



## Host specificity and coevolution of Flavobacteriaceae endosymbionts within the siphonous green seaweed *Bryopsis*

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### ABSTRACT

The siphonous green seaweed *Bryopsis* harbors complex intracellular bacterial communities. Previous studies demonstrated that certain species form close, obligate associations with Flavobacteriaceae. A predominant imprint of host evolutionary history on the presence of these bacteria suggests a highly specialized association. In this study we elaborate on previous results by expanding the taxon sampling and testing for host–symbiont coevolution. Therefore, 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 *Bryopsis* samples, and 92 additional samples belonging to the Bryopsidales and other orders within the class Ulvophyceae. Results indicate that the Flavobacteriaceae endosymbionts are restricted to *Bryopsis*, and only occur within specific, warm-temperate and tropical clades of the genus. Statistical analyses (AMOVA) demonstrate a significant non-random host–symbiont association. Comparison of bacterial 16S rRNA and *Bryopsis* *rbcl* phylogenies, however, reveal complex host–symbiont evolutionary associations, whereby closely related hosts predominantly harbor genetically similar endosymbionts. Bacterial genotypes are rarely confined to a single *Bryopsis* species and most *Bryopsis* species harbored several Flavobacteriaceae, obscuring a clear pattern of coevolution.

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### 1. Introduction

Bacteria living within the body or cells of eukaryotes are extremely abundant and widespread (Dale and Moran, 2006; Ryan et al., 2008; Kikuchi, 2009). These endosymbiotic bacteria often contribute to diverse metabolic host functions, making their presence favorable or even essential (Relman, 2008). Eventually, both the bacterial partner and the host may lose their autonomy and become strictly dependent on each other, resulting in an obligate association (Dale and Moran, 2006; Toft and Andersson, 2010). 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 (Moran et al., 1993; Sachs et al., 2011). This process might give rise to coevolution or cospeciation, evolutionary processes resulting in congruent host and bacterial phylogenies (Peek et al., 1998; Clark et al., 2000; Legendre et al., 2002; Rosenblueth et al., 2012).

In seaweed–bacterial associations, coevolution has only been suggested between the red alga *Prionitis* and its gall-forming *Roseobacter* symbionts (Ashen and Goff, 2000). In the siphonous green seaweed *Bryopsis* (Chlorophyta: Ulvophyceae), bacteria have been observed by electron microscopy in both vegetative thalli and gametes, suggesting a close, specific association between the algal host and bacterial endophytes (Burr and West, 1970). Recently, molecular results showed that geographically diverse *Bryopsis* samples harbor well-defined and rather stable intracellular bacterial communities consisting of a mix of casually and more closely associated species (Hollants et al., 2011a,b, 2013a). Of these bacteria, Flavobacteriaceae symbionts displayed a putatively obligate endobiotic lifestyle and were never isolated from the *Bryopsis* surface and surrounding seawater (Hollants et al., 2011b). The Flavobacteriaceae is a large family of bacteria with diverse ecophysiological characteristics (Bernardet and Nakagawa, 2006). They are known to decompose polysaccharides such as agar, cellulose and carrageenans, making them key players in biotransformation and nutrient recycling processes in the marine environment (Bernardet and Nakagawa, 2006; Goecke et al., 2010; Hollants et al., 2013b). Because of these traits, species of this family often inhabit seaweed surfaces where they have been shown to fulfill antimicrobial (Penesyan et al., 2009; Wiese et al., 2009),

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pathogenic (Sunairi et al., 1995; Weinberger et al., 1997; Vairappan et al., 2008), algal morphogenic, and zoospore settlement inducing (Tatewaki et al., 1983; Nakanishi et al., 1996; Matsuo et al., 2003; Patel et al., 2003; Marshall et al., 2006) roles. Many members of the Flavobacteriaceae, like *Algibacter*, *Fucobacter*, *Maribacter*, and *Ulvibacter* species, have been initially isolated from marine macroalgal surfaces (Goecke et al., 2010, 2013). In addition, several intracellular bacterial symbionts of insects belong to the family Flavobacteriaceae and were shown to affect the reproduction of their hosts (Bernardet and Nakagawa, 2006). In *Bryopsis*, the presence of Flavobacteriaceae was found to be highly congruent with the host phylogeny of two warm-temperate to tropical clades (Hollants et al., 2013a). Testing the hypothesis of non-random host-symbiont association and possibly coevolution, however, requires a rich and geographically diverse sampling.

In this study, we aimed to assess the host-symbiont specificity and possible coevolution of Flavobacteriaceae endosymbionts in *Bryopsis*. Since, the experimental design used previously, i.e. labor-intensive unialgal culturing, surface sterilization, clone libraries, and DGGE analyses (Hollants et al., 2010, 2011a,b, 2013a), was unsuitable for detailed screening of *Bryopsis*-associated Flavobacteriaceae endosymbionts, we developed a PCR protocol to specifically and exclusively 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 samples of other genera of green seaweeds. Phylogenetic and statistical analyses were performed to test for non-random host-symbiont association and possibly coevolution.

## 2. Materials and methods

### 2.1. Algal material

In total 238 green algal samples were screened for the presence of Flavobacteriaceae endosymbionts, including 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*) (Table S1). Algal samples were collected during different field expeditions and clean portions of the thalli were preserved in silica-gel.

### 2.2. DNA extraction and PCR amplification

Algal samples were subjected to total DNA-extraction following a CTAB protocol modified from Doyle and Doyle (1987). To create a *Bryopsis* host phylogeny, chloroplast-encoded *rbcl* genes were amplified as described by Hollants et al. (2011a). For the specific amplification of Flavobacteriaceae endosymbiont 16S rRNA genes, we designed species-specific primers in Kodon v3.5 (Applied Maths, Belgium) with as target group full length Flavobacteriaceae 16S sequences (JF521600–JF521604, HE648933, HE648935, HE648940, and HE648943) obtained in our previous studies (Hollants et al., 2011a, 2013a). Due to the large non-target group (i.e. all other bacterial 16S sequences) only one suitable region (position 690–720) for specific primer annealing was found. Consequently, we designed one species-specific primer which we used in both the forward (F695: 5'-GGCAGTGTGTAAGCCTAA-3') as well as reverse (R695: 5'-TTAAGCTTAGCAACCTGCC-3') direction together with the 16S

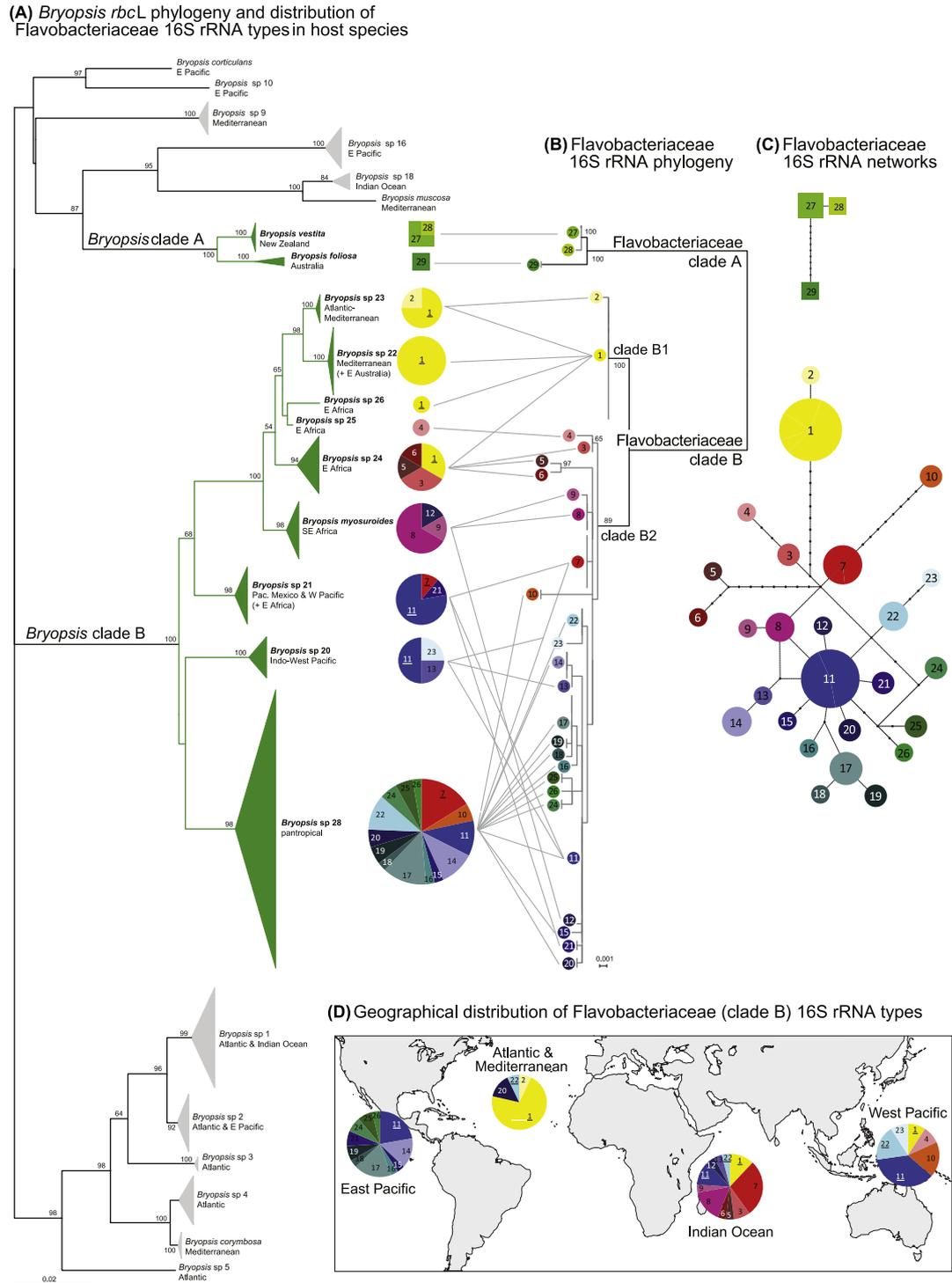
rRNA gene universal primers 1492R and 27F (Lane, 1991), respectively. *Bryopsis* DNA extracts from previous studies (Hollants et al., 2011a, 2013a), in which Flavobacteriaceae endosymbiont DNA was known to be present or absent, were used as templates for the initial PCR optimization experiments. Thermocycling conditions were investigated using gradient-PCR with the following reaction mix: 1× AmpliTaq Gold reaction buffer (Applied Biosystems), 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.5 μM of each primer and 1.25 U/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 by Hollants et al. (2011a). Flavobacteriaceae endosymbiont 16S 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 (Huber et al., 2004). Bacterial and algal sequences were submitted to EMBL under accession numbers HE775438–HE775517 and HF583293–HF583423, respectively.

### 2.3. Phylogenetic analyses of host and symbiont

Two alignments were created for phylogenetic analyses. The *Bryopsis* alignment consisted of 146 *rbcl* 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 (Edgar, 2004). 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 (Posada, 2008). Phylogenetic trees were reconstructed by maximum likelihood (ML) using PhyML v3.0 (Guindon and Gascuel, 2003), via the University of Oslo Biportal website (Kumar et al., 2009). The *Bryopsis* *rbcl* and bacterial 16S rRNA gene alignment were analyzed under a GTR+G model. Trees were visualized in Mega 4.0 (Tamura et al., 2007) 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 (Hudson and Coyne, 2002; Leliaert et al., 2009). Phylogenetic analysis of the Flavobacteriaceae 16S dataset resulted in a tree with three well supported clades (Fig. 1B: clades 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 (Posada and Crandall, 2001). Statistical parsimony networks (Templeton et al., 1992) were constructed with TCS 1.21 (Clement et al., 2000), 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).

### 2.4. Analysis of host-symbiont coevolution and biogeography

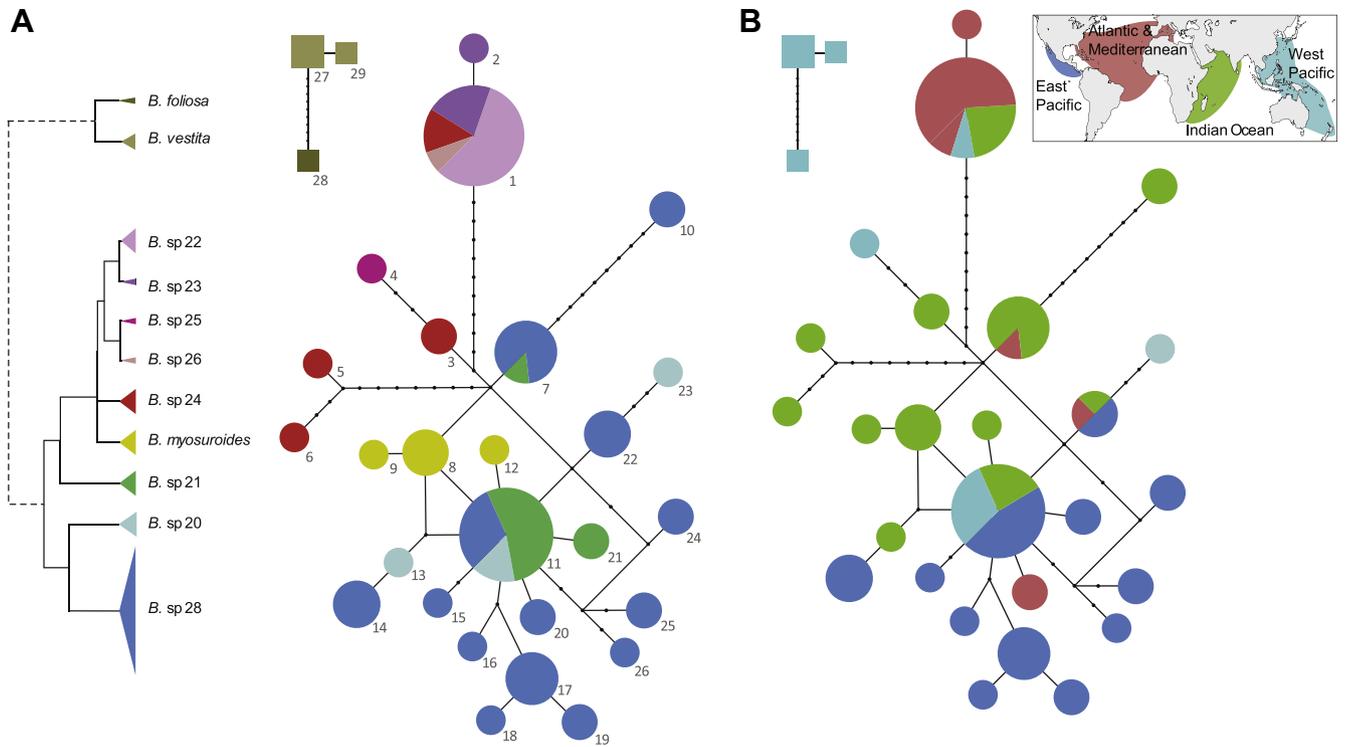
We used different statistical techniques to assess coevolution between Flavobacteriaceae endosymbionts of clade B and the *Bryopsis* host, and to investigate to which degree the bacterial genetic variation was geographically structured. Analysis of molecular variance (AMOVA) of Flavobacteriaceae 16S sequences was used to investi-



**Fig. 1.** 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 phylogenetic 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.

gate the percentage of variation within and between populations, which were predefined as the different host species (*Bryopsis* sp. 20, 21, 22, 23, 24, 28 and *B. myosuroides*) or geographical regions (Atlantic–Mediterranean, East Pacific, Indian Ocean and West Pacific). Because of small sample sizes, *Bryopsis* sp. 25 and 26 were

excluded from the analyses. Patterns of genetic structuring among *Bryopsis* species and between geographical regions were estimated using Arlequin v3.5.1.3 (Excoffier and Lischer, 2010). Population pairwise  $\phi_{ST}$  values, a measure of population differentiation or genetic distance, were calculated using Tamura–Nei distances.



**Fig. 2.** 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 1**  
Pairwise  $\phi_{ST}$  values of Flavobacteriaceae endosymbionts between *Bryopsis* host species (clade B).

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

Values in bold are significantly different from zero after Bonferroni correction.

### 3. Results and discussion

#### 3.1. 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 (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* (Hollants et al., 2011a, 2013a). None of the other Bryopsidales or Ulvophyceae algal samples yielded positive amplifications (Table S1), indicating a strong host specificity and an intimate association of the Flavobacteriaceae endosymbionts with *Bryopsis*.

**Table 2**  
Pairwise  $\phi_{ST}$  values of Flavobacteriaceae endosymbionts between four geographical regions.

	Atlantic–Mediterranean	East Pacific	Indian Ocean	West Pacific
Atlantic–Mediterranean				
East Pacific	<b>0.66</b>			
Indian Ocean	<b>0.45</b>	<b>0.26</b>		
West Pacific	<b>0.44</b>	<b>0.18</b>	0.05	

Values in bold are significantly different from zero after Bonferroni correction.

Mapping of the positive amplifications on the *Bryopsis* host phylogram revealed that the presence of Flavobacteriaceae endosymbionts was restricted to two clades (green branches, Fig. 1A): 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 host–endosymbiont associations evolved twice

independently, or that the association has been lost in one or more *Bryopsis* clades (Fig. S1).

Although our data suggest a preference of Flavobacteriaceae endosymbionts for high temperatures, it is difficult to distinguish whether 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 predominantly determined by seawater temperature regimes (Breeman, 1988). For *Bryopsis*, variation partitioning analysis showed that the presence or absence of Flavobacteriaceae endosymbionts could be largely explained by host phylogenetic factors, which are inevitably interrelated with environmental factors as a result of phylogenetic niche conservatism (Losos, 2008; Hollants et al., 2013a). These results are in agreement with specific host–symbiont associations (Hollants et al., 2013a). Niche conservatism of hosts resulting in temperature-dependent variation of endosymbionts has also been described in other eukaryotes, including sponges, squids and insects (Taylor et al., 2005; Erwin and Thacker, 2008; Toju and Fukatsu, 2011; Zamborsky and Nishiguchi, 2011).

### 3.2. Flavobacteriaceae genetic diversity

The 80 *Bryopsis*-associated Flavobacteriaceae 16S rRNA gene sequences formed a distinct and well supported clade that included two other sequences from sponge- and coral-associated uncultured bacteria (Thiel et al., 2007; Sunagawa et al., 2009) (Fig. S1). The clade was distantly related to cultured Flavobacteriaceae type strains (85–87% 16S rRNA gene similarity), confirming our previous observation that the Flavobacteriaceae endosymbionts likely represent a new genus (Hollants et al., 2011a). The *Bryopsis*-associated Flavobacteriaceae fell into two smaller clades (Fig. 1B, Fig. S1). Clade A consisted of endosymbionts from *Bryopsis vestita* and *B. foliosa*; clade B included the endosymbionts from the other nine *Bryopsis* species (*Bryopsis myosuroides* and *Bryopsis* sp. 20, 21, 22, 23, 24, 25, 26 and 28). Clade B consisted 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. Statistical parsimony analysis resulted in two unconnected networks, corresponding to clade A (three 16S genotypes) and B (26 genotypes). The unresolved relationships within clade B were reflected in a highly interconnected network (Fig. 1C), which may result from homoplasies or recombination (Posada and Crandall, 2001) (see Section 3.3). 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 a maximum of 96.1% between clades A and B (Fig. S1).

### 3.3. Host–symbiont coevolution and biogeography

We applied different methods for examining the association between Flavobacteriaceae endosymbionts and *Bryopsis* hosts. 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. 1) or vice versa (Fig. 2A). Strict topological congruence was observed between *Bryopsis vestita* and *B. foliosa* (clade A) and their associated endosymbionts. However, within clade B, correlation between the phylogenies of Flavobacteriaceae and *Bryopsis* was more complex for three reasons. First, several bacterial genotypes were present in different *Bryopsis* hosts. For example, genotype 1 was found in four *Bryopsis* species (sp. 22, 23, 24 and 26), genotype 11 was present in three species (sp. 20, 21 and 28), and genotype 7 in two species (sp. 21 and 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 coevolution. 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 1), indicating genetic differentiation of endosymbionts between *Bryopsis* species. Pairwise  $\phi_{ST}$ -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 1). Our data also indicated that genetic diversity of endosymbionts was to a large extent geographically structured, with most 16S genotypes being restricted to one geographical region (Fig. 1D, Fig. 2B). This was supported by AMOVA and pairwise  $\phi_{ST}$ -values that showed significant genetic differentiation between the East Pacific, Atlantic–Mediterranean and Indo-Pacific (Table 2). However, this geographical signal may in part be due to dispersal limitation of the host, which results 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 were restricted to two *Bryopsis* clades (clades 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 harbored Flavobacteriaceae endosymbionts (Fig. 1A). A similar strict phylogenetic distribution of endosymbionts was 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 was also apparent when examining specific Flavobacteriaceae genotypes within *Bryopsis* clade B. For example, genotype 1 is widely distributed in the Atlantic, Mediterranean and Indo-Pacific, but clearly restricted to a single clade including *Bryopsis* sp. 22, 23, 24 and 26.

There are several potential and non-exclusive explanations for this complex host–symbiont evolutionary association, invoking uptake of bacteria from the environment and/or vertical transmission of bacterial endosymbionts (Burr and West, 1970; Hollants et al., 2011b). First, related *Bryopsis* species may have evolved similar traits that select for the uptake of specific Flavobacteriaceae from the environment. This uptake may be selective and specific to a certain extent only, depending on habitat-specific physiological requirements, *Bryopsis* plants and availability of certain Flavobacteriaceae genotypes in the seawater. Second, the occurrence of specific Flavobacteriaceae genotypes in different *Bryopsis* species may also be explained by lateral transfer of endosymbionts between host species (host-switching). Sea slugs, which are known to graze on siphonous green algae, could act as effective carriers of bacteria between different *Bryopsis* species (Händeler et al., 2010). The observation that *Bryopsis* endosymbionts are related to bacteria encountered in sponge and coral hosts (Fig. S1) might be indicative for host-switching among distantly related eukaryotes (Weinert et al., 2009). Third, the presence of Flavobacteriaceae in related hosts may be explained through vertical inheritance of bacteria either during sexual reproduction or asexual proliferation by fragmentation or extruded protoplasts that regenerate into new *Bryopsis* plants. The diversity of Flavobacteriaceae genotypes within a single *Bryopsis* species could then be explained by recent and ongoing divergence of endosymbionts. The observation that some endosymbiont genotypes (genotypes 1, 7 and 11, Fig. 1) are found in different *Bryopsis* species may be the result of persistence of ancestral Flavobacteriaceae genotypes in different host lineages. Finally, incongruent host–symbiont coevolution patterns might

be biased by ambiguous algal host and endosymbiont species delimitation. For example, the low level of 16S sequence variability proves that this molecular marker offers limited phylogenetic resolution at lower taxonomic levels (Erwin and Thacker, 2008). Faster evolving markers would provide more polymorphic sites and suitable information to assess coevolution patterns.

In conclusion, our results provide strong evidence for a non-random association between *Bryopsis* and its Flavobacteriaceae endosymbionts, whereby more closely related host species predominantly harbor genetically similar endosymbionts, suggestive of coevolution. The physiological ground for this alliance remains unknown from both the host and endosymbiont perspective. It is possible that Flavobacteriaceae endosymbionts offer the algal host an adaptive advantage. Future studies focusing on functional diversity of the endosymbionts should bring additional insights in these little studied algal–bacterial associations.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymp.2013.02.025>.

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