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Using Nuclear-encoded LSU and SSU rDNA Sequences to Identify the Eukaryotic Endosymbiont in *Amphisolenia bidentata* (Dinophyceae)

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The marine dinoflagellate *Amphisolenia bidentata* possesses complete intracellular symbionts of prokaryotic and eukaryotic origin. This was confirmed ultrastructurally little over 20 years ago when it was showed that the eukaryotic endosymbiont had a nucleus, a chloroplast and mitochondria. We collected *Amphisolenia bidentata* cells in the Indian Ocean and the identity of the eukaryotic endosymbionts was investigated using both microscopical and molecular methods. Individual specimens of *Amphisolenia bidentata* were identified by light microscopy and selected for single-cell PCR. Host and endosymbiont nuclear-encoded LSU and SSU rDNA sequences were determined by PCR cloning. Blast searches showed the endosymbiont LSU sequence to have affinity to Pelagophyceae, an algal class within Chromalveolata that also includes dinoflagellates. Since more SSU rDNA sequences from pelagophytes are available we performed a SSU based phylogeny of chromalveolates. The eukaryotic endosymbiont clustered within a clade comprising flagellated and coccoid pelagophytes whereas *Amphisolenia bidentata* formed a sister taxon to other dinophysioids. Molecular data therefore resolved the endosymbiont in *A. bidentata* being a pelagophyte and thus identified the ninth novel chloroplast type in dinoflagellates and a new species association. Based on sequence divergence estimates and phylogenetic inference the endosymbiont in *A. bidentata* likely represents an undescribed genus of pelagophytes.

Key words: *Amphisolenia bidentata*; dinophysioid; eukaryotic endosymbiont; new species association; Pelagophyceae; tropical dinoflagellate.

Introduction

Unicellular dinoflagellates inhabit all temperature regimes from polar to tropical waters. The vast majority is found in marine environments and approximately half of the species lack chloroplasts and consequently are obligate heterotrophs. Diverse and complex feeding strategies are displayed and include total food uptake (i.e. phagotrophy), extrasomal digestion invoking a pallium and partial uptake using a microtubule-supported

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feeding tube (i.e. a peduncle) (e.g. Hansen and Calado 1999). The remaining dinoflagellates possess chloroplasts and there is growing evidence that mixotrophy plays an important role in these photosynthetic species (Burkholder et al. 2008 and references herein; Hansen 2011). The photosynthetic dinoflagellates comprise a heterogeneous group harboring a diverse assemblage of chloroplasts, which are believed to originate from at least eight independent endosymbiotic events (Moestrup and Daugbjerg 2007). Some of these have led to the establishment of permanent chloroplasts controlled by the host nucleus. Others are transient in being functional only for a certain period of time and eventually digested by the host, i.e. kleptochloroplasts (Gast et al. 2007; Schnepf and Elbrächter 1999).

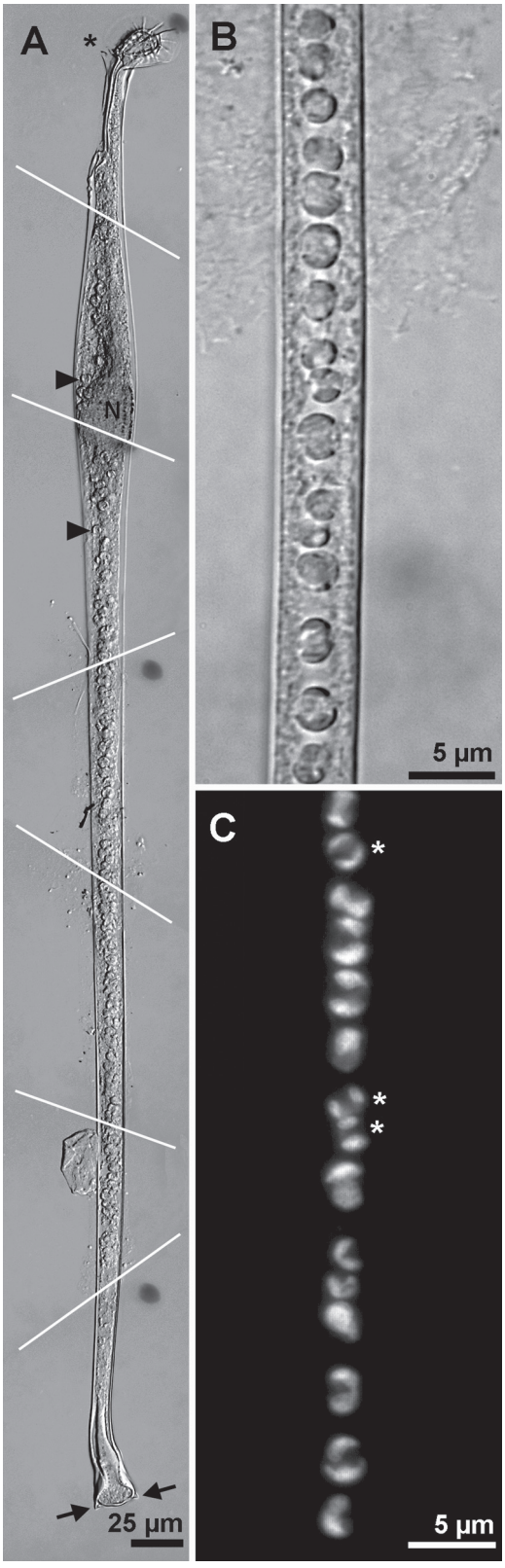
Intracellular or extracellular symbionts of either prokaryotic or eukaryotic origin are housed temporarily or permanently by several dinoflagellates. Especially in tropical, oligotrophic waters various types of symbioses are relatively frequent and they have been speculated to be of nutritious value for the host cell (Lucas 1991). The intriguing order Dinophysiales includes numerous warm temperate to tropical species in which several types of symbioses have been recorded (Hallegraeff and Jeffrey 1984; Hallegraeff and Lucas 1988; Kofoid and Skogsberg 1928; Lucas 1991; Taylor 1976). The accommodation of cyanobacterial cells in an extracellular cavity observed in *Ornithocercus*, *Histioneis* and *Citharistes* represents the most characteristic symbiosis (e.g. Hallegraeff and Jeffrey 1984; Hallegraeff and Lucas 1988; Kofoid and Skogsberg 1928; Lucas 1991; Schütt 1895; Taylor 1976). A different kind of symbiosis is seen in *Amphisolenia*, another dinophysioid genus, where the symbiotic cells are intracellular and displaced along the characteristically elongated host cell. In earlier studies these were reported as chloroplasts of different form, size and color (e.g. Kofoid and Skogsberg 1928). A more recent ultrastructural examination has shown them as complete endosymbiotic cells of either prokaryotic or eukaryotic origin (Lucas 1991). The prokaryotic and eukaryotic nature was also alluded to by a molecular phylogenetic study based on 16S rDNA sequence data (Foster et al. 2006).

The objective of this study was to use molecular data (*viz*. ribosomal gene sequences) to unravel the identity of the eukaryotic endosymbiont in *Amphisolenia bidentata*. Light and epifluorescence microscopy was performed to examine the gross morphology of the endosymbionts including its chloroplast. Nuclear-encoded ribosomal gene sequences (LSU and SSU rDNA) were determined from the host and its eukaryotic endosymbionts by PCR amplification and subsequent cloning of PCR products into *E. coli*. This was performed in order to separate the co-occurring ribosomal PCR products originating from the host and its endosymbionts. The LSU rDNA sequences were compared using a nucleotide Blast search in GenBank and the SSU rDNA sequences were added to a data matrix comprising a diverse assemblage of Chromalveolates for phylogeny inference. Since *A. bidentata* could not be cultured in the laboratory, all investigations were performed on single cells isolated from Lugol-fixed material. The cells used for sequence determinations were photo documented and shown here for reasons of identification.

Results

Light Microscopy

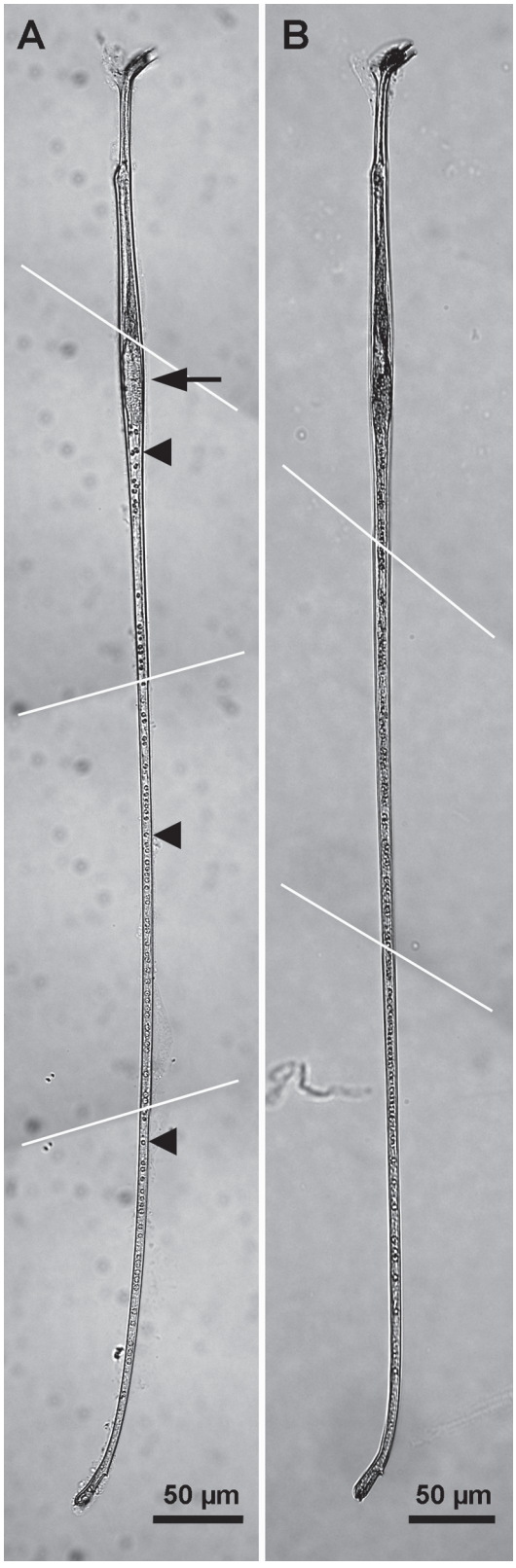
Lugol-fixed cells of *Amphisolenia bidentata* were identified in the light microscope by cell shape and the two small spinules in the antapical end (Fig. 1A). Thus, they were identical to previously described material by Kofoid and Skogsberg (1928) and Taylor (1976). Numerous small sized endosymbiotic cells were present both above and below the large nucleus, which was located ca. 1/3 from the apex (Fig. 1A). In fixed material these endosymbiont cells were more or less circular in outline (Fig. 1B) and with a cell diameter measuring 1.6-2.6 μm (n = 15). Lugol-fixation can cause a considerable increase in volume, and the morphometric measurements may therefore be biased compared to measurements of live material (Menden-Deuer et al. 2001). Contrary to previous studies (Nishino 1986) we were able to perform epifluorescence microscopy on cells stored at 4 °C in the dark for more than two years in acid Lugol's iodine. Even though the epifluorescence signal was weak the endosymbiotic cells clearly revealed the presence of chloroplasts (Fig. 1C). In each cell the single arc-shaped chloroplast was positioned at the cell perimeter (Fig. 1B). Chloroplasts in live cells observed during our cruise across the Indian Ocean were yellow-green (Daugbjerg, pers. observation). In three examined cells of *Amphisolenia bidentata* the number of endosymbionts varied between 91 and 101. The endosymbionts were always observed as complete and variation in the epifluorescence intensity of symbiotic cells within the same host cell was never observed. Food vacuoles were not observed in *A. bidentata*. Neither did we notice prokaryotic cells inside *A. bidentata*.



Cloning of PCR Products

The isolated *Amphisolenia bidentata* cells used as template in single-cell PCR amplifications and subsequent cloning of ribosomal genes into *E. coli* are illustrated in Figure 2A-B. Specifically the cell in Figure 2A was used to obtain LSU rDNA sequences and the cell in Figure 2B was used to obtain SSU rDNA sequences from the host and its endosymbiont, respectively. However, to obtain visible PCR products when run on an agarose gel semi-nested PCR amplifications for both the LSU and SSU rDNA genes had to be performed (Fig. 3). For this we used the primer combinations shown in Table 1. PCR cloning followed by sequence determination of the LSU rDNA products using the same primer combinations resulted in 981 base pair long fragments for the dinoflagellate host (Fig. 3, Lane 2) whereas the LSU rDNA fragments from the eukaryotic endosymbiont were only 875 base pairs long (Fig. 3, Lane 3). The variation in fragment length is also visible in Figure 3. The dinoflagellate LSU rDNA sequence was identical to a previously reported sequence of *A. bidentata* also collected from the Indian Ocean and given GenBank accession number FJ808682 in Jensen and Daugbjerg (2009). We used this independently obtained sequence as a quality control for the species identification and as a duplicate check for the sequence determination based on single cells. SSU rDNA sequences from the host and its eukaryotic endosymbiont were determined twice but we only show one of the cells used for this purpose (Fig. 2B). The two host SSU rDNA sequences were identical as were the two eukaryotic endosymbiont SSU rDNA sequences. The resulting DNA fragments based on cloning of PCR products followed by sequence determination resulted in equally long fragments, i.e. 1484

Figure 1. Light micrographs (Nomarski interference contrast) of *Amphisolenia bidentata* fixed in Lugol's iodine. (**A**) *Amphisolenia bidentata* with a minute epitheca (*) and a strikingly elongated hypotheca that ends with a foot-like appearance possessing two antapical spinules (arrows). Note a large nucleus (N) in the midbody. The endosymbiotic cells are positioned throughout the majority of the hypotheca and two are marked by arrowheads. The cell shown in A is a composite of 7 images taken of the same cell. Individual images marked by separating white lines; (**B**) Large magnification of eukaryotic endosymbionts in *Amphisolenia bidentata*; (**C**) Same area of the cell as in B but in epifluorescence microscopy, revealing chloroplast auto-fluorescence in two-year old material. Note the arc-shaped chloroplasts (*).



base pairs for both the host and its endosymbiont (Fig. 3A, Lanes 5-6).

Endosymbiont Identity Based on LSU rDNA

A nucleotide Blast search of the endosymbiont LSU rRNA sequence showed it to be most similar to four species assigned to the Pelagophyceae (i.e. *Pelagomonas calceolata*, *Aureococcus anophagefferens*, *Pelagococcus subviridis* and *Ankylochrysis lutea*). The two next most similar species based on the lowest E-values were the heterotrophic flagellate *Developayella elegans* and the brown alga *Chorda filum*, respectively. To further elucidate the identity of the eukaryotic endosymbiont of *Amphisolenia bidentata* sequence divergence estimates for the 6 most similar species were calculated (Table 2). Based on this the endosymbiont had a sequence divergence of ca. 10% to *Pelagomonas*, *Pelagococcus* and *Aureococcus* whereas it was slightly more divergent to *Ankylochrysis* (sequence divergence 14 to 16% depending on the method used to calculate the estimate; Table 2). The sequence divergence between the endosymbiont and *Developayella* and *Chorda* was significantly higher (21 to 27.5%). The lowest sequence divergence for any of the pelagophytes was between the flagellated species *Pelagomonas calceolata* and the coccoid species *Pelagococcus subviridis* as they diverged by ca. 5% (Table 2).

Molecular Phylogeny

Compared to nuclear-encoded LSU rDNA the SSU rDNA gene has been determined from many more protists, including relevant species of the Pelagophyceae. Therefore we also determined this gene from the endosymbiont and its dinoflagellate host allowing us to further elucidate the

Figure 2. Light micrographs (Nomarski interference contrast) of two Lugol-fixed specimens of *Amphisolenia bidentata* containing ca. 100 eukaryotic endosymbionts. The cells were used as template in single-cell PCR. (**A**) Specimen used for sequence determination of nuclear-encoded LSU rDNA in the host and its endosymbiont. Note the large nucleus (arrow) and individual eukaryotic endosymbionts (arrowheads); (**B**) Specimen used for sequence determination of nuclear-encoded SSU rDNA in the host and its endosymbiont. A is a composite of 4 images taken of the same cell and B is a composite of 3 images taken of the same cell. Individual images marked by separating white lines.

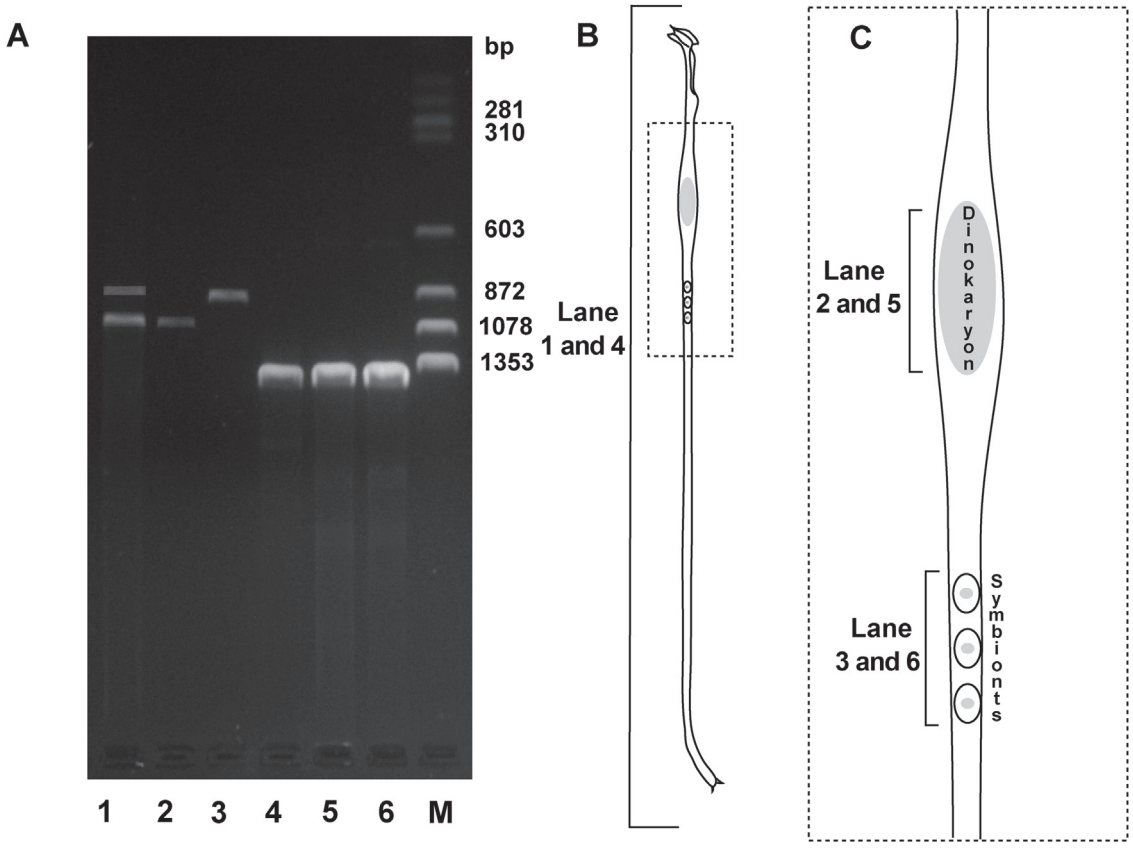


Figure 3. Diagram revealing separation of genes encoding LSU and SSU rDNA in *Amphisolenia bidentata* using PCR and cloning of PCR products into *E. coli*. (**A**) Agarose gel. Lane 1-3: LSU rDNA. Lane 4-6: SSU rDNA. Lane M: DNA marker ΦX174 DNA/BsuRI (HaeIII) (Fermentas). Lane 1 and 4: PCR products prior to cloning, Lane 2 and 5 cloned PCR products of *Amphisolenia*, Lane 3 and 6 cloned PCR products of endosymbionts. (**B**) Drawing of *Amphisolenia bidentata* with a reduced set of endosymbionts. (**C**) Magnification of B (boxed in area) showing dinokaryon and three endosymbionts with their nuclei. B and C are not drawn to scale.

identity of the eukaryotic endosymbiont. The two ribosomal SSU sequences (one from the host, the other from the eukaryotic endosymbiont) were added to a data matrix comprising a diverse assemblage of protists representing the chromalveolates (98 taxa), red algae (2 taxa) and green algae (3 taxa). Two glaucophytes rooted the tree. The phylogeny illustrated in Figure 4 was inferred from Bayesian analysis and revealed six highly supported clades representing the Chlorophyta (support: PP = 1.0, BS = 100), Rhodophyta (support: PP = 0.96, BS = 65), Heterokontophyta (support: PP = 1.0, BS = 100), Dinophyta (support: PP = 1.0, BS = 100), Haptophyta (support: PP = 1.0,

Table 1. LSU and SSU rRNA primer sequences and the combinations used to obtain host and endosymbiont sequences, respectively.

	Primary PCR	Semi-nested PCR
LSU primer combination	D1R[1] and ND28-1483R[2]	D1R and D3B[4]; D3A[3] and ND28-1483R
SSU primer combination	ND1F[5] and ND6R[6]	ND2F[7] and ND6R

[1]D1R 5′-ACCCGCTGAATTTAAGCATA-3′; [2]ND28-1483R 5′-GCTACTACCACCAAGATCTGC-3′; [3]D3A 5′-GACCCGTCTTGAAACACGGA-3′; [4]D3B 5′-TCGGAGGGAACCAGCTACTA-3′; [5]ND1F 5′-AACCTGGTTGATCCTGCCAG- 3′; [6]ND6R 5′-GATCCTTCTGCAGGTTCACC-3′; [7]ND2F 5′-GATTCCGGAGAGGGAGCCTG 3′.

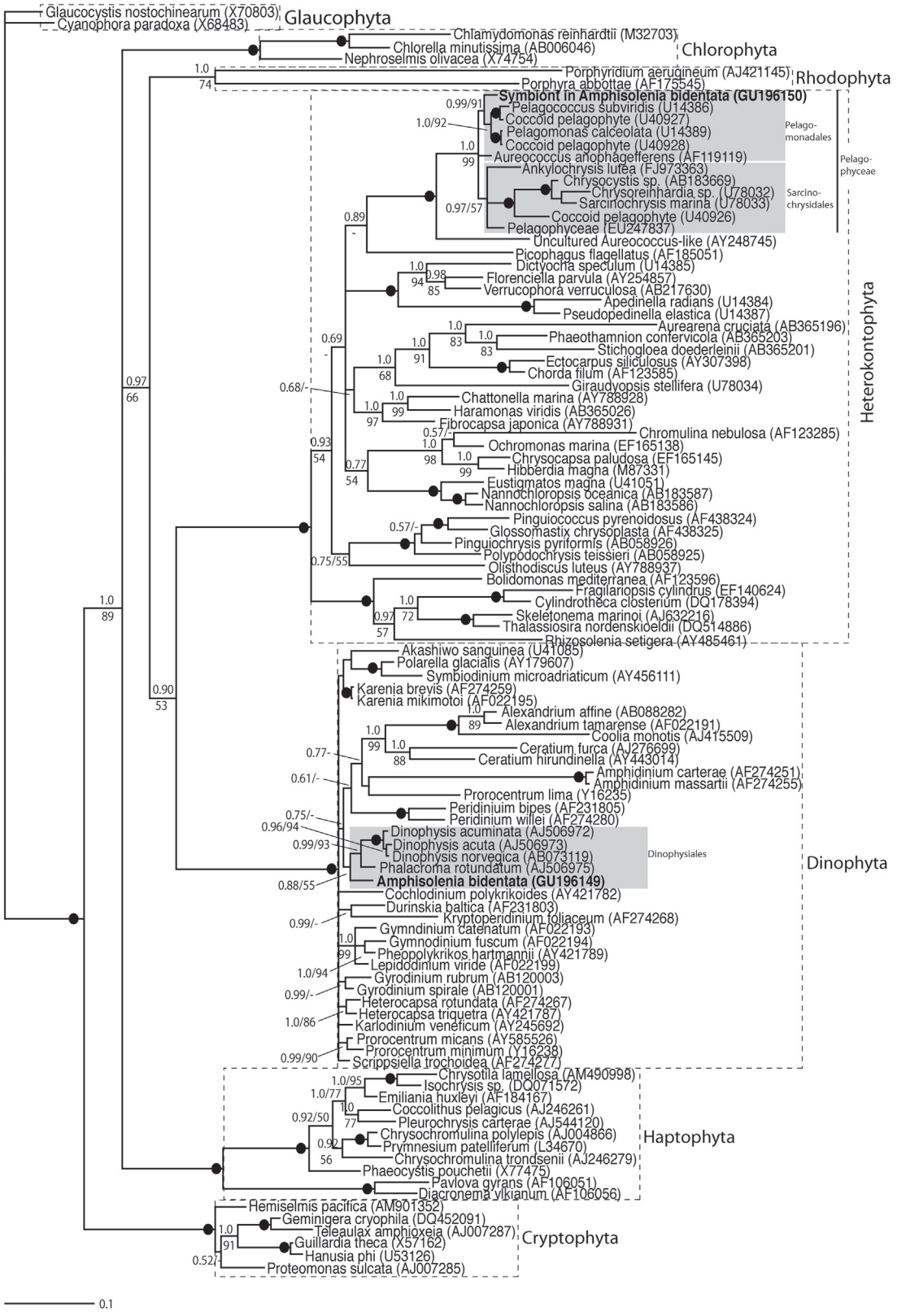

Glaucocystis nostochinearum (X70803)
Cyanophora paradoxa (X68483)
Glaucophyta
Chlamydomonas reinhardtii (M32703)
Chlorella minutissima (AB006046)
Nephroselmis olivacea (X74754)
Chlorophyta
Porphyridium aerugineum (AJ421145)
Porphyra abbottae (AF175545)
Rhodophyta
Symbiont in Amphisolenia bidentata (GU196150)
Pelagococcus subviridis (U14386)
Coccoid pelagophyte (U40927)
Pelagomonas calceolata (U14389)
Coccoid pelagophyte (U40928)
Aureococcus anophagefferens (AF119119)
Pelago-monadales
Pelago-phyceae
Ankylochrysis lutea (FJ973363)
Chrysocystis sp. (AB183669)
Chrysoreinhardia sp. (U78032)
Sarcinochrysis marina (U78033)
Coccoid pelagophyte (U40926)
Sarcino-chrysidales
Pelagophyceae (EU247837)
Uncultured Aureococcus-like (AY248745)
Picophagus flagellatus (AF185051)
Dictyocha speculum (U14385)
Florenciella parvula (AY254857)
Verrucophora verruculosa (AB217630)
Apedinella radians (U14384)
Pseudopedinella elastica (U14387)
Aurearena cruciata (AB365196)
Phaeothamnion confervicola (AB365203)
Stichogloea doederleinii (AB365201)
Ectocarpus siliculosus (AY307398)
Chorda filum (AF123585)
Giraudyopsis stellifera (U78034)
Chattonella marina (AY788928)
Haramonas viridis (AB365026)
Fibrocapsa japonica (AY788931)
Chromulina nebulosa (AF123285)
Ochromonas marina (EF165138)
Chrysocapsa paludosa (EF165145)
Hibberdia magna (M87331)
Eustigmatos magna (U41051)
Nannochloropsis oceanica (AB183587)
Nannochloropsis salina (AB183586)
Pinguiococcus pyrenoidosus (AF438324)
Glossomastix chrysoplasta (AF438325)
Pinguiochrysis pyriformis (AB058926)
Polypodochrysis teissieri (AB058925)
Olisthodiscus luteus (AY788937)
Bolidomonas mediterranea (AF123596)
Fragilariopsis cylindrus (EF140624)
Cylindrotheca closterium (DQ178394)
Skeletonema marinoi (AJ632216)
Thalassiosira nordenskioeldii (DQ514886)
Rhizosolenia setigera (AY485461)
Heterokontophyta
Akashiwo sanguinea (U41085)
Polarella glacialis (AY179607)
Symbiodinium microadriaticum (AY456111)
Karenia brevis (AF274259)
Karenia mikimotoi (AF022195)
Alexandrium affine (AB088282)
Alexandrium tamarense (AF022191)
Coolia monotis (AJ415509)
Ceratium furca (AJ276699)
Ceratium hirundinella (AY443014)
Amphidinium carterae (AF274251)
Amphidinium massartii (AF274255)
Prorocentrum lima (Y16235)
Peridiniuim bipes (AF231805)
Peridinium willei (AF274280)
Dinophysis acuminata (AJ506972)
Dinophysis acuta (AJ506973)
Dinophysis norvegica (AB073119)
Phalacroma rotundatum (AJ506975)
Dinophysiales
Amphisolenia bidentata (GU196149)
Cochlodinium polykrikoides (AY421782)
Durinskia baltica (AF231803)
Kryptoperidinium foliaceum (AF274268)
Gymndinium catenatum (AF022193)
Gymnodinium fuscum (AF022194)
Pheopolykrikos hartmannii (AY421789)
Lepidodinium viride (AF022199)
Gyrodinium rubrum (AB120003)
Gyrodinium spirale (AB120001)
Heterocapsa rotundata (AF274267)
Heterocapsa triquetra (AY421787)
Karlodinium veneficum (AY245692)
Prorocentrum micans (AY585526)
Prorocentrum minimum (Y16238)
Scrippsiella trochoidea (AF274277)
Dinophyta
Chrysotila lamellosa (AM490998)
Isochrysis sp. (DQ071572)
Emiliania huxleyi (AF184167)
Coccolithus pelagicus (AJ246261)
Pleurochrysis carterae (AJ544120)
Chrysochromulina polylepis (AJ004866)
Prymnesium patelliferum (L34670)
Chrysochromulina trondsenii (AJ246279)
Phaeocystis pouchetii (X77475)
Pavlova gyrans (AF106051)
Diacronema ylkianum (AF106056)
Haptophyta
Hemiselmis pacifica (AM901352)
Geminigera cryophila (DQ452091)
Teleaulax amphioxeia (AJ007287)
Guillardia theca (X57162)
Hanusia phi (U53126)
Proteomonas sulcata (AJ007285)
Cryptophyta
0.1

Table 2. LSU rDNA sequence difference in percent of the eukaryotic endosymbiont in *Amphisolenia bidentata* and the six most similar taxa based on the lowest E-value in a Genbank nucleotide blast search. The comparison was based on 1035 base pairs (including introduced gaps). Uncorrected distances (p values in PAUP*) are given above the diagonal and Kimura 2-parameter model distance values are given below the diagonal.

	1	2	3	4	5	6	7
Eukaryotic endosymbiont (1)	—	9.92	10.05	9.96	14.39	21.1	22.91
Pelagomonas calceolata (2)	10.66	—	4.43	12.35	16.8	22.42	24.89
Pelagococcus subviridis (3)	10.8	4.57	—	11.76	17.52	22.23	25.14
Aureococcus anaphagefferens (4)	10.72	13.56	12.84	—	14.23	20.27	23.25
Ankylochrysis lutea (5)	16.15	19.14	20.09	15.83	—	23.04	25.0
Developayella elegans (6)	24.94	26.81	26.58	23.75	27.81	—	21.68
Chorda fillum (7)	27.5	30.34	30.75	27.93	30.65	25.74	—

BS = 100) and Cryptophyta (support: PP = 1.0, BS = 100). According to this single gene analysis the super group Chromalveolata was not monophyletic as the rhodophytes were placed in between the cryptophytes and the remaining chromalveolates. The relationship within the dinoflagellate clade was not well resolved as most of the lineages formed a basal polytomy. However, these were supported by moderately high posterior probabilities and many of them also by bootstrap support from maximum likelihood analyses. The dinoflagellate of interest in this study *Amphisolenia bidentata* formed the earliest branching taxon within the Dinophysiales. This topology was moderately supported by posterior probabilities (0.88) but hardly by bootstrap analyses (55%). Within the phylum Heterokontophyta (= chromists), the class Pelagophyceae formed a sister group to *Picophagus flagellatus*, a picoplanktonic, heterotrophic flagellate tentatively included in Chrysophyceae by Guillou et al. (1999). Similarly to the results of the LSU rDNA nucleotide Blast Search the endosymbiont of *Amphisolenia bidentata* clustered within the 12 pelagophytes included. Here it formed a basal branch among the pelagophytes belonging to the order Pelagomonadales (Fig. 4). The other pelagophytes included clustered in the order Sarcinochrysidales. Though *Aureococcus anophagefferens* is considered a member of the Pelagomonadales (Bailey and Andersen 1999) its phylogeny was unresolved in our analysis. The uncultured *Aureococcus*-like isolate from Long Island Sound (USA) given GenBank accession number AY248745 formed the most basal branch within the Pelagophyceae. Pelagophytes with coccoid and flagellated thallus types were positioned in both Pelagomonadales and Sarcinochrysidales.

Sequence Divergence of SSU rDNA

Sequence divergence estimates between members of the Pelagomonadales (including the eukaryotic endosymbiont of *Amphisolenia*) varied between 0.6 and 2.1% (Table 3). Interestingly the SSU rDNA sequences of the flagellated *Pelagomonas* and an unidentified coccoid pelagophyte CCMP 1145 (both assigned to Pelagomonadales) were identical. The divergence estimates between members of the Sarcinochrysidales varied between 1.8 and 5.9% (Table 3), thus somewhat higher compared to the Pelagomonadales. Only slightly higher sequence divergence estimates were observed across members belonging to the two different orders (values ranged from 2.3 to 7.1%) compared to the Sarcinochrysidales. The endosymbiont of *Amphisolenia* was least divergent to *Pelagomonas calceolata* (1.7%) and two unidentified coccoid pelagophytes CCMP 1145 and 1395 (both with a 1.7% sequence divergence) and most divergent to *Pelagococcus subviridis* (1.97 or 1.99%) depending on the method used to calculate the sequence divergence (see Methods). Due to the

Figure 4. Phylogeny of 95 members of the Chromalveolata (sensu Cavalier-Smith 1998) based on 1484 base pairs of nuclear-encoded SSU rDNA sequences and inferred from Bayesian analysis (MrBayes ver. 3.2). The Glaucophyta comprised the outgroup. Branch support was obtained from Bayesian posterior probabilities of 39.600 trees and bootstrap with 100 replications in maximum likelihood analyses (PhyML ver. 3.0). At internal nodes posterior probabilities ($\leq$ 1) are written first followed by bootstrap values in percentage from maximum likelihood. Only bootstrap values $\geq$50% are shown. A filled black circle indicates the highest possible posterior probability (1.0) and a bootstrap value of 100%. The SSU rDNA sequences determined in this study are bold-faced. GenBank accession numbers are given in parentheses.

Table 3. SSU rDNA sequence difference in percent of the endosymbiont in *Amphisolenia bidentata* and 12 pelagophytes available in Genbank. The comparison was based on 1379 base pairs (including a few introduced gaps). Uncorrected distances (p values in PAUP*) are given above the diagonal and Kimura 2-parameter model distance values are given below the diagonal.

	1	2	3	4	5	6	7	8	9	10	11	12	13
Eukaryotic endosymbiont (1)	—	1.77	1.99	1.7	3.82	5.38	6.24	6.16	1.7	1.7	4.43	2.6	8.68
Aureococcus anophagefferens (2)	1.75	—	2.07	1.92	3.44	5.29	6.23	5.68	1.92	1.92	4.82	2.37	7.46
Pelagococcus subviridis (3)	1.97	2.04	—	1.47	3.52	5.93	7.05	6.31	1.47	0.59	5.14	2.83	8.52
Pelagomonas calceolata (4)	1.67	1.89	1.46	—	3.67	5.94	6.65	6.16	0	1.17	5.06	2.75	8.52
Ankylochrysis lutea (5)	3.72	3.35	3.43	3.57	—	5.22	6.17	5.69	3.67	3.29	5.15	3.14	9.2
Chrysocystis sp. (6)	5.17	5.1	5.68	5.68	5.03	—	2.67	1.77	5.94	5.69	4.51	5.23	10.61
Chrysoreinhardia sp. (7)	5.97	5.97	6.7	6.33	5.91	2.62	—	2.6	6.66	6.64	5.21	5.94	11.88
Sarcinochrysis marina (8)	5.9	5.46	6.04	5.9	5.47	1.75	2.55	—	6.16	6	5.36	5.62	11.11
Coccoid pelagophyte (CCMP 1145) (9)	1.67	1.89	1.46	0	3.57	5.68	6.33	5.9	—	1.18	5.06	2.75	8.52
Coccoid pelagophyte (CCMP 1395) (10)	1.67	1.89	0.58	1.16	3.21	5.46	6.33	5.75	1.16	—	5.14	2.45	8.34
Coccoid pelagophyte (CCMP1410) (11)	4.3	4.66	4.95	4.88	4.96	4.37	5.03	5.17	4.88	4.95	—	4.75	11.12
"Pelagophyceae" (CCMP 2097) (12)	2.55	2.33	2.77	2.69	3.07	5.03	5.68	5.39	2.69	2.4	4.59	—	8.61
Uncultured *Aureococcus*-like (13)	8.17	7.08	8.02	8.02	8.63	9.86	10.95	10.29	8.02	7.88	10.3	8.11	—

unknown identity of the uncultured *Aureococcus*-like pelagophyte it was not included in these comparisons. However, one may argue that based on the branch length leading to the *Aureococcus*-like pelagophyte and the sequence divergence to other pelagophytes it represents a new order within the Pelagophyceae. Alternatively it may not be a pelagophyte.

Discussion

Endosymbiont Identity

The eupelagic *Amphisolenia bidentata* is one of the most common species of its genus (Kofoid and Skogsberg 1928). We successfully obtained molecular sequence data of its eukaryotic endosymbiont by cloning into *E. coli* PCR amplified nuclear genes encoding LSU and SSU, respectively. Sequence divergence estimates based on LSU rDNA (Table 2) as well as a phylogenetic inference based on SSU rDNA (Fig. 4) clearly indicated that the endosymbiont was a member of the order Pelagomonadales in the class Pelagophyceae. Interestingly all described pelagophytes have been characterized using nuclear-encoded small subunit ribosomal sequences since Andersen et al. (1993) proposed the Pelagophyceae. This allowed a detailed molecular comparison and based on this we propose that the eukaryotic endosymbiont in *Amphisolenia bidentata* represents a new genus within the Pelagomonadales. However, we are reluctant to describe it as a new genus and species until detailed ultrastructural data has been obtained from freshly collected material. Unfortunately the scarcity of *A. bidentata* cells in the live samples collected during our cruise did not allow for a detailed ultrastructural study using transmission electron microscopy. Species assigned to Pelagophyceae are autotrophic, with presently eight monotypic genera of marine algae (Adl et al. 2005). Due to their minute cell size a number of pelagophytes (viz. *Pelagomonas*, *Aureococcus*, *Aureoumbra* and *Pelagococcus*) belong to the picoplanktonic eukaryotes (0.2 - 2 μm) or the lower range of nanoplankters (2-20 μm). The eukaryotic endosymbiont in *A. bidentata* also belongs to the size range between the pico- and nanoplankton. Yet no pelagophyte is known to form symbiotic associations. Hence, resolving the identity of the endosymbiont in *Amphisolenia bidentata* adds to the ecological understanding of the Pelagophyceae.

Notes on Chloroplast Types in Dinoflagellates

Molecular identification of the endosymbiont in *Amphisolenia bidentata* represents the ninth novel type of chloroplast association in dinoflagellates. Most dinoflagellates form stable symbiotic relationships with previously free-living eukaryotic algae. Such consortia between two eukaryotes have resulted in stable symbioses for the peridinin-containing dinoflagellates where the endosymbiont is considered to be a previously free-living red alga. The Kareniaceae forms another example of a permanent symbiosis as the chloroplast is all that is left of the haptophyte cell originally engulfed. Less stable symbioses are seen in *Amphidinium poecilochroum* (Larsen 1988) and an Antarctic dinoflagellate (Gast et al. 2007) where the chloroplasts need to be renewed repeatedly (kleptoplastidy). A remarkable association is seen between the green form of the unarmoured marine dinoflagellate *Noctiluca scintillans* and its photosynthetic symbiont *Pedinomonas noctilucae*. Here thousands of free-swimming cells of *P. noctilucae* are harbored inside the cytoplasm of the host. The green *Noctiluca* can only survive a few weeks in the laboratory without the ingestion of food (Hansen et al. 2004), suggesting that this symbiotic association as for *A. poecilochroum* and the Antarctic dinoflagellate is an early stage in the establishment of a permanent symbiosis. For a complete description of chloroplast types in dinoflagellates, see Moestrup and Daugbjerg (2007).

Stability of Symbiosis

Most of our observations were performed on fixed cells and not on cultured material. Here we may add that no one has yet successfully established a viable culture of any species of *Amphisolenia*. Therefore we can only add a few remarks on the stability of the symbiosis between *A. bidentata* and the pelagophyte endosymbiont. The three host individuals for which the number of endosymbionts were counted, all had a similar gross morphological outline and number of eukaryotic cells (between 91-101) in the cytoplasm. The symbionts observed by us seemed functional (i.e. fluoresced with the same intensity), and we found no signs of breakdown of symbionts inside *A. bidentata* cells. The lack of symbionts in food vacuoles inside *A. bidentata* cells was also noted by Lucas (1991) and Schnepf and Elbrächter (1999). This seems to indicate a stable relationship between the symbionts and the host, and there is no evidence available at this point that suggest the continual selective uptake of free-living pelagophytes from the surrounding waters. Thus, based on admittedly indirect evidence we favor the arguments of a stable symbiosis based on a self-sustaining intracellular population of a single pelagophyte species in *Amphisolenia bidentata*. Future studies may reveal the presence of additional endosymbiont diversity. Obviously, this symbiotic relationship has only reached the initial step in being fully integrated as they still represent a complete cell with nucleus, mitochondria and a single chloroplast as shown in the study by Lucas (1991). However, the hypothesis of a self-sustaining symbiont population of a single species in the cytoplasm of the host has to be tested. This probably requires the successful culturing of both *A. bidentata* and its eukaryotic endosymbiont(s).

Ecological Significance of Endosymbionts in *Amphisolenia*

Generally, productivity and biomass build-up of microbial communities in the open oceans is limited due to low concentrations of major nutrients, trace metals and minerals. Yet microorganisms seem to be well adapted to oligotrophic waters. One way to overcome the deficiency of nutrients and trace metals has obviously been to establish symbiotic associations. There are numerous examples of heterotrophic organisms harboring complete plant-like cells in nutrient-poor environments. Probably the best-known example is the coral reef forming polyps (colonial cnidarians). Here polyps harbor zooxanthellae (viz. single-celled dinoflagellates belonging to the genus *Symbiodinium*). The zooxanthellae provide a significant proportion of the polyps energy demand. Individual cells of *Amphisolenia bidentata* comprised ca. 100 eukaryotic endosymbionts that fluoresced even in 2-year old material fixed in Lugol's iodine (Fig. 1 C). The physiological significance of the association between *A. bidentata* and its pelagophyte symbiont has yet to be elucidated in detail but it seems reasonable that it represents an ecological advantage. All chloroplasts in the endosymbionts seem to facilitate photosynthesis and thus the production of organic matter. A proportion of this may be released as dissolved organic matter by passive leakage across the cell membrane to the cytoplasm of the dinoflagellate host. These compounds therefore likely support the metabolism of the dinoflagellate host.

Interestingly, in an ultrastructural study *Amphisolenia bidentata* and *A. thrinax* was also shown to contain either coccoid or short

rods of bacterial symbionts (Lucas 1991; fig. 3E) whereas *A. globifera* possessed cyanobacterial endosymbionts identified as *Synechococcus carcerarius* (Lucas 1991; fig. 3g-f). In a molecular study, Foster et al. (2006) showed that *A. bidentata* contained two cyanobacteria; one with a sequence similar to *Prochlorococcus* stains and the other with a sequence similar to heterocystous *Nostoc* spp. Since heterocyst-like cells were never observed in *A. bidentata* the sequence determination of this type of cyanobacteria was explained by engulfment prior to isolation. Still this adds to the advantage of the dinoflagellate host if the cyanobacteria can convert N_2 into ammonia by nitrogen fixation. Foster et al. (2006) also revealed a different eukaryotic endosymbiont in the three-part symbiotic association of another species of *Amphisolenia*, viz. *A. globifera*. This finding means that the eukaryotic endosymbionts have been acquired at least twice during the evolutionary history of the genus *Amphisolenia*. The functional biology of *Amphisolenia bidentata* therefore seems rather complex. Future studies should aim at quantifying the energy contribution to the host from the symbionts living inside *Amphisolenia* and establish the taxonomy of the pelagophyte species.

Methods

Sampling site and collection of material: Samples were collected during a cruise across the Indian Ocean from South Africa to Western Australia in the period October-November 2006. The cruise was part of the Danish Galathea 3 Expedition onboard the navy vessel HDMS Vædderen. Water samples were collected using a 20-μm plankton net attached to a 130 m long rope. A small amount (*ca.* 10 mL) of the collected sample was immediately fixed with Acid Lugol's iodine (Merck, Darmstadt, Germany) (final concentration ca. 3%) and kept cold and dark until returning to Copenhagen. Single cells of *Amphisolenia bidentata* with eukaryotic endosymbionts used as template for PCR amplification and gene cloning in this study were isolated from a fixed sample collected on 16 November, 2006 at 16°01′668S, 119°20′233E.

Light microscopy (LM): Lugol-fixed cells were observed using an Olympus Provis AX70 microscope (Olympus, Tokyo, Japan) equipped with Nomarski interference contrast. Digital micrographs were taken with an Axio Cam (Zeiss, Oberkochen, Germany). Chloroplasts in the symbiotic cells were visualized by epifluorescence microscopy using a blue excitation filter.

Single-cell isolation: Lugol-fixed single cells were isolated using hand drawn Pasteur pipettes under an Olympus stereomicroscope SZX 12 (Olympus, Tokyo, Japan). Micrographs were obtained as described for LM. Following the documentation step the single cell was washed at least three times in ddH_2O under the stereomicroscope, and finally transferred to a 0.2 mL PCR tube (StarLab, Ahrensburg, Germany) and kept frozen at -20 °C until further processing.

PCR amplification and rDNA sequence determinations: To ensure cell disruption, physical treatments were applied prior to PCR. This was conducted using a sterile needle as described in Moestrup et al. (2006). Using single cells as template, PCR amplifications were preformed as previously outlined in Hansen and Daugbjerg (2004). Table 1 provides the combination of primers used to amplify SSU and LSU rDNA. The amplified PCR products were examined by 1.5% agarose gel electrophoresis (Fig. 3 A). The fresh PCR-amplified rDNA fragments were cloned following the protocol for chemical transformation with TOPO TA cloning kit (Part no 45-0641, Lot no. 316690, Invitrogen, USA). PCR conditions, purification of PCR products and rRNA sequence determination were as described in Moestrup et al. (2008). For determination of SSU rRNA from the dinoflagellate host and its endosymbiont a total of 20 clones were sequenced. Of these 18 identical sequences were assigned to the host and 2 clones with identical sequences were assigned to the endosymbiont.

Alignment and phylogenetic analyses of nuclear-encoded SSU rDNA: Using BioEdit (Hall 1999) SSU rDNA sequences from the eukaryotic endosymbiont and *Amphisolenia bidentata* were added to an alignment comprising sequences retrieved from GenBank of two glaucophytes, three green algae, two red algae and 95 chromalveolates. The Chromalveolata included cryptophytes (6 taxa), dinoflagellates (34 taxa), haptophytes (11taxa) and heterokonts (stramenopiles = chromists, 44 taxa). The sequences were aligned using ClustalX (Thompson et al. 1997) and comprised 1463 base pairs including introduced gaps. Following major kingdom level phylogeny of eukaroytes, the Glaucophyta constitute a sister group to the Chromalveolata (Hackett et al. 2007). Hence, Glaucophyta was used as the outgroup. The data matrix was analyzed by Bayesian analysis (MrBayes; Ronquist and Huelsenbeck 2003) and maximum likelihood (ML) as it is implemented in PhyML 3.0 (Guindon and Gascuel 2003). Bayesian analysis was carried out on the freely available Bioportal (www.bioportal.uio.no) and used two simultaneous Monte Carlo Markov chains (MCMC; Yang and Rannala 1997) that were run from random trees for a total of 2 million generations (metropolis-coupled MCMC). Trees were sampled for every 50th generation and the "burn-in" was evaluated as described in Hansen et al. 2007. AWTY (Wilgenbusch et al. 2004) was used to graphically evaluate the extent of the MCMC analysis. Burn-in occurred after 20.050 generations. Hence, the first 401 trees were discarded, leaving 39.600 trees for estimating posterior probabilities (PP). Thus, PP, values were obtained from a 50% majority rule consensus of the kept trees. In PhyML we used the parameter settings estimated by Modeltest ver. 3.7 (Posada and Crandall 1998) for the proportion of invariable sites and the among site rate heterogeneity. PhyML analyses were run using the online version available on the Montepellier bioinformatics platform at http://www.atgc-montpellier.fr/phyml. Evaluation of branch support was by bootstrapping with 100 replications.

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