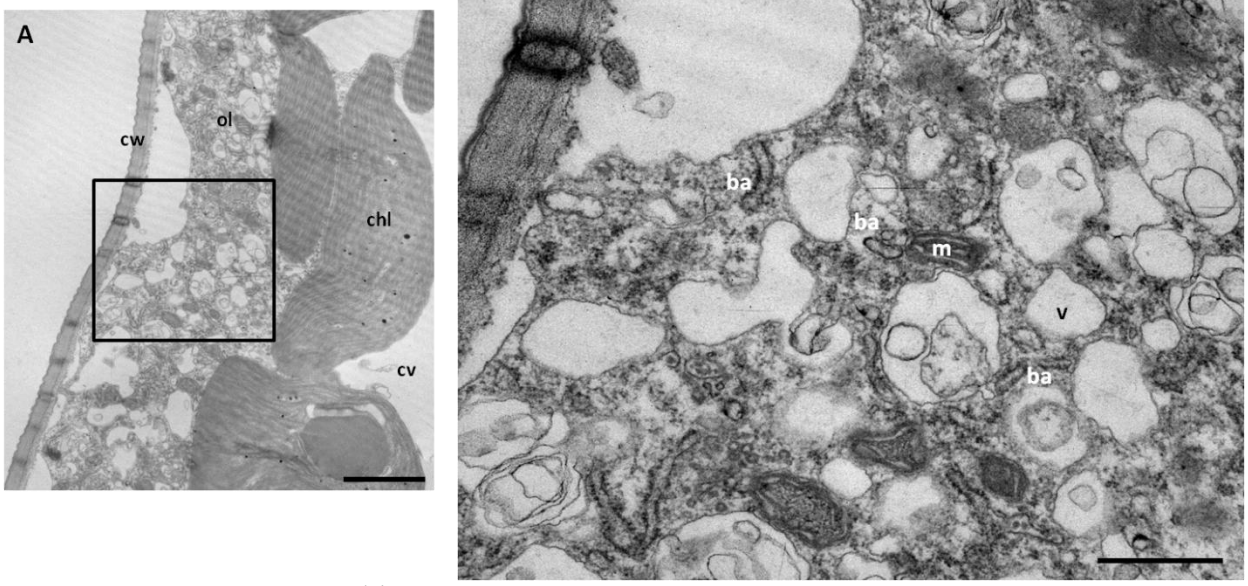
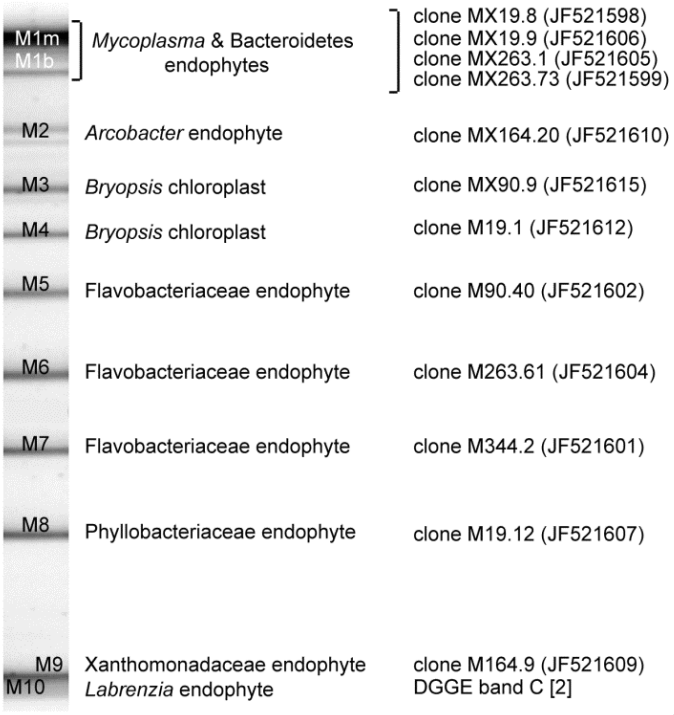


Supplementary Figure S3.1: Transmission electron micrograph of vegetative *Bryopsis* thallus in longisection. Fig. A: the outer cytoplasmic layer (ol) adjacent to the *Bryopsis* cell wall (cw) contains most of the organelles excluding only the chloroplasts (chl), which are present in the inner layer next to the central vacuole (cv). Magnification: x 8000, scale bar: 3 μm. Fig. B (detail of Fig. A): besides mitochondria (m), endoplasmic reticulum and vacuolar evaginations (v), endogenous bacteria (ba) are present in the outer cytoplasmic layer. Magnification: x 25000, scale bar: 1 μm.

Supplementary Figure S3.2: The marker used as a normalization and identification tool in all DGGE analyses. This marker covers the full range of endophytic (including chloroplast) sequences previously obtained from *Bryopsis* samples MX19, MX90, MX164, MX263 and MX344 (see section 3.2.1 [225]). For each marker band, the band name (M1m, M1b, M2-M10), taxonomic identification, clone reference and accession number are represented.



3.2.3. Disentangling host phylogenetic, environmental and geographic signals in intracellular bacterial communities of *Bryopsis*

Joke Hollants, Frederik Leliaert, Heroen Verbruggen, Anne Willems and Olivier De Clerck. Permanent residents or temporary lodgers: characterizing intracellular bacterial communities of siphonous green algae. *Manuscript submitted to Mol Ecol.* **Author contributions:** The first two authors have equally contributed to the study. JH designed and performed the experiments and wrote the manuscript. FL maintained the algal cultures, analyzed the data and outlined the figures. FL, HV and ODC collected the *Bryopsis* samples. FL, HV, AW and ODC commented on the manuscript.

Abstract

The ecological success of giant celled, siphonous green algae has repeatedly been linked to endophytic bacteria living within the cytoplasm of the hosts. Yet, virtually nothing is known about the relative importance of evolutionary and ecological factors controlling the intracellular bacterial flora of these seaweeds. Using the siphonous alga *Bryopsis* as a model, we explore the diversity of the intracellular bacterial communities and investigate whether their composition is controlled by ecological and biogeographical factors rather than the evolutionary history of the host. Using a combination of 16S rDNA clone libraries and DGGE analyses, we show that siphonous algae harbor a diverse and complex mixture of generalist and specialist bacteria. Variation partitioning analyses show a strong impact of local environmental factors on bacterial community composition for generalist species, while specialists reflect a predominant imprint of evolutionary history. The results highlight the importance of interpreting the presence of individual bacterial phylotypes in the light of ecological and evolutionary principles such as phylogenetic niche conservatism to understand complex endobiotic communities and the parameters shaping them.

Introduction

Variation in traits across species or populations is influenced by their ecology and evolutionary history [240]. Organisms are shaped by the environment in which they live, with species residing in similar environments having common adaptations [241]. They are also the product of their evolutionary history, and closely related species have the tendency to be more similar than distantly related species [242]. This tendency for related species to resemble each other more in a trait than expected by chance is referred to as phylogenetic signal or phylogenetic conservatism [243]. Applying these principles to host-bacterial relationships, one might presume that obligate, vertically inherited bacteria (specialists) are phylogenetically structured, while facultative endobiotic bacteria (generalists) are expected to be more randomly dispersed among host species [244] (Fig. 3.15). In this study, we assess for the first time the combined effect of host dependency, ecology and biogeography on the structure of a complex endobiotic community in an algal model.

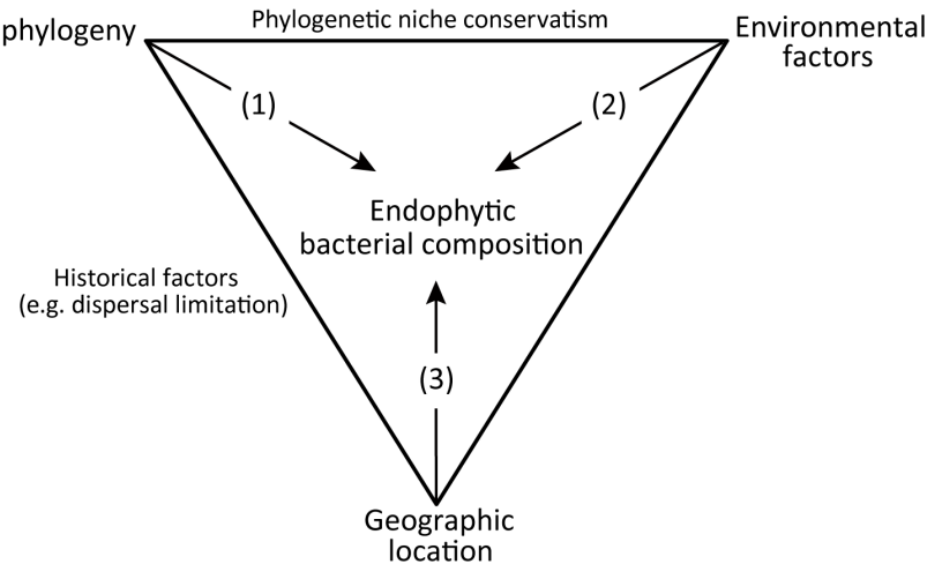


Figure 3.15: Relationships between host phylogeny, environment and geography on endophytic bacterial composition and relations between these three factors. 1: phylogenetic structured variation, 2: ecological structured variation and 3: geographic structured variation. The shared influence of phylogeny and environment (1+2) is known as "phylogenetically structured environmental variation".

Marine macroalgae (seaweeds) are commonly associated with bacteria that either live on the surface or in the cytoplasm and/or vacuolar systems of the cells [4]. These bacteria are able to influence the morphogenesis and life cycle of their algal host [80, 83, 86] and are linked with various metabolic functions such as the production of growth factors, fixed nitrogen and antimicrobial

compounds [54, 73, 93]. Siphonous green seaweeds, consisting of a single giant tubular cell, form a benevolent biotic environment for endobiotic bacterial communities [39, 124]. The siphonous cells, which range from centimeters to meters in length, typically exhibit vigorous cytoplasmic streaming to transport organelles, photosynthates and nutrients [107]. Chisholm *et al.* [75] demonstrated that siphonous algae take up nutrients from the sediment by a root-like system containing intracellular bacteria and translocate them throughout the thallus. These cellular innovations alongside unique mechanisms of wounding response [111, 112] and the close interactions with bacteria may provide a physiological explanation for the successful spread of siphonous green algae in marine coastal habitats [75, 125, 158].

Very little is known about the factors controlling the presence of bacteria inside siphonous seaweeds. Two host-related mechanisms may affect the intracellular bacterial composition. Firstly, siphonous seaweeds readily regenerate form protoplasts, facilitating environmental uptake of bacteria into the cell [234]. Secondly, endogenous bacteria can persist by vertical inheritance through gametes [36]. Beside the question of whether the endobionts are acquired vertically or from the environment, ecological parameters and geographic aspects may also need to be considered to explain the bacterial composition, as some bacteria (or hosts) are likely to be geographically restricted or occur only in particular niches. Although a previous study suggested that seaweed-associated bacterial communities are biogeographically structured [125], it is not known whether ecological or historical factors cause this structure.

The goal of this study is to investigate the relative roles of host, environment and geography in determining the intracellular bacterial flora of siphonous seaweeds, focusing on the genus *Bryopsis* as a case study. This genus is known to harbor several types of endogenous bacteria and protocols are in place to study them (see sections 3.1.2 and 3.2.1 [187, 225]). *Bryopsis* is known to possess mechanisms for environmental uptake (see section 3.2.2 [245]) as well as vertical inheritance of bacteria [36]. This combination of features, combined with the large collection of available cultures, makes the genus an ideal case study to address our goal. The experimental approach consisted of molecular characterization of host samples and their intracellular bacterial flora. The molecular identification of bacterial phylotypes, along with the host phylogeny and environmental data, were explored and analyzed with statistical techniques designed to disentangle the effects of host phylogeny, geography and the external environment on the intracellular bacterial composition.

Material and methods

Algal material

The 20 *Bryopsis* samples analyzed in this study are listed in Supplementary Table S3.2 (p. 100) and their sampling sites are depicted in Figure 3.16. All samples were transferred to and maintained as unialgal cultures under the conditions described in section 3.2.1 [225].

Figure 3.16: Map of *Bryopsis* sampling sites. The collection sites are marked by black circles and labelled with the *Bryopsis* sample name. In addition to the 15 *Bryopsis* samples analyzed in this study, also the five Mexican *Bryopsis* samples MX19, MX90, MX164, MX263 and MX344, which were previously studied (see section 3.2.1 [225]), are depicted.

Molecular approach

Bryopsis samples were subjected to a surface sterilization step to eliminate epiphytic bacterial contamination (see section 3.1.2 [187]) prior to total DNA extraction [160]. The host *rbc*L and bacterial 16S rRNA genes were PCR amplified as described in section 3.2.1 [225]. The endophytic bacterial diversity was assessed by creating 16S rRNA gene clone libraries and performing nested PCR denaturing gradient gel electrophoresis (DGGE) analyses as described in sections 3.2.1 and 3.2.2 [225, 245]. Sequences were submitted to EMBL under accession numbers HE648924-HE648948.

Sequence data analyses

*Bryopsis rbc*L and bacterial 16S rRNA gene sequences were assembled, checked for chimeras, compared with nucleotide databases and aligned as described in section 3.2.1 [225]. Phylogenetic trees were inferred with maximum likelihood (ML) implemented in PhyML v3.0 [195] and Bayesian inference (BI) using MrBayes [246], via the University of Oslo Bioportal website [196]. Both analyses were performed under a HKY+G model as determined by the Akaike Information Criterion in JModeltest v0.1.1 [194].

Statistical analysis

The influence of environmental, geographic, and host phylogenetic factors on the endophytic bacterial diversity in *Bryopsis* was analyzed using multivariate statistical and comparative phylogenetic approaches. The response table was represented by a presence/absence matrix of the seven bacterial phylotypes in the 20 host samples (Fig. 3.17). The three explanatory matrices (environment, geography and phylogeny) were prepared as follows. The environmental component was represented by seven macro-ecological variables extracted from Bio-ORACLE [247], a global environmental dataset of satellite-based and *in situ* measured marine geophysical, biotic and climate information at a final spatial resolution of 5 arcmin (9.2 km) (Supplementary Table S3.3, p. 100). The geographic component was represented by a set of orthogonal spatial variables extracted from geographic coordinates by Moran's Eigenvector Maps (MEM) analysis [248] using 'codep' in R [249]. The geographic matrix was represented by the first two eigenvectors, which were the only ones having positive eigenvalues (6.54 and 1.52). The phylogenetic component was expressed as principal coordinates via a principal coordinate analysis (PCoA) [250] computed from a distance matrix [251]. A corrected distance matrix of the *Bryopsis rbc*L alignment was calculated in MEGA [197]; the PCoA analysis was performed in PCO [252]. The phylogenetic matrix was represented by the first four principal coordinates, representing 98% of the total variation.

To study the influence of environment, geography and host phylogeny on the endophytic bacterial diversity, we first performed data ordinations and calculated phylogenetic signals of the bacterial community composition. Ordination of *Bryopsis* samples based on endophytic bacterial community composition was performed using a principal component analysis (PCA) in CANOCO for Windows 4.5 [253]. Environmental variables were plotted on the PCA graph as supplementary information. Phylogenetic signal was assessed for (i) the environmental variables, (ii) geography, (iii) the total endophytic bacterial community (i.e. represented by principal components 1 and 2

calculated as described above) and (iv) the presence/absence of the seven endophytic bacterial OTUs. P-values were calculated using randomizations of the K-statistic [254] in the R package Picante [255] (for i-iii) and the D statistic [256] in the R package 'caper' (http://cran.r-project.org/web/packages/caper/) (for iv). We quantified the common and unique influences of host phylogeny, geography and environment on the endophytic flora variation by variation partitioning analysis using the varpart function in the R package 'vegan' (http://cran.r-project.org/web/packages/vegan/). This statistical technique partitions the variation of a response matrix with respect to different explanatory matrices using redundancy analysis ordination (RDA) (or partial regression when the response table contains a single vector) [241, 257]. The technique is widely used in ecological studies to establish the relationships between species distributions and predictors of interest, such as environmental and spatial variables, but has also been applied in a phylogenetic context [258]. The total bacterial diversity, as well as presence/absence data of the seven individual phylotypes was considered as response tables. We performed variation partitioning analyses using three (phylogeny, environment and geography) and two (phylogeny, environment) explanatory tables, respectively.

Results

Bryopsis host phylogeny

Based on the phylogenetic analysis of host *rbc*L sequences (Fig. 3.17) we assigned the seaweed samples to nine *Bryopsis* species, numbered sp. 1 through 9. The host phylogeny shows three main clades. Clades A and B include *Bryopsis* samples isolated from cold to temperate regions, whereas clade C is warm-temperate to tropical. The phylogenetic signal in annual mean sea surface temperature, as well as annual mean PAR and dissolved oxygen levels, which are inversely proportional to each other, is statistically significant ($P < 0.01$, Table 3.4), suggesting that the structure of the *Bryopsis* phylogeny reflects temperature-related environmental variables. Conversely, geographic location (represented by Moran's Eigenvector Maps) did not show a significant phylogenetic structure (Table 3.4).

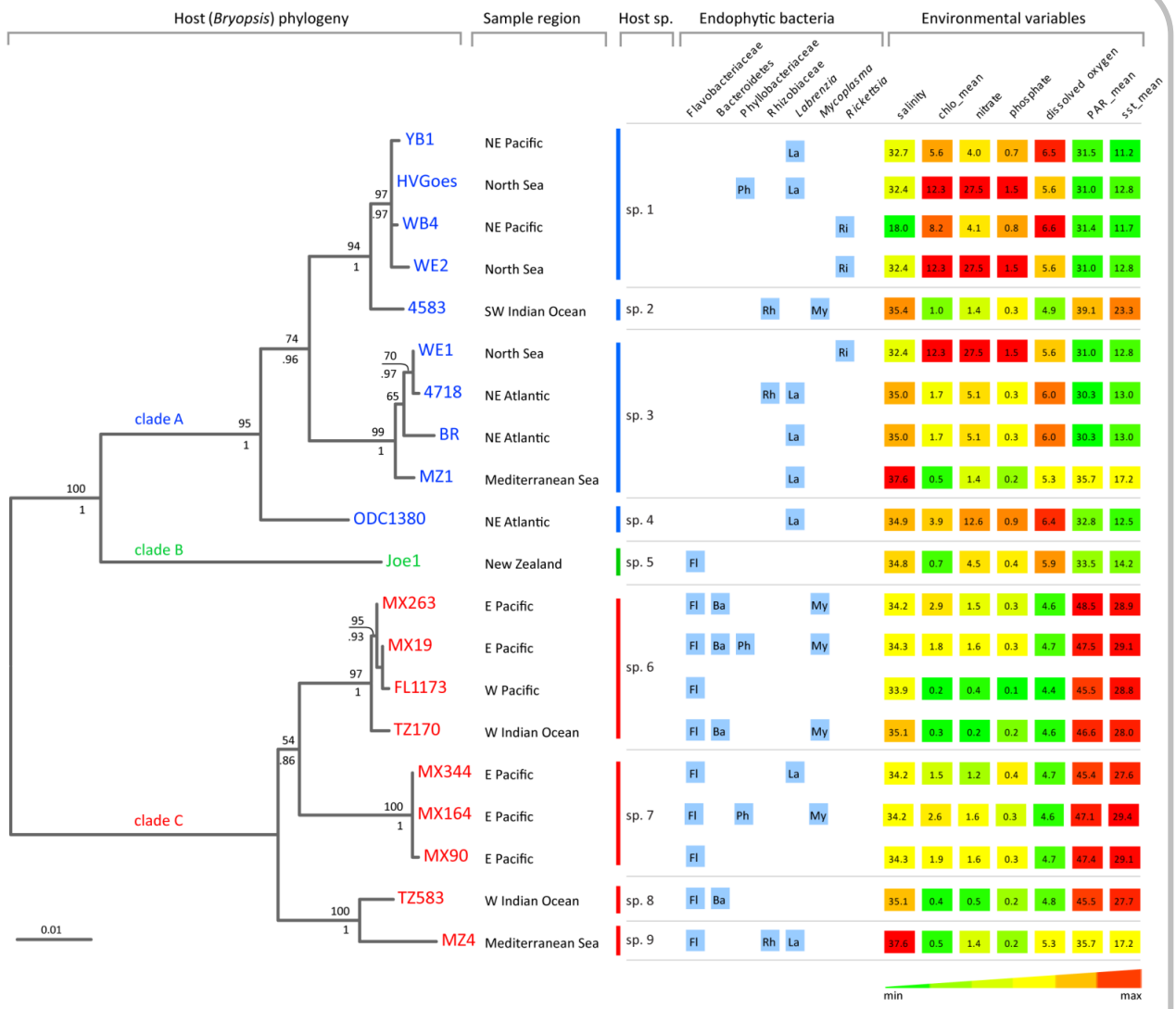


Figure 3.17: Endophytic diversity results, geographic data and environmental variables plotted against the *Bryopsis* host phylogram. The endophytic bacterial diversity displayed by blue boxes summarizes the diversity results from the 16S rRNA gene clone libraries and DGGE analyses. Environmental variables were extracted from the host sampling sites using Bio-ORACLE: salinity (PSS); chlo_mean: annual mean chlorophyll (mg m^{-3}); nitrate (µmol l^{-1}); phosphate (µmol l^{-1}); dissolved oxygen (ml l^{-1}); PAR_mean: annual mean photosynthetically available radiation (µmol m^{-2} s^{-1}); sst_mean: annual mean sea surface temperature (°C) (see also Supplementary Table S3.3, p. 100). The phylogram on the left classifies the 20 algal samples for which endophytic bacterial data ara available in nine different *Bryopsis* species and three distinct clades (i.e. A, B and C). These clades seem more consistent with the ecology of the host samples (environmental variables depicted on the right) than with their geographic origin (sample region). ML bootstrap values and BI posterior probabilities, respectively, are indicated above and below the branch nodes. The scale bar indicates 0.01 nucleotide changes per nucleotide position.

Table 3.4: Phylogenetic signal values calculated for the environmental variables (Fig. 3.17), geography (Moran's eigenvector maps, MEM 1 and 2), total bacterial composition (principal components 1 and 2) (Fig. 3.18) and the presence of the seven endophytic bacterial OTUs (Fig. 3.17). P values were calculated from randomizations using Blomberg et al.'s K (K) and Fritz and Purvis' D statistic (D). Statistical significant p-values ≤ 0.01 are indicated in bold.

		Phylogenetic signal	P-value
Environmental variables	chlo_mean	0.07	0.18 (K)
	dissolved oxygen	0.16	**0.00**(K)
	nitrate	0.04	0.49(K)
	PAR_mean	0.73	**0.00**(K)
	phosphate	0.05	0.29(K)
	salinity	0.04	0.55(K)
	sst_mean	0.70	**0.00**(K)
Geography	MEM 1	0.07	0.07(K)
	MEM 2	0.08	0.20(K)
Total bacterial composition	PC 1	0.07	0.09(K)
	PC 2	0.03	0.74(K)

		Phylogenetic signal	P-value
Presence endophytic phylotypes	Bacteroidetes	-0.03	**0.01** (D)
	Flavobacteriaceae	-0.54	**0.00**(D)
	Labrenzia	1.16	0.67(D)
	Mycoplasma	0.63	0.12(D)
	Phyllobacteriaceae	1.75	0.98(D)
	Rhizobiaceae	1.19	0.61(D)
	Rickettsia	1.24	0.66(D)

Endophytic bacterial diversity

The results from the clone libraries and DGGE analyses showed the presence of seven unique endophytic bacterial phylotypes or operational taxonomic units (OTUs) within *Bryopsis* (Table 3.5). Five could be identified as Flavobacteriaceae (OTU-1), *Mycoplasma* (OTU-2), Bacteroidetes (OTU-3), Phyllobacteriaceae (OTU-4) and *Labrenzia* (OTU-7) species, which were previously shown to occur in *Bryopsis* (see section 3.2.1 [225] , Table 3.5 and Supplementary Figure S3.3 on page 101). In addition, two new endophytic phylotypes were identified, OTU-5 and OTU-6 (Table 3.5 and Supplementary Figure S3.3, p. 101). OTU-5 showed high sequence similarities with Rhizobiaceae strains isolated from root nodules of leguminous plants, and represents two distinct clusters that include *Rhizobium leguminosarum* and *Ensifer meliloti* type strains, respectively. OTU-6 is allied to

uncultured Rickettsiales bacteria associated with the coral *Montastraea faveolata* and the marine ciliate *Diophrys appendiculata*. All OTU-6 sequences formed a distinct and well-supported clade closely related to the genus *Rickettsia* and most likely represent at least a new species based on their low sequence similarities (≤ 93%) with *Rickettsia* type strains.

Endophytic bacterial composition

Figure 3.17 schematizes the endophytic bacterial diversity (blue boxes) in *Bryopsis*. Composition of the endophytic community varied between host species, and samples from the same host species harbored diverse combinations of one to four different endophytic phylotypes. Different host species with the same geographic origin commonly displayed differences in their intracellular bacterial community composition (e.g. samples MZ1 and MZ4). This apparent lack of correlation between total bacterial diversity and *Bryopsis* host species and geography is confirmed by the PCA plot which illustrates that the ordination of the different *Bryopsis* species is not fully explained by their similarity in endophytic bacterial community composition (Fig. 3.18). This PCA plot, however, clearly indicates a correlation between the presence of individual endophytic phylotypes and certain environmental variables. Flavobacteriaceae, Bacteroidetes and *Mycoplasma* endophytes were only present in *Bryopsis* species isolated from tropical or warm-temperate seas, *Labrenzia* species were more often found in algal samples isolated from temperate regions, and *Rickettsia* endophytes were only present in *Bryopsis* species inhabiting seas with a low mean sea surface temperature (11.7-12.8°C) and high chlorophyll, nitrate and phosphate levels (Figs. 3.17 and 3.18). These correlations suggest that the distribution of individual bacterial OTUs may be more predictable than the total bacterial community composition. Individual bacterial endophyte groups also appear to be more strongly correlated with the host phylogeny than the overall bacterial composition. Flavobacteriaceae and Bacteroidetes species displayed a significant phylogenetic signal ($P \leq 0.01$, see Table 3.4) while Rhizobiaceae, Phyllobacteriaceae, *Mycoplasma, Rickettsia* and *Labrenzia* species did not. Because the host phylogeny is correlated with ecological features as a consequence of niche conservatism (see Fig. 3.15), it is not obvious whether the latter pattern is due to ecological preferences of the endophytic bacteria or their host.

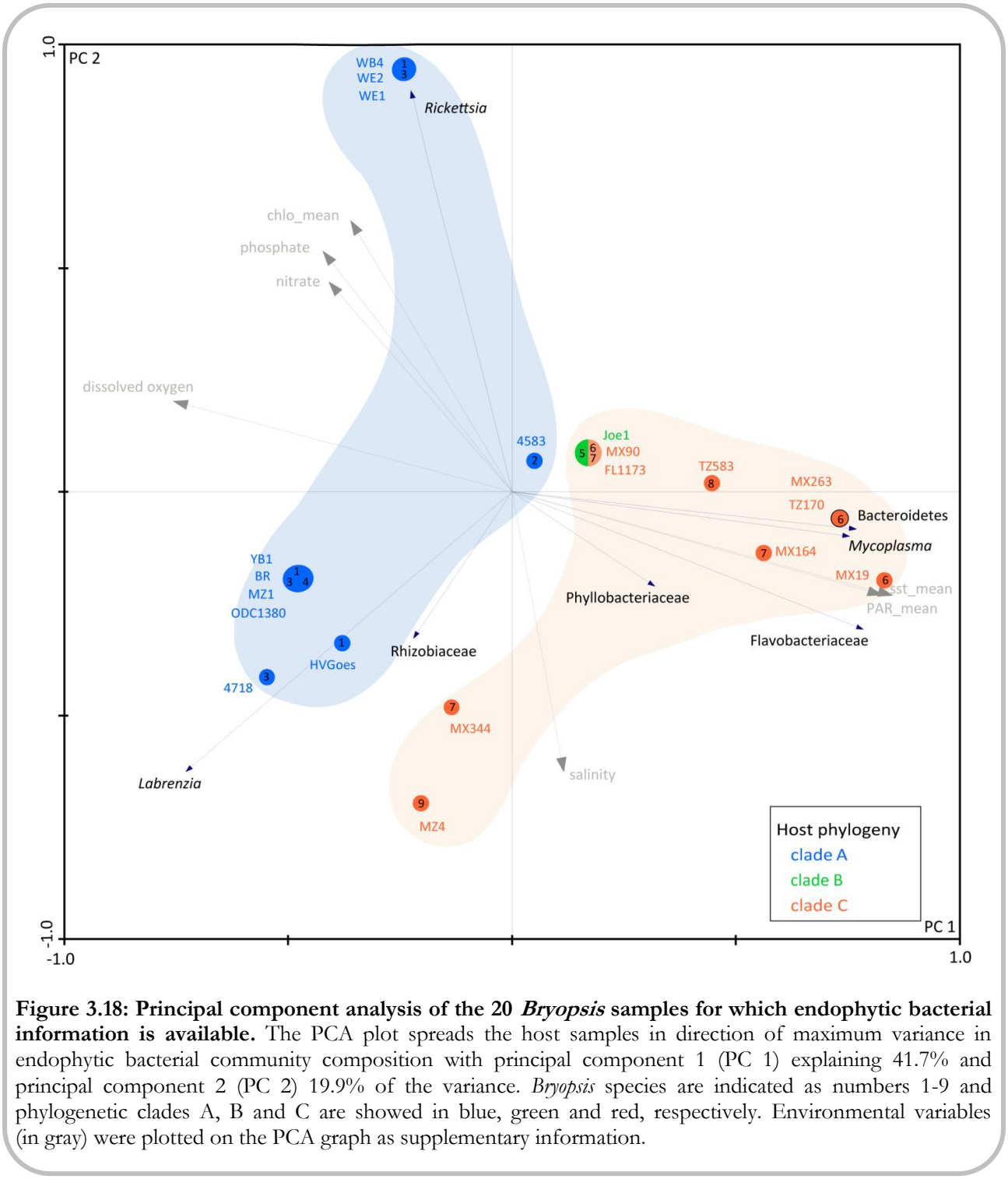


Figure 3.18: Principal component analysis of the 20 *Bryopsis* samples for which endophytic bacterial information is available. The PCA plot spreads the host samples in direction of maximum variance in endophytic bacterial community composition with principal component 1 (PC 1) explaining 41.7% and principal component 2 (PC 2) 19.9% of the variance. *Bryopsis* species are indicated as numbers 1-9 and phylogenetic clades A, B and C are showed in blue, green and red, respectively. Environmental variables (in gray) were plotted on the PCA graph as supplementary information.

Table 3.5: Taxonomic affiliation of the clones and DGGE bands representing the endophytic bacterial OTUs, sorted per *Bryopsis* sample.

Host	16S rRNA gene sequence analysis of bacterial clones and DGGE bands					
***Bryopsis* sample**	**OTU* no.**	**OTU representative clone/DGGE band**	**Accession no.**	**Higher taxonomic ranks**	**Closest NCBI match**	**Accession no. (Query coverage/Maximum identity)**
4583	OTU-2	DGGE band 4583a	HE648924	Mollicutes, Mycoplasmatales, Mycoplasmataceae	Uncultured *Mycoplasma* sp. clone MX19.9	JF521606 (100/100)
	OTU-5	DGGE band 4583I	HE648925	Alphaproteobacteria; Rhizobiales; Rhizobiaceae	*Ensifer meliloti* strain RMP66	AB665549 (100/100)
	OTU-5	DGGE band 4583II	HE648926	Alphaproteobacteria; Rhizobiales; Rhizobiaceae	*Rhizobium leguminosarum* strain IPR-Pv1097	JN208903 (100/100)
4718	OTU-5	Clone 4718.68	HE648927	Alphaproteobacteria; Rhizobiales; Rhizobiaceae	*Ensifer medicae* WSM419	CP000738 (100/99)
	OTU-7	Clone 4718.108	HE648928	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	Uncultured bacterium clone SGUS723	FJ202588 (100/99)
BR	OTU-7	Clone BR.63	HE648929	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	*Labrenzia alba* strain CECT 5094	NR_042378 (100/99)
FL1173	OTU-1	DGGE band FL1173b	HE648930	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured Flavobacteriaceae bacterium clone MX19.14	JF521603 (100/100)
HVGoes	OTU-4	DGGE band HVGoesII	HE648931	Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	Uncultured Phyllobacteriaceae bacterium clone MX164.59	JF521608 (100/100)
	OTU-7	Clone HVGoes.14	HE648932	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	Uncultured bacterium clone SGUS723	FJ202588 (100/99)
Joe1	OTU-1	Clone Joe1.40	HE648933	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured Flavobacteriaceae bacterium clone MX19.14	JF521603 (100/96)
MX19	OTU-1	Clone MX19.14	JF521603	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
	OTU-2	Clone MX19.9	JF521606	Mollicutes, Mycoplasmatales, Mycoplasmataceae	Uncultured bacterium clone GB96	GU070687 (100/97)
	OTU-3	Clone MX19.8	JF521598	Bacteroidetes; unclassified Bacteroidetes	Uncultured bacterium clone Dstr_N15	GU118164 (99/94)
	OTU-4	Clone MX19.12	JF521607	Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	Uncultured Rhizobiales bacterium clone PRTBB8661	HM799061 (99/99)
MX90	OTU-1	Clone MX90.40	JF521602	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
MX164	OTU-1	Clone MX164.14	JF521600	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
	OTU-2	DGGE band MX164 B	HE599214	Mollicutes, Mycoplasmatales, Mycoplasmataceae	Uncultured bacterium clone GB96	GU070687 (100/97)
	OTU-4	Clone MX164.59	JF521608	Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae	Phylobacteriaceae bacterium strain DG943	AY258089 (97/99)
MX263	OTU-1	Clone MX263.61	JF521604	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
	OTU-2	Clone MX263.1	JF521605	Mollicutes, Mycoplasmatales, Mycoplasmataceae	Uncultured bacterium clone GB96	GU070687 (100/97)
	OTU-3	Clone MX263.73	JF521599	Bacteroidetes; unclassified Bacteroidetes	Uncultured bacterium clone Dstr_N15	GU118164 (99/94)

Host	16S rRNA gene sequence analysis of bacterial clones and DGGE bands					
***Bryopsis* sample**	**OTU* no.**	**OTU representative clone/DGGE band**	**Accession no.**	**Higher taxonomic ranks**	**Closest NCBI match**	**Accession no. (Query coverage/Maximum identity)**
MX344	OTU-1	Clone MX344.2	JF521601	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured bacterium clone SHFH601	FJ203530 (99/96)
	OTU-7	DGGE band MX344 C	HE599215	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	*Labrenzia alba* isolate CMS163	FR750958 (100/100)
MZ1	OTU-7	Clone MZ1.9	HE648934	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	*Labrenzia alba* type strain CECT 5094^T	AJ878875 (100/99)
MZ4	OTU-1	Clone MZ4.22	HE648935	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured Flavobacteriaceae bacterium clone MX19.14	JF521603 (100/99)
	OTU-5	Clone MZ4.102	HE648936	Alphaproteobacteria; Rhizobiales; Rhizobiaceae	*Ensifer meliloti* SM11	CP001830 (100/99)
	OTU-5	Clone MZ4.43	HE648937	Alphaproteobacteria; Rhizobiales; Rhizobiaceae	*Rhizobium leguminosarum* bv. *viciae* strain BIHB 1160	EU730590 (100/99)
	OTU-7	DGGE band MZ4δ	HE648938	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	Uncultured *Labrenzia* sp. DGGE band MX344 C	HE599215 (100/100)
ODC1380	OTU-7	DGGE band ODC1380e	HE648939	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	*Labrenzia aggregata* strain KMO25	JF514325 (100/100)
TZ170	OTU-1	Clone TZ170.53	HE648940	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured Flavobacteriaceae bacterium clone MX19.14	JF521603 (100/99)
	OTU-2	Clone TZ170.27	HE648941	Mollicutes, Mycoplasmatales, Mycoplasmataceae	Uncultured *Mycoplasma* sp. clone MX19.9	JF521606 (100/99)
	OTU-3	Clone TZ170.55	HE648942	Bacteroidetes; unclassified Bacteroidetes	Uncultured Bacteroidetes bacterium clone MX19.8	JF521598 (100/99)
TZ583	OTU-1	Clone TZ583.13	HE648943	Bacteroidetes; Flavobacteria; Flavobacteriales	Uncultured Flavobacteriaceae bacterium clone MX19.14	JF521603 (100/99)
	OTU-3	DGGE band TZ583c	HE648944	Bacteroidetes; unclassified Bacteroidetes	Uncultured Bacteroidetes bacterium clone MX19.8	JF521598 (100/99)
WE1	OTU-6	Clone WE1.5	HE648945	Alphaproteobacteria; Rickettsiales	Uncultured bacterium clone SHFG464	FJ203077 (99/98)
WE2	OTU-6	Clone WE2.2	HE648946	Alphaproteobacteria; Rickettsiales	Uncultured bacterium clone SHFG464	FJ203077 (99/97)
WB4	OTU-6	Clone WB4.44	HE648947	Alphaproteobacteria; Rickettsiales	Uncultured bacterium clone SHFG464	FJ203077 (99/98)
YB1	OTU-7	Clone YB1.1	HE648948	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae	*Labrenzia aggregata* strain 2PR58-2	EU440961 (100/99)

* OTUs were delineated at 97% sequence similarity

Host versus environmental influences

In order to disentangle the influences of different factors shaping the endophytic bacterial diversity, we performed variation partitioning analyses. In the first set of analyses we partitioned the variation of the bacterial diversity data with respect to the ecological, geographic and host-phylogenetic factors into different portions: a part strictly influenced by environmental variables, a part strictly influenced by the *Bryopsis* host phylogeny, a part strictly explained by geography, four parts explained by the shared influence of these three factors, and an unexplained part of the variation. When considering the total endophytic bacterial diversity, more or less equal parts of the variation (ca. 30%) were explained by environmental and phylogenetic factors, while the strict influence of geography was low; most of the variance, however, remained unexplained (Fig. 3.19A). Analyses of the seven bacterial phylotypes separately showed that the influence of environment, phylogeny and geography was very different between the seven phylotypes. The influence of geography was, in most cases, low and highly correlated with environment and/or host phylogeny (Fig. 3.19A and Supplementary Table S3.4, p. 103). For this reason, we excluded geography in a second set of analyses (Fig. 3.19B). The independent effects of host phylogeny and environment had little influence on the presence of Phyllobacteriaceae, Rhizobiaceae and *Labrenzia* phylotypes. The shared influence of host phylogeny and environment was larger than their individual effects for these bacterial types. The occurrence of *Mycoplasma* and *Rickettsia* species, on the other hand, was in part strictly determined by environmental factors, whereas the distribution of Bacteroidetes could to a large extent be explained by host phylogenetic factors only. Most of the variance in presence of these six endophytic phylotypes, however, remained unexplained, suggesting that factors other than host phylogeny and environment determine their occurrence within particular *Bryopsis* samples (Fig. 3.19). This is in contrast with the situation for Flavobacteriaceae endophytes, whose presence could be entirely explained by host phylogenetic factors, which partly overlapped with environmental factors.

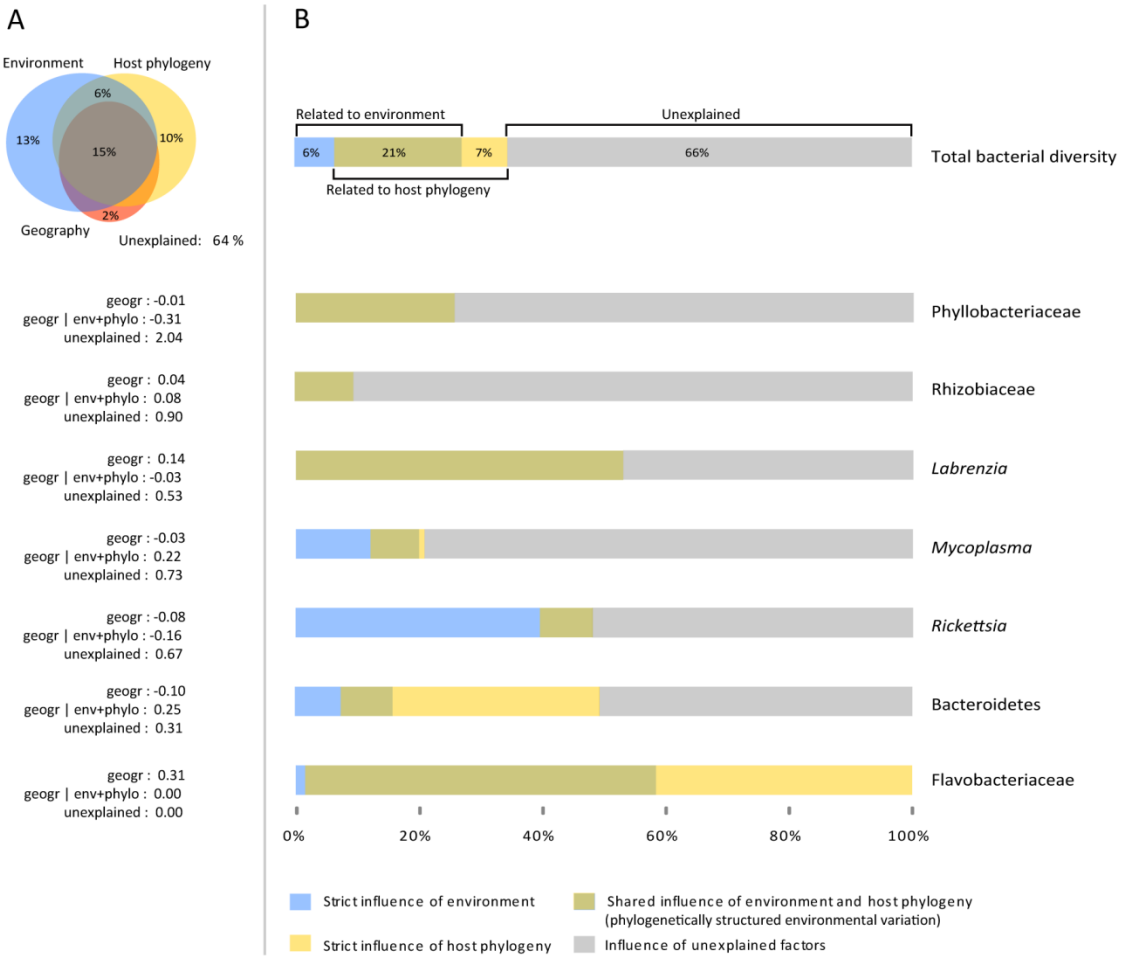


Figure 3.19: Variation partitioning. Adjusted R^2 values are given or illustrated. **A.** Results of the analysis with three explanatory tables: phylogeny, environment and geography. Venn diagram shows the influence of the three factors on the total bacterial diversity. Below are the variation explained by geography and the unexplained variation given for the seven bacterial phylotypes. **B.** Results of the analysis with two explanatory tables: phylogeny and environment. Diagrams show the unique and shared influence of both factors on the variation in total endophytic bacterial diversity and the individual endophytic phylotypes. Negative fractions (which indicate that two explanatory variables have strong and opposite effects on the dependent variable) are treated as zeros in the graphs. We refer to Supplementary Table S3.4 on page 103 for a detailed overview of the variation partitioning results.

Discussion

Community structure and variation in traits across species are the outcome of environmental, geographical and historical factors which are clearly interwoven with each other. Bacterial communities associated with eukaryotic hosts are influenced by similar factors which need to be identified separately. Besides serving as baseline knowledge of the bacterial diversity occurring inside the cells of siphonous seaweeds, our results provide insights into the various elements that contribute to the composition of the endogenous bacterial flora of siphonous green seaweeds.

Diversity of endogenous bacteria

Besides the five bacterial phylotypes that were previously characterized in *Bryopsis* (*Labrenzia, Mycoplasma,* Phyllobacteriaceae, Bacteroidetes and Flavobacteriaceae, see section 3.2.1 [225]), we identified two additional phylotypes related to Rhizobiaceae and *Rickettsia* species. These bacteria have been especially well studied from terrestrial habitats [202, 232], but have also been reported from marine habitats. Rhizobiales are common epiphytes of *Ulva* seaweeds [13, 59, 61, 203] and have also been isolated from the surface of kelps where they display antimicrobial activity [93]. Additionally, a *Rhodopseudomonas* species with the potential to fix nitrogen was isolated from the rhizoidal cytoplasm of the siphonous green seaweed *Caulerpa taxifolia* [75]. We presume that also *Bryopsis* hosts Rhizobiaceae species with nitrogen fixing capacities as we were able to amplify *Ensifer*-like nitrogenase reductase genes (EMBL accession numbers HE649370-HE649371) from *Bryopsis* samples 4718 and MZ4 by the *nifH* protocol described by De Meyer *et al.* [170]. Obligate intracellular *Rickettsia* species, on the other hand, have not previously been described from macroalgae but have been characterized through 16S rRNA gene analysis within freshwater green algae [259], marine ciliates [260] and coral tissue [218].

Factors affecting bacterial composition

Even though each bacterial phylotype was encountered in at least three *Bryopsis* samples, the total endophytic bacterial diversity per host sample showed no clear pattern. All algal samples harbored diverse combinations of one to four endophytic phylotypes regardless of their phylogenetic affiliation, geographic origin or macro-ecological niche. On the other hand, when the presence of individual endophytic phylotypes rather than the total bacterial composition was analyzed, host phylogenetic, geographic and environmental influences could be determined more clearly. These three factors, however, are inevitably interrelated as a result of phylogenetic niche conservatism, i.e. the tendency of closely related species to be ecologically similar [261], and historical factors such as dispersal limitation, resulting in geographic proximity of closely related species (Fig. 3.15). Disentangling the effects of host phylogeny, geography and environment shed light on the symbiotic nature and transmission mode of the individual endophytic phylotypes.

The presence of endophytic Phyllobacteriaceae, Rhizobiaceae and *Labrenzia* phylotypes was not separately determined by host phylogenetic, geographic and ecological factors, suggesting these endophytes are true generalists adapted to both free-living and host associated lifestyles along with a wide variety of environmental conditions. This is consistent with our previous observations that

Labrenzia and Phyllobacteriaceae endophytes can survive outside their *Bryopsis* host and are reacquired from the local environment after repeated wounding events in culture (see section 3.2.2 [245]). Also the close phylogenetic relatedness of all three endophytic phylotypes with sequences from free-living bacteria (Supplementary Figure S3.3, p. 101) indicates a recently initiated, facultative relationship with the *Bryopsis* host. These generalist phylotypes may be selectively acquired by *Bryopsis* hosts to fulfill specific metabolic requirements, such as nitrogen-fixation (Rhizobiaceae, [202]), anoxygenic photosynthesis (Phyllobacteriaceae, see section 3.2.1 [225]) or CO-oxidation (*Labrenzia*, [199]).

The occurrence of *Mycoplasma* and *Rickettsia* endophytes was to some extent strictly influenced by environmental factors. *Mycoplasma* endophytes were only present in *Bryopsis* samples from tropical regions, whereas *Rickettsia* bacteria were only found in algal samples isolated from temperate seas. This environmental influence suggests the acquisition of habitat-specific endophytes by *Bryopsis* hosts. In addition, the phylogenies of these more specialized endophytic phylotypes show a close relatedness with symbiotic *Rickettsia* and *Mycoplasma* species isolated from the cytoplasm of the marine ciliate *Diophrys appendiculata* [260] and the intestinal bacterial flora of the *Bryopsis*-feeding abalone *Haliotis diversicolor* [221], respectively, suggesting the uptake of these endophytes could be vector dependent. This hypothesis is likely as both endophytes belong to orders that are well-known as obligate intracellular parasites of plants and animals [262, 263]. Also within sponge hosts, horizontal symbiont transmission has been proposed to occur through vectors including sponge-feeding animals [264].

The presence of Bacteroidetes species within *Bryopsis* was to a large degree influenced by host phylogenetic factors, indicating that these endophytes may be vertically transmitted. This may take place through reproduction via fragmentation of the *Bryopsis* thallus or by extruded protoplasts that regenerate and develop into new *Bryopsis* plants [115]. Such asexual reproductive stages may act as vehicles by which bacteria are inevitably transmitted without being exposed to strict host phylogenetic influences only [265].

The presence of Flavobacteriaceae was found to be influenced by host phylogenetic factors only, suggesting that these bacteria are true specialized and obligate endosymbionts, which are entirely vertically transmitted via asexual and sexual reproductive stages [266]. This is in line with results from culture experiments, which showed that these bacterial species are strictly dependent on the *Bryopsis* host for their growth and survival (see section 3.2.2 [245]).

In conclusion, characterization of *Bryopsis* algae sampled worldwide revealed the presence of complex endobiotic bacterial communities. Evaluation of host phylogenetic, geographic and ecological factors revealed the presence of a mix of generalist and specialist bacteria. These observations, however, were only evident when subdividing the total endophytic diversity into its individual bacterial phylotypes, suggesting that both the whole community and individual community members need to be considered in host-symbiont studies.

Acknowledgements

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Supplementary Table S3.2: **Overview of the *Bryopsis* samples analyzed in this study, their collection sites and collection dates.**

***Bryopsis* sample**	**Collection site**	**Collection date**
Bryopsis 4583	Umhlanga Rocks KwaZulu Natal, South Africa	August 2005
Bryopsis 4718	Roscoff, Brittany, France	April 2008
Bryopsis BR	Roscoff, Brittany, France	July 2008
Bryopsis FL1173	Negros Oriental, Apo Island, Philippines	September 2007
Bryopsis HVGoes	Sas van Goes, The Netherlands	June 2007
Bryopsis Joe1	Moa Dt, Wellington, New Zealand	October 2008
Bryopsis MX19	Playa el Panteon, Puerto Angel, Oaxaca, Mexico	February 2009
Bryopsis MX90	Mazunte Beach, Mazunte, Oaxaca, Mexico	February 2009
Bryopsis MX164	Acapulco, Guerrero, Mexico	February 2009
Bryopsis MX263	Playa las Gatas, Zihuatanejo, Guerrero, Mexico	February 2009
Bryopsis MX344	Playa Careyero, Punta de Mita, Nayarit, Mexico	February 2009
Bryopsis MZ1 and MZ4	Begur, Catalogna, Spain	January 2008
Bryopsis ODC1380	Pointe de la Crèche, Boulogne, Nord-Pas-de-Calais, France	April 2007
Bryopsis TZ170	N tip of peninsula, Ruvula, Mtwara, Tanzania	January 2008
Bryopsis TZ583	E of lighthouse, Nungwi, Zanzibar, Tanzania	February 2008
Bryopsis WB4	Willapa Bay, SW Washington, USA	May 2008
Bryopsis WE1 and WE2	Wemeldinge, The Netherlands	May 2008
Bryopsis YB1	Yaquina Bay, Oregon, USA	May 2008

Supplementary Table S3.3: Overview of the macro-ecological variables extracted from Bio-ORACLE [247], their units, data handling and source.

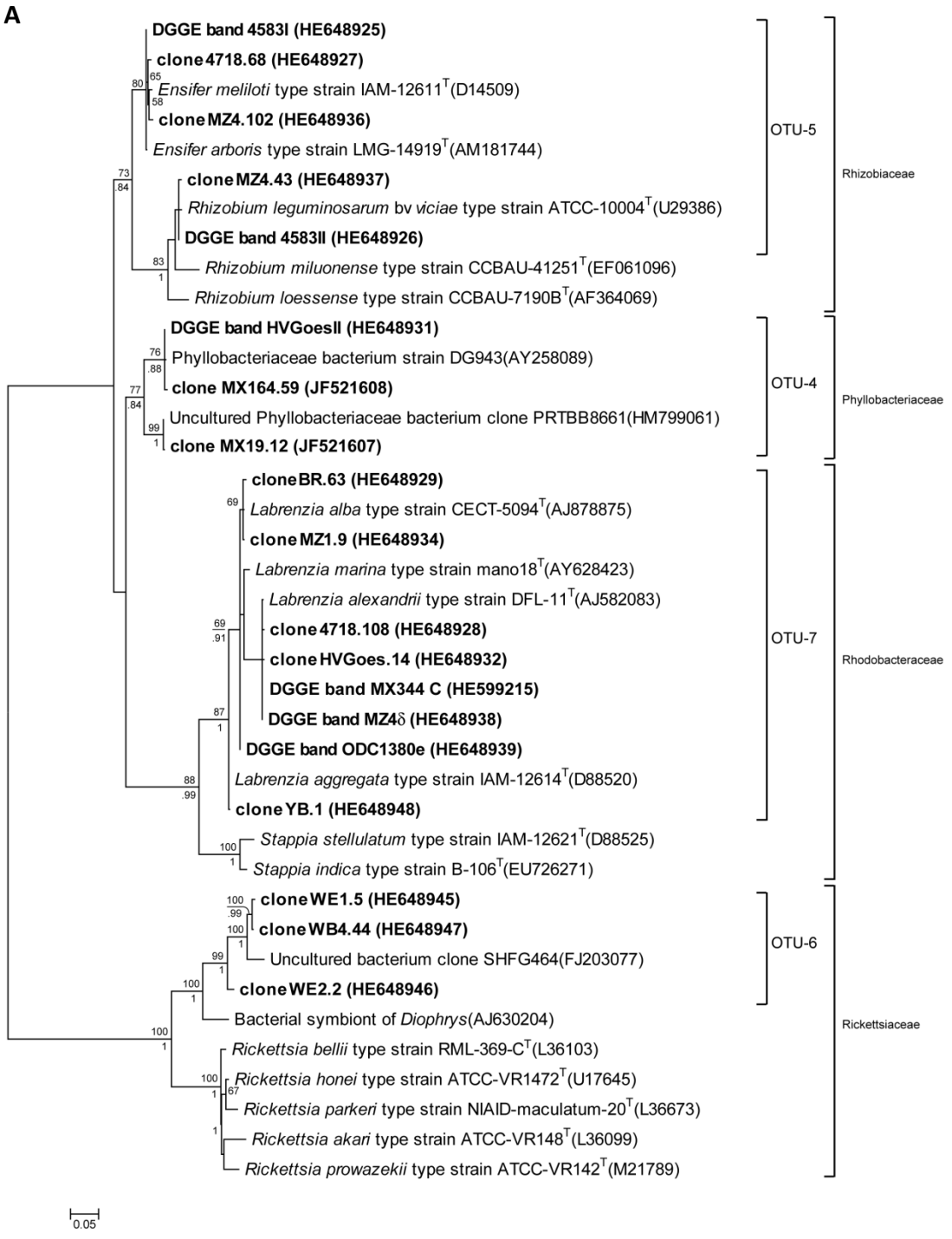
Environmental variable	**Units**	**Bio-ORACLE data handling**	**Source**
Annual mean chlorophyll (chlo_mean)	mg m^{-3}	Temporal mean from monthly climatologies (2002-2009)	Satellite data from Aqua-MODIS[2]
Annual mean photosynthetically available radiation (PAR_mean)	µmol m^{-2} s^{-1}	Temporal mean from monthly climatologies (1997-2009)	Satellite data from SeaWiFS[2]
Annual mean sea surface temperature (sst_mean)	°C	Temporal mean from monthly climatologies (2002-2009)	Satellite data from Aqua-MODIS[2]
Dissolved oxygen	ml l^{-1}	DIVA[1] interpolation of *in situ* measurements	*In situ* data from WOD 2009[3]
Nitrate	µmol l^{-1}	DIVA[1] interpolation of *in situ* measurements	*In situ* data from WOD 2009[3]
Phosphate	µmol l^{-1}	DIVA[1] interpolation of *in situ* measurements	*In situ* data from WOD 2009[3]
Salinity	PSS	DIVA[1] interpolation of *in situ* measurements	*In situ* data from WOD 2009[3]

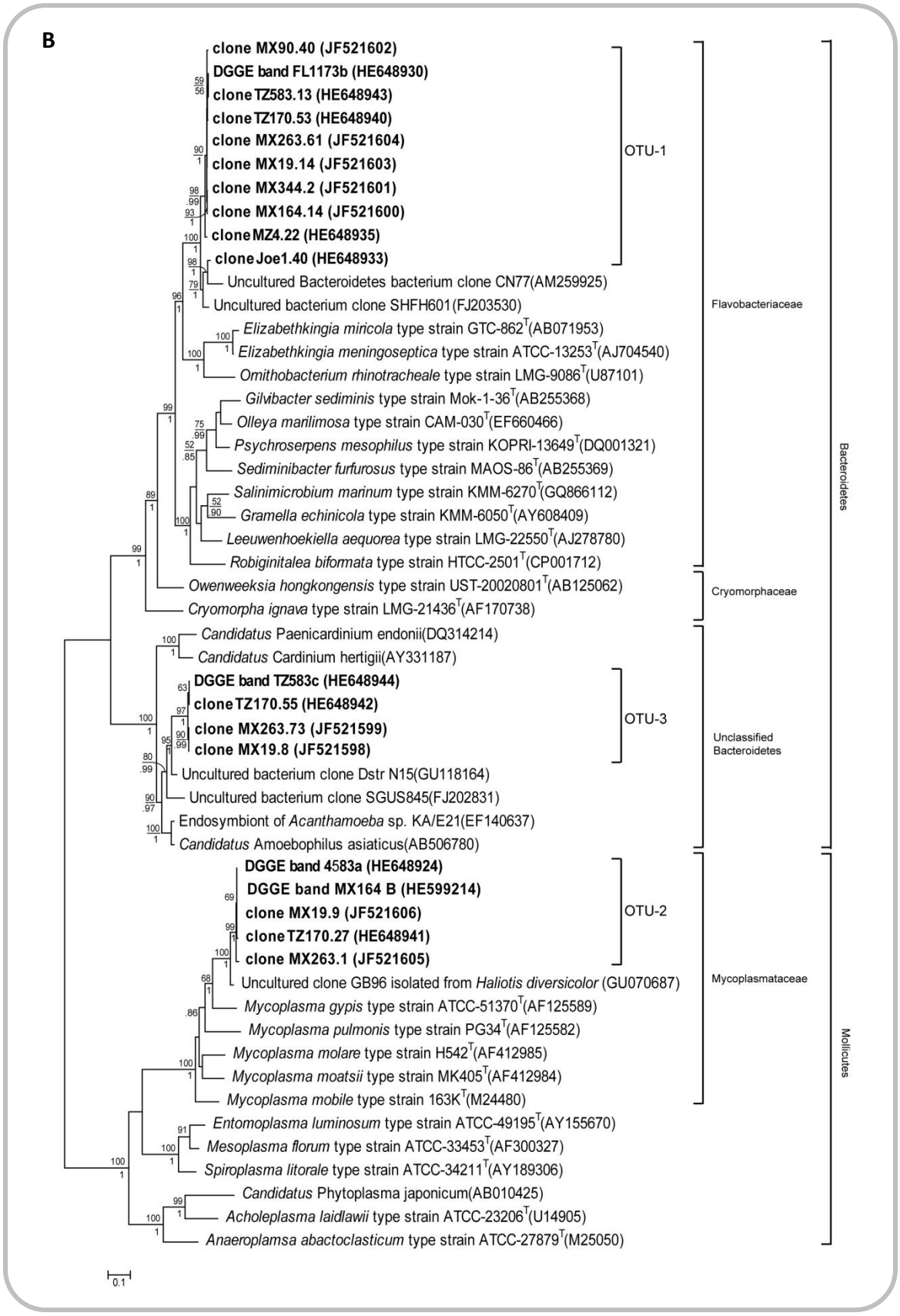
[1] Data interpolating variational analysis [267]

[2] Ocean-observing satellite sensors available at: http://oceancolor.gsfc.nasa.gov/

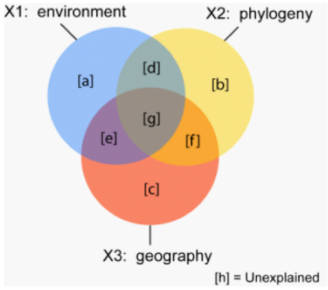
[3] World Ocean Database 2009 available at: http://www.nodc.noaa.gov/OC5/WOD09/pr_wod09.html

Supplementary Figure S3.3: Wide-range ML/BI trees showing the phylogenetic positions of endophytic bacterial clones and DGGE bands. Phylogenies were inferred from 16S rRNA gene sequences determined in this and our previous study (in bold, see section 3.2.1), BLAST hits (see Table 3.5), and Alphaproteobacterial (**A**) as well as Bacteroidetes and Mollicutes (**B**) type strains. Phylograms were generated using ML and BI under a GTR+G model. ML bootstrap values above 50% and BI posterior probabilities above 0.8, respectively, are indicated on top and beneath the branch nodes. The scale bar shows 5 (**A**) and 10 (**B**) nucleotide substitutions per 100 nucleotides.

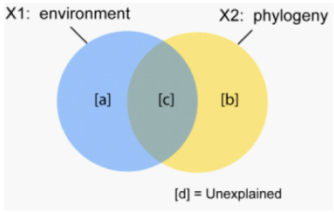



B
clone MX90.40 (JF521602)
DGGE band FL1173b (HE648930)
clone TZ583.13 (HE648943)
clone TZ170.53 (HE648940)
clone MX263.61 (JF521604)
clone MX19.14 (JF521603)
clone MX344.2 (JF521601)
clone MX164.14 (JF521600)
clone MZ4.22 (HE648935)
clone Joe1.40 (HE648933)
OTU-1
Uncultured Bacteroidetes bacterium clone CN77(AM259925)
Uncultured bacterium clone SHFH601(FJ203530)
Elizabethkingia miricola type strain GTC-862T(AB071953)
Elizabethkingia meningoseptica type strain ATCC-13253T(AJ704540)
Ornithobacterium rhinotracheale type strain LMG-9086T(U87101)
Flavobacteriaceae
Gilvibacter sediminis type strain Mok-1-36T(AB255368)
Olleya marilimosa type strain CAM-030T(EF660466)
Psychroserpens mesophilus type strain KOPRI-13649T(DQ001321)
Sediminibacter furfurosus type strain MAOS-86T(AB255369)
Salinimicrobium marinum type strain KMM-6270T(GQ866112)
Gramella echinicola type strain KMM-6050T(AY608409)
Leeuwenhoekiella aequorea type strain LMG-22550T(AJ278780)
Robiginitalea biformata type strain HTCC-2501T(CP001712)
Owenweeksia hongkongensis type strain UST-20020801T(AB125062)
Cryomorpha ignava type strain LMG-21436T(AF170738)
Cryomorphaceae
Candidatus Paenicardinium endonii(DQ314214)
Candidatus Cardinium hertigii(AY331187)
DGGE band TZ583c (HE648944)
clone TZ170.55 (HE648942)
clone MX263.73 (JF521599)
clone MX19.8 (JF521598)
OTU-3
Uncultured bacterium clone Dstr N15(GU118164)
Uncultured bacterium clone SGUS845(FJ202831)
Endosymbiont of Acanthamoeba sp. KA/E21(EF140637)
Candidatus Amoebophilus asiaticus(AB506780)
Unclassified Bacteroidetes
Bacteroidetes
DGGE band 4583a (HE648924)
DGGE band MX164 B (HE599214)
clone MX19.9 (JF521606)
clone TZ170.27 (HE648941)
clone MX263.1 (JF521605)
OTU-2
Uncultured clone GB96 isolated from Haliotis diversicolor (GU070687)
Mycoplasma gypis type strain ATCC-51370T(AF125589)
Mycoplasma pulmonis type strain PG34T(AF125582)
Mycoplasma molare type strain H542T(AF412985)
Mycoplasma moatsii type strain MK405T(AF412984)
Mycoplasma mobile type strain 163KT(M24480)
Mycoplasmataceae
Entomoplasma luminosum type strain ATCC-49195T(AY155670)
Mesoplasma florum type strain ATCC-33453T(AF300327)
Spiroplasma litorale type strain ATCC-34211T(AY189306)
Candidatus Phytoplasma japonicum(AB010425)
Acholeplasma laidlawii type strain ATCC-23206T(U14905)
Anaeroplamsa abactoclasticum type strain ATCC-27879T(M25050)
Mollicutes
0.1

Supplementary Table S3.4: Results of the variation partitioning analyses using three (phylogeny, environment and geography) and two (phylogeny, environment) explanatory tables. Adjusted R^2 values are shown, with values > 20% indicated in bold. Negative fractions indicate that two explanatory variables have strong and opposite effects on the dependent variable [268].



Fraction	Total bacterial diversity	Phyllobacteriaceae	Rhizobiaceae	*Labrenzia*	*Mycoplasma*	*Rickettsia*	Bacteroidetes	Flavobacteriaceae
[a+d+f+g] = X1	**0,27**	-0,19	0,16	**0,25**	0,04	**0,57**	0,14	**0,68**
[b+d+e+g] = X2	**0,28**	-0,11	-0,06	0,16	0,08	-0,01	**0,42**	**0,99**
[c+e+f+g] = X3	0,07	-0,01	0,04	0,14	-0,03	-0,08	-0,10	**0,31**
[a+b+d+e+f+g] = X1+X2	**0,34**	-0,73	0,02	**0,50**	0,05	**0,49**	**0,44**	**1,00**
[a+c+d+e+f+g] = X1+X3	**0,26**	-0,33	0,11	**0,26**	-0,07	**0,49**	**0,33**	**0,73**
[b+c+d+e+f+g] = X2+X3	**0,23**	-0,17	-0,19	0,06	0,06	-0,16	**0,47**	**0,99**
[a+b+c+d+e+f+g] = All	**0,36**	-1,05	0,10	**0,47**	**0,27**	**0,33**	**0,69**	**1,00**
[a] = X1 | X2+X3	0,13	-0,87	**0,29**	**0,41**	**0,21**	**0,49**	**0,22**	0,00
[b] = X2 | X1+X3	0,10	-0,71	-0,01	**0,22**	**0,34**	-0,16	**0,36**	**0,27**
[c] = X3 | X1+X2	0,02	-0,31	0,08	-0,03	**0,22**	-0,16	**0,25**	0,00
[d]	0,06	**0,54**	-0,22	-0,29	-0,24	0,08	**0,21**	**0,41**
[e]	-0,03	0,17	-0,13	0,03	-0,33	0,08	-0,06	0,04
[f]	-0,07	**0,25**	-0,21	-0,06	-0,24	0,01	-0,20	0,00
[g]	0,15	-0,11	**0,29**	0,20	**0,32**	-0,02	-0,09	**0,27**
[h] = Residuals	**0,64**	**2,05**	**0,90**	**0,53**	**0,73**	**0,67**	**0,31**	0,00



Fraction	Total bacterial diversity	Phyllobacteriaceae	Rhizobiaceae	*Labrenzia*	*Mycoplasma*	*Rickettsia*	Bacteroidetes	Flavobacteriaceae
[a+b] = X1	**0,30**	-0,03	0,09	**0,21**	**0,20**	**0,48**	0,16	**0,58**
[b+c] = X2	**0,23**	-0,11	-0,06	0,16	0,08	-0,01	**0,42**	**0,99**
[a+b+c] = X1+X2	**0,36**	-0,39	-0,07	**0,53**	**0,21**	**0,38**	**0,49**	**1,00**
[a] = X1|X2	0,13	-0,28	0,00	**0,38**	0,12	**0,40**	0,07	0,01
[b]	0,17	**0,26**	0,09	-0,16	0,08	0,08	0,08	**0,58**
[c] = X2|X1	0,06	-0,36	-0,16	**0,32**	0,00	-0,10	**0,34**	**0,42**
[d] = Residuals	**0,64**	**1,39**	**1,07**	**0,47**	**0,79**	**0,62**	**0,51**	0,00

3.2.4. Axenic cultivation of the *Bryopsis* host and *in vitro* isolation of intracellular bacteria

Abstract

Interactions between different organisms can exhibit diverse forms of interdependency among the partners. The symbiosis can be obligate, resulting in associates which are mutually dependent for survival and can no longer be cultivated separately. In this case, the host cannot be 'cured' of its symbionts by treatment with antibiotics without severe consequences for its fitness and/or development, and neither can the symbionts themselves be isolated in pure culture. Many associations among organisms, however, have a more transient nature. In these facultative symbioses, the partners can survive without each other and can be cultivated separately *in vitro* [8, 232]. Likewise, the *Bryopsis*-intracellular bacterial partnership shows several interdependency modes. The algal endophytic bacterial community consists of both generalist (e.g. *Labrenzia*, Rhizobiaceae and Phyllobacteriaceae) and specialist (e.g. Flavobacteriaceae and Bacteroidetes) species which display a facultative and more obligate endobiotic lifestyle, respectively (see sections 3.2.2 [245] and 3.2.3). To examine the interdependency of the *Bryopsis* host and the endophytic bacterial partners in more detail, several attempts were made to culture them separately. This section describes preliminary experiments to 'cure' *Bryopsis* algae of endophytes and tryouts to culture the (facultative) endophytic bacteria on solid and liquid media mimicking the algal host. While antibiotics seemed not sufficient to 'cure' the *Bryopsis* host from its intracellular bacteria, cultivation attempts in liquid media supplement with *Bryopsis* extract and inhibitors for gram-positive bacteria yielded the growth of *Labrenzia* and Phyllobacteriaceae bacteria. In addition, bacterial epi- and periphytes associated with *Bryopsis* algae which were cultured during the cultivation tests are reported.

Materials and methods

Axenic cultivation of the Bryopsis host

The interdependency among *Bryopsis* algae and bacteria as well as the physiological role of the endophytic flora can only be determined when the host is free of epiphytic contamination and (partially) 'cured' of its endophytes. Therefore, it is essential to cultivate *Bryopsis* axenically. A successful surface sterilization protocol has been developed for *Bryopsis* cultures based on a combined mechanical, chemical and enzymatic approach (see section 3.1.2 [187]). The utility of this for cultivation purposes, however, is limited as non-viable algal samples are obtained at the end of the treatment. Axenic culturing techniques that make use of antibiotics [31, 177, 178, 269], on the other hand, might eliminate bacteria without affecting the viability of algal cells, allowing subsequent *in vitro* experiments. To obtain axenic *Bryopsis* cultures, unialgal samples 4583, 4718, FL1173, ODC1380 and TZ170 (see Supplementary Table S3.2, p. 100) were treated with an antibiotic mixture following a protocol by Droop [177]. The antibiotic stock solution was prepared by adding Penicillin (5000 μg/ml), Streptomycin (800 μg/ml), Chloramphenicol (400 μg/ml) and Kanamycin (100 μg/ml) to 1x modified Provasoli enriched seawater (PES) [181]. This stock solution was sterilized through a 0.2 μm cellulose filter and serial diluted, i.e. 1:1, 2:1, 4:1, 8:1 and 16:1 (PES medium:antibiotic stock solution). *Bryopsis* samples were treated with antibiotic dilutions for 24 hours under the culture conditions described in section 3.1.2 [187] and subsequently transferred to fresh, sterile PES medium for one week. Axenicity of *Bryopsis* cultures was tested by plating small algal filaments on PES medium supplemented with 1% agar. Inoculated plates were incubated for two weeks under the culture conditions described above and checked for bacterial growth and algal viability by means of a binocular microscope. To verify the cultivation results at a molecular level, denaturing gradient gel electrophoresis (DGGE) analyses were performed on algal extracts as described in section 3.1.2 [187].

Cultivation of Bryopsis (facultative) endophytic bacteria

Experiment 1: Plating of *Bryopsis* cytoplasm on solid agar media

In this experiment the cytoplasm of rinsed *Bryopsis* samples 4583, 4718, BR, HVGoes, MX164, MX344 and ODC1380 (see Supplementary Table S3.2, p. 100) was isolated by centrifugation as described by Berger & Kaever [180] and subsequently plated on four different agar media, i.e. Marine Agar (MA, Becton Dickinson), Nutrient Agar (NA, Oxoid), Tryptone Soya Agar (TSA, Oxoid) and Yeast Mannitol Agar (YMA), a medium proposed by Vincent [270] to isolate traditional rhizobia. All

plates were incubated at 20°C and colonies with different morphologies were isolated and purified. Pure cultures were cryopreserved at -80°C in broth medium supplemented with 15% glycerol. Genomic DNA of each isolate was extracted using the alkaline lysis method following Baele *et al.* [271] and these DNA extracts were subsequently subjected to DGGE analyses as described in section 3.1.2 [187] to dereplicate the isolates and to obtain an initial identification. To achieve this latter, DGGE analyses were performed with a known marker containing V3 16S rRNA gene fragments of *Bryopsis* bacterial endophytes as a standard (see Supplementary Figure S3.2, p. 81). DGGE banding patterns were normalized and comparatively clustered using Dice similarity coefficients in the software package BioNumerics v5.1 (Applied Maths, Belgium). To determine the phylogenetic position of the isolates, full length 16S rRNA gene sequences of representatives of each cluster were amplified and sequenced as described in section 3.2.1 [225].

Experiment 2: Plating of *Bryopsis* cytoplasm on solid agar media mimicking the algal host

Based on the results of experiment 1, *Bryopsis* samples HVGoes and ODC1380 as well as the media NA and TSA were omitted from this new experiment. Accordingly, the cytoplasm of samples 4583, 4718, BR, MX164 and MX344 was re-isolated by centrifugation as described above and plated on MA and YMA plates supplemented with *Bryopsis* extract. Inoculated plates were incubated at 20°C and bacterial isolates were screened as described above.

Experiment 3: Cultivation of *Bryopsis* endophytes in liquid media mimicking the algal host

To simulate the *Bryopsis* host environment even more and to tackle epiphytic contamination, attempts were made to culture the bacterial endophytes in liquid media (Table 3.6) supplemented with *Bryopsis* extract and a component (i.e. eosine/methylene blue or deoxycholic acid sodium salt) that inhibits growth of Gram-positive bacteria. Since it has been shown that *Bryopsis* facultative bacterial endophytes are also present in the epiphytic and surrounding cultivation water (see section 3.2.2 [245]), liquid media were inoculated with four subsamples of *Bryopsis* cultures 4718 and MX19 (see Supplementary Table S3.2, p. 100): (i) cytoplasm, (ii) cytoplasm + epiphytes, (iii) cultivation water and (iv) washing water. Subsample (i) (cytoplasm) was obtained by washing the *Bryopsis* thalli by repeatedly vortexing in sterilized artificial seawater (ASW) for two minutes. Surface sterilization was performed following a protocol modified from Burke *et al.* [171]. *Bryopsis* thalli were placed into 485 µl phosphate buffered saline (PBS) supplemented with 10 µl ethylenediaminetetraacetic acid (EDTA) and 5 µl filter-sterilized multienzyme cleaner (ox-gall soap, Dr. Beckmann). Samples were incubated

for 2 hours at room temperature and 80 rpm and subsequently vortexed for 2 min. Algal material was separated from the remaining liquid, rinsed and crushed with a pestel to release the cytoplasm. To acquire subsample (ii) (cytoplasm + epiphytes), *Bryopsis* thalli were washed as described above and directly crushed. The washing water of both subsamples (i) and (ii) was collected to form subsample (iv) (washing water). The cultivation water subsample (iii) was obtained by collecting seawater in which *Bryopsis* samples were cultured for a period of one month. Of subsample (iii) and (iv) 150 ml was centrifuged and the pellet obtained was resuspended in 4 ml ASW and used as inoculum. One ml of each subsample was homogenized in 9 ml of all liquid media (see Table 3.6). Subsequently, serial dilutions were made (10^{-1} till 10^{-3} for subsamples i and ii, and 10^{-1} till 10^{-5} for subsamples iii and iv) and incubated at 20°C. Liquid cultures were screened for (endophytic) bacterial growth by DGGE as described above and 100 µl of the 'endophytic growth positive' tubes was plated on solid agar media. Inoculated plates were incubated at 20°C and bacterial isolates were screened as described above. In addition, DGGE bands at positions of endophytic marker bands were excised, sequenced and identified as described in section 3.2.1 [225]. To verify their true correspondence with *Bryopsis* endophytes, the 16S rRNA gene sequences of the excised bands and isolates were aligned and clustered with previously obtained endophytic bacterial sequences (see sections 3.2.1 [225] and 3.2.3) using BioNumerics.

Table 3.6: Composition of liquid cultivation media applied in experiment 3.

Medium	Nutrient composition	Gram-positive growth inhibitor	pH
A	720 ml water 0.274 g Marine broth (Difco) 90 ml *Bryopsis* extract	0.324 g eosine Y 0.0526 g methylene blue	7
B	720 ml natural seawater 90 ml *Bryopsis* extract	0.324 g eosine Y 0.0526 g methylene blue	7
C	720 ml *Bryopsis* cultivation water 90 ml *Bryopsis* extract	0.324 g eosine Y 0.0526 g methylene blue	7
D	720 ml water 0.274 g Marine broth (Difco) 90 ml *Bryopsis* extract	2 g deoxycholic acid sodium salt	7

Results and discussion

Axenic cultivation of the Bryopsis host

Only *Bryopsis* samples treated with the lowest antibiotic dilution (i.e. 1 part PES medium at 1 part antibiotic stock solution) showed no bacterial growth on agar plates. Higher dilutions 2:1, 4:1, 8:1 and 16:1 were ineffective in eliminating epiphytic bacterial contamination. Microscopic observations revealed all samples were viable after the antibiotic treatment and showed no reduction in growth rate. However, when *Bryopsis* samples treated with the 1:1 antibiotic solution were screened with DGGE, some epiphytic bacteria still seemed present on the *Bryopsis* surface (Fig. 3.20). The antibiotic treatment was effective in reducing the epiphytic communities (treated sample versus untreated control sample, Fig. 3.20), but the DGGE fingerprints of treated samples still showed several non-endophytic DGGE bands (sample treated with antibiotics versus chemical and enzymatic sterilized sample, Fig. 3.20). These remaining epiphytic bacterial bands (asterisks on Fig. 3.20) mainly correspond with gammaproteobacterial, i.e. *Alteromonas, Pseudoalteromas* and *Thalassomonas*, species as determined by sequence analysis.

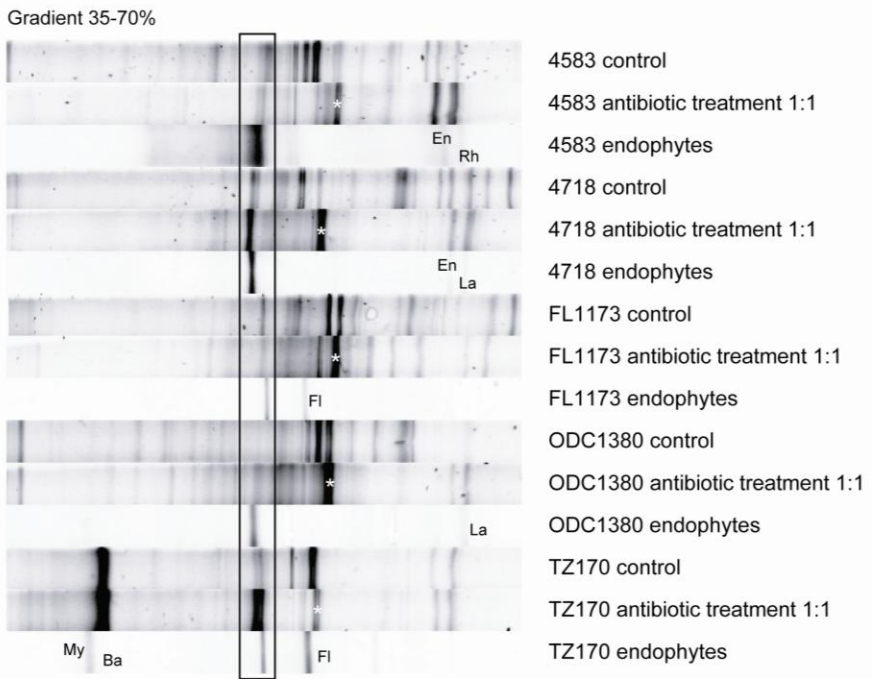


Figure 3.20: **Visual comparison of normalized DGGE fingerprints obtained from untreated control, treated (antibiotic treatment 1:1) as well as surface sterilized (endophytes) *Bryopsis* samples.** Bacterial endophytes are indicated with Ba (Bacteroidetes), En (*Ensifer*), Fl (Flavobacteriaceae), La (*Labrenzia*), My (*Mycoplasma*) and Rh (*Rhizobium*). White asterisks denote bands which were excised from the polyacrylamide gel and subsequently sequenced. The black box indicates chloroplasts V3 16S rRNA gene fragments as verified by DNA sequencing.

Significantly, none of the *Bryopsis* endophytes present (e.g. *Ensifer, Labrenzia, Mycoplasma, Rhizobium*, Bacteroidetes and Flavobacteriaceae species) were eliminated by the antibiotic mixture used, proving this antibiotic treatment is inadequate to 'cure' *Bryopsis* algae of their endophytic flora. Longer exposure times, other antibiotic mixtures and concentrations, or a combination of antibiotic use with cell wall penetrating substances might be more effective in future axenicity experiments. Also the giant-cell morphology and special regeneration mechanisms of *Bryopsis* (see Chapter 1, Box 3) may offer some axenic cultivation options. Although it has been shown that these characteristics are insufficient to generate true axenic algal cultures (section 3.1.2 [187]), a combined approach of these features with antibiotic use might successfully eliminate epiphytes and (partially) 'cure' *Bryopsis* algae from bacterial endophytes. Furthermore, the observation that *Bryopsis* cultures can lose their Phyllobacteriaceae endophytes after one year cultivation (section 3.2.2 [245]), may hint at a potential to 'cure' algal samples from endophytes by adjustments of the cultivation medium and/or serial wounding events. Instead of axenicity experiments on *Bryopsis* gametophytes only, also other life cycle phases, such as gametes, could be axenically cultivated. Wichard and colleagues (unpublished data), for example, developed an effective protocol to obtain axenic *Ulva* gametes by means of a phototaxis regulated separation between gametes and bacterial cells.

Cultivation of Bryopsis (facultative) endophytic bacteria

All cultivation experiments yielded growth of mostly *Bryopsis* epi- and periphytic bacteria (Fig. 3.21A-C, Table 3.7). Only the liquid culture attempts of experiment 3 were successful in growing *Labrenzia* and Phyllobacteriaceae endophytes *in vitro.* Of these latter bacterial species, merely *Labrenzia* could be isolated in pure culture. Endophytic *Labrenzia* isolates were cultivated on media A, B and C (see Table 3.6) inoculated with all types of *Bryopsis* subsamples and cluster together with *Labrenzia* sequences previously obtained from *Bryopsis* samples (see section 3.2.3 and Fig. 3.22). Phyllobacteriaceae endophytes, on the other hand, could be grown in a mixed liquid culture (medium D) as shown by clustering of DGGE band 'liquid culture MX19 (i) D' with endophytic Phyllobacteriaceae sequences in Figure 3.22, but could not be subsequently isolated on agar plates. This suggests Phyllobacteriaceae endophytes may require some metabolites and/or signaling molecules from other co-cultivated bacteria for their growth. Consequently, diffusion chambers [272, 273] or the augmentation of solid growth media with specific nutrients and/or cell-free extracts derived from helper strains [274] might stimulate the monoculture of these Phyllobacteriaceae endophytes in further experiments.

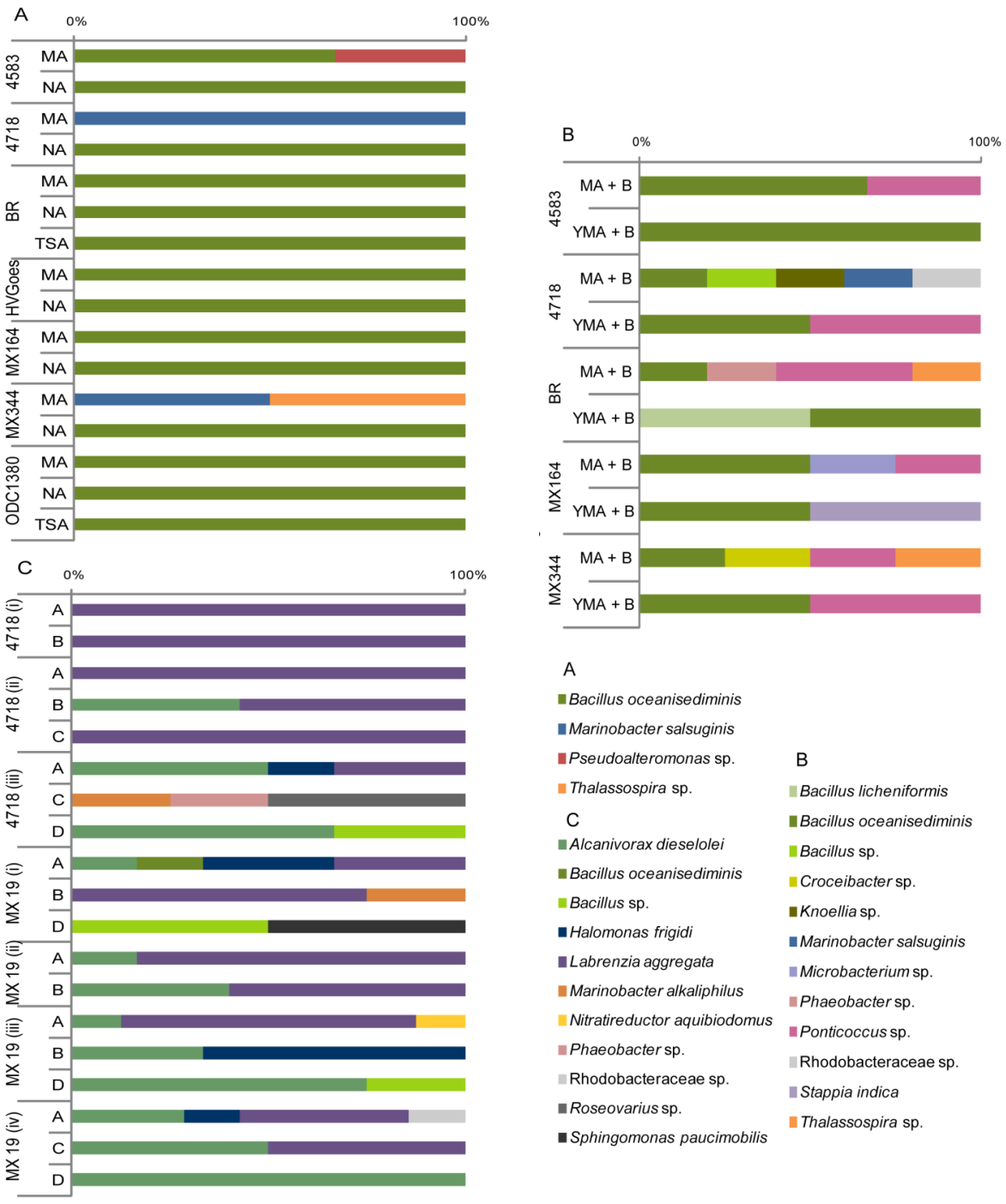


Figure 3.21: **Overview of bacterial isolates obtained from cultivation experiments on solid media (A), on solid media with *Bryopsis* extract (B) and on solid media after an initial liquid culture step (C).** Media applied: Marine Agar (MA), Marine Agar supplemented with *Bryopsis* extract (MA + B), Nutrient Agar (NA), Tryptone Soya Agar (TSA), Yeast Mannitol Agar (YMA), Yeast Mannitol Agar supplemented with *Bryopsis* extract (YMA + B), and three newly designed media A, B and C (see Table 3.6). Inocula: (A and B) cytoplasm of *Bryopsis* samples 4583, 4718, BR, HVGoes, MX164, MX344 and ODC1380; and (C) subsamples of *Bryopsis* cultures 4718 and MX19: (i) cytoplasm, (ii) cytoplasm + epiphytes, (iii) cultivation water and (iv) washing water.

Over all, experiment 3 allowed the most growth of *Bryopsis* associated bacteria (Fig. 3.21C). Not unexpected, as liquid cultivation techniques using media with low organic matter concentrations have been demonstrated to considerably improve the bacterial culturability [275 and references therein]. The solid media used in experiments 1 and 2 were apparently too nutrient-rich, resulting in an overgrowth of mainly Gram-positive *Bacillus* species at the expense of endophytic bacterial cultivation [274]. This drawback has been overcome in experiment 3 by the use of Gram-positive growth inhibitors such as eosine/methylene blue and deoxycholic acid sodium salt as well as dilute nutrient media. Nevertheless, cultivation attempts could be further elaborated and optimized in the future to better suit the growth of *Bryopsis* endophytic bacteria. For example, the natural environment of the endophytic bacteria, i.e. the host internal conditions, could be mimicked even more by adding photosynthetic metabolites to the culture media. Watanabe *et al.* [276] developed such an effective artificial medium supplemented with organic carbon and nitrogen which imitates the nutritional conditions surrounding algae to favor the growth of photosynthate-dependent epiphytic bacteria. The cultivation of bacteria with an intracellular life-style, however, presents a particular challenge as it remains difficult to determine and reproduce the environmental conditions required for metabolic activity [274].

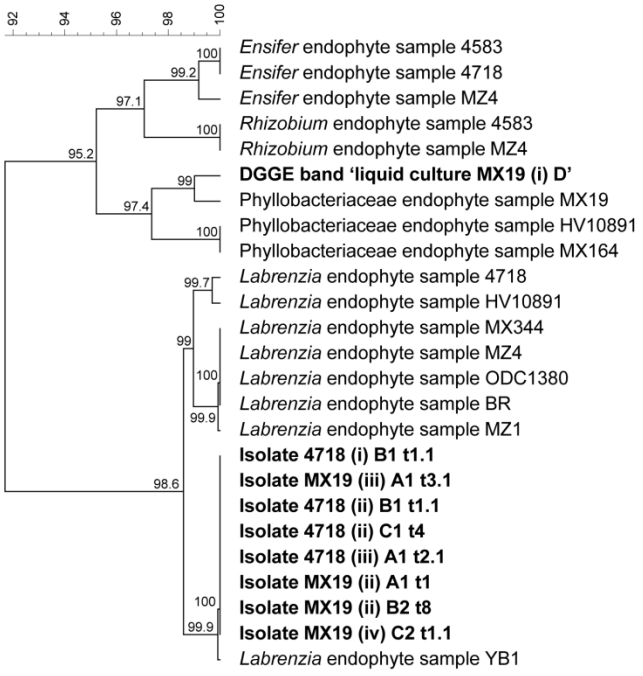


Figure 3.22: **UPGMA dendrogram of endophyte 16S rRNA gene sequences previously determined (in bold, see section 3.2.3) as well as sequenced endophytic isolates and liquid culture DGGE bands.** Numbers at the branch nodes represent sequence similarity values.

Cultivation of Bryopsis epi- and periphytic bacteria

Besides the targeted endophytic bacterial growth, cultivation experiments also yielded the growth of *Alcanivorax, Bacillus, Croceibacter, Halomonas, Knoellia, Marinobacter, Microbacterium, Nitratireductor, Phaeobacter, Ponticoccus, Pseudoalteromonas, Roseovarius, Sphingomonas, Stappia, Thalassospira*, and undetermined Rhodobacteraceae bacterial species associated with the *Bryopsis* surface and/or surroundings (Fig. 3.21A-C, Table 3.7). Most of these bacterial species have been previously isolated from seaweed surfaces (see Chapter 1) and are known to possess cell wall degrading (e.g. *Alcanivorax* [277], *Bacillus* [278], *Halomonas* [279], *Marinobacter* [280], *Pseudoalteromonas* [279] and *Sphingomonas* [58]), pathogenic (*Halomonas* [120] and *Pseudoalteromonas* [120, 144]), morphogenic (*Bacillus* [80] and *Pseudoalteromonas* [80]), growth promoting (*Bacillus* [74] and *Pseudoalteromonas* [73]) and antimicrobial (*Bacillus* [29, 93, 134], *Microbacterium* [134], *Phaeobacter* [29, 90] and *Pseudoalteromonas* [29, 88, 93]) properties. As several of these isolates are related to up to now uncultivated clones and possibly represent new bacterial species (Table 3.7), they may offer great opportunities for future biodiscovery research [272].

In conclusion, it is only through the isolation of individual (facultative) symbiotic partners that a comprehensive characterization of their interdependency and physiological properties can be undertaken. Axenic cultivation of the host as well as the isolation of the bacterial partners *in vitro* may open the way for further infection studies [281] which address the interdependency of the *Bryopsis*-bacterial association and can give an insight into the uptake mode and potential function(s) of the intracellular bacteria. In addition, even in this age of high-throughput sequencing, the establishment of pure bacterial cultures remains essential for a full physiological and taxonomic characterization and provides almost the only way to discover the applied potential of bacterial species [272].

Acknowledgements

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Table 3.7: **Taxonomic identification of cluster representative isolates obtained in all three cultivation experiments.**

Isolate	Closest matching strains in BLAST	AN	QC	MI	Ecology
4718 (iii) A1 t3	*Halomonas* sp. BCw150	FJ889581	99%	99%	Arctic seawater
R-49267	*Halomonas* sp. BCw077	FJ889579	99%	99%	Arctic seawater
	Halomonas sp. Ko501	AF550585	99%	99%	Basalt at Kolbeinsey Ridge
MX19 (i) D2 t1	*Sphingomonas* sp. SKJH-30	AY749436	100%	100%	LN2 vessels for long term banking of genome resources
R-49268	Uncultured bacterium clone S25_1428	EF575084	99%	100%	Site S25 near Coco's Island (Costa Rica)
	Uncultured bacterium clone sb21.43	HQ904214	99%	100%	Anaerobic degradation of *Microcystis* blooms
4718 (ii) B1 t1.2	*Alcanivorax* sp. TE-9	AB055207	100%	100%	Sea water sampled from the Sea of Japan
R-49269	*Alcanivorax dieselolei* strain 2W806	AB453732	99%	100%	Ago Bay (Japan)
	Alcanivorax dieselolei strain N1203	AB453731	99%	100%	Ago Bay (Japan)
4718(iii) C5 t5	*Marinobacter alkaliphilus* strain aa-11	EU652042	99%	99%	Ocean sediment (hot springs close to Xiamen Sea)
R-49270	*Marinobacter* sp. E407-9	FJ169969	99%	99%	Sediment of the South China sea
	Marinobacter alkaliphilus strain 2PR56-13	EU440994	99%	99%	Southwest Indian Ocean deep sea water column
4718 MA + B f	*Marinobacter* sp. PR52-13	EU440976	99%	100%	Southwest Indian Ocean deep sea water column
R-49271	*Marinobacter* sp. 2PR57-9	EU440985	99%	99%	Southwest Indian Ocean deep sea water column
	Marinobacter sp. MARC4S	DQ768634	99%	99%	Deep sea sediments of the Middle Atlantic Ridge
4583 MA c	Uncultured *Pseudoalteromonas* sp. clone IerC24-21	HQ161521	100%	100%	Chemotactic enrichment + carbon substrates sample
R-49272	Bacterium 4746K8-B14	HQ640932	100%	100%	Cold-water coral *Lophelia pertusa*
	Bacterium 4873K4-B11	HQ640926	100%	100%	Cold-water coral *Lophelia pertusa*
MX164 YMA + B f	Uncultured bacterium clone OTU9	AB576903	100%	99%	Denitrifying PEG pellet samples
R-49273	Uncultured *Stappia* sp. clone MJ33	GU212810	100%	99%	Ballast water at Ningbo port
	Uncultured bacterium clone 15A7	FJ711763	100%	99%	Tropical brackish water system
4718 (i) B1 t1	Alphaproteobacterium MBIC3993	AB025419	99%	100%	Isolated from Ponape Island (western Pacific Ocean)
R-49274	Alphaproteobacterium MBIC1535	AB023435	99%	100%	Isolated from Ponape Island (western Pacific Ocean)
	Labrenzia aggregata strain 2PR58-2	EU440961	99%	100%	Southwest Indian Ocean deep sea water column
MX19 (iii) A1 t3.2	*Nitratireductor aquibiodomus* strain PR57-9	EU440986	100%	99%	Southwest Indian Ocean deep sea water column
R-49275	*Nitratireductor aquibiodomus* type strain NL21T	AF534573	100%	99%	Marine methanol-fed denitrification reactor
	Mesorhizobium sp. TUT1018	AB098586	100%	99%	Fed-batch reactor
MX19 (iv) A1 t3	*Roseovarius aestuarii* type strain SMK-122T	EU156066	100%	95%	Tidal flat of the Yellow Sea in Korea
R-49276	Uncultured marine bacterium clone BM1-F-85	FJ826183	99%	95%	Sea water after diatom bloom in the Yellow Sea
	Pseudoruegeria aquimaris type strain SW-255T	DQ675021	100%	95%	Seawater of the East Sea in Korea
4718 (iii) C5 t3	Uncultured bacterium clone A8W_70	HM057815	99%	99%	Ocean water from the Yellow Sea
R-49277	Bacterium DG874	AY258075	96%	100%	*Gymnodinium catenatum*
	Bacterium DG878	AY258077	95%	100%	*Gymnodinium catenatum*
4718 (iii) C5 t2	Uncultured Rhodobacteraceae bacterium clone xmg-9	HM116852	99%	98%	Phenathrene-enriched consortium in sea water
R-49278	*Phaeobacter caeruleus* type strain LMG 24369T	AM943630	98%	97%	Marine electroactive biofilm
	Leisingera aquimarina type strain LMG 24366T	AM900415	98%	97%	Marine electroactive biofilm

Isolate	Closest matching strains in BLAST	AN	QC	MI	Ecology
MX344 MA + B b	Rhodobacteraceae bacterium CSQ-8	EF512131	95%	99%	*Isochrysis galbana*
R-49279	*Ponticoccus litoralis* type strain CL-GR66	EF211829	95%	98%	Coastal seawater
	Uncultured alpha proteobacterium clone 06-03-31	DQ153131	100%	96%	Surface biofilm in estuarine seawater
MX344 MA + B c	*Thalassospira* sp. H94	FJ903195	100%	99%	Seawater
R-49280	*Thalassospira* sp. H88	FJ903193	100%	99%	Seawater
	Thalassospira sp. MCCC 1A02060	EU440820	99%	99%	Ocean seawater
4718 MA + B b	*Knoellia subterranea* strain CCGE2276	EU867301	100%	99%	*Phaseolus vulgaris* endophyte
R-49281	Uncultured actinobacterium clone D3E05	EU753661	98%	99%	Dry stromatolite
	Knoellia sp. DMZ1	HQ171909	97%	99%	Soil sample in Korea
MX164 MA + B e	*Microbacterium schleiferi* strain 2PR54-18	EU440992	99%	98%	Southwest Indian Ocean deep sea water column
R-49282	Catechol-degrading *Microbacterium* sp. Atl-19	EF028128	99%	98%	Unknown
	Microbacterium lacticum strain 3388	EU714364	99%	98%	Clinical specimen
MX344 MA + B h	*Croceibacter atlanticus* type strain HTCC2559T	AY163576	100%	100%	Atlantic Ocean
R-49283	Uncultured Bacteroidetes bacterium clone DBS1h1	GQ984357	100%	99%	Surface water in the Northern Bering Sea
	Marine arctic deep-sea bacterium FI7	AJ557873	99%	99%	Arctic deep sea
MX19 (i) D1 t1	*Bacillus* sp. 3559BRRJ	JF327782	100%	99%	Unknown
R-49284	*Bacillus* sp. BZ85	HQ588864	100%	99%	Soil containing high amounts of oil and heavy metals
	Bacillus arsenicus strain S8-14	EU624418	99%	99%	Palk Bay sediments
MX19 (i) A2 t3	*Bacillus* sp. strain CCMM B645	FR695470	100%	99%	Salt marsh Lower Loukkos (Larache, Morocco)
R-49285	*Bacillus* sp. strain CCMM B655	FR695469	100%	99%	Salt marsh Lower Loukkos (Larache, Morocco)
	Bacillus firmus strain MC1B-14	AY833571	100%	99%	Epilithic biofilms from a subtropical rocky shore
4718 (iii) D1 t2	*Bacillus* sp. F15(2011)	HQ323453	99%	98%	Air in the Mogao Grottoes, Dunhuang, China
R-49286	*Bacillus* sp. 19495	AJ315063	100%	97%	Mural paintings in the Servilia tomb
	Bacillus sp. 19493	AJ315061	100%	97%	Mural paintings in the Servilia tomb
4718 MA + B c	Uncultured Firmicutes bacterium clone M0027_082	EF071363	100%	99%	Human colonic mucosal biopsy
R-49287	Eubacterium sp. 11-12	EU571159	99%	99%	Soil from the Amazonas, outside the city of Manaus
	Uncultured bacterium clone Hg1bB9	EU236314	98%	99%	*Haliclona* cf. *Gellius* sp.
BR YMA + B h	*Bacillus licheniformis* strain AIS70	GU967451	100%	99%	Clay mine
R-49288	*Bacillus licheniformis* strain AIS53	GU967448	100%	99%	Clay mine
	Bacillus licheniformis strain AIS39	GU967447	100%	99%	Clay mine

AN: accession number
QC: query coverage
MI: maximum identity

Part 3.3 Endophytic bacterial communities of natural *Bryopsis* samples

3.3.1. Flavobacteriaceae endosymbionts within natural *Bryopsis* samples: host specificity and cospeciation

Joke Hollants, Frederik Leliaert, Heroen Verbruggen, Olivier De Clerck and Anne Willems. Complex pattern of coevolution of a Flavobacteriaceae endosymbiont and its green algal host, *Bryopsis*. *Manuscript in preparation.*
Author contributions: The first two authors have equally contributed to the study. JH designed and performed the experiments. FL analyzed the data and outlined the figures. JH and FL wrote the manuscript. FL, HV and ODC collected the algal specimens. ODC and AW commented on the manuscript.

Abstract

The siphonous green seaweed *Bryopsis* harbors complex intracellular bacterial communities, of which certain Flavobacteriaceae species form a close, obligate association with the algal host. Culture studies have indicated a strict vertical transmission of Flavobacteriaceae endosymbionts from one host generation to the next, suggestive of host-symbiont cospeciation. To address this hypothesis we optimized a PCR protocol to directly and specifically amplify Flavobacteriaceae endosymbiont 16S rRNA gene sequences, which allowed us to screen a large number of algal samples without the need for cultivation or surface sterilization. We analyzed 146 samples belonging to the genus *Bryopsis*, and 92 additional samples belonging to the Bryopsidales and other orders within the class Ulvophyceae. Our results indicate that the Flavobacteriaceae endosymbionts are restricted to *Bryopsis*, and only occur within specific warm-temperate and tropical clades of the genus. Comparative analyses of the bacterial 16S rRNA and *Bryopsis rbc*L gene datasets show a complex host-symbiont evolutionary association with some degree of cospeciation. Our results provide evidence for a tight, highly specific symbiosis between the partners in which the endosymbionts are likely to fulfill significant functions.

Introduction

Bacteria living within the body or cells of eukaryotes are extremely abundant and widespread [216, 230, 232]. These endosymbiotic bacteria often contribute to diverse metabolic host functions, making their presence favorable or even essential [8]. Eventually, both the bacterial partner and the host may lose their autonomy and become dependent on each other, resulting in an obligate association [232, 282]. Obligate endosymbiotic bacteria have been shown to form highly host-specific interactions that are maintained across host generations over long periods of time by vertical transmission [283, 284]. This latter process might give rise to cospeciation, an evolutionary process resulting in congruent host and bacterial phylogenies [285].

In seaweed-bacterial associations, coevolution has only been suggested between the red alga *Prionitis* and its gall-forming *Roseobacter* symbionts [123]. In the siphonous green seaweed *Bryopsis*, bacteria have been visualized by electron microscopy in both vegetative thalli and gametes, suggesting a close, specific association between the algal host and bacterial endophytes [36]. Recently, molecular results showed that geographically diverse *Bryopsis* samples harbor well-defined and rather stable endophytic bacterial communities consisting of a mix of generalist and specialist species (sections 3.2.1, 3.2.2 [225, 245] and 3.2.3). Of these bacteria, only Flavobacteriaceae symbionts displayed an obligate endobiotic lifestyle and were never isolated from the *Bryopsis* surface and surrounding seawater (section 3.2.2 [245]). The family Flavobacteriaceae is a large group of bacteria with diverse eco-physiological characteristics [286]. They are known to decompose polysaccharides such as agar, cellulose and carrageenan, making them key players in biotransformation and nutrient recycling processes in the marine environment [4 and references therein, 286]. Because of these traits, Flavobacteriaceae bacteria often inhabit seaweed surfaces where they have been shown to possess antimicrobial [29, 93], pathogenic [94, 151, 287], and algal morphogenic as well as zoospore settlement inducing [78-81, 86] capabilities. Many members of the family Flavobacteriaceae, like *Algibacter*, *Fucobacter*, *Maribacter*, and *Ulvibacter* species, have been initially isolated from marine macroalgal surfaces [4]. In addition, several intracellular bacterial symbionts of insects belong to the family Flavobacteriaceae and were shown to affect their hosts' reproduction [286 and references therein]. In *Bryopsis*, the presence of Flavobacteriaceae was found to be highly congruent with the host phylogeny of two warm-temperate to tropical clades (see section 3.2.3). Testing the hypothesis of cospeciation, however, requires a rich and geographically diverse sampling. The experimental design used previously, i.e. labor-intensive unialgal culturing, surface sterilization, clone libraries, and DGGE analyses (see Chapter 3, Part 2 [187, 225, 245]), prevented high throughput screening of *Bryopsis* associated Flavobacteriaceae endosymbionts.

In this study, we aimed to assess the host specificity and evolution of Flavobacteriaceae endosymbionts in *Bryopsis*. To achieve this goal we developed a PCR protocol to specifically amplify Flavobacteriaceae endophytic sequences in non-surface sterilized, natural *Bryopsis* samples. To assess the distribution of these Flavobacteriaceae endosymbionts outside *Bryopsis*, we also screened a large number of Bryopsidales and other ulvophytes. Phylogenetic and statistical analyses were performed to address the *Bryopsis*-Flavobacteriaceae cospeciation hypothesis.

Material and methods

Algal material

Supplementary Table S3.5 (p. 130) lists the algal samples which were screened for the presence of Flavobacteriaceae endophytes in this study. The list contains 146 *Bryopsis* samples covering 23 different species and 92 additional samples of Bryopsidales (genera *Avrainvillea, Boodleopsis, Caulerpa, Chlorodesmis, Codium, Derbesia, Halimeda, Rhipilia, Tydemania* and *Udotea*), Dasycladales (*Acetabularia, Bornetella* and *Neomeris*), Cladophorales *(Aegagropila, Anadyomene, Apjohnia, Boergesenia, Boodlea, Chaetomorpha, Cladophora, Cladophoropsis, Dictyosphaeria, Ernodesmis, Microdictyon, Rhizoclonium, Siphonocladus* and *Valonia*) and Ulvales *(Ulva)*. Algal samples were collected during different field expeditions and clean portions of the thalli were preserved in silica-gel.

DNA extraction and PCR amplification

Algal samples were subjected to a total DNA-extraction following a CTAB protocol modified from Doyle and Doyle [160]. To create a *Bryopsis* host phylogeny, chloroplast-encoded *rbc*L genes were amplified as described in section 3.2.1 [225]. For the specific amplification of Flavobacteriaceae endosymbiont 16S rRNA genes, we designed species-specific primers in Kodon v3.5 (Applied Maths, Belgium) with as only target group full length Flavobacteriaceae 16S rRNA gene sequences (JF521600-JF521604, HE648933, HE648935, HE648940, and HE648943) obtained in our previous studies (sections 3.2.1 [225] and 3.2.3). However, due to the large non-target group (i.e. all other bacterial 16S rRNA gene sequences other than Flavobacteriaceae endosymbiont sequences) during primer design, only one suitable region (from position 690 to 720) for specific primer annealing could be found. Consequently, we designed one species-specific primer which we subsequently used in both the forward (F695: 5'-GGCAGGTTGCTAAGCCTTAA-3') as well as reverse (R695: 5'-TTAAGGCTTAGCAACCTGCC-3') direction together with the 16S rRNA gene universal

primers 1492R and 27F [164], respectively. *Bryopsis* DNA extracts from previous studies (see sections 3.2.1 [225] and 3.2.3), with Flavobacteriaceae endosymbiont DNA present or not present, were used as templates for the initial PCR optimization experiments. Thermocycling conditions were investigated using gradient-PCR with the following reaction mix: 1x AmpliTaq Gold reaction buffer (Applied Biosystems), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer and 1.25 units/μl AmpliTaq Gold DNA polymerase (Applied Biosystems). Optimized thermocycling conditions were as follows: one cycle of 95°C for 5 min; 25 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 1 min; one final extension cycle at 72°C for 10 min. PCR amplicons were purified using a Nucleofast 96 PCR clean up membrane system (Machery-Nagel, Germany) according to the manufacturer's instructions and sequenced as described in section 3.2.1 [225]. Flavobacteriaceae endosymbiont 16S rRNA gene sequences were assembled using the BioNumerics 5.1 software (Applied Maths, Belgium), compared with nucleotide databases via BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and chimera-checked using Bellerophon [192]. Sequences were submitted to EMBL under accession numbers HE775438-HE775517.

Phylogenetic analyses of host and symbiont

Two alignments were created for phylogenetic analyses. The *Bryopsis* alignment consisted of 146 *rbc*L sequences and was 1363 bp long, including 100 variable and 85 parsimony informative positions. The 80 Flavobacteriaceae 16S rRNA gene sequences obtained from *Bryopsis* samples were aligned with 15 Flavobacteriaceae type strains and closest BLAST hits using MUSCLE [193]. The resulting alignment was 1470 bp long, including a small number of gaps, and 500 variable and 398 parsimony informative positions.

Models of nucleotide substitution were selected using the Akaike information criterion with JModelTest v0.1.1 [194]. Phylogenetic trees were reconstructed by maximum likelihood (ML) using PhyML v3.0 [195], via the University of Oslo Bioportal website [196]. The *Bryopsis rbc*L and bacterial 16S rRNA gene alignment were analysed under a GTR + G model. Trees were visualized in Mega 4.0 [197] and annotated with Adobe® Illustrator® CS5.

Based on the resulting *Bryopsis* phylogram, 23 species were identified as clades of closely related sequences that are preceded by relatively long, well supported branches [288, 289]. Phylogenetic analysis of the Flavobacteriaceae 16S rRNA gene dataset resulted in a tree with three well supported clades (Fig. 3.23B: A, B1 and B2). Because the internal branches of clade B2 were largely unresolved, the genetic variation within this clade could be represented more appropriately by a network [290]. Statistical parsimony networks [291] were constructed with TCS 1.21 [292], with calculated maximum

connection steps at 95% and alignment gaps treated as missing data. Sequence similarity between the 16S rRNA gene sequences was determined in BioNumerics v5.1 (Applied Maths, Belgium).

Analysis of host-symbiont co-evolution and biogeography

We used different statistical techniques to assess codivergence between Flavobacteriaceae endosymbionts of clade B and the *Bryopsis* host, and to investigate to which degree the bacterial genetic variation was geographically structured.

First, analysis of molecular variance (AMOVA) of Flavobacteriaceae was used to investigate the percentage of variation within and between populations, which were predefined as the different host species (*Bryopsis* spp. 20, 21, 22, 23, 24, 28 and *B. myosuroides*) or geographical regions (E Pacific, Atlantic-Mediterranean, Indian Ocean and W Pacific). Patterns of genetic structuring among *Bryopsis* species and between geographical regions were estimated using Arlequin v3.5.1.3. [293]. Population pairwise Φ_{ST} values, a measure of population differentiation or genetic distance, were calculated using Tamura–Nei distances. Because of small sample sizes, *Bryopsis* spp. 25 and 26 were excluded from the analyses.

Results and discussion

Restricted phylogenetic distribution of Flavobacteriaceae endosymbionts

The newly designed PCR protocol was successful in amplifying Flavobacteriaceae sequences directly from algal DNA extracts. Sequencing resulted in unambiguous electropherograms, indicating the primer designed (i.e. F/R695) is highly specific for the targeted endosymbionts, and suggesting the exclusive presence of one flavobacterial genotype per host plant. This allowed for screening of a large number of algal samples without the need for culturing, surface sterilization, or molecular cloning. Of the 146 *Bryopsis* samples examined, 80 displayed an amplicon on agarose gel. The 16S rRNA gene sequences were most similar (99% BLAST similarity) to Flavobacteriaceae endosymbiont sequences previously obtained from *Bryopsis* (see sections 3.2.1 [225] and 3.2.3). None of the other Bryopsidales and Ulvophyceae algal samples yielded positive amplifications (Supplementary Table S3.5, p. 130), indicating a strong host specificity and an intimate association of the Flavobacteriaceae endosymbionts towards *Bryopsis*.

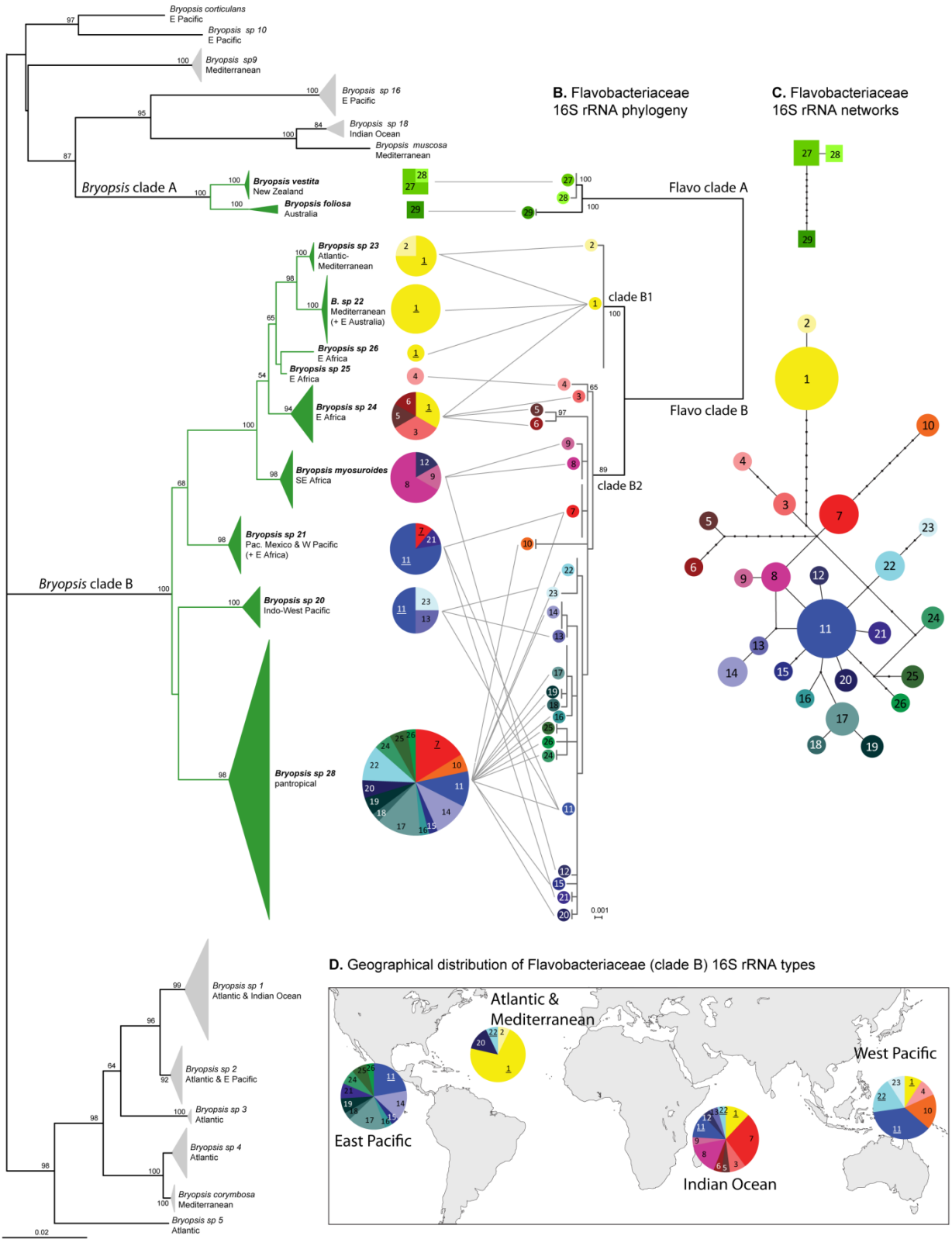
A. Bryopsis rbcL phylogeny and distribution of Flavobacteriaceae 16S rRNA types in host species
Bryopsis corticulans
E Pacific
Bryopsis sp 10
E Pacific
Bryopsis sp9
Mediterranean
Bryopsis sp 16
E Pacific
Bryopsis sp 18
Indian Ocean
Bryopsis muscosa
Mediterranean
Bryopsis clade A
Bryopsis vestita
New Zealand
Bryopsis foliosa
Australia
Bryopsis sp 23
Atlantic-Mediterranean
B. sp 22
Mediterranean (+ E Australia)
Bryopsis sp 26
E Africa
Bryopsis sp 25
E Africa
Bryopsis sp 24
E Africa
Bryopsis myosuroides
SE Africa
Bryopsis sp 21
Pac. Mexico & W Pacific (+ E Africa)
Bryopsis clade B
Bryopsis sp 20
Indo-West Pacific
Bryopsis sp 28
pantropical
B. Flavobacteriaceae 16S rRNA phylogeny
Flavo clade A
clade B1
clade B2
Flavo clade B
0.001
C. Flavobacteriaceae 16S rRNA networks
Bryopsis sp 1
Atlantic & Indian Ocean
Bryopsis sp 2
Atlantic & E Pacific
Bryopsis sp 3
Atlantic
Bryopsis sp 4
Atlantic
Bryopsis corymbosa
Mediterranean
Bryopsis sp 5
Atlantic
0.02
D. Geographical distribution of Flavobacteriaceae (clade B) 16S rRNA types
Atlantic & Mediterranean
West Pacific
East Pacific
Indian Ocean

Figure 3.23: Flavobacteriaceae endosymbiont data (B and C) plotted on the *Bryopsis* host phylogram (A) and geographical distribution of Flavobacteriaceae 16S rRNA types (D). Green colored branches denote positive amplification of Flavobacteriaceae endosymbiont 16S rRNA genes within the respective algal samples. The TCS parsimony network (C) visualizes phylogentic relations among the different Flavobacteriaceae 16S rRNA gene types (numbers 1-29) and each black node represents 1 nucleotide mutation separating genotypes. Colored circles (numbers on these circles refer to sequence types) on pictures B and C indicate endosymbiont genotypes and are in picture C proportionally sized to the number of sequences (i.e. Flavobacteriaceae strains) they represent. These distributions are also represented in the pie charts (B and D) in which the numbers again correspond to the endosymbiont 16S rRNA gene types. ML bootstrap values are indicated at the branch nodes (A and B). The scale bar indicates 0.02 (A) and 0.001 (B) nucleotide changes per nucleotide position.

Mapping of the positive amplifications on the *Bryopsis* host phylogram revealed that Flavobacteriaceae endosymbiont presence is restricted to two separate clades (green branches, Fig. 3.23A): a large clade (B) containing *Bryopsis* species from tropical and warm-temperate regions and a smaller clade (A) including *B. vestita* and *B. foliosa* samples from New Zealand and southern Australia, respectively. The non-monophyly of the *Bryopsis* species containing Flavobacteriaceae (although not strongly supported) either indicates that these endosymbionts were acquired twice independently, or that these bacteria have been lost in one or more *Bryopsis* lineages. Regardless of the robustness of the host phylogeny, the close relationships among the endosymbionts (Fig. 3.24) would suggest a single acquisition of Flavobacteriaceae in *Bryopsis*.

Although our data suggest a preference of Flavobacteriaceae endosymbionts for high temperatures, it is difficult to distinguish if this results from an actual temperature preference of the bacteria or ecological preferences of the host. Host ecological preferences likely play an important role as seaweed species distributions are known to be overwhelmingly limited by seawater temperature regimes [294]. For *Bryopsis*, we previously showed (see section 3.2.3), using variation partitioning analysis, that most of the Flavobacteriaceae endosymbiont presence variation could be explained by host phylogenetic factors, which are inevitably interrelated with environmental factors as a result of 'phylogenetic niche conservatism' [261]. This indicates that the occurrence of Flavobacteriaceae endobionts is mainly structured by their algal host phylogeny instead of an individual ecological preference, and is in agreement with the presumably vertical transmission of these endosymbionts from one *Bryopsis* generation to the next by sexual reproductive stages (see section 3.2.3). Niche conservatism of hosts resulting in temperature-dependent variation of endosymbionts has been described in other eukaryotes, including sponges, squids and insects [295-298].

Flavobacteriaceae genetic diversity

The 80 *Bryopsis*-associated Flavobacteriaceae 16S rRNA gene sequences formed a highly supported monophyletic group, which also included two other sequences from a sponge- and coral-associated uncultured bacterium [217, 218] (Fig. 3.24). This clade was distantly related to cultured Flavobacteriaceae type strains (≤ 87% 16S rRNA gene similarity), confirming our previous observation that the Flavobacteriaceae endosymbionts most likely represent a new genus (section 3.2.1 [225]). The *Bryopsis* associated Flavobacteriaceae fell into two distinct, well supported clades (Fig. 3.23B, Fig. 3.24). Clade A consists of endosymbionts from *Bryopsis vestita* and *B. foliosa*; clade B includes the endosymbionts from the other nine *Bryopsis* species (*B. myosuroides*, and *Bryopsis* spp. 20, 21, 22, 23, 24, 25, 26 and 28). Clade B consists of two subclades: a small clade B1 and a large clade B2 with unresolved internal branches, which can be better represented as a phylogenetic network.

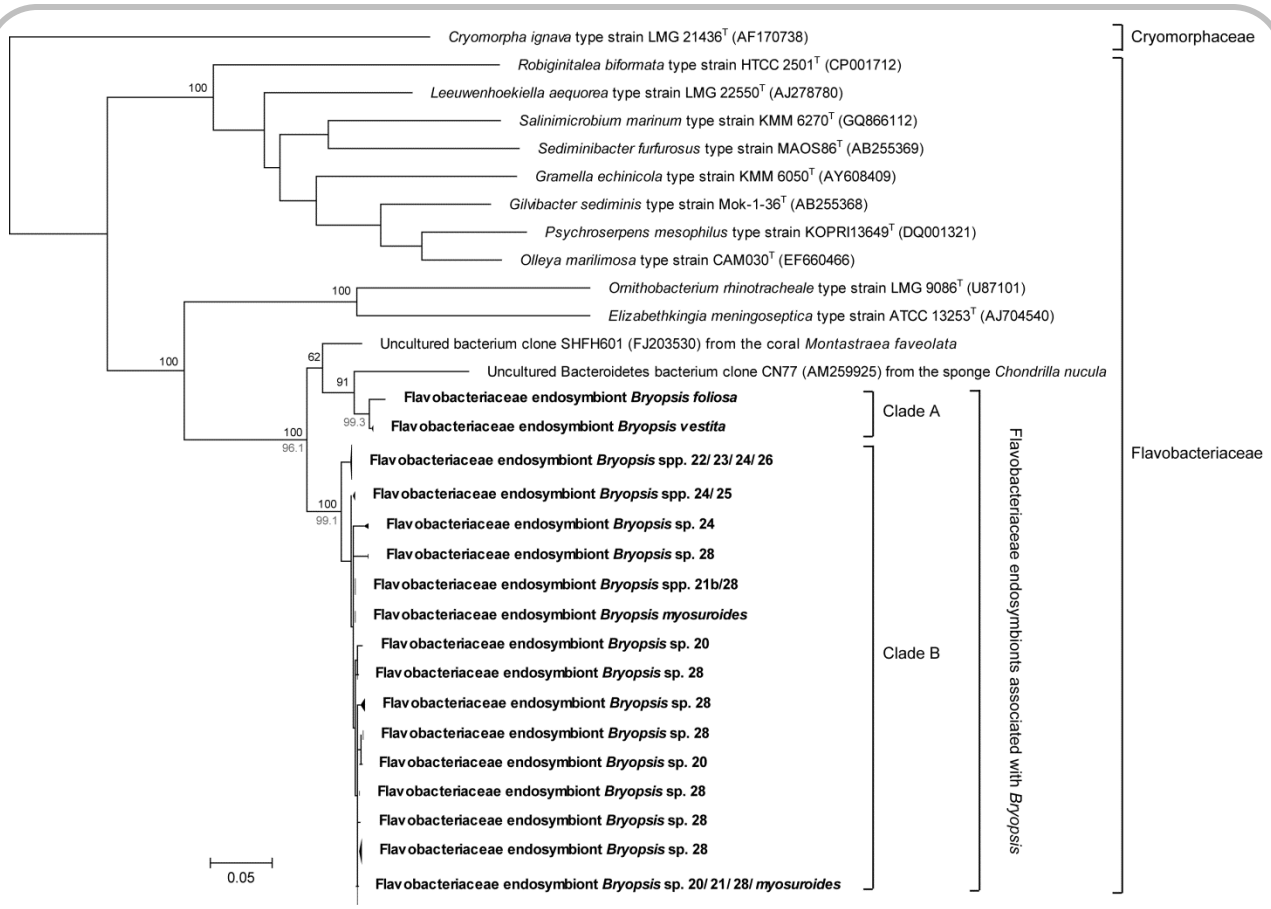

Figure 3.24: Maximum likelihood tree showing the phylogenetic position of *Bryopsis* Flavobacteriaceae endosymbionts. Phylogenies were inferred from 16S rRNA gene sequences determined in this study (in bold), BLAST hits and Flavobacteriaceae type strains. Bootstrap values and sequence similarity values are indicated at the branch nodes in black and grey, respectively. The scale bar shows 5 nucleotide substitutions per 100 nucleotides.

Statistical parsimony analysis resulted in two unconnected networks, corresponding to clade A (three 16S genotypes) and B (16 genotypes). The unresolved relationships within clade B were reflected in a highly interconnected network (Fig. 3.23C), which either result from homoplasies or may be indicative of recombination [290] (see below). Pairwise sequence similarity of the 16S rRNA gene sequences (1445 bp) was 99.3-99.9% within clade A, 99.1-100% within clade B, and 96.1% between clades A and B (Fig. 3.24).

Host-symbiont coevolution and biogeography

We applied different methods for examining possible coevolution between Flavobacteriaceae endosymbionts and *Bryopsis* hosts, and for assessing the role of geography on codivergence. First, a possible correlation between endosymbiont and host genetic variation was visually explored by comparing host and symbiont trees and by mapping the Flavobacteriaceae genotypes on the host phylogeny (Fig. 3.23) or vice versa (Fig. 3.25A). Strict topological congruence was observed between *Bryopsis vestita* and *B. foliosa* (clade A) and their associated endosymbionts. However, correlation between Flavobacteriaceae of clade B and *Bryopsis* was less obvious for three reasons. First, several bacterial genotypes were present in different *Bryopsis* hosts. For example, genotype 1 was found in four *Bryopsis* species (spp. 22, 23, 24 and 26), genotype 11 was present in three species (spp. 20, 21 and 28), and genotype 7 in two species (spp. 21, 28). Secondly, most *Bryopsis* species contained multiple Flavobacteriaceae genotypes, with *Bryopsis* sp. 28 possessing as much as 14 different genotypes. Thirdly, relationships among Flavobacteriaceae genotypes were largely unresolved, hampering the reconstruction of reconciled trees.

Because of these complicating factors, we applied statistical approaches that do not require a well resolved host and symbiont phylogeny for assessing codivergence. AMOVA revealed that 57% of the genetic variation in endosymbiont 16S rRNA gene sequences was attributable to the host species clade divisions and subsequent permutation tests pointed out that this difference was significant ($p < 0.0001$, Table 3.8A), indicating genetic differentiation between *Bryopsis* species. Pairwise Φ_{ST}-values between the species are highest between more distantly related species, while genetic differentiation was found to be insignificant between some closely related species (Table 3.8A). On the other hand, our data indicate that genetic diversity of endosymbionts is also to a large extent geographically structured, with most 16S genotypes being restricted to one geographical region (Fig. 3.23D, 3.25B). This is supported by AMOVA and pairwise Φ_{ST}-values that showed significant genetic differentiation between the East Pacific, Atlantic-Mediterranean and Indo-Pacific

(Table 3.8B). This geographical signal, however, is at least partly due to dispersal limitation of the host, resulting in confined geographical ranges for most host species. Several observations favor the hypothesis that endosymbiont genetic diversity is primarily structured by host phylogeny. As described above, Flavobacteriaceae endosymbionts are restricted to two *Bryopsis* clades (clade A and B), irrespective of host biogeography. For example, of the five *Bryopsis* species from the Mediterranean Sea, only the two species from clade A harbor Flavobacteriaceae endosymbionts (Fig. 3.23A). A similar strict phylogenetic distribution of endosymbionts is observed for the different *Bryopsis* species from Pacific Mexico, Pacific Nicaragua, South Africa and the Seychelles. A phylogenetic rather than geographic effect on endosymbiont genetic differentiation is also apparent when examining specific Flavobacteriaceae genotypes within *Bryopsis* clade B. For example, genotype 2 is widely distributed in the Atlantic, Mediterranean and Indo-Pacific, but clearly restricted to a single clade including *Bryopsis* spp. 22, 23, 24 and 26.

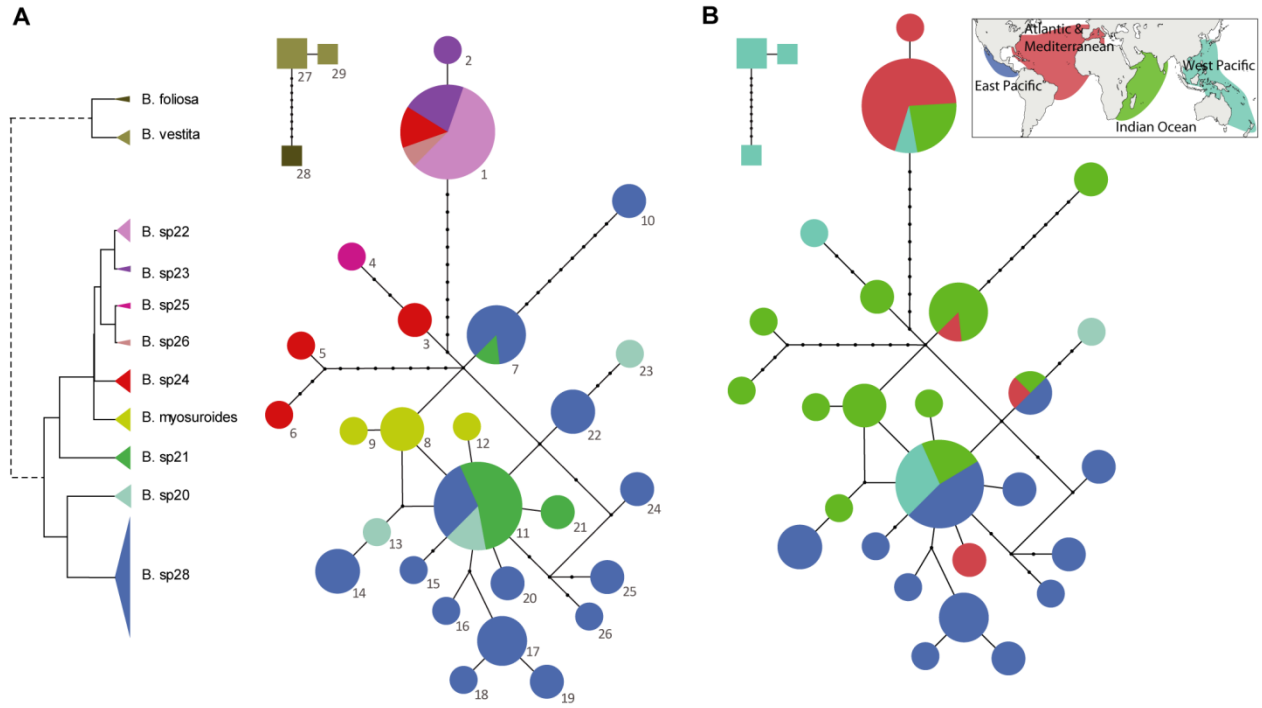

Figure 3.25: TCS parsimony network of 16S rRNA gene sequences of Flavobacteriaceae endosymbionts. Circles depict endosymbiont genotypes and are proportionally sized to the number of sequences (i.e. Flavobacteriaceae strains) they represent. Colors within the network correspond to (A) *Bryopsis* species as depicted in the host phylogram on the left and (B) geographical location of the host samples as depicted in the map on the right. Each black node represents 1 nucleotide mutation separating genotypes.

Table 3.8: A: Pairwise Φ_{ST} values of Flavobacteriaceae endosymbionts between host species (clade B).

	***B.* sp 22**	***B.* sp 23**	***B.* sp 24**	***B. myosuroides***	***B.* sp 21**	***B.* sp 20**
***B.* sp 22**						
***B.* sp 23**	0.10					
***B.* sp 24**	**0.51**	0.41				
B. myosuroides	**0.94**	**0.91**	**0.27**			
***B.* sp 21**	**0.96**	**0.94**	**0.45**	**0.59**		
***B.* sp 20**	**0.92**	**0.88**	**0.27**	**0.32**	0.03	
***B.* sp 28**	**0.74**	**0.72**	**0.36**	**0.19**	0.04	0.02

B: Pairwise Φ_{ST} values of Flavobacteriaceae endosymbionts between the four geographical regions.

	Atl -Med	**E Pacific**	**Indian Ocean**
Atlantic-Mediterranean			
E Pacific	**0.66**		
Indian Ocean	**0.45**	**0.26**	
W Pacific	**0.44**	**0.18**	0.05

Values in bold are significantly different from zero after Bonferroni correction

The observation that phylogenetically related *Bryopsis* species harbor the same or closely related endosymbiont genotypes is suggestive of a vertical inheritance of Flavobacteriaceae species from one host generation to the next, suggesting that cospeciation does occur to some extent. However, as described above, several factors within the *Bryopsis*/Flavobacteriaceae clade B are obscuring this pattern of cospeciation. The diversity of Flavobacteriaceae genotypes within a single *Bryopsis* species can be explained by recent and ongoing divergence of endosymbionts. Also contact with co-occurring facultative endophytes (see sections 3.2.1, 3.2.2 [225, 245] and 3.2.3) and/or with other bacteria during asexual reproductive stages (i.e. thallus fragmentation and protoplast formation, see Chapter 1, Box 3) might increase Flavobacteriaceae heterogeneity through recombination [299]. The fact that some endosymbiont genotypes (genotypes 1, 7 and 11, Fig. 3.23) are distributed among different *Bryopsis* species can be explained by two different scenarios. A first explanation might be the persistence of ancestral Flavobacteriaceae genotypes in different host lineages (Fig. 3.26, scenario 1). A second, alternative scenario is lateral gene transfer of Flavobacteriaceae endosymbionts between different *Bryopsis* species, known as host-switching (Fig. 3.26, scenario 2). Given that the Flavobacteriaceae endophytes have never been encountered free-living in seawater (see section 3.2.2 [245]), host-switching would require host-specific mechanisms. For example, hybridization between closely related *Bryopsis* species could result in a mixing effect of the associated Flavobacteriaceae genotypes. Hybridization in green algae is not well studied, but might be more widespread than

anticipated [300]. Also sea slugs, which are known to graze on siphonous green algae, could act as effective carriers of bacteria between different *Bryopsis* species [301]. The observation that *Bryopsis* Flavobacteriaceae endosymbionts are related to bacterial sequences encountered in sponge and coral hosts (see above, Fig. 3.24), might even suggest this host-switching may occur among distantly related eukaryotes [262]. However, the fact that Flavobacteriaceae endosymbionts are phylogenetically, rather than geographically, restricted, makes the host-switching scenario between *Bryopsis* species less plausible.

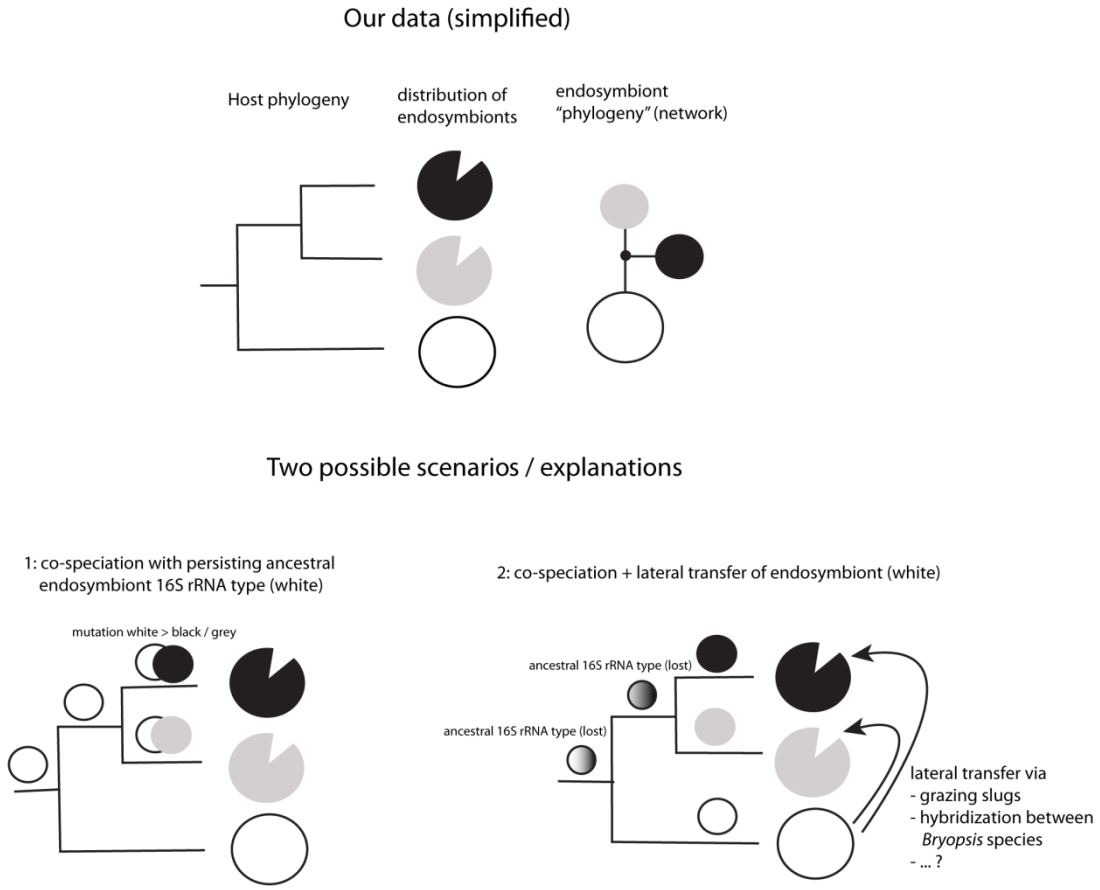

Figure 3.26: Two possible scenarios for the occurrence of the same Flavobacteriaceae endosymbiont 16S rRNA gene type in different *Bryopsis* species.

In addition, these incongruent host-symbiont coevolution patterns might also be biased by ambiguous algal host and endosymbiont species delimitation. For example, the low level of 16S rRNA gene sequence variability proves that this molecular marker offers limited phylogenetic resolution at lower taxonomic scales [296]. Other markers may exist, which are evolving faster than the 16S rRNA region, and their sequences would provide more polymorphic sites and suitable information to assess coevolution patterns.

Despite these limitations, our results strongly point toward a tight, highly specific association between the algal host and Flavobacteriaceae endosymbionts. The physiological ground for this alliance, however, remains unknown from both the *Bryopsis* host and obligate Flavobacteriaceae endosymbiont viewpoint. It is possible that Flavobacteriaceae endosymbionts offer the algal host some adaption to elevated sea water temperatures. Such endosymbionts affecting host tolerance to temperature stresses have been reported within various insect hosts [298, 302-304].

Acknowledgements

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Supplementary Table S3.5: Overview of algal samples (taxonomic affiliation, sample name and geographic location) that were screened for the presence of Flavobacteriaceae endophytes.

Order	Genus/species	Sample name	Country	Geography
Bryopsidales	*Avrainvillea asarifolia*	LL0044	Belize	Atlantic Ocean
Bryopsidales	*Avrainvillea nigricans*	LL0005	Belize	Atlantic Ocean
Bryopsidales	*Avrainvillea silvana*	LL0045	Belize	Atlantic Ocean
Bryopsidales	*Boodleopsis pusilla*	LL0046	Belize	Atlantic Ocean
Bryopsidales	*Bryopsis corticulans*	HV1535	USA	Pacific Ocean
Bryopsidales	*Bryopsis corymbosa*	HEC4772	France	Mediterranean Sea
Bryopsidales	*Bryopsis corymbosa*	HV1237	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis corymbosa*	ODCMZ1	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis corymbosa*	ODCMZ2	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis foliosa*	F0001	Australia	Indian Ocean
Bryopsidales	*Bryopsis foliosa*	F0002	Australia	Indian Ocean
Bryopsidales	*Bryopsis muscosa*	HV1238	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis myosuroides*	F.0172	South Africa	Indian Ocean
Bryopsidales	*Bryopsis myosuroides*	F.0175	South Africa	Indian Ocean
Bryopsidales	*Bryopsis myosuroides*	KZN0156	South Africa	Indian Ocean
Bryopsidales	*Bryopsis myosuroides*	ODC1185	South Africa	Indian Ocean
Bryopsidales	*Bryopsis myosuroides*	ODC1186	South Africa	Indian Ocean
Bryopsidales	*Bryopsis myosuroides*	ODC1187a	South Africa	Indian Ocean
Bryopsidales	*Bryopsis myosuroides*	F.0174	South Africa	Indian Ocean
Bryopsidales	*Bryopsis myosuroides*	KZN2318	South Africa	Indian Ocean
Bryopsidales	*Bryopsis* sp. 1	F.0173	South Africa	Indian Ocean
Bryopsidales	*Bryopsis* sp. 1	FL62	South Africa	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	HEC10851	South Africa	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	HEC10881	South Africa	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	KZN0920	South Africa	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	TS133	South Africa	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	TS172	South Africa	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	F0006	Argentina	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	SEY477	Seychelles	Indian Ocean
Bryopsidales	*Bryopsis* sp. 1	KZN931	South Africa	Indian Ocean
Bryopsidales	*Bryopsis* sp. 1	SEY382	Seychelles	Indian Ocean
Bryopsidales	*Bryopsis* sp. 1	Sn10839	Indonesia	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 1	JH001	France	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	JH002	France	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	JH003	France	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 1	West4583	South Africa	Indian Ocean
Bryopsidales	*Bryopsis* sp. 10	MX0359	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 16	F.0112	Nicaragua	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 16	HV1559	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 16	HV1757	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 16	HV1779	Mexico	Pacific Ocean

Order	Genus/species	Sample name	Country	Geography
Bryopsidales	*Bryopsis* sp. 16	HV1780	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 16	MX0254	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 18	HEC15265	Madagascar	Indian Ocean
Bryopsidales	*Bryopsis* sp. 18	KZN0800	South Africa	Indian Ocean
Bryopsidales	*Bryopsis* sp. 18	ODC1187b	South Africa	Indian Ocean
Bryopsidales	*Bryopsis* sp. 2	EE4 (FJ715718)	Netherlands	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 2	HVGoes	Netherlands	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 2	WB3	USA	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 2	WB4	USA	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 2	WE2	Netherlands	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 2	WE3	Netherlands	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 2	YB2	USA	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 2	YB1	USA	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 20	F.0176	Malaysia	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 20	HEC14151	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 20	HEC8671	Kenya	Indian Ocean
Bryopsidales	*Bryopsis* sp. 20	KE1	Kenya	Indian Ocean
Bryopsidales	*Bryopsis* sp. 20	HEC14796	Mauritius	Indian Ocean
Bryopsidales	*Bryopsis* sp. 20	HEC16048	Sri Lanka	Indian Ocean
Bryopsidales	*Bryopsis* sp. 21	FL1173	Philippines	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 21	HV1682	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 21	HV1686	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 21	MX0036	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 21	MX0156	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 21	MX0253	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 21	MX19 (JF521594)	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 21b	TZ170	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 22	HV2122	Australia	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 22	MZ4	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 22	ODCMZ3	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 22	ODCMZA	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 22	HV1227	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 22	HV1228	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 22	HV1229	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 22	HV1240	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 22	HV1241	Spain	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 23	HV967	USA	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 23	HV968	USA	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 24	HEC9474	Kenya	Indian Ocean
Bryopsidales	*Bryopsis* sp. 24	HEC10690	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 24	HEC11314	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 24	HEC12192	Tanzania	Indian Ocean

Order	Genus/species	Sample name	Country	Geography
Bryopsidales	*Bryopsis* sp. 24	HEC14026	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 24	HEC14932	Madagascar	Indian Ocean
Bryopsidales	*Bryopsis* sp. 24	TZ583	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 24	ODC679	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 25	HV1983	Japan	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 26	HEC9417	Kenya	Indian Ocean
Bryopsidales	*Bryopsis* sp. 26	HEC14026	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0096	New Caledonia	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0097	New Caledonia	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0104	Nicaragua	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0105	Costa Rica	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0107	Costa Rica	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0108	Costa Rica	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0109	Nicaragua	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0110	Nicaragua	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0111	Nicaragua	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0113	Nicaragua	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0114	Nicaragua	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0115	Panama	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0116	Panama	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0117	Panama	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0119	Panama	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	F.0120	Panama	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	HEC10527	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HEC10657	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HEC11198	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HEC6728	Kenya	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HEC9490	Kenya	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HEC9510	Kenya	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HOD-RUN98-33	Reunion	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HOD-RUN98-34	Reunion	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HV1609	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	HV1614	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	HEC14609b	Mauritius	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	ODC1747	Philippines	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	MX0086	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	MX0314	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	MX164 (JF521593)	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	MX344 (JF521596)	Mexico	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	HEC12942	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	SEY323	Seychelles	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	HV566	Philippines	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	PH167	Philippines	Pacific Ocean

Order	Genus/species	Sample name	Country	Geography
Bryopsidales	*Bryopsis* sp. 28	PH222	Philippines	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	HV679	Philippines	Pacific Ocean
Bryopsidales	*Bryopsis* sp. 28	SEY357	Seychelles	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	TZ0053	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 28	TZ0088	Tanzania	Indian Ocean
Bryopsidales	*Bryopsis* sp. 3	HV880 (FJ432637)	France	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 3	ODC1380	France	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 4b	BR	France	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 4c	BY	Netherlands	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 4c	HV1340	Spain	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 4c	HV1341	Spain	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 4c	HV1370	Spain	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 4c	WE1	Netherlands	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 4c	West4718	France	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 5	HV1388	France	Atlantic Ocean
Bryopsidales	*Bryopsis* sp. 9	HEC1637	France	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 9	JH021	France	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 9	JH022	France	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 9	JH023	France	Mediterranean Sea
Bryopsidales	*Bryopsis* sp. 9	JH025	France	Mediterranean Sea
Bryopsidales	*Bryopsis vestita*	Joe1	New Zealand	Pacific Ocean
Bryopsidales	*Bryopsis vestita*	Joe2	New Zealand	Pacific Ocean
Bryopsidales	*Bryopsis vestita*	Joe3	New Zealand	Pacific Ocean
Bryopsidales	*Bryopsis vestita*	F0082b	New Zealand	Pacific Ocean
Bryopsidales	*Caulerpa cupressoides*	MX0382	Mexico	Pacific Ocean
Bryopsidales	*Caulerpa mexicana*	LL0104	Bahamas	Atlantic Ocean
Bryopsidales	*Caulerpa peltata*	HV2030	Japan	Pacific Ocean
Bryopsidales	*Caulerpa prolifera*	LL0113	Belize	Atlantic Ocean
Bryopsidales	*Caulerpa racemosa*	MX0174	Mexico	Pacific Ocean
Bryopsidales	*Caulerpa racemosa*	LL0118	Martinique	Atlantic Ocean
Bryopsidales	*Caulerpa serrulata*	LL0010	Fiji	Pacific Ocean
Bryopsidales	*Caulerpa sertularioides*	MX0316	Mexico	Pacific Ocean
Bryopsidales	*Caulerpa taxifolia*	LL0131	Fiji	Pacific Ocean
Bryopsidales	*Chlorodesmis* sp.	HV1774	Sri Lanka	Indian Ocean
Bryopsidales	*Chlorodesmis* sp.	MX0081	Mexico	Pacific Ocean
Bryopsidales	*Codium arabicum*	TZ0517	Tanzania	Indian Ocean
Bryopsidales	*Codium decorticatum*	G.371	Brazil	Atlantic Ocean
Bryopsidales	*Codium duthiae*	HEC10919	South Africa	Atlantic Ocean
Bryopsidales	*Codium dwarkense*	TZ0818	Tanzania	Indian Ocean
Bryopsidales	*Codium fragile*	HEC1554	South Africa	Atlantic Ocean
Bryopsidales	*Codium fragile*	HV1099	USA	Atlantic Ocean
Bryopsidales	*Codium fragile*	HV1392	France	Atlantic Ocean
Bryopsidales	*Codium fragile*	HV1786	Mexico	Pacific Ocean

Order	Genus/species	Sample name	Country	Geography
Bryopsidales	*Codium geppiorum*	TZ0370	Tanzania	Indian Ocean
Bryopsidales	*Codium isabelae*	G.083	Panama	Pacific Ocean
Bryopsidales	*Codium spongiosum*	HV2489	Australia	Indian Ocean
Bryopsidales	*Derbesia* sp.	HV1600	Mexico	Pacific Ocean
Bryopsidales	*Derbesia* sp.	HV1448	Netherlands	Atlantic Ocean
Bryopsidales	*Derbesia* sp.	TZ0612	Tanzania	Indian Ocean
Bryopsidales	*Derbesia* sp.	HV1079	Netherlands	Atlantic Ocean
Bryopsidales	*Derbesia* sp.	MX0021	Mexico	Pacific Ocean
Bryopsidales	*Halimeda borneensis*	W0168	Micronesia	Pacific Ocean
Bryopsidales	*Halimeda copiosa*	LL0417	Belize	Atlantic Ocean
Bryopsidales	*Halimeda cuneata*	G.905	South Africa	Indian Ocean
Bryopsidales	*Halimeda discoidea*	LL0020	Panama	Pacific Ocean
Bryopsidales	*Halimeda distorta*	H.0097	British Indian Ocean Territory	Indian Ocean
Bryopsidales	*Halimeda gigas*	W0162	Micronesia	Pacific Ocean
Bryopsidales	*Halimeda gigas*	HA0238	Australia	Pacific Ocean
Bryopsidales	*Halimeda macroloba*	LPT0034	Thailand	Pacific Ocean
Bryopsidales	*Halimeda opuntia*	LL0459	Panama	Atlantic Ocean
Bryopsidales	*Halimeda opuntia*	HA0373	Australia	Pacific Ocean
Bryopsidales	*Halimeda opuntia*	LPT0030	Thailand	Indian Ocean
Bryopsidales	*Halimeda tuna*	H.0086	British Indian Ocean Territory	Indian Ocean
Bryopsidales	*Halimeda tuna*	HV889	Spain	Mediterranean Sea
Bryopsidales	*Rhipilia orientalis*	HEC10402	Papua New Guinea	Indian Ocean
Bryopsidales	*Tydemania expeditionis*	HV873	Philippines	Pacific Ocean
Bryopsidales	*Udotea unistratea*	LL0051	Belize	Atlantic Ocean
Cladophorales	*Aegagropila* sp.	Aeg 1	probably Ukraine	Freshwater, aquarium trade
Cladophorales	*Anadyomene* sp.	FL1113	Philippines	Pacific Ocean
Cladophorales	*Anadyomene* sp.	TZ0177	Tanzania	Indian Ocean
Cladophorales	*Apjohnia laetevirens*	HV2291	Australia	Pacific Ocean
Cladophorales	*Apjohnia laetevirens*	HV2342	Australia	Pacific Ocean
Cladophorales	*Boergesenia forbesii*	Boerg1	Seychelles	Indian Ocean
Cladophorales	*Boergesenia forbesii*	FL1114	Philippines	Pacific Ocean
Cladophorales	*Boergesenia* sp.	JAP073	Japan	Pacific Ocean
Cladophorales	*Boodlea* sp.	Huisman nov2006 sn	Australia	Indian Ocean
Cladophorales	*Boodlea* sp.	FL1110	Philippines	Pacific Ocean
Cladophorales	*Boodlea* sp.	TZ0147	Tanzania	Indian Ocean
Cladophorales	*Chaetomorpha antennina*	MX275.branched_culture	Mexico	Pacific Ocean
Cladophorales	*Chaetomorpha crassa*	FL1132	Philippines	Pacific Ocean
Cladophorales	*Chaetomorpha crassa*	ODC1640	Kenya	Indian Ocean
Cladophorales	*Chaetomorpha* sp.	FL1092	Philippines	Pacific Ocean
Cladophorales	*Chaetomorpha* sp.	TZ0877	Tanzania	Indian Ocean
Cladophorales	*Cladophora vagabunda*	HEC15734	Madeira	Atlantic Ocean
Cladophorales	*Cladophora vagabunda*	Bernecker 73493	Costa Rica	Pacific Ocean

Order	Genus/species	Sample name	Country	Geography
Cladophorales	*Cladophora vagabunda*	TZ0203	Tanzania	Indian Ocean
Cladophorales	*Cladophoropsis vaucheriiformis*	TZ0826	Tanzania	Indian Ocean
Cladophorales	*Cladophoropsis vaucheriiformis*	HEC7547	Papua New Guinea	Pacific Ocean
Cladophorales	*Cladophoropsis vaucheriiformis*	HEC10097	Japan	Pacific Ocean
Cladophorales	*Dictyosphaeria cavernosa*	FL1091	Philippines	Pacific Ocean
Cladophorales	*Dictyosphaeria cavernosa*	TZ0197	Tanzania	Indian Ocean
Cladophorales	*Dictyosphaeria sericea*	HV2275	Australia	Pacific Ocean
Cladophorales	*Dictyosphaeria* sp.	Bernecker86	Costa Rica	Pacific Ocean
Cladophorales	*Dictyosphaeria versluysii*	TS253	Hawaii	Pacific Ocean
Cladophorales	*Dictyosphaeria versluysii*	TZ0156	Tanzania	Indian Ocean
Cladophorales	*Ernodesmis verticillata*	Bernecker 73483	Costa Rica	Pacific Ocean
Cladophorales	*Microdictyon boergesenii*	BW00392	Panama	Indian Ocean
Cladophorales	*Microdictyon tenuius*	HEC16007	Sri Lanka	Indian Ocean
Cladophorales	*Rhizoclonium africanum*	TZ0781	Tanzania	Indian Ocean
Cladophorales	*Rhizoclonium* sp.	FL1164	Philippines	Pacific Ocean
Cladophorales	*Siphonocladus tropicus*	Siph3	Canary Islands	Atlantic Ocean
Cladophorales	*Valonia macrophysa*	BW00825	Panama	Pacific Ocean
Cladophorales	*Valonia* sp.	FL1120	Philippines	Pacific Ocean
Cladophorales	*Valonia* sp.	IT028B	Hawaii	Pacific Ocean
Cladophorales	*Valonia* sp.	TZ0148	Tanzania	Indian Ocean
Dasycladales	*Acetabularia dentata*	HEC12349	Philippines	Pacific Ocean
Dasycladales	*Acetabularia ryukyuensis* var. *philippinensis*	HEC12329	Philippines	Pacific Ocean
Dasycladales	*Bornetella oligospora*	FL1108	Philippines	Pacific Ocean
Dasycladales	*Neomeris annulata*	HEC12327	Philippines	Pacific Ocean
Dasycladales	*Neomeris vanbosseae*	TZ0198	Tanzania	Indian Ocean
Ulvales	*Ulva* sp.	Qingdao1	China	Pacific Ocean
Ulvales	*Ulva* sp.	JH3epi	France	Atlantic Ocean

3.3.2. *In situ* hybridizations of *Bryopsis* intracellular bacteria with group- and species-specific fluorescent probes

Abstract

Fluorescence *in situ* hybridization (FISH) with oligonucleotide probes targeting bacterial 16S ribosomal RNA has been widely used in environmental microbiology [165-167]. Two applications of the technique are common: (i) the identification, quantification and/or localization of certain phylogenetic groups with previously designed and tested probes [127], and (ii) the *in situ* presence verification of sequences within their respective source of origin using newly designed species-specific probes [5]. In the search for intracellular bacteria within the green siphonous seaweed *Bryopsis,* the presence of metabolically active bacteria inside the algal cytoplasm was previously confirmed by means of the universal bacterial probe mix EUB338 (section 3.2.1 [225]). In this section, preliminary hybridization experiments are reported with alphaproteobacterial and CFB group-specific fluorescent probes to reveal the detailed location of certain bacterial endophytes within the *Bryopsis* cell. Also exploratory hybridizations with the newly designed F695 probe are described to specifically locate Flavobacteriaceae endosymbionts within the *Bryopsis* interior. Whereas *Labrenzia* and Rhizobiaceae endophytes are mainly located in the vacuole and in the compact cytoplasm at the tip of the *Bryopsis* plant, *Rickettsia* and Flavobacteriaceae endophytes are situated in the close vicinity of and/or within the algal chloroplasts. Besides providing some insight into the location and function of endogenous bacteria, hybridizations with endophyte specific probes may facilitate the future detection and identification of intracellular bacteria in natural *Bryopsis* samples.

Materials and Methods

To study the detailed location of certain endophytes (i.e. *Labrenzia, Rickettsia,* Rhizobiaceae and Flavobacteriaceae species) within the *Bryopsis* interior, fresh thalli of the algal samples 4718, MX90 and WB4 (see Supplementary Table S3.2, p. 100) were fixed, embedded in LR white resin and sectioned as described in section 3.2.1 [225]. Subsequently, six serial sections from each *Bryopsis* sample were hybridized with group-specific fluorescent probes which were selected by comparing the endophytic bacterial sequences (see sections 3.2.1 [225], 3.2.3 and 3.3.1) with previously designed and tested probes in probeBase [305]. *In situ* hybridization was performed according to Daims *et al.* [188] with FLUOS-labelled probes ALF968, CF319a and Rick_527 (Table 3.9) matching the alphaproteobacterial (i.e. Rhizobiaceae and *Labrenzia*), Flavobacteriaceae and *Rickettsia* endophytes, respectively. Additionally, the universal bacterial Cy3-labelled EUB338 probe mix [169] was used as a positive control. To exclusively locate the Flavobacteriaceae endosymbionts, the species-specific primer previously designed (see section 3.3.1) was transformed into a Cy3-labelled probe and trial hybridizations were performed with 200 ml formamide per ml hybridization buffer and an incubation of 90 min at 46°C. All hybridized sections were mounted in AF-1 antifadent (Citifluor, UK) and viewed with an Olympus BX51 epifluorescence microscope fitted with a DAPI/FITC/TRITC triple band filter. *Bryopsis* specimens were not surface-sterilized prior to hybridization due to potential loss of morphology (see section 3.2.1 [225]).

Table 3.9: Fluorescence *in situ* hybridization probes.

Probe	Target	Target site	Sequence 5'-3'	%FA*	Ref.
EUB338 mix **	All Bacteria	338-355	GCT GCC TCC CGT AGG AGT GCA GCC ACC CGT AGG TGT GCT GCC ACC CGT AGG TGT	0-50	[169]
ALF968	Alphaproteobacteria, except of Rickettsiales	968-985	GGT AAG GTT CTG CGC GTT	20	[306]
CF319a	Most Flavobacteria, some Bacteroidetes, some Sphingobacteria	319-336	TGG TCC GTG TCT CAG TAC	35	[307]
Rick_527	Members of the Rickettsiaceae family	527-542	CCC CTC CGT CTT ACC G	0	[260]
F695	Flavobacteriaceae endosymbiont of *Bryopsis*	695-714	GGC AGG TTG CTA AGC CTT AA	t.b.d.	This study

Probe targets, sequences and hybridization conditions were obtained from probeBase [305]
* Percent formamide in the hybridization buffer for optimal hybridization conditions in FISH experiments
** Use of probes EUB338 I, II and III in an equimolar mixture

Results and discussion

Figure 3.27 depicts the hybridization results of probes ALF968-FLUOS and EUB338-Cy3 on sections of *Bryopsis* sample 4718. This sample contains *Labrenzia* and Rhizobiaceae endophytes as previously determined by clone libraries and DGGE analyses (see section 3.2.3). After screening of the probeBase database [305], these *Labrenzia* and Rhizobiaceae sequences showed a match with the ALF968 alphaproteobacterial group-specific probe. Consequently, both endophytic phylotypes bind the ALF968 (green) as well as the universal bacterial EUB388 (red) fluorescent probe. This dual binding gives rise to a signal in both the green and red channel resulting in a green-yellowish colour in the right picture of Fig. 3.27. From this figure it can be deduced that *Labrenzia* and Rhizobiaceae endophytes are mainly located in the centre of the *Bryopsis* section (potential vacuole, V) and in the compact cytoplasm at the tip of the *Bryopsis* plant. Potential nitrogen fixing Rhizobiaceae as well as CO-oxidizing *Labrenzia* endophytes (see section 3.2.3) might be very useful to *Bryopsis* during periods of growth. This location at the thallus tip is thus perhaps not surprising as growth of *Bryopsis* thalli occurs at the tip of the main axis and side branches in a zone of apical cytoplasm called the meristemplasm [36].

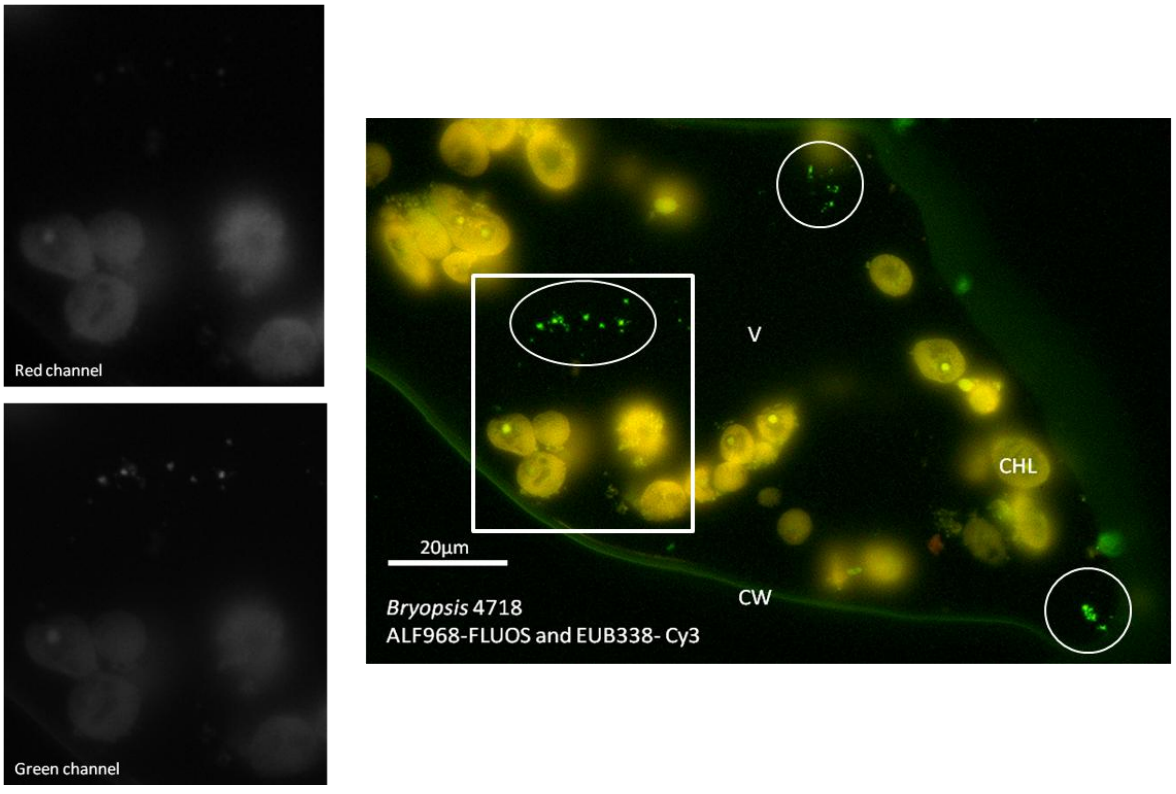

Figure 3.27: *Bryopsis* sample 4718 hybridized with the probes ALF968-FLUOS and EUB338-Cy3.
CW = cell wall, CHL = chloroplasts, V = vacuole, and white circles = bacteria.

Rickettsia endophytes, on the other hand, seem to be more closely associated with the *Bryopsis* chloroplast (Fig. 3.28), suggesting they and/or their biochemical pathways require the presence of photosynthetic metabolites. Figure 3.28 shows the shared binding of the Rick_527-FLUOS and EUB338-Cy3 probe on *Bryopsis* WB4 sections resulting in a signal in both the red and green channel. A yellowish (in this case rather orange due to the high Cy3 intensity) colour is observed in the right picture in Figure 3.28. In addition, the universal bacterial EUB338 probe hybridized with no other bacterial rRNA than *Rickettsia* sequences, confirming our previous molecular results that *Bryopsis* sample WB4 only harbors *Rickettsia* endophytes (section 3.2.3). This apparent mutual exclusion among *Rickettsia* and other bacterial endophytes has also been observed within insect hosts [308].

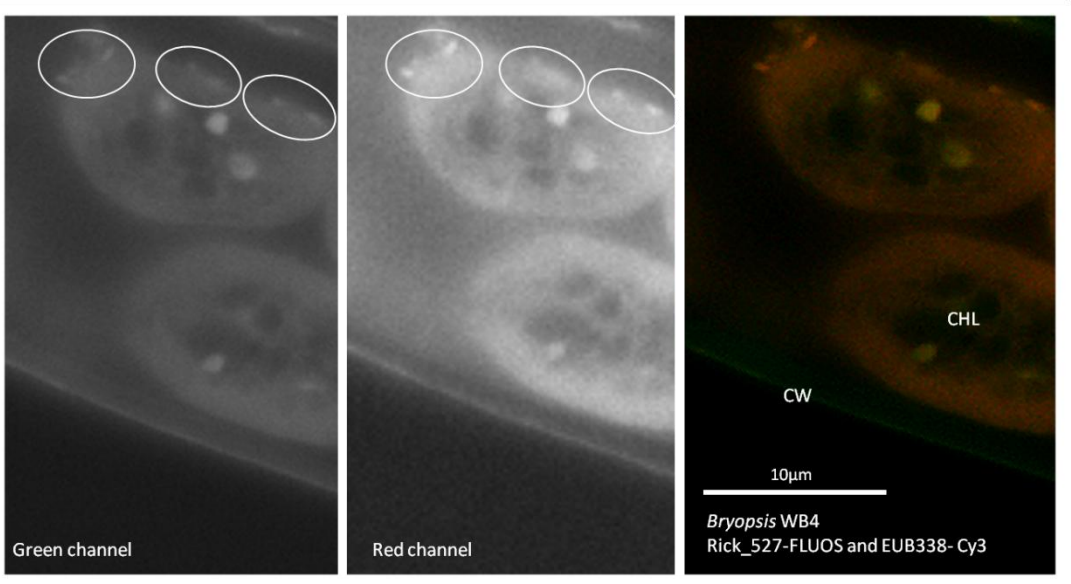

Figure 3.28: *Bryopsis* sample WB4 hybridized with the probes Rick_527-FLUOS and EUB338-Cy3. CW = cell wall, CHL = chloroplasts, and white circles = bacteria.

Figure 3.29A displays the hybridization results of probes CF319a-FLUOS and EUB338-Cy3 probe on sections of *Bryopsis* sample MX90 known to harbor only Flavobacteriaceae endosymbionts (sections 3.2.1 [225] and 3.2.3). Because the CF319a and EUB338 probe hinder each other sterically (i.e. the binding sites of both probes on the 16S rRNA are too close to each other), Flavobacteriaceae rRNA present only binds the CFB group-specific CF319a probe. Little or no binding of the EUB338 probe occurred (red channel, Fig. 3.29A), resulting in a vivid green signal in the picture on the right of Fig. 3.29A. This signal indicates that Flavobacteriaceae endosymbionts are located within the *Bryopsis* chloroplast and/or in close proximity with the chloroplastidal membranes. Also Figure 3.29B

which depicts the preliminary hybridization results of the Flavobacteriaceae endosymbiont specific probe F695-Cy3 (red) shows that Flavobacteriaceae RNA may be present within the chloroplasts and in the outer layer of the cytoplasm next to the cell wall which contains all other *Bryopsis* organelles. The location in the vicinity of nuclei, mitochondria, Golgi complexes and mainly chloroplasts may indicate that Flavobacteriaceae endosymbionts perform significant roles within their *Bryopsis* host. This endorses the previously postulated obligate symbiotic nature of the Flavobacteriaceae intracellular bacteria and their host specificity towards *Bryopsis* algae (sections 3.2.2 [245], 3.2.3 and 3.3.1). Moreover, the presence of Flavobacteriaceae endosymbionts within the *Bryopsis* chloroplast may contribute to the strong autonomy and activity of this algal organelle inside the body of some herbivorous sea slugs (see Chapter 1, Box 3), and might even provide a possible explanation for the production of bioactive kahalalides by both sea slugs and their *Bryopsis* food [176]. Likewise, a functional association between algal chloroplasts and intraplastidal bacteria has been reported within the diatom *Pinnularia* [309, 310].

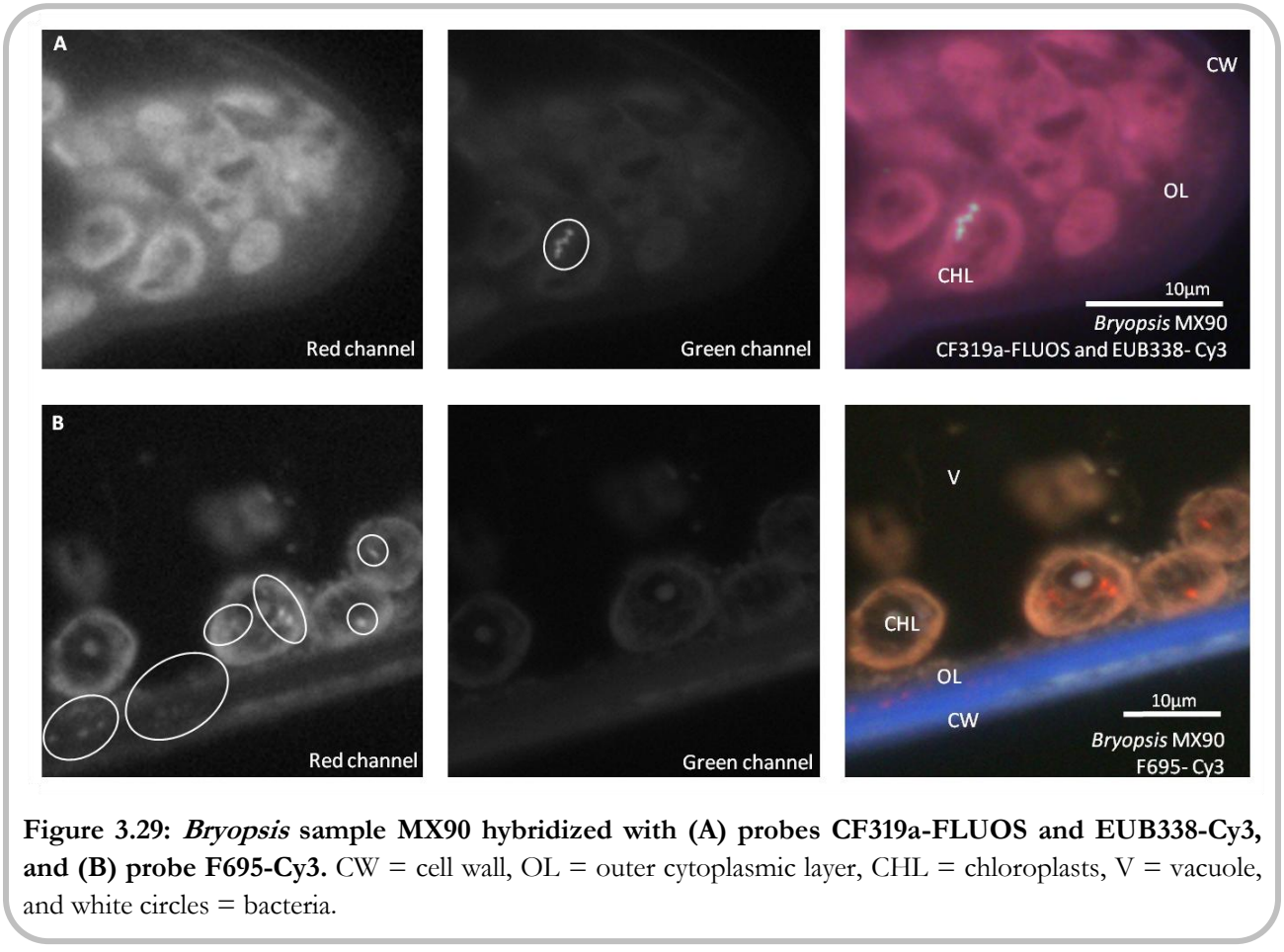

Figure 3.29: *Bryopsis* sample MX90 hybridized with (A) probes CF319a-FLUOS and EUB338-Cy3, and (B) probe F695-Cy3. CW = cell wall, OL = outer cytoplasmic layer, CHL = chloroplasts, V = vacuole, and white circles = bacteria.

Even though these FISH experiments provided a first insight into the specific location of *Labrenzia, Rickettsia*, Rhizobiaceae and Flavobacteriaceae endophytes within their *Bryopsis* hosts, repeated hybridizations with multiple group- and endophytic bacterial species-specific probes are necessary to support these preliminary results. Hybridization conditions of the Flavobacteriaceae specific probe F695 (and potential other endophyte species-specific probes) should be further optimized with Clone-FISH, a technique which validates the specificity of new probes designed to target uncultured bacteria [311]. In addition, the location results have shed some light on the potential function of the endophytes within *Bryopsis*, however, the true physiological role of these intracellular bacteria should be further investigated. *In situ* techniques which link functional gene presence (metabolic potential) to bacterial cell identity in environmental samples may be suitable to pursue this goal. Examples of such techniques include mRNA-FISH [312], gene-FISH [313] and *in situ* rolling circle amplification-FISH [314, 315]. Also fluorescent *in situ* hybridization combined with microautoradiography [FISH-MAR, 316], stable-isotope Raman spectroscopy [Raman-FISH, 317] and secondary-ion mass spectrometry [FISH-SIMS, 318], could provide a linkage between identity and function.

To summarize, exploratory FISH experiments with group- and species-specific probes on *Bryopsis* sections revealed that *Labrenzia, Rickettsia*, Rhizobiaceae and Flavobacteriaceae endophytes occupy distinct locations within the host cell which are consistent with the symbiotic nature and potential function of these intracellular bacteria. These and more functional FISH protocols may open the way to fully explore the *Bryopsis*-intracellular bacteria partnership in natural algal samples.

Acknowledgements

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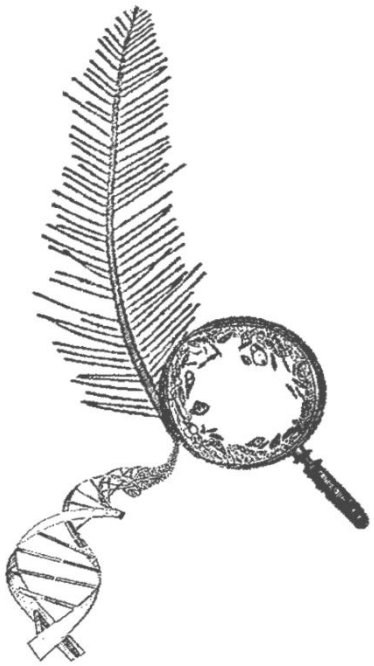
4 Chapter

Concluding discussion



"*All life on Earth today derived from common ancestors. The first to evolve – yet the last to be studied in detail – are bacteria. Scientists have now discovered that bacteria not only are the building blocks of life, but also occupy and are indispensable to every other living being on Earth. Without them, life's essential processes would quickly grind to a halt, and Earth would be as barren as Venus and Mars.*"

Carla Cole (*IN CONTEXT* **34**, p. 18, 1993), based on the work of Lynn Margulis and Dorion Sagan

Like all organisms, algae have developed in a world of microbes. It is therefore hardly surprising that many of these photosynthetic eukaryotes host a wide range of intracellular bacteria. Various microalgae such as dinoflagellates and diatoms are known to harbor bacterial endosymbionts which are linked with diverse metabolic functions [212, 229, 319, 320]. Within macroalgal hosts, however, bacterial endosymbiosis seems more restricted to certain seaweed lineages. Besides the occurrence of bacterial endophytes inside red algal galls and buds [74, 121, 123], true intracellular, non-pathogenic bacteria have to date only been reported inside the giant cells of some green siphonous seaweeds such as *Bryopsis, Caulerpa, Chlorodesmis, Halimeda, Penicillus* and *Udotea* [36-41, 75, 124]. These macro, yet unicellular, algae display extraordinary physicochemical adaptations and wounding responses to overcome their perceived vulnerable single cell morphology (Chapter 1, Box 3). These survival and reproduction strategies alongside endobiotic interactions with potential nitrogen fixing and photosynthetic bacteria, have been suggested to play a role in the success of siphonous green algae in a range of marine habitats [75, 125]. To shed light on the endophytic bacterial partner, the *Bryopsis*-bacterial partnership was explored with a variety of culture-dependent and culture-independent techniques, which were optimized particularly for this study (Chapter 3, Part 1).

Main results and general conclusion

Presence, diversity, identity and uniqueness

Forty years after the initial reports of intracellular bacteria within *Bryopsis* [36, 37], the natural presence of true endophytic bacteria inside the algal cytoplasm was confirmed by electron microscopy and fluorescent *in situ* hybridization (sections 3.2.1, 3.2.2 and 3.3.2). Additional 16S rRNA gene-based techniques revealed that not just one, but several different bacterial phylotypes

reside within the algal host interior of which Bacteroidetes, Flavobacteriaceae, *Labrenzia, Mycoplasma,* Phyllobacteriaceae, Rhizobiaceae and *Rickettsia* species were encountered in three or more *Bryopsis* samples collected globally. Notwithstanding this similarity, the total bacterial diversity varied among different *Bryopsis* cultures with the presence of one to a maximum of four endophytic phylotypes per host sample (section 3.2.3). The co-occurrence of multiple bacterial partners underpins the recent assumption that the diversity of various host associated endobiotic communities has been greatly underestimated so far [216, 230, 321]. Harbouring multiple endobionts could permit further expansion of host capabilities and might even benefit the whole endobiotic flora by allowing syntrophy (i.e. cross-feeding) [322]. In addition, endophytic bacteria of *Bryopsis* algae show some similarity with those of other siphonous seaweeds as Alphaproteobacteria (mainly Rhodobacterales and Rhizobiales) and Bacteroidetes intracellular bacteria have also been characterized within *Caulerpa* [75, 124, 125]. Moreover, several Cladophorales algae (e.g. *Boergesenia* and *Boodlea*), were found to harbour *Labrenzia* and Rhizobiaceae species similar to *Bryopsis* (Leliaert *et al,* unpublished data), supporting the significance of these endophytes within siphonous seaweed hosts. Several of the *Bryopsis* endophytic phylotypes, however, are more closely related to known (endo)symbiotic bacteria of non-algal hosts such as amoeba, land plants, insects and marine animals [202, 214, 216, 221, 222, 286, 323, 324]. This may hint at the existence of a universal group of bacterial taxa which are particularly adapted to (but not necessary reliant on) an intracellular lifestyle. The clear distinctiveness between free-living and endophytic algal-associated bacterial communities (section 3.2.2) supports this hypothesis. Such an 'ecological coherence' has been proven in other niches [128 and references therein], and might also be applied to macroalgal surface associated bacterial communities (see Chapter 1).

Specificity, stability, interdependency and symbiotic nature

The allegiance of bacteria towards an intracellular lifestyle, however, displays diverse degrees of specificity. Some bacteria are (host-specific) obligate endosymbionts, while others are more facultative endobiotic and can survive without a host. Either way, specific modes of partner recognition are required which mainly rely on surface structures (e.g. peptidoglycans, lipopolysaccharides and lectins) and defense mechanisms of both the host and bacterial partner [325]. It has been postulated that high endobiont diversity (i.e. more than 2 à 3 species) goes together with high levels of flexibility in symbiont specificity and stability [322]; and *Bryopsis* seems no exception. Temporal stability experiments showed the loss of Phyllobacteriaceae endophytes in

Bryopsis samples after prolonged cultivation (section 3.2.2), and phylogenetic analyses pointed out that Rhizobiaceae as well as *Labrenzia* endobionts are closely related to sequences of free-living bacterial strains (section 3.2.3). Moreover, *Labrenzia* and Phyllobacteriaceae endophytes were also identified from *Bryopsis* surroundings (section 3.2.2) and could be isolated on artificial media (section 3.2.4). In contrast to these facultative endobiotic phylotypes, mainly Flavobacteriaceae endosymbionts showed a more obligate lifestyle with high specificity towards the *Bryopsis* host (sections 3.2.2 and 3.3.1). Flavobacteriaceae specific amplification revealed the exclusive presence of this endosymbiont in *Bryopsis* species from warm-temperate and tropical seas, and phylogenetic analyses indicated some degree of cospeciation (section 3.3.1).

Host/habitat influences, symbiont transmission modes and function

In addition to differences in intrinsic bacterial lifestyles characteristics, also host phylogeny, habitat and geography influence the diversity of endobiotic communities [322]. Statistical analyses revealed that the total *Bryopsis* endobiotic community composition could only be explained by a mix of host phylogenetic, geographic and environmental factors which had different (shared and/or independent) effects on the individual community members (section 3.2.3). Phyllobacteriaceae, Rhizobiaceae and *Labrenzia* species seemed true generalists which are laterally acquired by any *Bryopsis* host, regardless host species, habitat and geography, to possibly fulfill functions such as nitrogen-fixation, photosynthesis and CO-oxidation (Fig. 4.1). These metabolic processes might be very useful to *Bryopsis* during periods of growth, explaining the location of these facultative endophytes in the meristemplasm, i.e. a zone of apical cytoplasm at the thallus tip where proliferation occurs (section 3.3.2). The occurrence of *Mycoplasma, Rickettsia* and Bacteroidetes endophytes was to some extent influenced by environmental factors, suggesting an additional lateral acquisition of habitat-specific bacteria by *Bryopsis* hosts. This type of 'habitat-specific acquisition' has been argued to provide ecological flexibility by allowing the host to take up bacteria which are optimally adapted to their local environment [326]. Since *Mycoplasma, Rickettsia* and Bacteroidetes species are well-know obligate intracellular symbionts of other eukaryotes, these endophytes may be more likely taken up from co-occurring hosts (i.e. vector dependent acquisition) rather than from the surrounding seawater (Fig. 4.1). In addition, Bacteroidetes endophytes seemed more securely associated with *Bryopsis* as they also displayed some vertical transmission. Only Flavobacteriaceae endosymbionts, however, appeared strictly vertically transmitted from one *Bryopsis* generation to the next via presumable sexual (e.g. gametes and spores) reproductive stages. This confirms that

Flavobacteriaceae endophytes are true obligate symbionts which are entirely dependent on the algal host for their survival (Fig. 4.1). The location of these endosymbionts in and/or in the close vicinity of chloroplasts (section 3.3.2) might indicate these intracellular bacteria fulfill a significant role within the *Bryopsis* host. Nevertheless, their exact ecological function remains unclear.

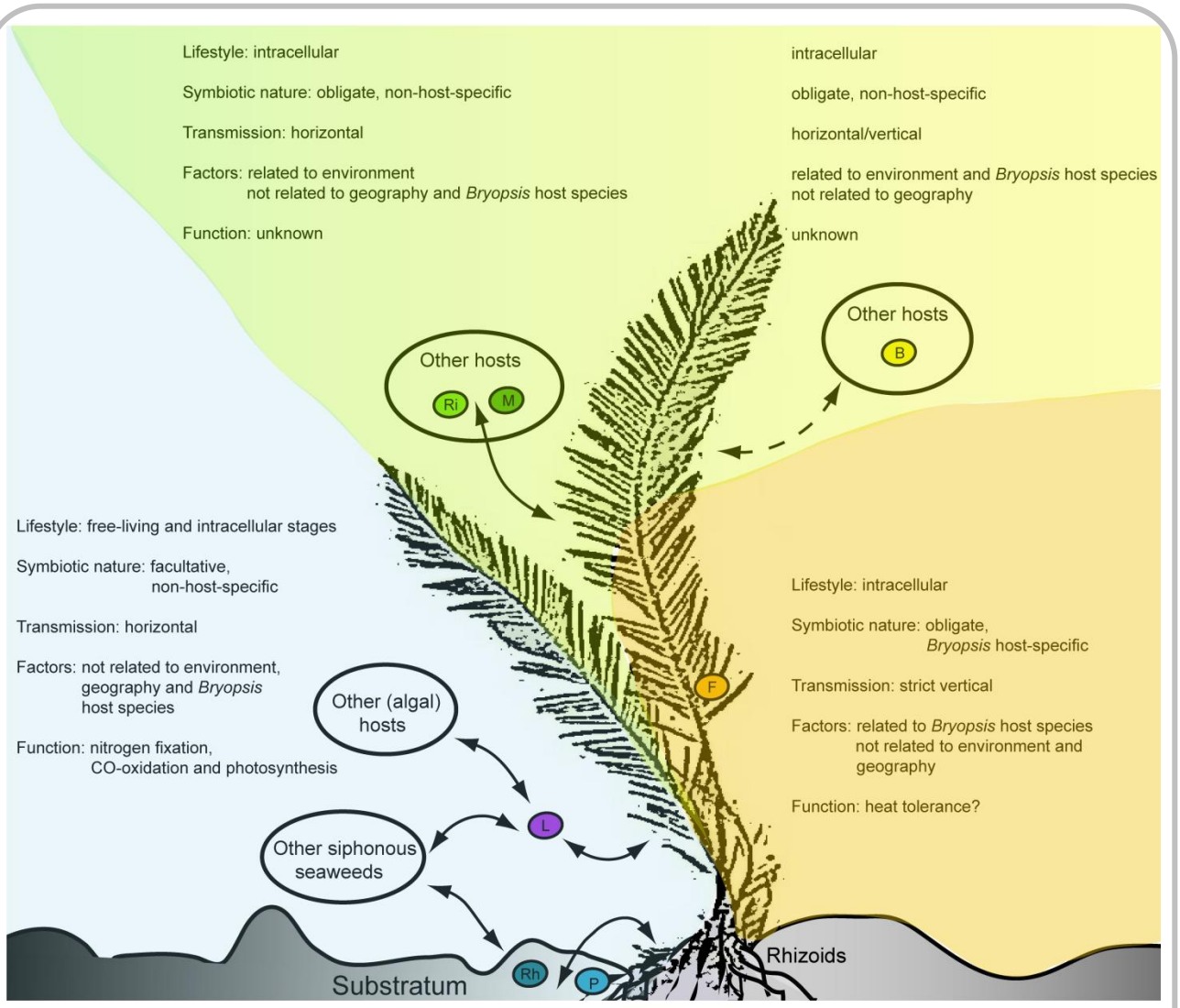


Figure 4.1: The *Bryopsis*-bacterial partnership. Schematic overview of the endophytic bacterial lifestyles and transmission modes to the *Bryopsis* host. Habitat and algal host influences on the bacterial flora as well as potential functions of the bacterial endophytes are described. B = Bacteroidetes, F = Flavobacteriaceae, L = *Labrenzia*, M = *Mycoplasma*, P = Phyllobacteriaceae, Rh = Rhizobiaceae, and Ri = *Rickettsia* endobionts.

Conclusion

Bryopsis algae harbour taxonomically diverse endophytic communities which consist of a mix of generalist and specialist bacterial species. Whereas *Labrenzia,* Phyllobacteriaceae, Rhizobiaceae, *Mycoplasma, Rickettsia* and Bacteroidetes are to a greater or lesser extent laterally acquired from the environment and/or other hosts, mainly Flavobacteriaceae appear *Bryopsis*-specific obligate endosymbionts which are vertically transmitted across generations of host species (Fig. 4.1). Either way, there seems to be a highly specific mode of partner recognition as *Bryopsis* selectively maintains and/or attracts the same endophytes globally, even though the algal interior is repeatedly exposed to various other marine bacteria during wounding events (i.e. thallus rupture and protoplast formation). With these observations, the *Bryopsis*-bacterial partnership fits into the general picture of eukaryote-prokaryote symbiosis systems. Also in various marine animal [264, 321, 322] and insect hosts [216, 232] complex, yet specific, endobiotic communities exist which consist of a mix of one to two primary (obligate) and several secondary (facultative) bacterial partners. This suggests the conservation of basic mechanisms and principles among symbioses of bacteria with hosts from the whole tree of life, possibly giving rise to a universal group of bacterial taxa which share a general intracellular lifestyle. The presence of, for example, nitrogen-fixing Rhizobiaceae species in both land plants and siphonous seaweeds, is a fine example of this universality.

Where to go from here?

Methodology

Even though all methods used in this study were optimized to examine the *Bryopsis*-bacterial partnership to the best possible extent (see Chapter 3, part 1), the rRNA approach applied has well-documented limitations in assessing the microbial diversity of environmental samples. Biases might be introduced in each step of the approach: e.g. insufficient DNA extraction, preferential PCR amplification, co-migration of DNA fragments during DGGE analysis, inadequate clone library screening and low sensitivity of FISH methods [228, 327-329]. Due to these restraints, the full-cycle 16S rRNA gene approach has been assumed to reveal the diversity of the dominant (abundant) community members only [128]. Although this might be not that restrictive in the study of less diverse endobiotic communities [228], diversity studies of endobionts comprise some specific challenges. Especially the initial surface sterilization, included to eliminate all epiphytic contamination, represents a critical step in the methodological approach. Despite the efficiency of the newly designed protocol (section 3.1.2), each surface sterilization method remains a balancing exercise between too much (i.e. neutralizing internal bacteria) and too little (i.e. outer surface bacteria being mistaken for endobionts). While the reproducibility of results (e.g. Fig. 3.10, section 3.2.2) supports their reliability, the surface sterilization protocol is accountable for some additional limitations. The constraint of living, uni-algal samples as starting material gave rise to extended cultivation of the algal samples prior to molecular processing (see section 3.2.1). This prolonged stay under artificial culture conditions might have unknown effects on the endophytic bacterial community, suggesting the diversity obtained from *Bryopsis* cultures might not fully represent the variety present within the alga in its natural environment. Nevertheless, the bacteria identified in this study are at least part of the natural *Bryopsis* endobiotic flora and supplementary species-specific amplifications as well as fluorescent *in situ* hybridizations (Chapter 3, part 3) underline their true contribution in natural algal samples. Further, 16S rRNA gene-based analyses of photosynthetic eukaryote-prokaryote symbiosis systems are also restricted by 'symbiosis' itself: the cyanobacterial origin of chloroplasts interferes with the characterization of bacteria through massive coamplification of the algal host's chloroplast DNA with universal bacterial primers [228, 330]. Elimination of chloroplast DNA and/or removal of intact chloroplasts before extraction would greatly simplify future 16S rRNA gene-based as well as metagenomic analyses (see below) of the endophytic bacterial community. Diverse protocols are described to selectively amplify bacterial 16S rRNA genes [163, 331], chemically/enzymatically eliminate chloroplast DNA [330, 332], and

separate bacteria form host organelles by density gradient centrifugation [333, 334]; these methods, however, should be optimized to be applicable on *Bryopsis* hosts [330]. In addition, as surface sterilization as well as subsequent DGGE and cloning protocols are labor-intensive and time-consuming, only a limited number of samples could be processed.

Ready-to-use surface sterilization methods and sequenced-based metagenomic analyses in combination with high-throughput next-generation sequencing technologies would be required to examine the endophytic bacterial diversity in a more effective way. Besides looking at 'who is (in) there', also the question 'what are they doing there?' should be tackled more profoundly in future research. Whole-genome sequencing and functional metagenomics could reveal insight into the role of endophytic bacteria within the *Bryopsis* host. Sequence-based analyses of complete genome sequences may shed light on the metabolic potential of the endophytes [334-336], and functional screening of metagenome libraries may identify new genes and/or novel natural products of endobiotic origin [337, 338]. To fully elucidate symbiosis systems, however, it will be necessary to go beyond endobiont genome studies alone by integrating data at all levels (genes, transcripts and proteins) from all symbiosis partners, including the host, as well as information on the interaction of these molecules at a systems biology level [333, 336]. Despite the potential of 'omics' technologies and high-throughput screening methods in generating data, the extraction of useful biological information from these datasets remains a significant (computational) challenge [334]. It has been suggested that the true 'omics' power will be realized when these technologies are integrated with 'classical' approaches that examine gene expression or functional activity *in vivo* such as certain FISH techniques (see section 3.3.2) and stable isotope analyses [339]. Also efforts to culture previously unculturable bacteria – still a prerequisite for their full characterization (see section 3.2.4) – will likely be facilitated by clues about their physiology derived from 'omics' data [339].

Significance of seaweed-bacterial studies

There is a major lack of knowledge about marine symbioses and the impact that these associations have on their hosts' ecology and on global biogeochemical processes of essential nutrients such as carbon, nitrogen and sulfur [340]. Exploring the *Bryopsis*-bacterial partnership may lead to a better understanding of the significance of these marine symbioses. More specifically, *Bryopsis* associated endophytes can provide an insight into the metabolic interchange which underlies the ability of marine hosts and, in particular, siphonous seaweeds to inhabit oligotrophic waters [75, 125, 340]. As this successful spread can have a negative impact on the native biota, host associated bacteria might

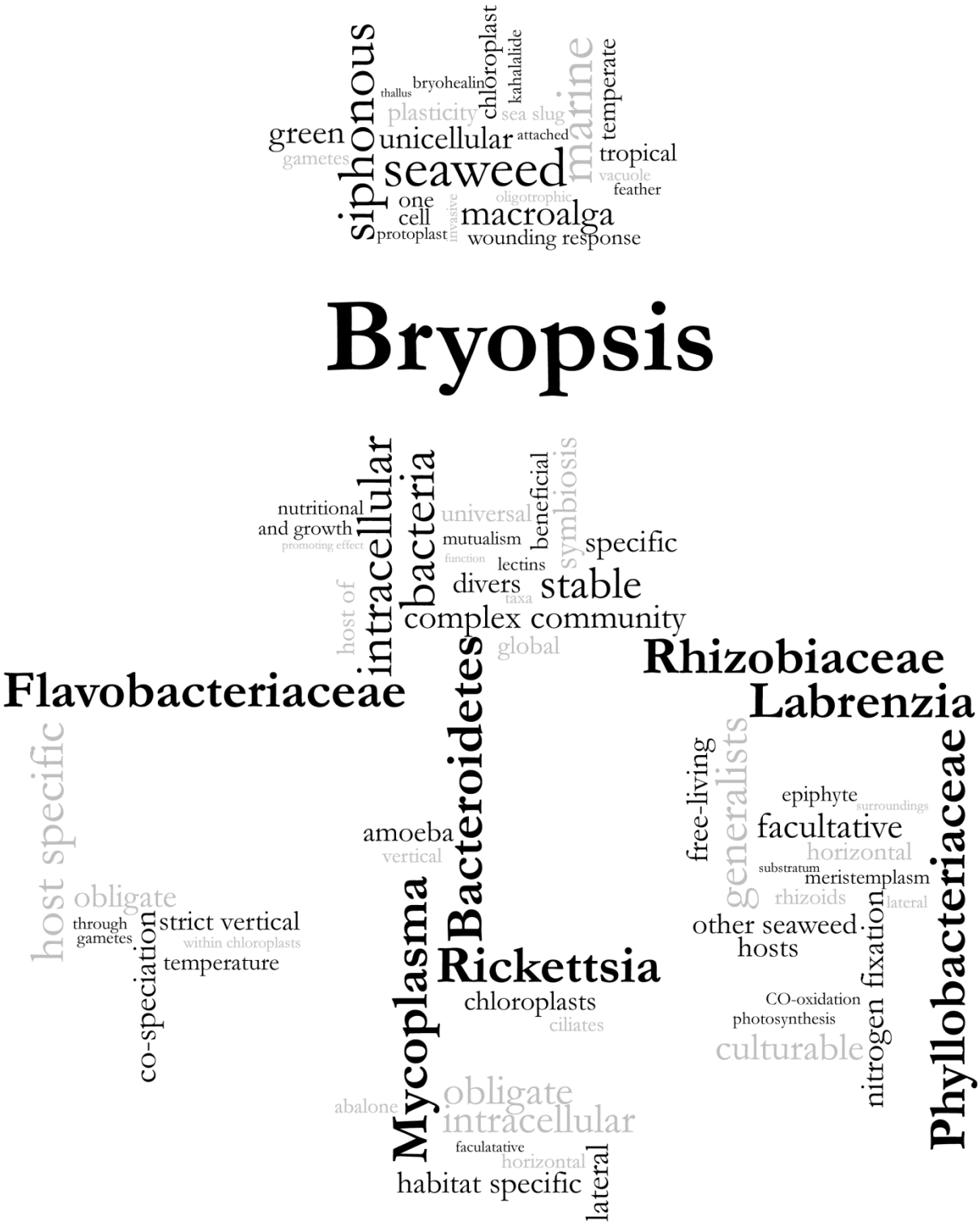
be a target by which to control the process [9]. Moreover, (in part) biogeographically structured bacterial communities can provide a clue on the origin of introduction [125]. Phylogenetically structured obligate endobionts, on the other hand, might be used as part of an integrative taxonomical approach for species delimitation in cryptic (algal) hosts [341]. On a grander scale, whole genome analyses of (obligate) endobionts may bring to light major evolutionary patterns [336]. Furthermore, seaweed-bacterial studies have importance for the characterization of bacterial pathogens in mariculture industry and the discovery of novel natural products (Chapter 1, Box 2).

General reflections

The omnipresence and universality of symbiosis render the term to be a synonym for biology instead of a concept within. Thinking about symbiosis raises issues concerning the boundaries of self and the definition of species, and leads to discussions about holism versus egoism. In their turn, current efforts to understand symbiosis reflect a tension between the reductionist approaches of molecular biologists and the holistic approaches of ecologists [8]. Therefore, the largest obstacle for symbiosis studies may stem from the culture gaps among the different disciplines. The infrastructure as it stands would not foster this process: academic departments, federal agencies and scientific societies are structured in such a way as to frustrate opportunities for productive interactions and creative eccentricity [9]. To achieve the full potential of this field, however, a vast array of technical, cultural and social hurdles must be overcome. Luckily, we can learn a lot from the efficient cross-talk between seaweeds and bacteria!

"*Acknowledging that our ancestors are bacteria is humbling and has disturbing implications. Besides impugning human sovereignty over the rest of nature, it challenges our ideas of individuality, uniqueness, and independence. It even violates our view of ourselves as discrete physical beings separated from the rest of nature and – still more unsettling – it challenges the alleged uniqueness of human intelligent consciousness. Those who speak only for the special interests of human beings fail to see how interdependent life on Earth really is. Without the microbial life forms, we would sink in feces and choke on the carbon dioxide we exhale. […] The ancient, vast, and fundamental nature of our interdependence with other forms of life may be humbling, but it provides a basis for facing the future free of crippling delusions. Despite all our conceits, we are as much exploited as exploiters, as much consumed as consumers. The lesson of evolutionary history is that it will be through conservation, interaction, and networking, not domination, that we avert a premature end to our species.*"

Carla Cole (*IN CONTEXT* **34**, p. 18, 1993), based on the work of Lynn Margulis and Dorion Sagan


siphonous
seaweed
macroalga
green
unicellular
marine
tropical
temperate
chloroplast
kahalalide
bryohealin
thallus
plasticity
sea slug
attached
gametes
vacuole
feather
oligotrophic
one
cell
invasive
protoplast
wounding response
Bryopsis
intracellular
bacteria
symbiosis
beneficial
nutritional
and growth
promoting effect
universal
mutualism
function
lectins
specific
divers
taxa
stable
complex community
host of
global
Flavobacteriaceae
Rhizobiaceae
Labrenzia
Bacteroidetes
host specific
obligate
through
gametes
strict vertical
within chloroplasts
temperature
co-speciation
amoeba
vertical
Mycoplasma
Rickettsia
chloroplasts
ciliates
free-living
generalists
epiphyte
surroundings
facultative
horizontal
substratum
meristemplasm
rhizoids
lateral
other seaweed
hosts
CO-oxidation
photosynthesis
culturable
nitrogen fixation
Phyllobacteriaceae
abalone
obligate
intracellular
faculatative
horizontal
habitat specific
lateral

The term 'symbiosis' described in 1879 as 'a beneficial alliance between two dissimilar organisms' has been rewritten over the last decade. Symbiosis no longer defines a concept in biology; it basically ís biology. Symbioses are widespread covering diverse forms of relationships among multiple partners and support fundamentally important processes. Endosymbiosis with one symbiotic partner (the endosymbiont) living intracellularly within the other (the host), is the most intimate form of symbiosis. The host typically provides a nutrient-rich, sheltered environment for the endosymbiont. In turn, endosymbionts expand the physiological capacities of their hosts, enabling them to invade novel metabolic and ecological niches. In view of this, it has been suggested that interactions with bacterial endosymbionts play a role in the success of siphonous (i.e. single giant cell) seaweeds in a range of marine habitats. This dissertation aimed to explore the partnership between siphonous seaweeds and their intracellular bacterial communities, focusing on the green alga *Bryopsis* as host organism. The identity, diversity, uniqueness, stability, specificity, function, symbiotic nature and transmission modes of the endophytic bacterial communities within *Bryopsis* were examined by a culture-independent full-cycle 16S rRNA gene approach. Statistical analyses were performed to identify the factors (e.g. host phylogeny, geography and environment) shaping the endobiotic bacterial community composition and culture-dependent techniques were implemented to investigate the interdependency among the symbiotic partners.

Results indicate *Bryopsis* harbours rather stable and taxonomically diverse endophytic communities composed of certain Bacteroidetes, Flavobacteriaceae, *Labrenzia*, *Mycoplasma,* Phyllobacteriaceae, Rhizobiaceae and *Rickettsia* species. Although the algal interior is repeatedly exposed to various other marine bacteria during wounding events, a highly specific mode of partner recognition seems to exist as *Bryopsis* selectively maintains and/or attracts the same bacteria globally. This specificity is confirmed by the clear distinctiveness of the intracellular bacterial communities from those occurring in the surrounding seawater, even while the endophytic *Labrenzia*, Phyllobacteriaceae and Rhizobiaceae phylotypes are closely related to free-living bacterial strains and could be isolated on artificial media. These *Labrenzia*, Phyllobacteriaceae and Rhizobiaceae endophytes seem true generalists which are laterally acquired from the environment by any *Bryopsis* host, regardless of host species, habitat and geography, to possibly fulfill functions such as nitrogen-fixation, photosynthesis and CO-oxidation. Also *Mycoplasma, Rickettsia* and Bacteroidetes bacteria appear to a greater or lesser extent horizontally transmitted to *Bryopsis* algae. The habitat-specific lateral acquisition of these obligate intracellular bacteria, however, more likely takes place from co-occurring hosts (e.g. microalgae, ciliates, amoebae and sea slugs) rather than from the surrounding seawater.

Flavobacteriaceae species, on the other hand, are closely associated to *Bryopsis* as they seem vertically transmitted, obligate endosymbionts which show some degree of cospeciation. The unique, host specific presence of these bacteria within *Bryopsis* species from warm-temperate and tropical seas and their internal location in and/or in the close vicinity of algal chloroplasts, might indicate Flavobacteriaceae endosymbionts fulfill a significant role within the *Bryopsis* host. Their exact ecological function, however, remains unclear.

Taken together, these results indicate that the *Bryopsis*-bacterial partnership matches the universal eukaryote-prokaryote symbiosis picture in which a mix of one to two specialist and several generalist bacteria reside within a single host. These diverse and complex endosymbiotic communities might permit further expansion of host capabilities, suggesting that *'it takes two to tango, but a whole crowd to stage dive'*. Also in future (*Bryopsis*-bacterial) symbiosis research, the efficient teamwork between multiple scientists from diverse disciplines might eventually create a major leap forward in the understanding of our symbiotic planet.

De term 'symbiose' gedefinieerd in 1879 als 'een gunstige en/of noodzakelijke vorm van samenleven tussen twee verschillende organismen' is de laatste jaren herschreven. Symbiose definieert niet langer een begrip in de biologie, maar ís ronduit biologie. Symbioses zijn wijdverspreid in de natuur en omvatten diverse, zowel neutrale als goed- en slechtaardige, relaties tussen meerdere partners. Bovendien liggen deze doorgedreven vormen van samenleven aan de basis van fundamenteel belangrijke, evolutieve processen. Endosymbiose waarbij een partner (de endosymbiont) huist binnenin de andere (de gastheer), is de meest intieme vorm van samenleven. De gastheer biedt een voedselrijke, beschutte omgeving voor de endosymbiont die op zijn beurt de fysiologische mogelijkheden van de gastheer uitbouwt zodat deze nieuwe metabolische en ecologische niches kan bewandelen. Zo is geopperd dat interacties met bacteriële endosymbionten een rol spelen in het succes van sifonale (macroscopische maar toch eencellige) zeewieren in diverse mariene milieus. Dit proefschrift had tot doel de samenwerking tussen sifonale zeewieren en hun inwendige (intracellulaire) bacteriën onder de loep te nemen, met de focus op het groene vederwier *Bryopsis* als gastheer. De identiteit, diversiteit, uniekheid, stabiliteit, specificiteit, functie, symbiontische aard en transmissie wijze van de endofytische (m.n. inwendig levend in een plant) bacteriën in *Bryopsis* werden onderzocht aan de hand van een cultuur-onafhankelijke benadering. Statistische analyses werden uitgevoerd om na te gaan welke factoren (bv. gastheersoort, geografie en omgeving) de aanwezigheid van bepaalde bacteriën binnenin het wier beïnvloeden. Ook de wederzijdse afhankelijkheid van beide symbiosepartners, wier en bacteriën, werd onderzocht met cultuur-afhankelijke technieken.

Resultaten tonen aan dat *Bryopsis* een relatief stabiele en taxonomisch diverse endofytische flora bevat bestaande uit bepaalde Bacteroidetes, Flavobacteriaceae, *Labrenzia*, *Mycoplasma,* Phyllobacteriaceae, Rhizobiaceae and *Rickettsia* bacteriën. Niettegenstaande het wier, zowel van buiten als van binnen, herhaaldelijk wordt blootgesteld aan een (letterlijke) zee van bacteriën, oogt de interactie tussen *Bryopsis* en inwendige bacteriën toch zeer specifiek. *Bryopsis* huist wereldwijd dezelfde bacteriële soorten en bovendien verschillen de intracellulaire bacteriën beduidend van deze in het omringende zeewater. Desalniettemin bleken de endofytische *Labrenzia*, Phyllobacteriaceae en Rhizobiaceae bacteriën juist nauw verwant aan vrij-levende bacteriële soorten en konden ze worden geïsoleerd *in vitro*. Deze *Labrenzia*, Phyllobacteriaceae en Rhizobiaceae endofyten zijn dus echte generalisten die uit de omgeving worden opgenomen door om het even welke *Bryopsis* gastheer, ongeacht soort, plaats en/of omgeving, om mogelijk stikstof-fixatie, fotosynthese en CO-oxidatie functies te vervullen. Ook *Mycoplasma, Rickettsia* en Bacteroidetes bacteriën lijken in meer of mindere mate transient

(herhaaldelijk en tijdelijk) opgenomen door vederwieren die in een welbepaald milieu (bepaalde temperatuur, zoutgehalte, nitraatconcentratie, etc) groeien. Deze obligaat intracellulaire bacteriën worden echter niet opgenomen uit het omgeringde zeewater, maar veeleer uitgewisseld tussen *Bryopsis* en andere gastheren zoals bijvoorbeeld microalgen, ciliaten, amoeben en zeeslakken. Flavobacteriaceae endosymbionten daarentegen zijn veel inniger geassocieerd met *Bryopsis*. Ze worden verticaal overgedragen van de ene wiergeneratie naar de andere en vertonen zelfs enige mate van co-speciatie, m.n. als *Bryopsis* evolueert, evolueren de Flavobacteriaceae bacteriën mee. De unieke aanwezigheid van deze bacteriën in *Bryopsis* soorten van warm-gematigde en tropische zeeën en hun interne locatie in en/of in de nabijheid van alg chloroplasten doet vermoeden dat Flavobacteriaceae endosymbionten veelbetekenende functies vervullen binnenin hun gastheer. De exacte ecologische functie van deze bacteriën blijft echter onduidelijk.

Deze bovenstaande resultaten wijzen erop dat het *Bryopsis*-bacteriële partnerschap past in het universele symbiose plaatje waarin een mix van één tot twee specialist en verschillende generalist bacteriën verblijven binnenin een enkele gastheer. Een diverse en complexe endosymbiontische flora kan er immers voor zorgen dat de gastheer meer mogelijkheden krijgt om te overleven. Met andere woorden: '*it takes two to tango, but a whole crowd to stage dive*'. Ook in toekomstig (*Bryopsis*-bacterie) symbiose onderzoek, kan de efficiënte samenwerking tussen meerdere wetenschappers uit diverse disciplines uiteindelijk zorgen voor een grote sprong voorwaarts in het begrijpen van onze symbiontische planeet.

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- 2000-2002: Sint-Jozefsinstituut, Ternat (Belgium)
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- 2002-2006: Ghent University (Belgium)

  Master in Biomedical Sciences

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  Master in Journalism

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- 2008-2012: Ghent University (Belgium)

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Additional education

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Professional employment

- September - December 2007: Data Manager
  Belgian Cancer Registry, Brussels

List of publications

- T. De Baere, J. Hollants, A. Waeytens, J. Huyghe, C. Cuvelier, R. Verhelst, P. Deschaght, M. Vaneechoutte and I. Dhooge. (2009) Otitis media microbes: culture, PCR, and confocal laser scanning microscopy. *B-ENT* **5:** 65-72.
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- J. Hollants, F. Leliaert, H. Verbruggen, A. Willems and O. De Clerck. Permanent residents or temporary lodgers: characterizing intracellular bacterial communities of siphonous green algae. *Manuscript submitted to Mol Ecol*
- J. Hollants, F. Leliaert, O. De Clerck and A. Willems. What to learn from sushi: a review on seaweed-bacterial associations. *Manuscript submitted as a mini-review to FEMS Microbiol Ecol*
- J. Hollants, F. Leliaert, H. Verbruggen, O. De Clerck and A. Willems. Complex pattern of coevolution of a Flavobacteriaceae endosymbiont and its green algal host, *Bryopsis*. *Manuscript in preparation*

Oral presentations

- J. Hollants, O. Leroux, F. Leliaert, O. De Clerck and A. Willems. The art nouveau of biology: Exhibiting seaweed-bacterial symbioses. VLIZ young marine scientists' day. Bruges (Belgium), February 2011

Abstracts and poster presentations

- J. Hollants, F. Leliaert, O. De Clerck and A. Willems. Endosymbiotic bacteria in siphonous green algae - Exploration of a partnership. Ecole Thématique - Les endosymbioses trophiques et leur rôle dans l'évolution passée et contemporaine des Eucaryotes. Roscoff (France), October 2008
- J. Hollants, F. Leliaert, O. De Clerck and A. Willems. Endosymbiotic bacteria in siphonous green algae - Exploration of a partnership. Meeting of the Belgian Society for Microbiology, Stress responses in the microbial world. Brussels (Belgium), December 2008
- J. Hollants, F. Leliaert, O. De Clerck and A. Willems. Endosymbiotic bacteria within *Bryopsis* species - Naming the actors. Doctoraatssymposium Faculteit Wetenschappen. Ghent (Belgium), April 2009
- J. Hollants, F. Leliaert, O. De Clerck and A. Willems. Endosymbiotic bacteria within *Bryopsis* species - Naming the actors. 6th International Symbiosis Society Congress. Madison (USA), August 2009
- J. Hollants, F. Leliaert, O. De Clerck and A. Willems. Endosymbiotic bacteria within *Bryopsis* species - Naming the actors. Meeting of the Belgian Society for Microbiology, Analyzing complex microbial communities and their host microbe interactions. Brussels (Belgium), December 2009
- J. Hollants, F. Leliaert, O. De Clerck and A. Willems. The who, where, when and why of endosymbiotic bacteria within the green alga *Bryopsis*. Meeting of the Belgian Society for Microbiology, Molecular dialogue in host-parasite interaction. Brussels (Belgium), November 2010
- J. Hollants, F. Leliaert, O. De Clerck and A. Willems. The who, where, when and why of endosymbiotic bacteria within the green alga *Bryopsis*. Young Botanists' Forum 2010. Meise, November 2010.

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