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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 rbc*L 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.

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 *rbc*L 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′-GGCAGGTTGCTAAGCCTTAA-3′) as well as reverse (R695: 5′-TTAAGGCTTAGCAACCTGCC-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 $MgCl_2$, 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.

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2.3. 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 (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 Bioportal website (Kumar et al., 2009). The Bryopsis *rbc*L 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-