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Putative N_2-fixing heterotrophic bacteria associated with dinoflagellate–*Cyanobacteria* consortia in the low-nitrogen Indian Ocean

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ABSTRACT: Heterotrophic dinoflagellates bearing unicellular cyanobacterial symbionts are common within the order Dinophysiales. However, the ecological role of these symbionts is unclear. Due to the occurrence of such consortia in oceanic waters characterized by low nitrogen concentrations, we hypothesized that the symbionts fix gaseous nitrogen (N_2). Individual heterotrophic dinoflagellates containing cyanobacterial symbionts were isolated from the open Indian Ocean and off Western Australia, and characterized using light microscopy, transmission electron microscopy (TEM), and nitrogenase (*nifH*) gene amplification, cloning, and sequencing. *Cyanobacteria*, heterotrophic bacteria and eukaryotic algae were recognized as symbionts of the heterotrophic dinoflagellates. *nifH* gene sequences were obtained from 23 of 37 (62%) specimens of dinoflagellates (*Ornithocercus* spp. and *Amphisolenia* spp.). Interestingly, only 2 specimens contained cyanobacterial *nifH* sequences, while 21 specimens contained *nifH* genes related to heterotrophic bacteria. Of the 137 *nifH* sequences obtained 68% were most similar to *Alpha-*, *Beta-*, and *Gammaproteobacteria*, 8% clustered with anaerobic bacteria, and 5% were related to second alternative nitrogenases (*anfH*). Twelve sequences from 5 host cells formed a discrete cluster which may represent a not yet classified *nifH* cluster. Eight dinoflagellates contained only 1 type of *nifH* sequence (>99% sequence identity) but overall the putative N_2-fixing symbionts did not appear host specific and mixed assemblages were often found in single host cells. This study provides the first insights into the *nifH* diversity of dinoflagellate symbionts and suggests a symbiotic co-existence of non-diazotrophic cyanobacteria and N_2-fixing heterotrophic bacteria in heterotrophic dinoflagellates.

KEY WORDS: Symbionts · Nitrogen fixation · Nitrogenase · Heterotrophic bacteria · *Ornithocercus* · *Amphisolenia* · *Histioneis* · Dinoflagellates · Indian Ocean · Galathea 3

INTRODUCTION

Cyanobacterial symbionts (cyanobionts) of non-photosynthetic dinophysoids (Dinophyceae) were first observed more than 100 yr ago (Schütt 1895). They are thought to function as photosynthetic partners in their relationship with the host (Taylor 1982), but despite many different types of cyanobacterial and bacterial symbionts having been described for several dinophysoid genera (such as *Amphisolenia*, *Histioneis*, *Ornithocercus* and *Parahistioneis*), little is known about the identity and diversity of these symbionts. Further, their ecological significance is essentially unknown.

Recently, we found that symbiont-bearing dinoflagellates were most common in the photic zone of the Indian Ocean characterized by low nutrients and low

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phytoplankton biomass (Tarangkoon et al. 2010). This is consistent with previous observations from the Indian Ocean (Jyothibabu et al. 2006) and the Red Sea (Gordon et al. 1994). Due to this distribution it has been suggested that the photosynthetic symbionts are N_2-fixers (diazotrophs), providing their hosts with reduced N (Gordon et al. 1994, Jyothibabu et al. 2006). To date only a single study has demonstrated nitrogenase in a cyanobiont of a heterotrophic dinoflagellate (Foster et al. 2006a); however, several studies indicate that cyanobionts may be diazotrophic. For instance, even though most of the 65 cyanobacterial *16S rRNA* gene sequences retrieved from individual heterotrophic eukaryotic host cells were related to *Synechococcus* sp., 3 sequences from 2 *Histioneis* sp. hosts were related to the unicellular N_2-fixing cyanobacterium *Cyanothece* sp. (Foster et al. 2006b). Further, using fluorescent *in situ* hybridization, putative unicellular diazotrophic cyanobionts associated with dinoflagellates have been observed in the Mediterranean Sea (Le Moal & Biegala 2009) and the Southwest Pacific Ocean (Biegala & Raimbault 2008).

In tropical and subtropical waters the ubiquitous filamentous cyanobacterium *Trichodesmium* sp. (Capone et al. 1997) and intracellular cyanobacterial symbionts of diatoms (*Richelia intracellularis*, a symbiont of diatoms, e.g. *Rhizosolenia* spp.; Carpenter et al. 1999) were long believed to be solely responsible for pelagic N_2 fixation. However, recent molecular studies targeting the nitrogenase (*nifH*) gene, encoding the iron protein component of the nitrogenase enzyme, have shown that free-living unicellular cyanobacteria are also abundant and can account for a significant fraction of the N_2 fixation (Zehr et al. 2001, Montoya et al. 2004). Similarly, it has recently been recognized that non-cyanobacterial diazotrophs, mostly heterotrophic bacteria, are widespread in marine waters, and their ecological function and importance is currently unknown (Farnelid & Riemann 2008).

In the present study we sought to identify potential N_2-fixing symbionts of heterotrophic dinoflagellates in the Indian Ocean. The symbionts were characterized using light microscopy and transmission electron microscopy (TEM) and *nifH* genes were amplified from individual symbiont-bearing dinoflagellate hosts using a nested PCR approach. Our study points to a hitherto unrecognized importance of heterotrophic bacteria for N acquisition in dinoflagellate–*Cyanobacteria* consortia in tropical waters.

MATERIALS AND METHODS

Sample collection. Sampling was carried out onboard the Danish Navy surveillance frigate 'F359 Vædderen' during Leg 7 of the 3rd Danish Galathea expedition (October to November 2006). Samples were obtained from 21 stations located across the Indian Ocean and along a transect perpendicular to Broome in North Western Australia (see Fig. 1 in Tarangkoon et al. 2010). For *nifH* gene analysis samples were obtained at stations BR5 to BR9 (BR5: 17° 03′ S, 120° 49′ E; BR6: 16° 50′ S, 120° 34′ E; BR7: 16° 26′ S, 119° 56′ E; BR8: 16° 15′ S, 119° 38′ E; BR9: 16° 01′ S 119° 19′ E) in the Broome transect. Live plankton samples were collected at each station by vertical hauls from about 70 m depth to the surface with a 20 µm mesh size plankton net, or from water samples (30 l) from 10 m and 30 m depths collected by Niskin bottles attached to a conductivity, temperature and depth profiler rosette. Subsequently, plankton was concentrated using a 20 µm mesh size Nitex filter. The filters were kept immersed during the filtration to facilitate the retention of live cells. The concentrated samples were transferred to 100 ml of filtered seawater from which single cells were isolated using a drawn Pasteur pipette. Cells for *nifH* gene analysis were then successively washed in 3 baths of 2 ml 0.2 µm filtered seawater, placed individually in a 0.2 ml PCR tube, and immediately frozen at –20°C. The cells included a range of dinoflagellate species (see Table 3), although no *Histioneis* spp. were obtained.

Microscopy. Dinoflagellates were examined shortly after sampling using an Olympus BX51 light microscope fitted with a Soft-Imaging ColorView III digital camera and identified as described in Tarangkoon et al. (2010). In total ~100 cells were examined in the study. Seven cells of *Ornithocercus magnificus* and 2 of *O. quadratus* were collected at Stn 5 in the Indian Ocean transect (29° 35′ S, 95° 15′ E), preserved and further processed in the laboratory for TEM (Tarangkoon et al. 2010). Briefly, sections performed on an Ultracut E ultramicrotome using a diamond knife were collected on slot grids, placed on Formvar film, and stained in uranyl acetate and lead citrate. The sections were examined in a JEOL JEM-1010 electron microscope operated at 80 kV and micrographs were taken using a GATAN 792 digital camera.

DNA extraction and *nifH* amplification. DNA was extracted from individual heterotrophic dinoflagellates with symbionts using an enzyme/phenol-chloroform protocol (Riemann et al. 2008) and 200 µl SET lysis buffer (20% sucrose, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0). An extraction without added sample served as a control on the purity of the extraction chemicals. Seven of the samples were not subjected to the extraction procedure but to 3 cycles of freeze/thawing (–80°C for 1 min and 75°C for 1 min constituted 1 cycle), which lyses the cells (Sebastian & O'Ryan 2001). To amplify *nifH*, degenerate primers

purified by high performance liquid chromatography and polyacrylamide gel electrophoresis (Sigma-Aldrich; Zehr & McReynolds 1989, Zani et al. 2000) were used according to a nested PCR protocol (Zehr & Turner 2001) using Pure Taq Ready-To-Go PCR Beads (GE Healthcare). A negative control reaction with UV-treated water was included in each PCR batch. To minimize the risk of contamination, mixing of reagents was done in a UV-treated sterile flow bench in a UV-treated room, template was added in a PCR/UV workstation in a separate room, and single tubes (not strips) were used. For the initial PCR reaction, 3 to 6 µl of the extracted DNA or freeze/thawed solution was added as template and 1 µl PCR product was transferred to the subsequent PCR reaction. Five µl from the second PCR reaction was examined on a 1% agarose gel, and for samples that produced a ~359 bp product the remaining 20 µl was gel purified (E.Z.N.A Gel extraction kit, VWR). The negative PCR control and the negative extraction control were always blank. For the negative control PCR reaction, although there was no visible product, the gel region corresponding to the correct product size was excised, gel purified and cloned. All purified products were cloned using the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was obtained using the R.E.A.L Prep96 Plasmid Kit (Qiagen) according to manufacturer's protocol and sequencing was done commercially (Macrogen, Korea).

Sequence and phylogenetic analysis. Vector sequences and primers were removed manually and the sequences were translated and aligned using the Lasergene 7 package (DNASTAR). The most similar uncultured and cultured relatives as identified from BLASTN comparisons from the NCBI GenBank database were added to the dataset and a neighbor-joining phylogenetic tree was constructed in MEGA4 (Tamura et al. 2007). The partial *nifH* sequences have been deposited in GenBank under accession numbers GU196835 to GU196971.

RESULTS

Microscopy analyses of symbionts

The morphologies (e.g. color, shape and size) of symbionts of heterotrophic dinoflagellates (~100 cells) were compared to published data on ectosymbionts (Table 1) and endosymbionts (Table 2). All *Ornithocercus* spp. cells had orange and elongated cyanobacterial ectosymbionts located within the cingulum, while some also had large rod-shaped non-cyanobacterial prokaryotes on their sulcal lists (Fig. 1A, Table 1). *Histioneis* spp. contained 2 other types of cyanobacterial ectosymbionts (Fig. 1B, Table 1). In *Amphisolenia* spp. only endosymbiotic spheres of 3 to 7 µm were found (Fig. 1C, Table 2). The endosymbionts in *A. bidentata* contained a single yellow chloroplast and a nucleus demonstrating its eukaryotic origin. The symbionts of *A. thrinax* had a more brownish color, but whether these symbionts were of a eukaryotic origin is unclear (not shown).

TEM revealed that the ectosymbionts of *Ornithocercus magnificus* and *O. quadratus* were *Cyanobacteria* and heterotrophic bacteria (Fig. 2A). Cyanobacterial ectosymbionts were observed in the cingulum of both species (Fig. 2A), though most were lost during the preparation process for TEM. These all appeared to be of the same type, i.e. containing 1 to 3 peripheral thylakoid bands in addition to several bands traversing the cell (Fig. 2B,E). Clusters of polyhedral granules, carboxysomes (Lucas 1991), were present in all the cyanobacterial ectosymbionts examined (Fig. 2B,D). In some, electron translucent granules were present (Fig. 2E), similar to putative cyanophycin granules (Lucas 1991). A typical eubacterial Gram-negative wall, consisting of a thin wall inbetween 2 membranes, surrounded the cyanobiont cells (Fig. 2C). Small rod-shaped, 1.5 × 0.2 µm, heterotrophic bacteria were observed in the cingulum of some *Ornithocercus* cells (Fig. 2A,F,G). Unfortunately, the large rod-shaped heterotrophic bacteria seen on the sulcal list by light microscopy (Fig. 1A) were lost during the TEM preparation procedure.

nifH sequence composition and phylogeny

nifH amplicons were obtained from 23 of the 37 analyzed symbiont-bearing heterotrophic dinoflagellates. The 137 *nifH* sequences obtained were related to *nifH* Cluster I (*Cyanobacteria* and *Alpha-*, *Beta-*, and *Gammaproteobacteria*), Cluster II (alternative nitrogenases; *anfH*) and Cluster III (anaerobic bacteria) as defined by Chien & Zinder (1996). Sixty-eight percent of the sequences (originating from 17 samples) were most similar to proteobacterial *nifH* sequences (Fig. 3). All 10 sequences from the negative control formed a cluster within *Betaproteobacteria* (>98% within-cluster sequence identity, Fig. 3) related to a previously reported PCR reagent contaminant sequence. Four sample sequences (*A. bidentata*, samples P60 and P62, Table 3) were affiliated with this cluster, but were not identical to the negative control sequences. Other sequences clustering with *Betaproteobacteria* were most similar to *Ideonella dechloratans* (18 sequences from 5 samples; 91 to 92% sequence identity) and *Burkholderia vietnamiensis* (12 sequences from 5 samples; 97 to 98%

Table 1. Types and characteristics of ectosymbionts of heterotrophic dinoflagellates

Group	Cell shape	Length (µm)	Width (µm)	Characteristics/internal structure of symbionts	Heterotrophic dinoflagellates	Source (name type)
Indian Ocean						
Synechococcus carcerarius	Ellipsoid, short rod, cylindrical	8–10	3–5	Light pink to purplish-red color	*Ornithocercus formosus*, *O. heteroporus*, *O. magnificus*, *O. quadratus*, *O. thumii*, *Histioneis carinata*, *H. pacifica*, *H. striata*, *Parahistioneis* sp.	Norris (1967)
Synechocystis consortia	Spherical	6–8		Grey-blue color	*H. carinata*, *Parahistioneis* sp.	Norris (1967)
Cyanobacteria	Rod/ ellipsoid	1.5–2.8	1.2–1.5	3–4 concentric thylakoids, carboxysomes central	*Ornithocercus* sp., *Histioneis* sp., *Parahistioneis* sp.	Lucas (1991) (Type I)
Cyanobacteria	Rod/ ellipsoid	1.0–1.7	2	Peripheral/central thylakoids, occasional carboxysomes	*Ornithocercus* sp., *Citharistes apsteinii*	Lucas (1991) (Type II)
Cyanobacteria	Spherical	3.5–4.8		Short, irregular thylakoids, few large carboxysomes, many cyanophycin granules	*Histioneis* sp., *Parahistioneis* sp.	Lucas (1991) (Type III)
Cyanobacteria	Elongate	8–10	1.7–3.3	2–3 peripheral thylakoids, several transverse thylakoids, cluster of carboxysomes, occasional putative cyanophycin granule	*O. magnificus*, *O. quadratus*	Present study, Tarangkoon et al. (2010)
Cyanobacteria	Ellipsoid	1.25		Orange color	*Histioneis* spp.	Present study, Tarangkoon et al. (2010)
Cyanobacteria	Spherical	2.5–5		Pale light greenish color	*Histioneis* spp.	Present study, Tarangkoon et al. (2010)
Pacific Ocean						
Cyanobacteria	Spherical/ oblong	1.6 ± 0.6[a]	1.3 ± 0.4[a]	Prominent glycogen clusters throughout the cytoplasm, carboxysomes scattered often in clusters	*Ornithocercus* sp.	Foster et al. (2006a) (Type 1)
Cyanobacteria	Ellipsoid	2.4 ± 0.6[a]	1.9 ± 0.5[a]	Sheath, 4–5 concentric peripheral/central thylakoids, carboxysomes, and glycogen stores central	*Ornithocercus* sp.	Foster et al. (2006a) (Type 2)
Cyanobacteria	Spherical	3.7 ± 0.7[a]	2.3 ± 0.8[a]	Sheath, large glycogen inclusions throughout cytoplasm, no distinct thylakoids	*H. depressa*	Foster et al. (2006a) (Type 4)
Cyanobacteria	Rod/ spherical	1.4 ± 0.5[a]	1.0 ± 0.3[a]	Glycogen in smaller packets scattered, diffuse thylakoids and no carboxysomes apparent	*H. depressa*	Foster et al. (2006a) (Type 5)
Cyanobacteria	Rod	2.8 ± 0.2[a]	1.3 ± 0.2[a]	Sheath, 3–4 peripheral thylakoids, small packets of glycogen or occasional as larger inclusion, no visible carboxysomes	*Histioneis* sp.	Foster et al. (2006a) (Type 6)
Cyanobacteria	Ellipsoid	1.7 ± 0.6[a]	0.9 ± 0.1[a]	4–6 peripheral thylakoids with small packets of glycogen scattered in between, no visible carboxysomes	*Histioneis* sp.	Foster et al. (2006a) (Type 7)
Prochlorococcus	Spherical/ oblong	0.6 ± 0.2[a]	0.3 ± 0.1[a]	2–3 peripheral thylakoids, small scattered glycogen packets, no visible carboxysomes	*Histioneis* sp.	Foster et al. (2006a) (Type 8)

[a]Mean length

Table 1 (continued)

Group	Cell shape	Length (µm)	Width (µm)	Characteristics/internal structure of symbionts	Heterotrophic dinoflagellates	Source (name type)
Atlantic Ocean						
Cyanobacteria	Spherical/ rod	3.5±0.7[a]	2.8±0.3[a]	Thylakoids throughout the cytoplasm, carboxysomes scatted in clusters, glycogen in larger bands between thylakoids	*O. magnificus*	Foster et al. (2006a) (Type 3), Janson et al. (1995)
Cyanobacteria (Type I of Lucas)	Rod/ ellipsoid	2.5		3–4 concentric thylakoids	*O. magnificus*	Janson et al. (1995)
Cyanobacteria (Type II of Lucas)	Rod/ ellipsoid	1–2		Peripheral/central thylakoids	*O. magnificus*	Janson et al. (1995)
Cyanobacteria (Type IV of Lucas)	Rod	10	1.5	Peripheral thylakoids	*O. magnificus*	Janson et al. (1995)
Heterotrophic bacteria	Coccoid	0.4±0.1[a] – 0.5±0.2[a]	0.4±0.1[a] – 0.3±0.1[a]	Glycogen scattered throughout the cytoplasm	*O. magnificus*	Foster et al. (2006a) (Type b1)
Heterotrophic bacteria	Coccoid	1.0±0.4 [a] – 0.3±0.1[a]	0.4±0.1[a] – 0.3±0.3[a]	Glycogen scattered throughout the cytoplasm	*O. magnificus*	Foster et al. (2006a) (Type b2)

[a]Mean length

Table 2. Types and characteristics of endosymbionts of heterotrophic dinoflagellates in the Indian Ocean

Group	Cell shape	Length (µm)	Width (µm)	Characteristics/internal structure of symbionts	Heterotrophic dinoflagellates	Source (name type)
Synechococcus carcerarius	Ellipsoid, short rod, cylindrical	8–10	3–5	Light pink to purplish-red color	*Amphisolenia globifera*	Norris (1967)
Cyanobacteria	Rod	10	1.5	Peripheral thylakoids, carboxysomes in rosettes. Oblique division	*Amphisolenia* sp.	Lucas (1991) (Type IV)
Eukaryotic	Spherical			Golden cells, possibly Chrysophyceae or Dinophyceae	*A. thrinax*, A. palmata	Norris (1967)
Eukaryotic	Spherical	2–3		1–2 plastids, a nucleus, mitochondrion	*A. bidentata*, *A. thrinax*	Lucas (1991)
Eukaryotic	Spherical	3–5		Single yellow chloroplast and a nucleus	*A. bidentata*	Present study, Tarangkoon et al. (2010)
Eukaryotic	Spherical	4–7		Brownish color	*A. thrinax*	Present study
Heterotrophic bacteria	Coccoid, short rod		0.5	A central core of DNA fibrils, numerous ribosomes	*A. bidentata*, *A. thrinax*	Lucas (1991)

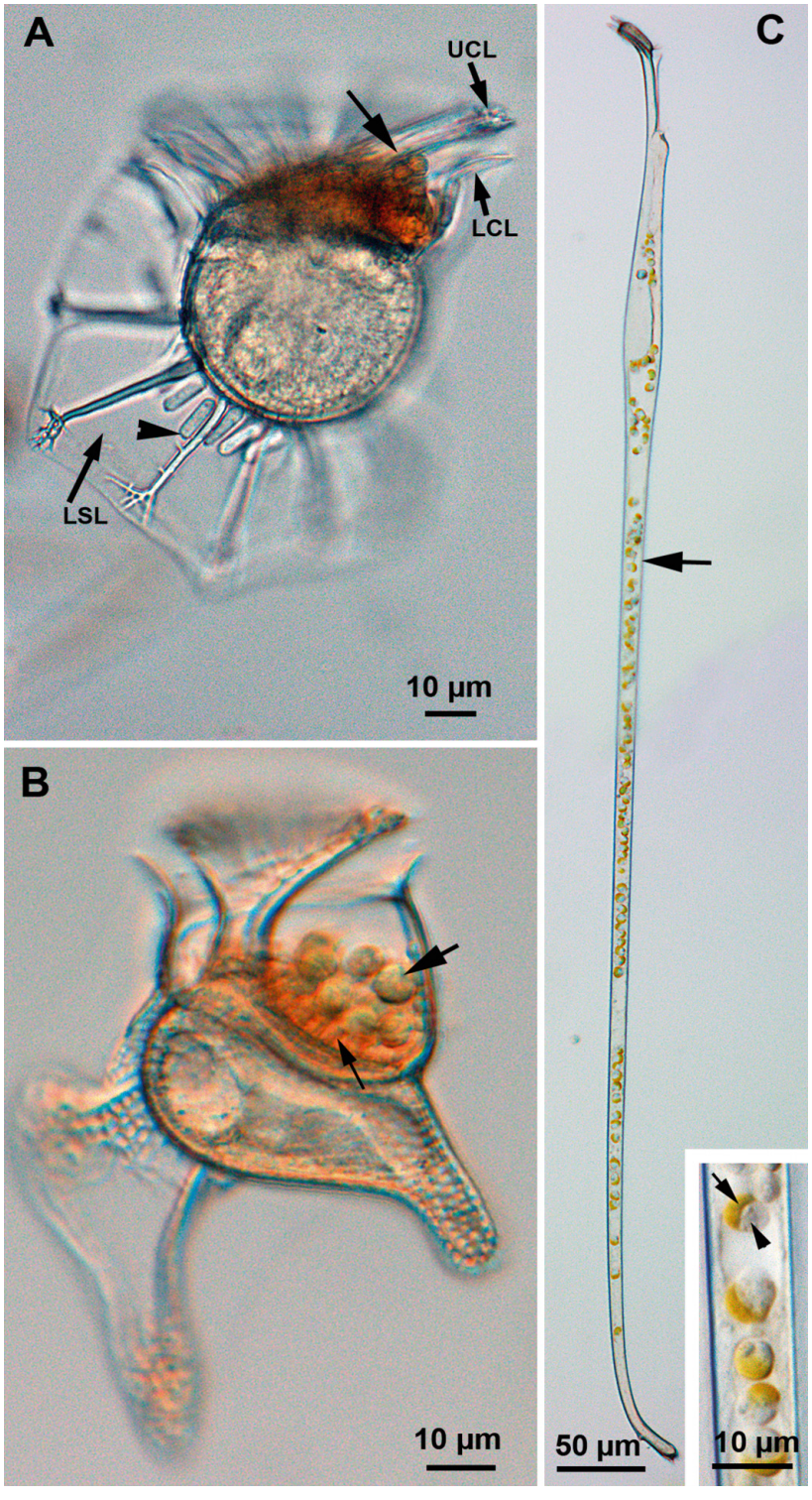

Fig. 1. Light microscopy of live cells. (A) *Ornithocercus thumii*; cyanobacterial ectosymbionts (large arrow). Notice large bacteria on the sulcal list (arrowhead). Small arrows indicate LCL (lower cingular list), UCL (upper cingular list), LSL (left sulcal list). (B) *Histioneis biremis*; 2 different types of cyanobacterial ectosymbionts are present (large and small arrow, respectively). (C) *Amphisolenia bidentata* with numerous eukaryotic endosymbionts (arrow). Inset: Details of the endosymbionts. Chloroplast (arrow), nucleus (arrowhead)

sequence identity) but were clearly distinguished from the negative control sequences (<89% sequence identity, <96% amino acid similarity, Fig. 3).

Two of the dinoflagellates contained *nifH* sequences clustering with *Cyanobacteria* (Fig. 3). Sample P7 contained sequences of 97% sequence identity to *Nostoc punctiforme* while the sequences from P1 were only distantly related to known phylotypes (<78% sequence identity). Three samples contained *nifH* sequences within *nifH* Cluster III and 2 samples contained sequences related to *anfH* genes, encoding the iron-only nitrogenase, within *nifH* Cluster II (Table 3). Twelve sequences, originating from 5 samples, formed a well supported cluster (bootstrap 99%, Fig. 3), which may represent a novel *nifH* cluster. These sequences clustered with *Caldicellulosiruptor saccharolyticus* (87 to 98% sequence identity) and with environmental *nifH* sequences (EU978414 and EU693383). Five sequences formed a separate cluster only distantly related to known *nifH* phylotypes (<69% sequence identity, Fig. 3).

To link sequence composition to dinoflagellate hosts, we examined whether a sample contained single or several *nifH* sequence types and whether specific *nifH* sequences were associated with specific host species. Eight host cells, among which all examined host species were represented, contained only 1 *nifH* sequence type (>99% sequence identity) while 9 host cells contained 2 or 3 *nifH* sequence types each (Table 3). In addition, similar *nifH* sequences were found in several hosts and different host species (Fig. 3). For example, 13 *nifH* gammaproteobacterial sequences from 4 samples of different species formed a distinct cluster with an uncultured *nifH* phylotype from the Pacific Ocean (DQ481270, 98 to 99% sequence identity) and 13 sequences originating from 3 samples of different species clustered with *Klebsiella pneumoniae* (98 to 99% sequence identity).

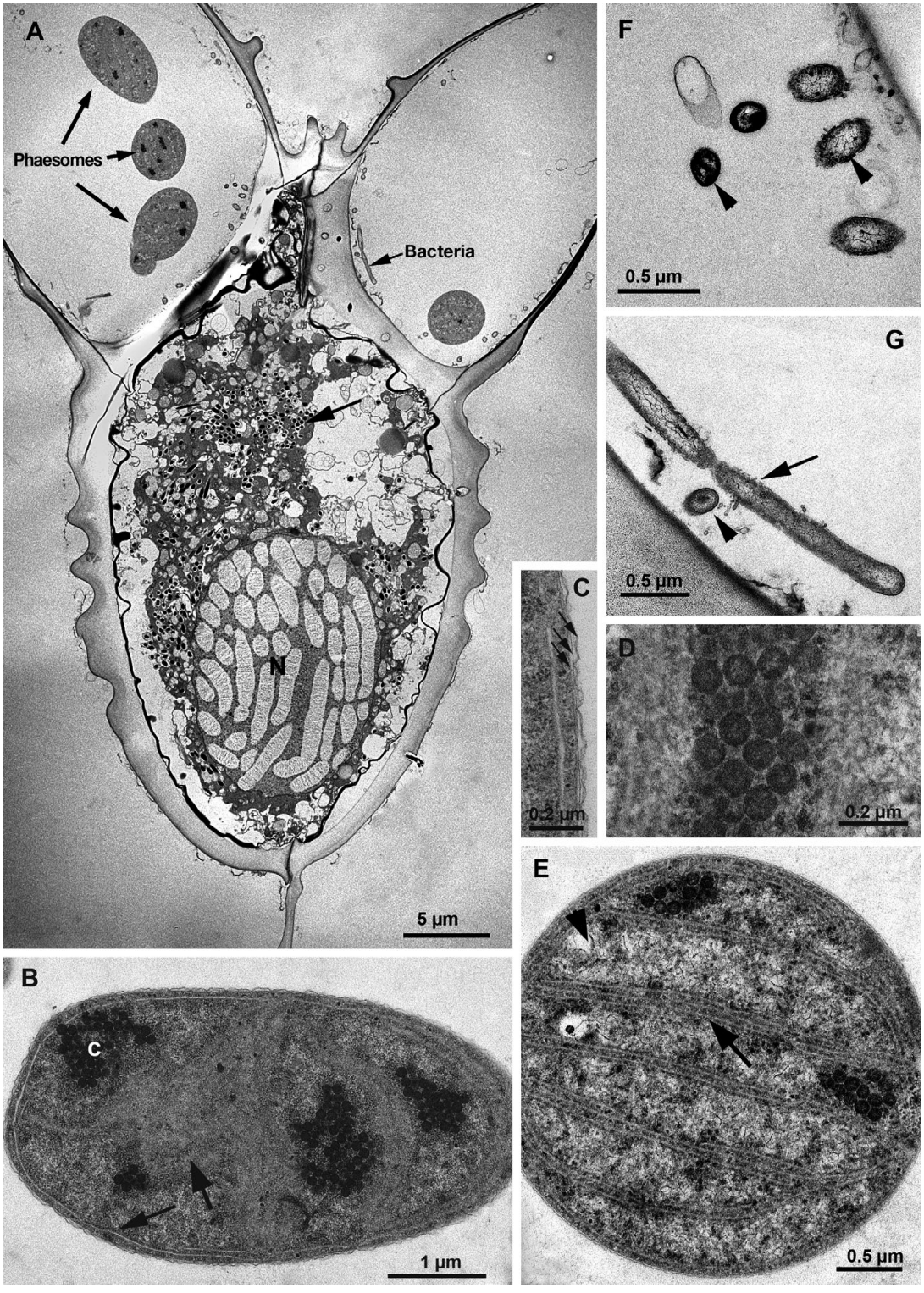

Fig. 2. TEM of *Ornithocercus magnificus*. (A) Longitudinal section of whole cell of *O. magnificus*. Four cyanobacterial ectosymbionts and small bacteria are present in the cingulum (large and small arrows, respectively). Numerous rhabdosomes are present within the cell (arrowhead). (B) Longitudinal section of a cyanobiont. Peripheral (small arrow) and central thylakoid membranes (large arrow); putative carboxysomes (c). (C) The triple-layered cyanobiont wall (arrows). (D) The putative carboxysomes. (E) Traversing thylakoids (arrow) and putative cyanophycin granule (arrowhead) of a cyanobiont. (F, G) Details of the bacterial ectosymbionts, in longitudinal (arrow) and transverse view (arrowhead)

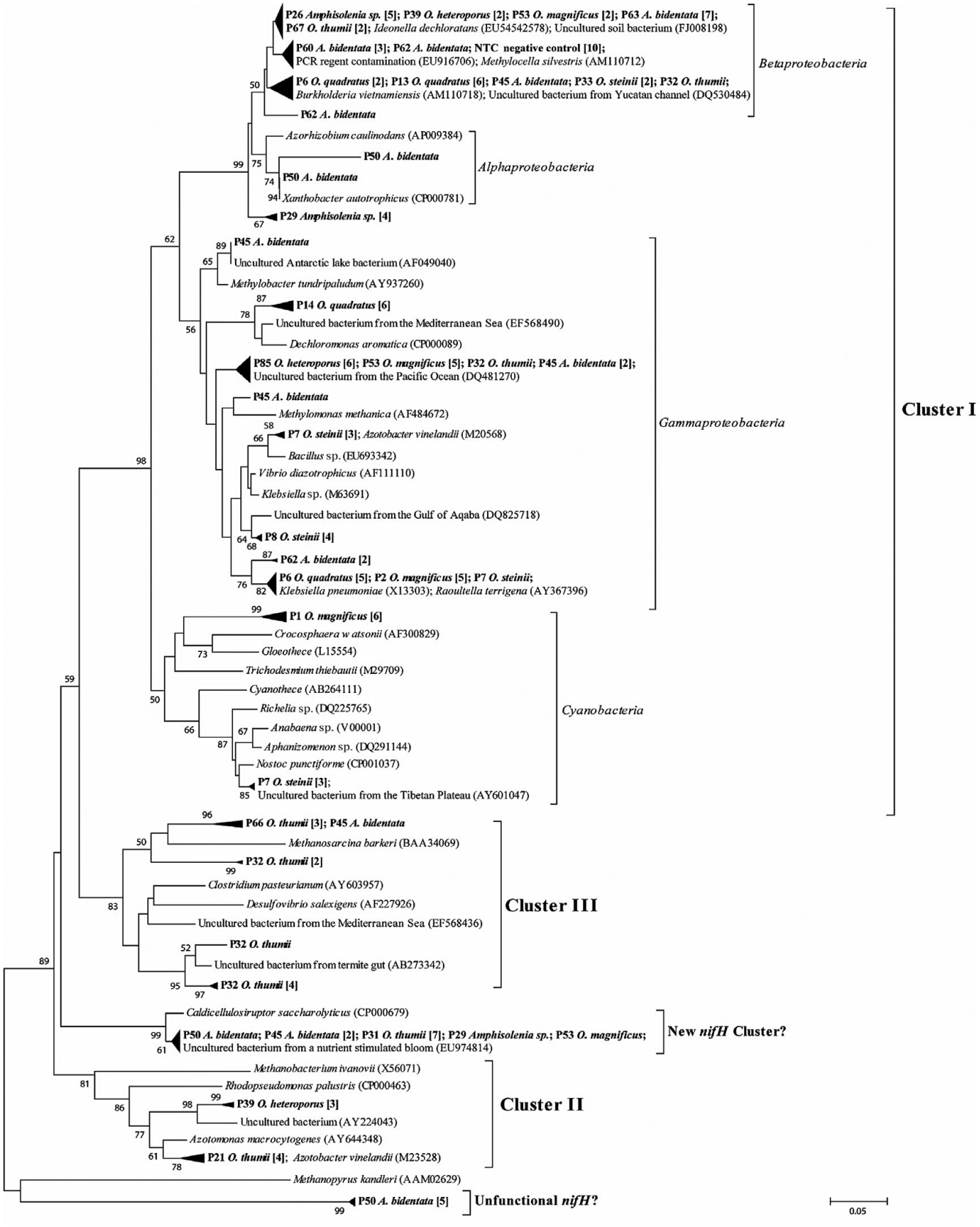

Fig. 3. Neighbor-joining phylogenetic tree of *nifH*-deduced amino acid sequences from symbiont-bearing heterotrophic dinoflagellates. Bootstrap values >50% (1000 replications) are shown. Scale indicates the number of amino acid substitutions per site. Multiple sequences clustering together are collapsed into triangles. Sample number and the individual host with symbionts are indicated in bold with the number of sequences in square brackets. Reference sequences are given with GenBank accession numbers in parentheses. *A.*: *Amphisolenia*; *O.*: *Ornithocercus*

Table 3. Phylogenetic affiliations of the nitrogenase gene (*nifH*) sequences obtained from various dinoflagellate species. The % sequence identity among the sequences clustering together is indicated in parentheses after the number of sequences per cluster. The *nifH* Cluster (I to III) is also given alongside the phylogenetic affiliation in parentheses (α: *Alphaproteobacteria*, β: *Betaproteobacteria*, γ: *Gammaproteobacteria*, C: *Cyanobacteria*) as designated in Fig. 3. ?: unknown cluster related to *Caldicellulosiruptor saccharolyticus*; ??: unknown cluster distantly related to known *nifH* phylotypes

Species	Sample ID	Accession numbers	Number of sequences per phylogenetic group						Total number of sequences per sample
Amphisolenia sp.	P26	GU196890–GU196894	5 (99.1) I (β)						5
	P29	GU196895–GU196899	4 (99.1) I (α)	1 ?					5
A. bidentata	P45	GU196923–GU196930	2 (99.7) ?	2 (98.2) I (γ)	1 I (γ)	1 I (γ)	1 I (β)	1 III	8
	P50	GU196931–GU196938	5 (99.7) ??	2 (96.3) I (α)	1 ?				8
	P60	GU196947–GU196949	3 (99.4) I (β)[a]						3
	P62	GU196950–GU196953	2 (92.7) I (γ)	1 I (β)	1 I (β)[a]				4
	P63	GU196954–GU196960	7 (99.1) I (β)						7
Ornithocercus heteroporus	P39	GU196918–GU196922	3 (98.1) II	2 (100) I (β)					5
	P85	GU196966–GU196971	6 (99.4) I (γ)						6
O. magnificus	P01	GU196845–GU196850	6 (97.8) I (C)						6
	P02	GU196851–GU196855	5 (98.8) I (γ)						5
	P53	GU196939–GU196946	5 (99.4) I (γ)	2 (99.4) I (β)	1 ?				8
O. quadratus	P06	GU196856–GU196862	5 (99.4) I (γ)	2 (98.5) I (β)					7
	P13	GU196874–GU196879	6 (96.9) I (β)						6
	P14	GU196880–GU196885	6 (98.8) I (γ)						6
O. steinii	P07	GU196863 - GU196869	3 (99.4) I (C)	3 (99.1) I (γ)	1 I (γ)				7
	P08	GU196870–GU196873	4 (99.1) I (γ)						4
	P33	GU196916–GU196917	2 (99.7) I (β)						2
O. thumii	P21	GU196886–GU196889	4 (98.1) II						4
	P31	GU196900–GU196906	7 (99.4) ?						7
	P32	GU196907–GU196915	4 (99.1) III	2 (98.8) III	1 III	1 I (?)	1 I (?)		9
	P66	GU196961–GU196963	3 (95.9) III						3
	P67	GU196964–GU196965	2 (99.4) I (β)						2
Negative control	NTC	GU196835–GU196844	10 (98.5) I (β)[a]						10
Total									137

[a]putative PCR reagent contaminant

DISCUSSION

The role of heterotrophic dinoflagellate symbionts has been a mystery for many years. Due to the apparent restriction of these consortia to marine waters deplete of inorganic reduced N, it has been suggested that the cyanobacterial symbionts provide their hosts with N through N_2 fixation (Gordon et al. 1994, Jyothibabu et al. 2006, Tarangkoon et al. 2010). In this first report of *nifH* genes from dinoflagellate–*Cyanobacteria* consortia, we show that 23 of the 37 investigated dinoflagellate cells carried putative diazotrophs, and that 21 of these carried *nifH* genes exclusively related to heterotrophic bacteria. Hence, our analysis suggests that heterotrophic diazotrophs rather than *Cyanobacteria* supply the dinoflagellates with reduced N.

Identification of symbionts through microscopy

The identification of symbionts of heterotrophic dinoflagellates has so far primarily been based on size, shape, pigmentation, and in some cases, ultrastructure. Heterotrophic bacterial ectosymbionts and/or cyanobacterial ectosymbionts of heterotrophic dinoflagellates have been described from the Indian, Pacific and Atlantic Oceans (Table 1). The types of ectosymbionts that we observed are similar to those previously described from the Indian Ocean (Table 1). For instance, the fairly large ectosymbionts (8–10 × 3–5 µm) in *Ornithocercus magnificus* and *O. quadratus* were similar in pigmentation, size and shape to *Synechococcus carcerarius* (Norris 1967; our Table 1). This was supported by a molecular study targeting cyanobacterial *16S rRNA* gene sequences from symbionts of eukaryotic hosts where the majority of the cyanobacterial sequences were closely related to *Synechococcus* (>96% similarity; Foster et al. 2006b). Further, the ectosymbionts of *Histioneis carinata* and *H. biremis* both contained at least 2 types of reddish cyanobacterial ectosymbionts; a large one (2.5 to 5 µm) and a smaller one (1.25 µm, Fig. 1B), similar to Types III and I, respectively (Table 2 in Lucas 1991).

In accordance with previous observations from the Indian Ocean, we found cyanobacterial and eukaryotic endosymbionts in *Amphisolenia* spp. (our Table 2; Tarangkoon et al. 2010). Photosynthetic endosymbionts were observed in both *A. bidentata* (Fig. 1C) and *A. thrinax*. So far only one type of prokaryotic endosymbiont, *S. carcerarius*, has been reported; originating from *A. globifera* (Norris 1967, Hallegraeff & Jeffrey 1984, Lucas 1991), while eukaryotic endosymbionts have been reported from different species of *Amphisolenia* (Norris 1967, Lucas 1991). We observed an additional type of eukaryotic symbiont in *A. bidentata*. Interestingly, Foster et al. (2006b) also recovered *16S rRNA* genes <92% identical to eukaryotic plastids from an *A. bidentata* host, which could represent the eukaryotic symbionts as reported by Lucas (1991) and/or in this study (Table 2).

Heterotrophic bacteria have previously been reported as both ecto- and endosymbionts of heterotrophic dinoflagellates (Tables 1 & 2). For instance, Foster et al. (2006a) detected 2 morphotypes of heterotrophic bacteria associated with *Ornithocercus magnificus*. Likewise, we observed heterotrophic bacterial ectosymbionts of *Ornithocercus* spp. (Fig. 1A) and both cyanobacterial and heterotrophic bacterial ectosymbionts for *O. magnificus* and *O. quadratus* (Fig. 2A). In *O. magnificus* and *O. steinii*, Janson et al. (1995) observed heterotrophic bacteria between the upper and the lower girdle list of the cingular groove in all samples examined by TEM. Also, groups of heterotrophic bacterial endosymbionts have been observed in the cytoplasm of *A. thrinrax* and *A. bidentata* (Lucas 1991). Interestingly, although using primers targeting cyanobacteria, 26% of the *16S rRNA* sequences recovered from eukaryotic marine hosts by Foster et al. (2006b) originated from heterotrophic bacteria; however, none of these could be directly linked to diazotrophic species (based on BLASTN search results on unpublished sequences provided by R. A. Foster). Thus, the occurrence of heterotrophic ecto- and endosymbionts of heterotrophic dinoflagellates is not unusual but to our knowledge, there has been no previous documentation of a N_2-fixing potential in these symbionts.

Identities of *nifH* genes obtained from symbiont-bearing dinoflagellates

Although cyanobacterial symbionts were visible in all examined dinoflagellates, only 2 of the 23 cells, which yielded *nifH* sequences, had sequences related to *Cyanobacteria*. Intriguingly, sample P7 (*Ornithocercus steinii*) contained *nifH* sequences of 97% sequence identity with the filamentous heterocystous cyanobacterium *Nostoc punctiforme*, which is known from freshwater and for its endosymbiotic associations with plants (Meeks et al. 2002). Similarly, *16S rRNA* gene sequences 92% identical to *Nostoc* spp. were found in an *Amphisolenia bidentata* host (Foster et al. 2006b). Taken together, these results may suggest that symbiosis facilitates the survival of *Nostoc* spp. in the saline marine environment. *nifH* sequences related to *Cyanobacteria* were also obtained from sample P1 (*O. magnificus*) but these were only distantly related to known phylotypes (Fig. 3). Since very few *nifH* sequences related to *Cyanobacteria* were found, we find it unlikely that the role of the *Cyanobacteria* in the

symbiosis should be to supply the host with reduced N. Importantly, in a parallel study (F. Farnelid & L. Riemann unpubl.) using the same primer sets, we detected representatives from the major groups of unicellular *Cyanobacteria* (e.g. *Crocosphaera watsonii*, *Cyanothece* sp. and Group A; Bergman et al. 1997, Stal & Zehr 2008). Thus, the lack of these known *Cyanobacteria* in the present data set is not due to a primer mismatch.

Twenty-one samples contained sequences clustering in *nifH* Cluster I, with *Alpha-*, *Beta-*, and *Gammaproteobacteria* (Fig. 3, Table 3). *nifH* gene contamination of PCR reagents, particularly with alpha- and betaproteobacterial sequences, may occur in the nested PCR (Zehr et al. 2003, Goto et al. 2005). However, the 10 *nifH* sequences we obtained from non-visible negative control samples clustered with only 4 sample sequences (Fig. 3, Table 3) and were not identical to any sample sequences. Hence, reagent contamination appeared negligible in the present study. However, hypothetical sources of error such as amplification of *nifH* genes derived from bacteria ingested by the host or from free-living bacteria which may have been passed through the 3 washing steps with 0.2 µm filtered seawater cannot be ruled out. In addition, as the detection limit of the nested *nifH* assay is unknown, samples which did not yield a *nifH* product could theoretically have contained putative diazotrophs.

Diverse *Proteobacteria* within *nifH* Cluster I are commonly detected in marine waters (e.g. Zehr et al. 1998, Church et al. 2005, Langlois et al. 2005, Hewson et al. 2007 Moisander et al. 2008). Associated with dinoflagellates, we found 8 diverse clusters of *nifH* sequences related to *Gammaproteobacteria* while only 2 sequences were related to *Alphaproteobacteria* (Fig. 3). Interestingly, 30 sequences (22% of all sequences) were affiliated with 2 betaproteobacterial clusters, distinct from the negative control sequences (Fig. 3). Similarly, bacteria associated with the photosynthetic dinoflagellate *Gyrodinium instriatum* were dominated by *Betaproteobacteria* (Alverca et al. 2002). Hence, although rare in marine ecosystems (Barberán & Casamayor 2010), *Betaproteobacteria* appear common as symbionts of dinoflagellates.

Sequences clustering in *nifH* Cluster II were obtained from 2 samples (*Ornithocercus heteroporus* P39 and *O. thumii* P21, Table 3). Molybdenum-independent nitrogenases are present in a diverse group of diazotrophs and second alternative nitrogenases are expressed under molybdenum- and vanadium-deficient conditions (Betancourt et al. 2008). Bacteria containing alternative nitrogenase genes have been isolated from diverse marine environments (Loveless et al. 1999) but interestingly, *anfH* related genes seem to be absent in sub-tropical and tropical open waters (e.g. Zehr et al. 1998, Church et al. 2005, Langlois et al. 2005, Hewson et al. 2007, Moisander et al. 2008). Thus, the recovery of *anfH* related genes suggests that symbionts of dinoflagellates may be an environmental niche in open water where second alternative nitrogenase genes can be used.

Sequences from Cluster III, which contains *nifH* genes from anaerobic bacteria, have been detected (Church et al. 2005) but appear uncommon in the open ocean (Langlois et al. 2005, 2008). The presence of Cluster III sequences in 3 dinoflagellates therefore suggests that the dinoflagellate–*Cyanobacteria* consortia provide low oxygen (O_2) habitats required for N_2 fixation (Paerl & Prufert 1987). Similarly, Cluster III sequences from strict anaerobes and nitrogenase activity have been detected in association with zooplankton (Braun et al. 1999). However, since our Cluster III sequences were only distantly related to cultivated anaerobic bacteria (76 to 87% sequence identity), the phenotypes they represent are rather uncertain. Survival of strict anaerobes associated with dinoflagellates would presumably require vertical inheritance of these symbionts as the dinoflagellate host divides. However, given the observed non-host specificity for the symbionts (see below), it may be more likely that the obtained Cluster III sequences derive from facultatively anaerobic bacteria.

Twelve sequences originating from 5 samples formed a discrete *nifH* cluster separate from the known *nifH* Clusters I to IV (Chien & Zinder 1996; our Fig. 3). These sequences had a sequence identity of 87 to 98% to the *nifH* gene of *Caldicellulosiruptor saccharolyticus* (van de Werken et al. 2008), which can grow in the absence of reduced N (van Niel et al. 2002). It is surprising to find sequences closely related to an anaerobic extreme thermophile in the pelagic zone; however, the cluster also contains *nifH* sequences from a marine bloom and from symbionts of corals. Hence, surface-associated growth in the marine environment, such as in association with dinoflagellates, may be characteristic for these bacteria.

Eleven clone libraries yielded 2 or 3 different *nifH* sequence types per dinoflagellate. This suggests the presence of mixed assemblages of diazotrophic symbionts in host cells (Table 3), consistent with previous microscopy observations of mixed populations of cyanobionts and/or bacterial cell types in one host cell (Foster et al. 2006a). In addition, observations of several specimens of the same dinoflagellate species with diverging *nifH* sequences and different species of dinoflagellates hosting identical *nifH* sequences suggested that the *nifH* phylotypes were not host specific. Similar patterns of non-host specific *16S rRNA* gene phylotypes were also observed for cyanobacterial sym-

bionts in tintinnids, dinoflagellates, and radiolarians (Foster et al. 2006b). In contrast, in the *Richelia intracellularis*-diatom symbiosis a divergence of *hetR* and *nifH* sequences of symbionts from different host genera was interpreted as an indication of host specificity (Janson et al. 1999, Foster & Zehr 2006). Thus, it appears that at any one time dinoflagellate hosts may contain multiple symbionts but the low degree of specificity also indicates that their dependence on specialized symbionts is not fundamental.

Putative ecological roles of the consortia

In dinoflagellate–*Cyanobacteria* consortia, the host's requirement for fixed carbon as well as N is presumably the driving force for the relationship. Our results show that heterotrophic bacterial symbionts rather than cyanobionts have the genetic potential for fixing N_2. Consequently, we speculate that the widespread, and somewhat counter-intuitive distribution of these large (50 to 1000 µm) species of heterotrophic dinoflagellates in the oligotrophic subtropical and tropical oceans is partly made possible by symbiont-mediated photosynthesis (*Cyanobacteria*) and N_2 fixation (heterotrophic bacteria).

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