Environmental Microbiology (2009) **11**(8), 2065–2077 doi:10.1111/j.1462-2920.2009.01928.x

A phylogenetic approach to detect selection on the target site of the antifouling compound irgarol in tolerant periphyton communities

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Summary

Using DNA sequence data for phylogenetic assessment of toxicant targets is a new and promising approach to study toxicant-induced selection in communities. Irgarol 1051 is a photosystem (PS) II inhibitor used in antifouling paint. It inhibits photosynthesis through binding to the D1 protein in PS II, which is encoded by the *psbA* gene found in genomes of chloroplasts, cyanobacteria and cyanophages. *psbA* mutations that alter the target protein can confer tolerance to PS II inhibitors. We have previously shown that irgarol induces community tolerance in natural marine periphyton communities and suggested a novel tolerance mechanism, involving the amino acid sequence of a turnover-regulating domain of D1, as contributive to this tolerance. Here we use a large number of *psbA* sequences of known identity to assess the taxonomic affinities of *psbA* sequences from these differentially tolerant communities, by performing phylogenetic analysis. We show that periphyton communities have high *psbA* diversity and that this diversity is adversely affected by irgarol. Moreover, we suggest that within tolerant periphyton the novel tolerance mechanism is present among diatoms only, whereas some groups of irgarol-tolerant cyanobacteria seem to have other tolerance mechanisms. However, it proved difficult to identify periphyton *psbA* haplotypes to the species or genus level, which indicates that the genomic pool of the attached, periphytic life forms is poorly studied and inadequately represented in international sequence databases.

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Introduction

Dramatic advances have been made in exploring microbial diversity using recently developed molecular techniques. DNA sequencing of clone libraries of specific genes, and later of the metagenome of natural communities has revealed a hitherto unknown microbial diversity of massive proportions. *Metagenomics* is a descriptive term for studies that in these ways address DNA sequences of all individuals in a community as a common genomic pool (Kowalchuk *et al*., 2007). The metagenomic approaches have so far had limited use in the study of periphyton communities. These communities consist of organisms growing on surfaces of submerged objects in aquatic environments (Wetzel, 1975). They are very diverse and are composed of different groups of organisms, e.g. bacteria, cyanobacteria, eukaryotic microalgae and microfauna. Although poorly described in a metagenomic context, periphyton communities have been extensively used in water quality assessments and in ecotoxicological research (e.g. see Niederlehner and Cairns, 1990; Patrick *et al*., 2007; Sabater *et al*., 2007). Since the sensitivity to a toxicant differs between species (Blanck *et al*., 1984; Vaal *et al*., 1997), exposure will result in a Toxicant-Induced Succession (TIS) where sensitive genotypes, strains or species are replaced by more tolerant ones. As a consequence, the tolerance of the whole community to the specific toxicant will increase, according to the concept of Pollution-Induced Community Tolerance (PICT) (Blanck *et al*., 1988). Thus, a pollution-tolerant community will have a different genotype, strain and/or species composition compared with a sensitive community. PICT in combination with community-structure change is an integrating endpoint for estimating ecologically relevant effects of toxicants (Blanck, 2002; Boivin *et al*., 2002).

Only few metagenomic studies have dealt with effects of toxicants on periphyton communities. Dorigo and colleagues (2002) compared rDNA composition in eukaryotic organisms from river periphyton with different exposure concentrations of atrazine and isoproturon, and Brummer and colleagues (2003) studied rDNA composition in the β-proteobacterial part of river periphyton with different exposure to various organic and inorganic pollutants. The

present study experimentally estimates the effects of a single toxicant and is therefore not confounded by other environmental variables. Moreover, it estimates effects on the composition of the entire oxygenic and phototrophic part of the communities including both pro- and eukaryotes. We studied the impact of the antifouling compound irgarol 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine) on the phylogenetic composition of the *psbA* gene in marine periphyton communities. The *psbA* gene encodes the D1 protein which is one of the components of photosystem (PS) II. Irgarol is used in antifouling paint to prevent settling of aquatic organisms on underwater surfaces (e.g. boat hulls and oil rigs). Since the first reports of irgarol contamination in 1992 (Readman *et al*., 1993) it has been found in numerous aquatic environments around the globe (reviewed by Konstantinou and Albanis, 2004 and later detected by Hall *et al*., 2005; Harino *et al*., 2005; Carbery *et al*., 2006; Gatidou *et al*., 2007; Blanck *et al*., 2009). This is consistent with observations that irgarol is quite persistent in the environment (Callow and Willingham, 1996; Hall *et al*., 1999) and suggests that wherever irgarol is used there is a considerable potential for contamination. Irgarol is a PS II inhibitor that binds to the D1 protein and thereby blocks photosynthetic electron transport in cyanobacteria, algae and higher plants (Moreland, 1980; Pfister *et al*., 1981; Fedtke, 1982; Vermaas *et al*., 1983). Therefore, the amino acid sequence of D1, and thus the DNA sequence of the *psbA* gene, is of obvious importance for irgarol binding and thus of great importance for irgarol tolerance. Moreover, since *psbA* is a functional gene that encodes the target protein for the studied toxicant, there is a direct link between the mechanism of action of the toxicant and the community composition of the *psbA* gene pool.

psbA is found in genomes of cyanobacteria, cyanophages and chloroplasts, and it is evolutionarily very conserved (Zurawski *et al*., 1982; Svensson *et al*., 1991; Trivedi *et al*., 1994). Therefore, sequences from as distantly related organisms as the early diverging cyanobacteria *Gloeobacter violaceus* and the conifer *Pinus contorta* can be aligned and compared in a satisfactory way. Genomes of cyanobacteria can contain two to five *psbA* genes: *psbA1–psbA5*. However, these genes only encode two forms of the D1 protein (Curtis and Haselkorn, 1984; Mulligan *et al*., 1984; Vrba and Curtis, 1990). These forms impart different functional characteristics to PS II and interchange of them is a means for light adaptation in cyanobacteria (Campbell *et al*., 1996). In most eukaryotic organisms there is only one *psbA* gene in each chloroplast genome and it typically lacks introns (Palmer, 1985; Dwivedi and Bhardwaj, 1995). There can, however, be up to hundreds of copies of the genome within a single chloroplast and, furthermore, several chloroplasts in one cell (Lee and Haughn, 1980; Bendich, 1987; Birky and Walsh, 1992). These genome copies may be polymorphic (Frey *et al*., 1999), resulting in a set of different haplotypes within the genomes. During selection, frequencies of such haplotypes can change among the chloroplast genomes and within the cell. This additional level of intraorganelle and intraindividual selection can induce changes in gene frequencies within the lifespan of an individual and provide a mechanism for very rapid evolution, which has been demonstrated as increases in the point mutation conferring tolerance to triazine herbicides in *Senecio vulgaris* (Frey, 1999). Perhaps the most interesting example of chloroplast genome organization is that of many dinoflagellate species. Their chloroplast genomes have been shown to be substantially reduced down to 16 functional genes, including *psbA*, and divided into so-called minicircles, containing only one to three genes each (Zhang *et al*., 1999; Hackett *et al*., 2004; Barbrook *et al*., 2006). Similarly to the copy number of chloroplast chromosomes in other species, the copy number of minicircles is variable at different growth stages (Koumandou and Howe, 2007), further demonstrating the variable nature of chloroplast genes. This variability in *psbA* gene organization and copy number among organisms suggests that in diverse periphyton communities (i) selection by irgarol might proceed at different rates in different taxa, and (ii) there may be only a weak correlation between the number of cells and the number of *psbA* genes. In addition to these biological reasons there are also methodological limitations, such as the inability to detect small species in the microscope, or primer mismatch in the polymerase chain reaction (PCR) producing the clone libraries, which might lead to preferential amplification and detection of sequences from some species. A strict correlation between clone libraries and morphologically distinct species should therefore not be expected. Instead, these two approaches should be viewed as complementary through which we can examine different aspects of the microbial community.

We have previously shown that richness in *psbA* haplotypes was very high in periphyton communities, and that irgarol-induced community tolerance was accompanied by a dramatic change in the composition of the *psbA* gene pool and of the predicted D1 protein types within the community (Eriksson *et al*., 2009). These changes indicate that the sequences of the D1 protein and *psbA* play important roles in organisms exposed to irgarol. In this study, we put the *psbA* haplotypes from two periphyton communities, one with low and one with elevated irgarol tolerance, in a phylogenetic context. The aims are to use phylogeny to (i) identify *psbA* haplotypes to lower taxonomic levels, e.g. genus or species, (ii) test whether phylogenetic affiliations of *psbA* are indicative of sensitivity or tolerance, and (iii) describe the diversity of periphyton species in a broad taxonomic context.

Results and discussion

We used natural marine surface water in an experimental flow-through system, and since irgarol is used as an antifouling agent in Sweden, it was not possible to achieve a true control community completely unexposed to irgarol. Instead, this community was exposed to the current background concentration of irgarol (for an overview of irgarol field contamination and periphyton community tolerance levels in the same area, see Blanck *et al*., 2009). In the following we therefore use the terms 'background' and 'irgarol' to couple sequences, species or communities to exposure to either background or experimentally elevated irgarol concentrations. The background concentration was 0.02 nM while the experimentally elevated concentration was 10 nM (Eriksson *et al*., 2009). The 10 nM exposure equals the upper range of detected irgarol concentrations in the environment (Konstantinou and Albanis, 2004; Lam *et al*., 2005; Carbery *et al*., 2006).

Since several authors have demonstrated that artefacts can be produced in PCR-produced clone libraries (e.g. see Liesack *et al*., 1991; Polz and Cavanaugh, 1998; Acinas *et al*., 2005), we developed a strategy to minimize these problems (see *Experimental procedures*). This resulted in exclusion of 23 haplotypes from further analyses and the establishment of libraries with very low probability of containing erroneous sequences. Within these revised libraries, 72 unique *psbA* haplotypes were found in the background and irgarol communities, but they were distributed very differently among the two groups. Only three haplotypes were common to both communities while 34 were found only in the background community and 35 only in the irgarol community. BLAST searches (Altschul *et al*., 1997) were performed against the nucleotide collection (nr/nt) in GenBank (Benson *et al*., 2008) in order to identify the haplotypes. Since there were no satisfactory BLAST matches to any known sequences, we used phylogeny to infer the periphyton *psbA* haplotypes to taxonomic groups.

We aligned periphyton *psbA* haplotypes to a large number of *psbA* sequences of known identity and determined their phylogenetic relationships. Some bacteriophage sequences of unknown identity were also included in order to increase taxon sampling in this group (Table S1). After a first phylogenetic analysis some taxa from *Embryophyta* and *Phaeophyceae* were excluded since they were identified as less relevant for assigning periphyton taxa to known taxonomic groups. Even though we used a fairly short segment of *psbA* (285 bp) the resulting tree identifies eukaryotes as a lineage separate from the prokaryotes (Fig. 1). The prokaryote part of the communities is very diverse, containing 56 haplotypes. Unfortunately, this part of the tree is largely unresolved and almost no periphyton haplotypes can be assigned to taxonomic groups (Figs 1 and 2B). This demonstrates that these organisms are poorly studied and poorly represented in the nucleotide databases. However, one periphytic prokaryote clade (clade 1; Fig. 2A), composed of 10 taxa from the background community, seems related to viral cyanophage sequences. The puzzling discovery that bacteriophages infecting *Synechococcus* and *Prochlorococcus* species have incorporated *psbA* in their genomes was made by Mann and colleagues (2003), and it has been studied by several authors since then (e.g. Lindell *et al*., 2004; Zeidner *et al*., 2005; Sharon *et al*., 2007). Although the cyanophage and periphyton prokaryote clade is only moderately supported (0.72 Bayesian posterior probability; clade 1 in Fig. 2A), the fact that only haplotypes from the background community are found in this clade is in agreement with earlier findings in virology. These studies showed that viral phage production depends on host photosynthesis during infection and that chemicals with the same biochemical mechanism of action as irgarol – inhibition of PSII – also have the effect of decreasing phage production (e.g. see Sherman and Haselkorn, 1971; Allen and Hutchison, 1976; Sherman, 1976).

Many studies of microbial communities have revealed high sequence microheterogeneity, meaning that even though many of the sequences are unique, a large proportion show high similarity to each other (e.g. see Ferris and Ward, 1997; Casamayor *et al*., 2002; Ferris *et al*., 2003). Similarly to what Field and colleagues (1997), Acinas and colleagues (2004) and Klepac-Ceraj and colleagues (2004) found for 16S rDNA sequences, we detected clades composed of many closely related prokaryote *psbA* haplotypes (e.g. clades 4 and 6–7 in Fig. 2B) in spite of substantial corrections for PCR artefacts. This pattern can partly originate from multiple paralogous copies of these genes. We evaluated this possibility by comparing sequence similarities of the periphyton prokaryote taxa with those of paralogous *psbA* copies within sequenced cyanobacterial genomes (Table S2) and with those of orthologous genes in non-periphytic cyanobacterial taxa through pairwise comparisons of the studied fragment. This analysis revealed that non-identical paralogous *psbA* fragments within prokaryote genomes have an average similarity of 90.6%, but also showed that it can be as low as 73.3% [*G. violaceus* (PCC 7421)]. The sequence similarities of the orthologous *psbA1* [in *Anabena* sp. (PCC 7120)], *Synechocystis* sp. (PCC 6714) and *Thermosynechococcus vulcanus*] and *psbA2* [in *Cyanobacterium* SG2, *Anabaena* sp. (PCC 7120), *Synechocystis* sp. (PCC 6803), *Prochlorothrix hollandica*, *Synechococcus* sp. (BL3) and *Anacystis nidulans* (R2)] gene fragments are in average 82.5% and 82.9% respectively. This indicates that in spite of the different

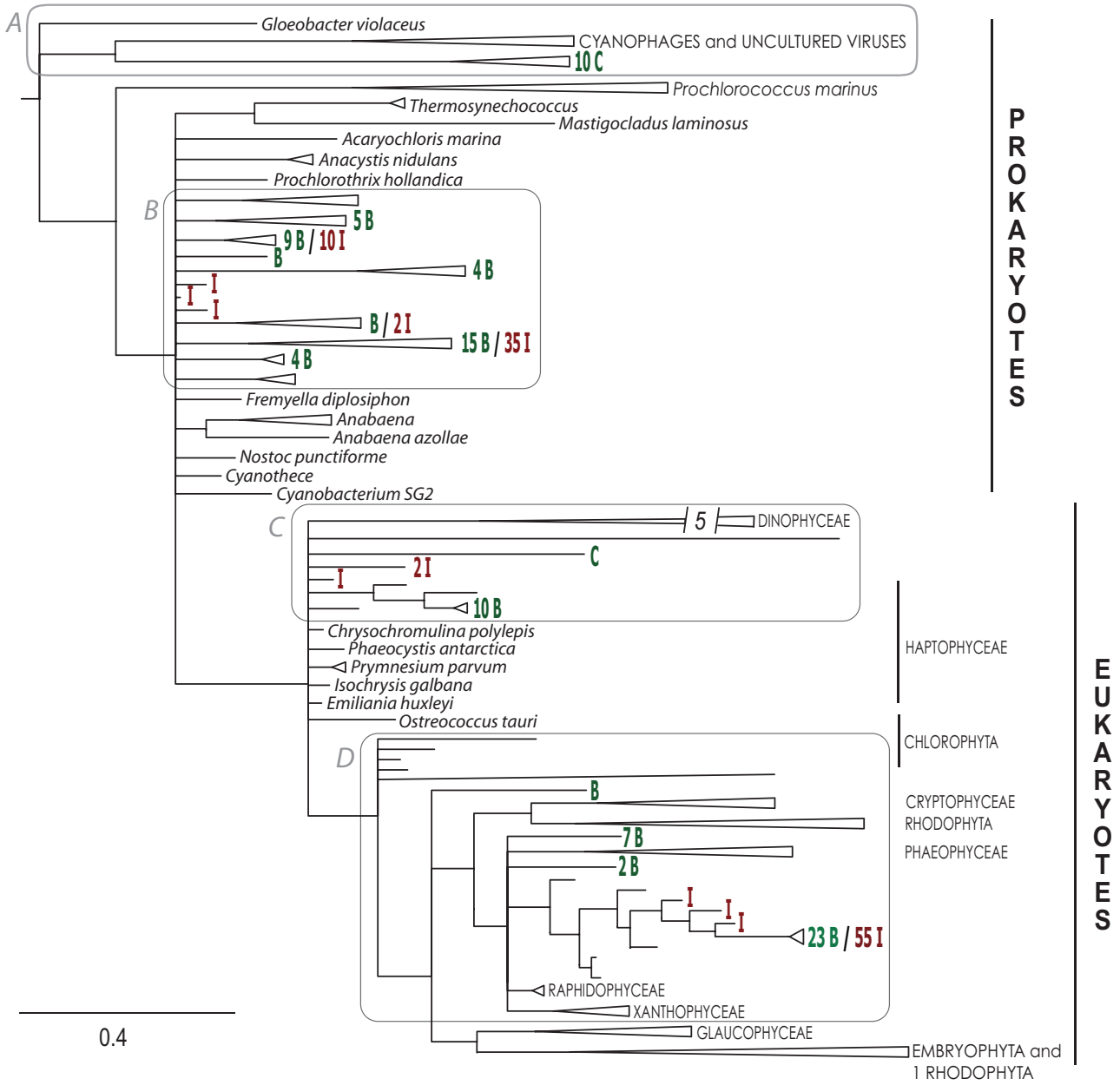

Fig. 1. Simplified tree showing the phylogenetic relationships of *psbA* haplotypes. Evolutionary distances were determined from an alignment of 285 nucleotides. The tree is a 50% majority-rule consensus of 32 000 trees from a Bayesian inference of phylogeny. *psbA* sequences amplified from the microcosms are indicated using the green letter B for sequences from microcosm with background exposure and the red letter I for sequences from microcosm with elevated irgarol exposure. The numbers before these letters indicate the total number of sequences found in the respective microcosm. The parts of the tree indicated with A, B, C and D are enlarged in Fig. 2. The scale bar represents the expected changes per site. The length of the *Dinophyceae* clade break is five scale bars.

functions of the paralogous genes (Campbell *et al.*, 1996), there is a higher degree of *psbA* divergence among species than among functions in one species. Moreover, even though the similarity of paralogous sequences on average is higher than that of orthologous sequences, sometimes these similarities overlap. For the periphyton prokaryote clades 4 and 6–7, the average sequence similarities were 95.7%, 86.3% and 97.8% respectively. Thus, this analysis and the topology of the tree indicate that these clades can contain paralogous as well as orthologous *psbA* genes.

The eukaryote part of the tree contains only 16 periphyton haplotypes but is more resolved than the prokaryote part (Fig. 1). Still, identification of periphyton haplotypes

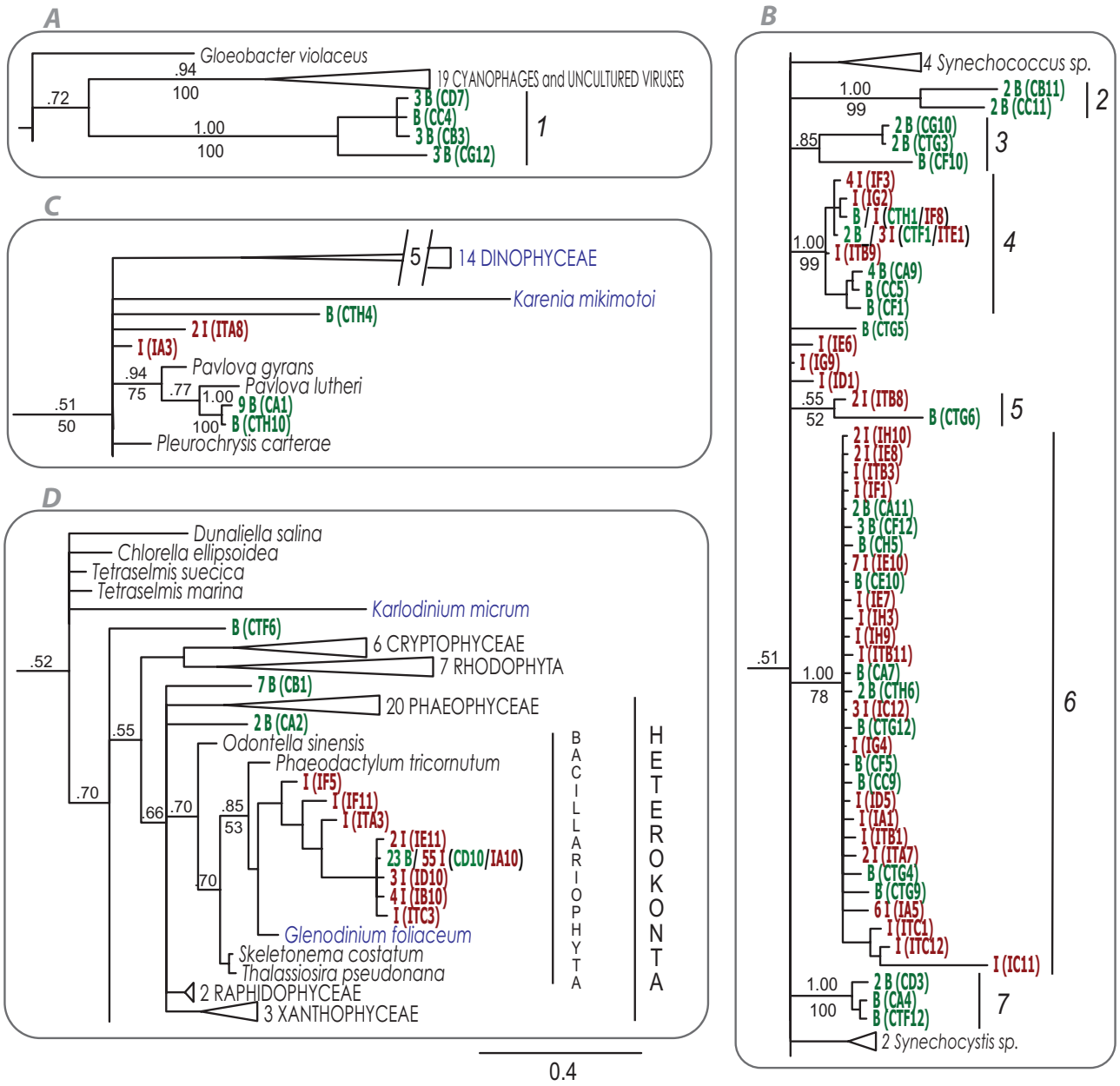

Fig. 2. Enlarged parts (A–D) of the phylogenetic tree shown in Fig. 1. The labels of the periphyton sequences are as in Fig. 1, but here the numbers indicate the detected number of sequences of a specific haplotype. Taxon names in blue indicate species belonging to *Dinophyceae*. The unidentified prokaryote periphyton clades in box (A) and (B) are numbered 1–7 to facilitate references in the text. Numbers above and below branches show posterior probability and jackknife support values, respectively, as applicable. The scale bar represents the expected changes per site. Branches of *Glaucophyceae* and *Embryophyta* were excluded from box (D) since they did not contribute to the identification of periphyton haplotypes.

to species or genus level was not particularly successful. The majority of the periphyton eukaryote haplotypes belong to the *Bacillariophyta* clade (Fig. 2D). This is consistent with data on relative abundance of species based on morphological characters in these communities (Eriksson *et al*., 2009). The *Bacillariophyta* clade also harbours the dinophycean species *Glenodinium foliaceum* (formerly named *Peridinium foliaceum*), which at first would appear to be an erroneous placement (Fig. 2D). However, the grouping of *G. foliaceum* in the diatom clade is consistent with the fact that this taxon previously has been shown to have acquired a diatom endosymbiont via tertiary endosymbiosis (Chesnick *et al*., 1996; 1997; Horiguchi and Takano, 2006). Such endosymbiotic events have

actually occurred many times within this group with an exceptionally complex chloroplast phylogeny as a result (Scherer *et al*., 1991; Moestrup and Daugbjerg, 2007). Our analysis is consistent with previous dinoflagellate phylogenies (Tengs *et al*., 2000; Ishida and Green, 2002; Patron *et al*., 2006) in that the species with fucoxanthin-pigmented chloroplasts, *Karenia mikimotoi* (formerly named *Gymnodinium mikimotoi*) and *Karlodinium micrum* (synonymous names: *Karlodinium veneficum* and *Gymnodinium micrum*), fall outside the clade composed of species with peridinin-pigmented chloroplasts, i.e. the *Dinophyceae* clade (Fig. 2C and D).

One of the most striking features of the trees shown in Figs 1 and 2C and D is the extremely long branches of dinoflagellate taxa. The branch lengths in the dinophycean clade consisting of 14 species (Figs 1 and 2C) range from 1.20 to 3.33 expected substitutions per site, with an average of 2.07, and that of *K. mikimotoi* (Fig. 2C) and *K. micrum* (Fig. 2D) correspond to 1.48 and 1.36 expected substitutions per site respectively. This can be compared with land plants, which in this tree have a range of branch lengths from 1.04 to 1.61 with an average of 1.28. Several studies (e.g. Holder and Lewis, 2003) have shown that Bayesian methods are less sensitive to long-branch attraction (Felsenstein, 1978) than parsimony methods, which suggests that this is a true result rather than an artefact of phylogenetic reconstruction. Moreover, these results are in agreement with previous studies (Cavalier-Smith, 1999; Zhang *et al*., 2000; Bachvaroff *et al*., 2006), which have also shown that dinoflagellates have long branches and thus elevated evolutionary rates as compared with other organisms. The complex dinoflagellate plastid phylogeny makes it difficult to determine whether some of the periphyton haplotypes are of dinoflagellate origin. Marine periphyton attached to glass surfaces have previously been shown to harbour dinoflagellates (Webster *et al*., 2006). Even though our analysis of morphologically different taxa only detected a very low abundance of the dinoflagellate *Prorocentrum lima* in the irgarol microcosm, it is still possible that some of the unidentified haplotypes with long branches, e.g. CTH4, CTF6 or ITA8, are of dinoflagellate origin.

There is a general taxonomic correspondence between morphologically distinct taxa and haplotypes in the microcosms. The general composition of the communities is the same in both data sets, i.e. the dominance of cyanobacteria and diatoms. There are also some examples of correspondence at lower taxonomic levels. There is one case of a likely identification to the level of genus in this analysis, namely the two background haplotypes (CA1 and CTH10) that group with two species of the genus of *Pavlova* (Fig. 2C). These may come from the irgarol-sensitive 'green-like' algal cells detected in the analysis of morphologically distinct taxa. There were also sprouts of brown algae in the data set of morphologically distinct taxa, which could be represented by the CB1 or CA2 haplotypes within the broader taxonomic group *Heterokonta* (Fig. 2D). There are also discrepancies between the data sets of morphologically distinct taxa and haplotypes. Obviously, the methodological limitations of the two approaches can give rise to such discrepancies. For example, cyanophages cannot be detected with a microscope but their DNA can readily be extracted, amplified and cloned. The amplification during PCR might also contribute to such discrepancies since primary and/or secondary structures of the template DNA will give differential primer affinity and dissociation of templates, which in turn can result in differential amplification efficiency and thus a biased composition of sequences (Wagner *et al*., 1994; Suzuki and Giovannoni, 1996; von Wintzingerode *et al*., 1997; Polz and Cavanaugh, 1998; Sipos *et al*., 2007). However, such discrepancies can also come from the inherent differences of the two levels of biological organization. Measuring gene and species frequency is simply not the same thing, for instance, due to differences in number of gene copies among different species.

Even though it is not entirely straightforward, our phylogeny of periphyton *psbA* can be compared with that made for marine phytoplankton *psbA* (Zeidner *et al*., 2003). These authors amplified a larger fragment of *psbA*, which theoretically should result in detection of greater haplotype richness. On the other hand they specifically selected for the picoplankton-sized ($< 3\ \mu m$) cells during cell fractionation, which in turn should lower the detected haplotype richness. In periphyton we found 72 haplotypes, belonging to diverse taxonomic groups (Figs 1 and 2), whereas Zeidner and colleagues (2003) found 3, 12 and 28 haplotypes from the Mediterranean Sea, the Red Sea and the central North Pacific Ocean respectively. The sampled picophytoplankton community was dominated by *Prochlorococcus* and *Synechococcus* haplotypes, even though some green algae, stramenopiles, haptophytes and one cryptophyte were found. Obvious differences between these picophytoplankton communities and the periphyton communities described in this study are the dominance of diatoms and the absence of *Prochlorochoccus* in periphyton. Although there are differences in sampling and in sequence length, it is noteworthy that higher richness was found when we sampled periphyton from two microcosms, at one occasion and one geographical area, as compared with picophytoplankton sampled at three different depths and at three locations around the world during three different years. We thus have to conclude that periphyton communities harbour a great diversity but also that the high haplotype richness from the two microcosms is a consequence of the experimental expo-

sure to irgarol, since this altered the composition of the *psbA* gene pool. Our results also support the conclusion by Zeidner and colleagues (2003) that *psbA* provides a reliable marker for picophytoplankton organisms and further extend this conclusion to include also diatoms.

The topology of the tree (Figs 1 and 2) shows that phylogenetic affiliations of periphyton haplotypes in some cases, but not in all, are correlated with sensitivity or tolerance to irgarol. Examples of sensitive taxonomic groups are prokaryote clade 1–3 and 7, the eukaryotic *Pavlova* clade and some single unidentified taxa (Fig. 2A–D). One larger phylogenetic group is unquestionably selected for by irgarol, namely the clade within *Bacillariophyta* (Fig. 2D). Although this clade harbours the most abundant haplotype in the background community, its abundance increased almost threefold (from 23 to 68 sequences) in the irgarol community. Moreover, the diversity in this grouping increased due to irgarol exposure (Fig. 2D). In contrast, the prokaryote clades 4–6 harbour both background and irgarol haplotypes. This suggests that the studied part of D1, corresponding to the irgarol-binding domain of D1 and the sequenced fragment of *psbA*, is less important for the ecological performance during irgarol exposure in the prokaryote clades than in the *Bacillariophyta* clade. Interestingly, the two different patterns of sensitivity/tolerance to irgarol in the *psbA* phylogeny are consistent with the proposal of two distinct tolerance mechanisms to irgarol in periphyton (Eriksson *et al*., 2009). Eriksson and colleagues (2009) showed that the only region of the irgarol-binding domain of D1 that differed between the background and irgarol community was the PEST domain. Although we cannot at this stage exclude the possibility that irgarol acts as a selection pressure also on other genes, or possibly other regions of *psbA*, the clear irgarol-induced change in distribution of PEST sequence types, and the suggested function of the PEST region to regulate degradation of proteins (Rogers *et al*., 1986; Rechsteiner and Rogers, 1996), imply that the tolerance mechanism involves increased degradation and turnover of the D1 protein. This mechanism of tolerating PS II-inhibiting herbicides has also been suggested previously by Trebst and colleagues (1988), Trebst (1996) and Draber and colleagues (1991). However, the two most irgarol-selected PEST sequence types differed in their occurrence pattern. The most abundant PEST sequence type in the irgarol community was present only in haplotypes that occurred in the irgarol community, which makes this PEST sequence type a likely irgarol tolerance-conferring trait. Importantly, all *Bacillariophyta* taxa have this PEST sequence type. In contrast, the second most abundant PEST sequence type in the irgarol community was present in haplotypes that occurred in both the irgarol and the background communities. Thus, the second most abundant PEST sequence type is less likely to contribute to irgarol tolerance in the irgarol community. According to our phylogenetic analysis, this PEST sequence type was present in haplotypes in prokaryote clade 6 and in the prokaryote single-sequence clades CTG5, IG9, IE6 and ID1 (Fig. 2B), which also occur in both background and irgarol communities. Thus the phylogenetic analysis detected irgarol selection in *Bacillariophyta* because their tolerance mechanism is likely to involve the PEST domain in D1, and thus depends on the sequence of *psbA*. In contrast, irgarol selection for prokaryote taxa was not detected by the phylogenetic analysis since their tolerance mechanism likely is directed towards some other trait. Even though no tolerance mechanism has been described in phytoplankton communities, the irgarol-induced selection for diatoms and cyanobacteria reported here is consistent with effects of irgarol in phytoplankton communities (Readman *et al*., 2004; Devilla *et al*., 2005).

Irgarol selection caused decreased diversity that was detected as the absence of some taxonomic groups in the irgarol community (Fig. 2A–D) and as lowered *psbA* sequence diversity from 0.216 (standard error of bootstrap analysis 0.040) for the background community to 0.184 (standard error of bootstrap analysis 0.043) in the irgarol community. As discussed above for the prokaryote part of the communities, a large fraction of the haplotypes are closely related, which can be seen in Fig. 3A as an almost exponential increase in the number of *psbA* clusters when similarity of sequences increases. The lower diversity in the irgarol community is also shown as a higher degree of sequence similarity in the restructured irgarol-tolerant community (Fig. 3A). When we analysed molecular selection by calculating non-synonymous and synonymous mutations (Hill and Hastie, 1987; Hughes and Nei, 1988) for the periphyton pool of *psbA* genes, no significant positive selection from irgarol could be detected. This is likely due to the high degree of conservation of this fragment of *psbA*. When we expanded the analysis to all taxa in the alignment, positive selection was detected only within the group of phage sequences (data not shown). Similar to most metagenomic studies, the number of detected genes accounted for only a small fraction of the total diversity, indicated by the steadily increasing rarefaction curves for both background and irgarol periphyton (Fig. 3B). Rarefaction analysis was also made on periphyton *psbA* data sets where sequences with decreasing degrees of similarity were grouped together. This analysis gave a similar result as shown in Fig. 3A, namely that the irgarol community contained less sequence diversity, which also decreased more when similar sequences were grouped together, compared with the background community (data not shown).

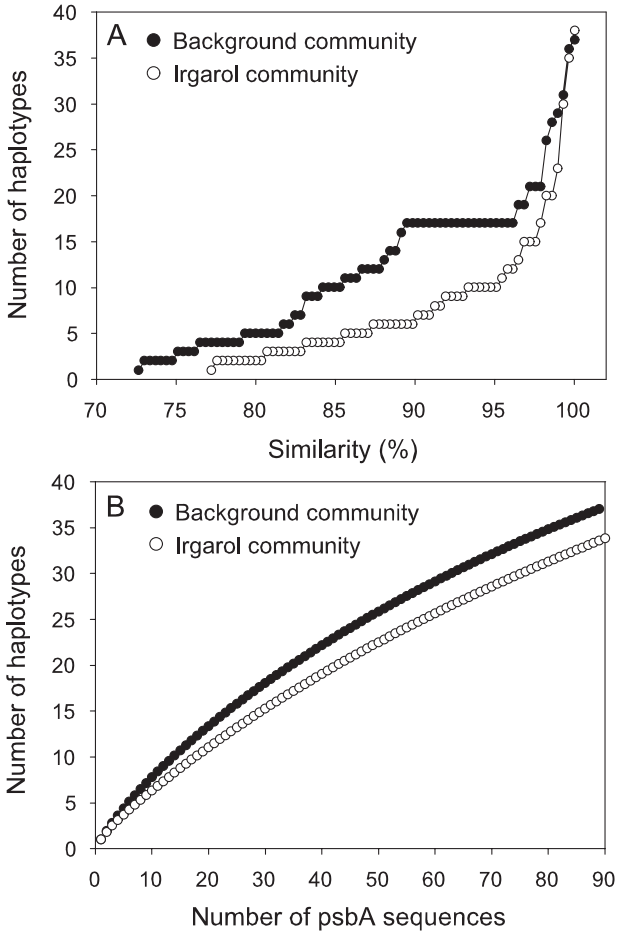

Fig. 3. Compositional pattern of periphyton *psbA* sequences from background and irgarol communities.
A. Relation between number of haplotypes and sequence similarity calculated with the UPGMA algorithm.
B. Rarefaction curves describing the number of haplotypes as a function of the number of *psbA* genes sampled.

In conclusion, the *psbA* gene seems promising for community studies in a taxonomic framework, since even such a short fragment as 285 base pairs gave a relatively high phylogenetic resolution. Still, it proved difficult to identify *psbA* haplotypes to lower taxonomic levels due to lack of annotated *psbA* sequences in international sequence databases. Consistent with the ecotoxicological community-tolerance response, specific haplotypes of *psbA* were shown to be irgarol-sensitive and the overall diversity of periphyton decreased after irgarol exposure. Phylogenetic affiliations of periphyton *psbA* sequences are indicative of irgarol sensitivity or irgarol tolerance for some taxa, but not for others. This is probably because the suggested tolerance mechanism, which is dependent on the PEST domain of D1 and thus encoded by the *psbA* gene, only seems to be present in the eukaryotic part of the community. The haplotype diversity of *psbA* in periphyton communities is very high and for higher taxonomic levels its composition is correlated to traditional measures of species composition. Thus, clone libraries and phylogenetic analysis of communities seem to be useful tools for tracing selection on the molecular targets of toxicants.

Experimental procedures

Microcosm experiment

The flow-through microcosm experiment was performed indoors at Sven Lovén Centre for Marine Sciences, Kristineberg on the west coast of Sweden during July and August in 2001 as described by Eriksson and colleagues (2009). In short, seawater with its indigenous microbiota was continuously pumped from 3 m depth in the Gullmar fjord into each microcosm at flow rate of approximately 220 ml min^{-1}. Larger organisms were prevented from entering the microcosms by a nylon net (1 mm mesh). Irgarol was delivered as water solution from the start of the periphyton colonization at a flow rate of 2 ml min^{-1}. The solution was made by adding 10 ml of stock solution to 10 l of de-ionized water. Irgarol stock solution was made in acetone at a concentration of 2.38 mM. An equal amount of acetone and de-ionized water was added to the background microcosm. Flow rates of the seawater and irgarol water solution were checked daily and adjusted when deviating more than 5% from the desired values. A stirring device in each aquarium ensured thorough mixing of the water. Each microcosm had two fluorescent tubes (Osram lumilux Daylight L 18W/12) as light source, giving a photon flux density of approximately 120 μmol photons m^{-2} s^{-1} at the water surface, and set to the light/dark regime in Sweden at this time of year. Periphyton communities colonized small glass discs (1.5 cm^2) that were mounted in polyethylene holders along the sides of the microcosms.

Clone libraries

Periphyton was carefully scraped off the glass discs with a scalpel and centrifuged at 6500 *g* for 10 min. The resulting pellets were snap-frozen in liquid nitrogen and stored at –80°C. Total periphytic DNA was extracted according to the protocol of the Plant DNAzol® Reagent (Invitrogen, Carlsbad, USA). PCR was performed with degenerate primers constructed from a sequence alignment with a diverse set of phototrophic taxa. The forward and reverse primer sequences were 5′-GTITTYCARGCIGARCAYAAYATIYTI ATGCAYCC-3′ and 5′-CCRTTIARRTTRAAIGCCATIGT-3′ respectively. Polymerase chain reaction conditions were an initial 2 min period at 95°C, followed by 45 cycles of 95°C for 30 s, 50°C for 45 s and 72°C for 30 s. There was then a final extension period of 5 min at 72°C. The produced *psbA* fragments were ligated into the pGEM®-T Easy Vector System II (Promega, Madison, USA) and transformed into competent *Escherichia coli* cells (JM109). One library each was constructed for the background and the irgarol microcosms. After excluding some sequences in order to minimize PCR artefacts (see *Sequence and phylogenetic analysis*) there was 68% efficiency in producing high-quality *psbA* sequences. The background and irgarol libraries contained 89 and 108 high-quality sequences respectively.

Accession numbers

The periphyton *psbA* sequences have GenBank accession numbers from AM933675 to AM933749. The accession numbers of the non-periphytic *psbA* sequences used in the phylogenetic analysis are given in Table S1.

Sequence and phylogenetic analysis

There have been a number of tools developed for detecting chimeric sequences within rDNA clone libraries [e.g. the Chimera Check program at the ribosomal database project II (Cole *et al*., 2003), the Mallard program (Ashelford *et al*., 2006), Bellerophon (Huber *et al*., 2004) or the Ccode program (Gonzalez *et al*., 2005)], but for other genes and especially for shorter fragments, these tools are not applicable. Therefore a strategy was developed to minimize PCR artefacts within our libraries. We removed sequencing errors by excluding clones with non-complementary forward and reverse sequences from further analysis. To avoid artefacts from polymerase errors we excluded sequences that fulfilled the two criteria of occurring only once in each library and differing by only one nucleotide from any other sequence within the library. Moreover, we manually identified possible recombination points among the sequences in each library, and checked whether any sequence could originate from other sequences, i.e. be of chimeric origin.

psbA sequences of known species, and some bacteriophage sequences with unknown identity, were retrieved from the EMBL-EBI database, and 161 sequences were aligned to the 72 periphyton *psbA* sequences. Sequences were aligned using Mafft 5.64 (Katoh *et al*., 2002; 2005) with the settings: FFT-NS-i (Slow; iterative refinement method), the gap opening penalty 1.53 and the offset value 0.123. Similarity and rarefaction calculations of periphyton *psbA* haplotypes were made with UPGMA in the Clusterer application (version 1) (Klepac-Ceraj *et al*., 2006). Sequence diversity within the communities was calculated using pairwise analysis of sequences and the Maximum Composite Likelihood method (Tamura *et al*., 2004). Standard error estimates were obtained by a bootstrap procedure with 1000 replicates. Positive molecular selection was tested with a codon-based *Z*-test under the test hypothesis of positive selection, using the Nei-Gojobori method (Nei and Gojobori, 1986). The *Z*-variance of the difference was computed using a bootstrap of 1000 replicates. For these analysis the software MEGA 4 (build number 4028) (Tamura *et al*., 2007) was used. When comparing similarities of paralogous and orthologous *psbA* sequences from non-periphytic cyanobacterial taxa and periphyton prokaryote *psbA* sequences, BioEdit 7.0.5.3 (Hall, 1999) was used.

For the phylogenetic analysis, both Bayesian and maximum parsimony methods were used as implemented in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) and PAUP* 4.0b10 (Swofford, 2002) respectively. We used *G. violaceus* as out-group since this cyanobacterium is a member of an early branching lineage (Nelissen *et al*., 1995; Turner *et al*., 1999; Cavalier-Smith, 2007). The evolutionary model suggested by the Akaike Information Criterion as implemented in MrModelTest 2.2 (Nylander, 2004) was incorporated in the input file of MrBayes. For each run, eight parallel Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) chains were performed, running 20 million generations, sampling every 1000 generations and saving branch lengths. The software Tracer 1.4 (Rambaut and Drummond, 2007) was used to determine when the tree sampling stabilized, and a burn-in value of 4000 was subsequently used when computing the results. The analysis gave a 50% majority-rule consensus tree from 32 000 sampled trees. Jackknife support values (Farris *et al*., 1996) were then estimated in PAUP* by running 10 000 replicates with 37% deletion, 20 random addition sequence replicates, TBR branch swapping and saving up to 10 trees per replicate.

Acknowledgements

This work has been supported by the European commission (ACE project MAS3-CT98-0178), the Swedish Research Council Formas (project No. 2004-2139), the University of Gothenburg Marine Research Centre, the Memorial Foundation of Birgit and Birger Wåhlström and the Platform Ecotoxicology – From Gene to Ocean within the University of Gothenburg all of which are gratefully acknowledged. We also thank the staff at Kristineberg, Sven Lovén Centre for Marine Sciences (formerly Kristineberg Marine Research Station) for their help and for providing excellent working conditions, and two anonymous reviewers for many constructive suggestions.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Organism names and accession numbers of the non-periphytic sequences in the phylogenetic analysis.
Table S2. Paralogous *psbA* sequences from prokaryote species.

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