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Molecular identification of diatom endosymbionts in nummulitid Foraminifera

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Abstract
The majority of extant families of larger Foraminifera are hosts for endosymbiotic diatoms, among them also Nummulitidae, which are the largest calcareous foraminiferans. Nummulitidae occur in reef environments and extend their depth distribution down to the base of the photic zone. They reach their highest diversity and abundance in the western Pacific. Some information about the diversity of nummulitid diatom symbionts has been gained by investigating morphological and ultrastructural features, but so far nothing has been known about the genetic diversity of their endosymbionts.
In the present work, the molecular diversity of diatom endosymbionts in 6 nummulitid genera has been examined by sequencing partial small subunit ribosomal RNA gene (SSU rDNA). Molecular analysis of the obtained sequences shows that the diatom endosymbionts in nummulitid Foraminifera are monophyletic and closely related to the genus *Thalassionema*. Diatom endosymbionts of deep water and shallow water nummulitids cluster in different groups. All diatom symbionts found in the nummulitid genus *Heterostegina* are characterized by the possession of spliceosomal introns.

Keywords: Endosymbiotic diatoms, Foraminifera, molecular identification

1. Introduction

Foraminifera belong to a major group of protists, comprising about 5,000 extant and 35,000 fossil species and are characterized by the possession of granuloreticulopodes, as well as by multichambered agglutinated or calcareous tests in most groups (Corliss, 1984). Representatives of this group can be found in every marine setting, from polar regions to tropical latitudes and from shallow water environments to deep sea basins, but some genera have also been described from freshwater environments and from a terrestrial habitat (Murray, 1991; Holzmann and Pawlowski, 2002).

The family Nummulitidae includes the largest extant calcareous Foraminifera, which are common in tropical and subtropical reef-environments. Recent nummulitid Foraminifera are restricted to the Indo-Pacific region with the exception of *Heterostegina* that shows a circumtropical distribution and can also be found in the Caribbean and as Lessepsian immigrants in the Mediterranean Sea (Hottinger, 1977; Murray, 1991; Langer and Hottinger, 2000). The systematics of nummulitid Foraminifera is mainly based on morphological studies (Hottinger, 1977; Banner and Hodgkinson, 1991). Molecular studies on the phylogenetic relationships in nummulitids have been carried out by Holzmann et al. (2003).

Symbiosis plays a key role in the evolution of large benthic Foraminifera. Algal symbionts provide their foraminiferal hosts with energy from photosynthesis necessary for survival and growth in oligotrophic environments and further promote calcification (Hallock, 1999). Most extant families of larger Foraminifera (4 out of 6) house small pennate diatoms as endosymbionts. Besides Nummulitidae, the families Amphisteginidae, Calcarinidae and Alveolinidae are hosts to diatoms, but contrary to Nummulitidae and Amphisteginidae, representatives of the latter 2 families are mostly restricted to the upper 50 m of the photic zone (Hohenegger, 2000; Hohenegger et al.,

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Table 1. Collection localities and host species of diatom endosymbionts.

Host species/DNA extraction number/ number of sequenced clones	Number of extracted host cells	Locality	Collection date	Depth (m)	Accession numbers
Nummulites venosus 301-1	1	Sesoko, Japan	Oct-96	50	AM233851
Nummulites venosus 301-2					AM233852
Nummulites venosus 302-1	1	Sesoko, Japan	Oct-96	50	AM233853
Nummulites venosus 303-1	1	Sesoko, Japan	Oct-97	50	AM233854
Operculinella cumingii 13-1	1	Sesoko, Japan	Jul-03	65	AM233849
Operculinella cumingii 13-2					AM233850
Operculina sp.1572-1	1	Guam, USA	Sept-99	2	AM233845
Operculina complanata 18-1	1	Sesoko, Japan	Jul-03	80	AM233867
Operculina complanata 20-1	1	Sesoko, Japan	Jul-03	80	AM233866
Operculina complanata 49-1	1	Sesoko, Japan	Jul-03	80	AM233855
Operculina elegans 53-1	2	Sesoko, Japan	Jul-03	40	AM233847
Operculina elegans 54-1	2	Sesoko, Japan	Jul-03	40	AM233848
Operculina ammonoides 232-1	1	Sesoko, Japan	Aug-96	18	AM233844
Operculina ammonoides 234-1	1	Sesoko, Japan	Aug-96	18	AM233846
Operculina ammonoides 494-1	1	Lizard Island, Australia	Aug-97	2	AM233843
Cycloclypeus carpenteri 6-1	1	Amami-O Shima, Japan	Jul-03	79	AM233842
Cycloclypeus carpenteri 21-1	0.5	Sesoko, Japan	Jul-03	80	AM233856
Cycloclypeus carpenteri 659-1	1	Sesoko, Japan	Nov-97	70	AM233857
Cycloclypeus carpenteri 659-2					AM233858
Cycloclypeus carpenteri 659-3					AM233859
Cycloclypeus carpenteri 660-1	1	Sesoko, Japan	Nov-97	70	AM233860
Planostegina operculinoides 16-1	1	Sesoko, Japan	Jul-03	80	AM233863
Planostegina operculinoides 662-1	2	Sesoko, Japan	Nov-97	80	AM233861
Planostegina operculinoides 662-2					AM233862
Planoperculina heterosteginoides 17-1	1	Sesoko, Japan	Jul-03	80	AM233864
Planoperculina heterosteginoides 19-1	1	Sesoko, Japan	Jul-03	80	AM233865
Heterostegina depressa 642-1	4	Maldives	Oct-97	3	AM233868
Heterostegina depressa 643-1	17	Maldives	Oct-97	3	AM233869
Heterostegina depressa 838-1	2	Florida Keys, USA	Jul-98	3	AM233870
Heterostegina depressa 1577-1	1	Guam, USA	Sept-99	2	AM233871

Table 2. List of amplification primers for the SSU rDNA gene in endosymbiotic diatoms. Note: EMBL/GenBank accession number of sequence used as reference for primer positions: X85396 (*Thalassiosira eccentrica*).

Primer	Sequence	Orientation	Specificity	Position in *T. eccentrica*	Position according to secondary structure model of *T. eccentrica*
Diaf1	cggagagggagcctgaga	Forward	Broad	383–400	Stem of helices 13–14
Diar1	agggcatcacagacctgt	Reverse	Broad	1429–1446	Stem of helix 36

2000). Based on morphological investigations, 22 different diatom species or varieties have been described from foraminiferal hosts, which are classified in 4 families belonging to several orders (Chai and Lee, 2000; Lee et al., 2000). Lee et al. (2000) have shown that symbiotic diatoms possess a 104 kDa surface glycoprotein, which is involved in host-symbiont recognition and is absent in all tested nonsymbiotic species.

Studies on the molecular diversity of foraminiferal symbionts have been previously carried out on two subfamilies of larger Foraminifera, Soritinae and Archaiasinae which are hosts to dinoflagellates and chlorophytes, respectively (Langer and Lipps, 1995; Pawlowski et al., 2001a,b; Pochon et al., 2001, 2006). The results confirmed the generic identification of chlorophyte (*Clamydomonas*) and dinoflagellate (*Symbiodinium*) symbionts proposed by previous ultrastructural and morphological studies. Chlorophyte endosymbionts do not seem to be strictly specific, as different foraminiferal species can share the same type of symbiont, but a strong host-symbiont specificity has been observed in the case of dinoflagellate symbionts (Pawlowski et al., 2001a; Garcia-Cuetos et al., 2005).

Molecular studies on the phylogenetic position of

diatoms among other organisms and relationships within the group have been carried out by Medlin et al. (1993, 1996a,b), Sorhannus (1996), Wiebe et al. (1996), Van der Auwera and De Wachter (1998), Sorhannus and Fox (1999), Ehara et al. (2000), Morton et al. (2002), and Scala et al. (2002). Molecular relationships between diatoms and their hosts have been investigated for two dinoflagellate species, which both contain a membrane-bound eukaryotic heterokont endosymbiont. Phylogenetic analyses of the data points to a pennate diatom ancestry for these symbionts (Chesnik et al., 1997). Apart from Foraminifera, these dinoflagellate species are so far the only reported case of symbiotic relationships among protists involving diatoms. In the present study, small subunit ribosomal RNA gene (SSU rDNA) sequences have been used to identify diatom symbionts in nummulitid Foraminifera and to examine their diversity and phylogenetic relationships.

2. Materials and Methods

Collection of specimens

Living nummulitid specimens were collected from the eastern and south-eastern Pacific, the Indian Ocean and the Caribbean Sea. Detailed information concerning collection sites and dates as well as GenBank accession numbers of new sequences are given in Table 1.

DNA extraction, amplification and sequencing

Prior to DNA extraction, each specimen was cleaned with a fine brush in sterile sea water to remove any debris and associated microorganisms. DNA extractions were performed by either using DOC lysis buffer (Holzmann and Pawlowski, 1996) or DNeasy Plant Mini Kit (Qiagen). Identification numbers of the DNA extractions are given in Table 1.

Partial SSU rDNA sequences of the endosymbionts were amplified in one fragment by using universal eukaryotic primers. A list of the primers used in our study is given in Table 2. PCR amplifications, cloning and sequencing were carried out as described in Holzmann and Pawlowski (2002). The 30 previously undescribed sequences reported in this paper were deposited in the EMBL/GenBank database under accession numbers AM233842-AM233871 (Table 1).

Sequence analysis

The 30 SSU rDNA gene sequences obtained in this study were aligned manually with 40 sequences of pennate diatoms using the BioEdit software (Hall, 1999), following the secondary structure model proposed by Wuyts et al. (2000). Two sequences of centric diatoms were used as an outgroup. Bayesian analyses were performed with MrBayes (Huelsenbeck and Ronquist, 2001) on 1,053 unambiguously aligned positions. Four simultaneous chains were run for 1,200,000 generations, and 12,000 trees were sampled, 2,000 of which were discarded as the burn-in. Posterior probabilities at all nodes were estimated from the 10,000 remaining trees. Additionally, the reliability of internal branches was determined using the bootstrap approach (Felsenstein, 1985). A maximum likelihood (ML) bootstrap analysis with 100 replicates was performed using PhyML (Guindon and Gascuel, 2003) jointly with the programs Seqboot and Consense from the Phylip package (Felsenstein, 2002). A minimum evolution (ME) bootstrap analysis with 10,000 replicates was also performed with PAUP* (Swofford, 1998), using the BioNJ option with ML-corrected estimates of the pairwise distances between sequences. In all cases, the GTR model of substitution (Lanave et al., 1984; Rodriguez et al., 1990) was used, taking into account a proportion of invariable sites, and a gamma-shaped distribution of the rates of substitution among variable sites, with eight rate categories. All parameters were estimated from the dataset.

3. Results

Sequence data

Partial SSU rDNA of diatom endosymbionts was sequenced for 25 isolates belonging to 9 nummulitid species (Table 1). The length of the sequences ranges from 1,066 basepairs (bp) in *Operculina* sp._1572 and *Operculina ammonoides*_232 to 1,535 bp in *Heterostegina depressa*_838. The G + C content ranges from 44.27% to 46.54%.

The fragment comprises a part of the SSU rDNA extending from position 383 to 1,446 in *Thalassiosira eccentrica* (X85396). It includes the stem regions 13 to 36 according to the secondary structure model of *T. eccentrica* (Medlin et al., 1996a) (Table 2).

Intraspecific variation has been examined by sequencing several clones from the same DNA extract (2 clones in *Nummulites venosus*_301, *Operculinella cumingii*_13, and *Planostegina operculinoides*_662 and 3 clones in *Cycloclypeus carpenteri*_659). The lowest values have been found in *N. venosus*_301 (0.5%), while the highest values have been detected in *P. operculinoides*_662 (0.9%).

Spliceosomal introns have been found in all sequences obtained from *H. depressa*. Information concerning the position and length of the introns are given in Table 3. Spliceosomal introns are stretches of non-coding DNA in eukaryotic genes. The majority of spliceosomal introns interrupt pre-mRNA in the nucleus and are removed by a ribonucleoprotein complex, which is called spliceosome (Bhattacharya et al., 2003).

Phylogenetic analysis

Fig. 1 presents the consensus tree of a Bayesian phylogenetic analysis of the SSU rDNA fragments, with the posterior probability values (PP) and bootstrap values (BV) of both ML and ME analyses indicated at the nodes. The trees inferred by ML and ME methods resulted in congruent phylogenetic trees (data not shown). The centric diatoms *Papiliocellulus elegans* and *Thalassiosira eccentrica* have been chosen as outgroup. Bacillariophyceae (supported by a PP of 1.00 and a ML BV of 89%) build a sister group to Fragilariophyceae (supported by a PP of 0.93 and a ML BV of 75%). Nummulitid endosymbionts build a monophyletic cluster (PP of 1.00, ML BV of 85%) among Fragilariophyceae, which includes two species of the genus *Thalassionema*.

Diatom endosymbionts of nummulitid species living in deep water (*C. carpenteri*, *O. complanata*, *P. heterosteginoides*, and *P. operculinoides*) build a weakly supported cluster (PP of 0.71, ML BV of 58%). Three sequences (*C. carpenteri*_21, *O. complanata*_18, and *O. complanata*_49) branch at the base of this cluster, while all others form a strongly supported subgroup (PP of 1.00, ML BV of 100%). Only one diatom sequence obtained from a deep water nummulitid (*C. carpenteri*_6) branches outside this cluster, as a sister to *Thalassionema* sp. AJ535140 (PP of 0.95, ML BV of 0.75).

Diatom sequences obtained from nummulitids living in shallower water group in three different clusters. The first one contains all sequences obtained from *Heterostegina* species (PP of 0.61, ML BV of 47%) and is basal to all other nummulitid endosymbionts. The second cluster contains sequences received from the nummulitid genera *Nummulites*, *Operculinella*, and *Operculina* (PP of 0.53, ML BV of 52%). The third cluster contains three *Operculina* symbionts and is a sister group to *Thalassionema nitzschoides* X77702 (PP of 1.00, ML BV of 100%); together with *Thalassionema* sp. AJ535140 and all symbionts from deep water nummulitids, they form a group supported by a PP of 0.70 and a ML BV of 63%. Two diatom sequences obtained from *N. venosus*_302 and *N. venosus*_303 branch separately at the base of this group.

4. Discussion

Diatom symbionts can be extracted from their foraminiferal hosts and grown in culture media where they regain their ability to form diagnostic cell envelopes and can therefore be identified morphologically (Lee et al., 2000). Morphological investigation of cultured diatom symbionts isolated from different foraminiferal families has revealed a certain bias in symbionts distribution: 6 diatom species belonging to 3 different genera (*Nitzschia, Fragilaria, Amphora*) were isolated from over 75% of the examined associations. Species of *Nitzschia* were found in 55% of the hosts, with *N. frustulum* var. *symbiotica* being the most abundant endosymbiont, found in 31% of the investigated species. 71% of individual Foraminifera are host to a single species of diatom, while a second symbiont species was found in the remainder (Lee, 1995; Lee et al., 1989, 1991, 1997, 2000).

Table 3. Position of spliceosomal introns in diatom SSU rDNA genes. Note: EMBL accession number for the alignment is ALIGN_000983.

Host species/diatom species	Donor site/position	Branch site/position	Acceptor site/position	Position according to secondary structure model of *T. eccentrica*
*Heterostegina depressa*_642	92–97	211–217	242–244	Loop between helices 16–17
	891–896	945–951	980–982	Loop of helix 29
*Heterostegina depressa*_643	92–97	211–217	242–244	Loop between helices 16–17
	894–899	958–964	993–995	Loop of helix 29
*Heterostegina depressa*_838	400–405	452–458	483–485	Loop of helix 21.7
	661–666	727–733	759–761	Loop of helix 22
	833–838	898–904	928–930	Loop between helices 25–26
	1023–1028	1078–1084	1115–1117	Loop of helix 29
	1164–1169	1243–1249	1251–1253	Loop between helices 30–31
*Heterostegina depressa*_1577	649–654	714–720	751–753	Loop between helices 25–26
	846–851	965–971	1001–1003	Loop of helix 29
*Cymatosira belgica*_X85387	959–964	1061–1067	1107–1109	Loop of helix 22
	1318–1323	1542–1548	1557–1559	Loop between helices 30–31
	1827–1832	1920–1926	1945–1947	Loop in stem region 36
*Extubocellulus spinifer*_AY485504	897–902	999–1005	1045–1047	Loop of helix 22
	1256–1261	1482–1488	1497–1499	Loop between helices 30–31
	1767–1772	1860–1866	1885–1887	Loop in stem region 36
*Amphora coffeaeformis*_AY485498	501–506	557–563	585–587	Loop in stem region 19
	1193–1198	1246–1252	1277–1279	Loop between helices 30–31
*Nanofrustulum shiloi*_AY485505	528–533	617–623	672–674	Loop in stem region 19

Within nummulitids, diatom endosymbionts have been isolated and examined from three species, *Heterostegina depressa, Operculina ammonoides* and *Cycloclypeus carpenteri*. It has been shown that Caribbean *Heterostegina* contain a different morphotype of *N. frustulum* var. *symbiotica*, which seems to be restricted to the western Atlantic region. More than 55% of the investigated *O. ammonoides* were host to *Achnanthes maceneryae* and *Nitzschia laevis*. The endosymbiotic diatoms of cultured specimens of *C. carpenteri* turned out to belong to a new genus related to *Nitzschia* (Lee et al., 1980, 1989, 1997).

In contrast with these results, our molecular analysis indicates that the symbionts in Nummulitidae are monophyletic and akin to *Thalassionema*. Similar symbiont sequences were obtained from different sampling localities in the eastern and south-eastern Pacific, the Indian Ocean and the Caribbean Sea. This suggests that host-symbiont specificity in nummulitid foraminifera and their diatom endosymbionts is stronger than has been suspected, as only one phylogenetically closely related cluster of symbionts could be detected. Our findings are supported by an ultrastructural study published by Leutenegger (1983). Fine structural analysis shows that diatom symbionts of *H. depressa*, sampled from the Gulf of Elat, Hawaii and the Maldives belong to the same symbiont type at all locations. The results indicate, that the symbiont type in *H. depressa* is characterized by invaginated pyrenoids, which is different from the two diatom species supposedly isolated from this host, i.e. *Nitzschia panduriformis* and *Nitzschia valdestriata* (Lee et al., 1980, 1989), which have simple internal or compound internal pyrenoids respectively. Leutenegger (1983) concluded, that morphological studies on symbionts *in situ* point out a higher specificity of host-symbiont associations than examination of cultured diatoms isolated from hosts.

Different nummulitid generations further seem to have a preference to stay with the same type of symbiont, as can be seen from the specimens collected in Sesoko in 1996, 1997 and 2003 (Fig. 1, Table 1). This corresponds to the results obtained by Leutenegger (1983), who has observed the same symbiont type in specimens of *H. depressa* sampled in Elat during the years 1971 to 1974 and the Maldives 1977 to 1980.

One could assume that the different results obtained by culturing diatom symbionts and sequencing them from host cell extractions originate in the diatom's sensitivity to culture conditions. It might be possible that those diatoms growing best in culture media are not identical to those living as endosymbionts within foraminifera. The living endosymbiont bearing foraminiferal specimens that are used for culturing or sequencing purposes are always collected in their natural habitat. Even if cleaned thoroughly prior to extraction, it is very difficult to obtain axenic conditions; Scanning electron microscopy (SEM) shows that diatom frustules can often be found on the exterior part of foraminiferal tests, in spite of careful brushing (personal observation of M.H.), although Lee et al. (1989) applied isolation procedures that should prevent contamination by nonsymbiont algae.

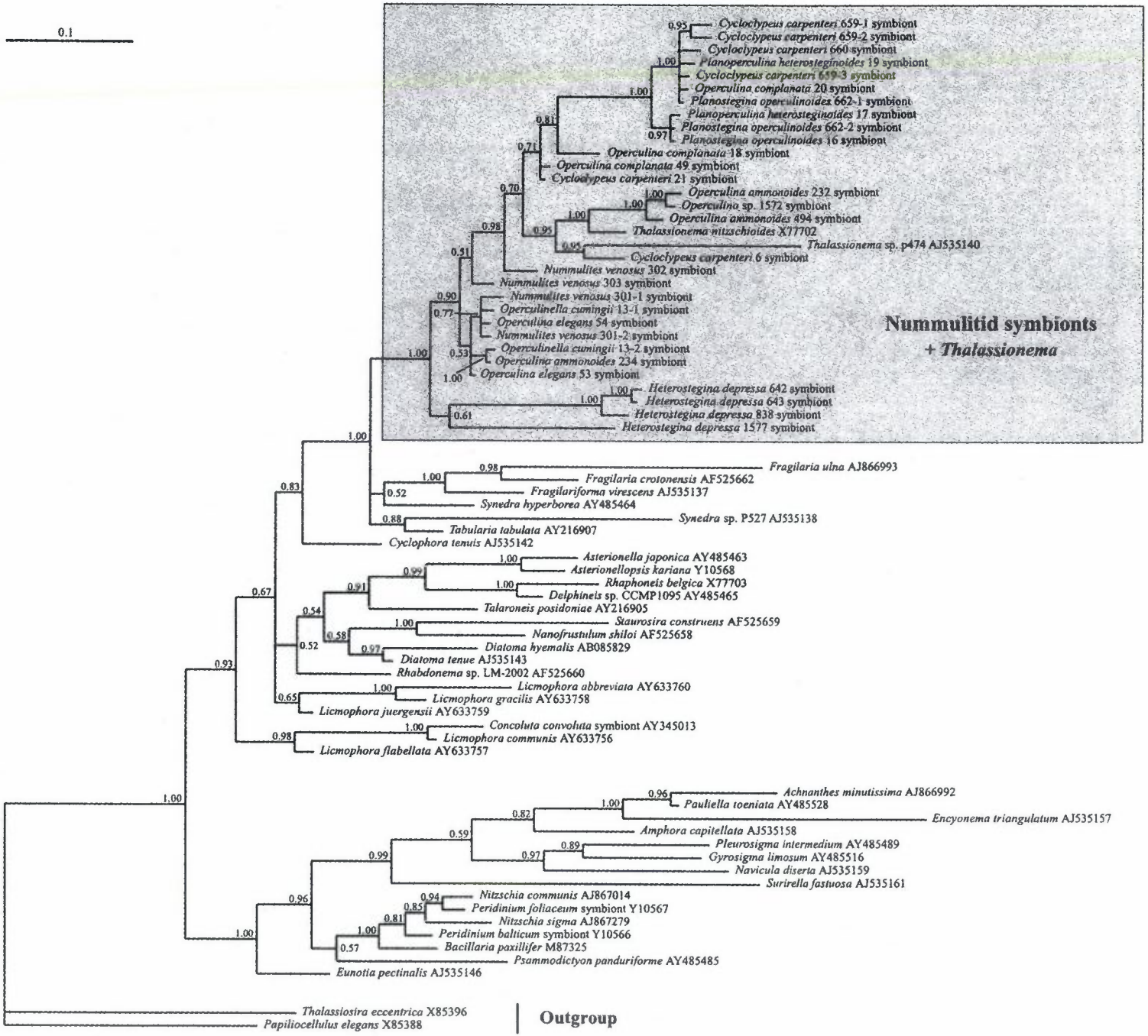
The strong monophyly of the analyzed diatom sequences corroborates the assumption that the obtained sequences stem from nummulitid endosymbionts and not from possible contaminations. This hypothesis is further supported by the fact that the nummulitid host specimens used in the present study were sampled in distant geographic localities over a period of several years.

SSU rDNA sequences were obtained by using broad eukaryotic primers (Table 2). No reamplifications were carried out. The primers used in this study would also amplify the 3 most commonly isolated diatom symbionts in Foraminifera (*Fragilaria*, *Amphora* and *Nitzschia*) (Lee, 2000), as well as Bacillariophycea. Due to the nature of the primers and because nummulitid DNA extractions are not axenic, about 44% of the obtained sequences belonged to a variety of other organism groups including fungi, Ciliophora, Cercozoa, amoeba, Haptophycea, Dinophycea and Viridiplantae. We could, however, not obtain any sequences belonging to *Fragilaria*, *Amphora* or *Nitzschia*, nor did we obtain any sequences clustering among Bacillariophycea. The monophyletic origin of diatom endosymbionts in Nummulitidae further suggests that their symbiotic relationships are due to a single evolutionary event.

Symbiont variability within the host has been examined by sequencing two to three clones for four DNA extracts and has been found less than 1% in each case. The results indicate that individual nummulitids do not seem to harbour markedly different symbiont types. This is in line with molecular studies on archaiasinid and soritid Foraminifera that have also identified monospecific endosymbiont assemblages for each of the investigated foraminiferal specimens (Pawlowski et al., 2001a; Pochon et al., 2004).

The genetic distances among different diatom genera displayed in the tree are quite low (e.g. genetic variability of 0.1% to 0.3% between the genera *Peridinium*, *Bacillaria* and *Nitzschia* at the base of the tree) (Fig. 1). The genetic variability between diatom symbiont sequences ranges from 0.1% to 3.5%, which would indicate that there exist different endosymbiont lineages in nummulitids, which may in fact represent different species or even genera (Fig. 1).

Distribution of nummulitid genera is correlated with depth (Hottinger, 1983; Hohenegger, 2004) and some diatom symbiont species were more frequently isolated from hosts living in shallow waters (<25 m), while others were only recovered in deeper waters (Leutenegger, 1983, 1984; Lee, 1990). Our results seem to confirm a depth zonation in nummulitid endosymbionts. The symbiont sequences obtained from deep-water nummulitids living from 70 to 100 m (*Cycloclypeus*, *Planostegina*,


0.1
Cyclocylpeus carpenteri 659-1 symbiont
Cyclocylpeus carpenteri 659-2 symbiont
Cyclocylpeus carpenteri 660 symbiont
Planoperculina heterosteginoides 19 symbiont
Cyclocylpeus carpenteri 659-3 symbiont
Operculina complanata 20 symbiont
Planostegina operculinoides 662-1 symbiont
Planoperculina heterosteginoides 17 symbiont
Planostegina operculinoides 662-2 symbiont
Planostegina operculinoides 16 symbiont
Operculina complanata 18 symbiont
Operculina complanata 49 symbiont
Cyclocylpeus carpenteri 21 symbiont
Operculina ammonoides 232 symbiont
Operculina sp. 1572 symbiont
Operculina ammonoides 494 symbiont
Thalassionema nitzschioides X77702
Thalassionema sp. p474 AJ535140
Cyclocylpeus carpenteri 6 symbiont
Nummulites venosus 302 symbiont
Nummulites venosus 303 symbiont
Nummulites venosus 301-1 symbiont
Operculinella cumingii 13-1 symbiont
Operculina elegans 54 symbiont
Nummulites venosus 301-2 symbiont
Operculinella cumingii 13-2 symbiont
Operculina ammonoides 234 symbiont
Operculina elegans 53 symbiont
Nummulitid symbionts
+ Thalassionema
Heterostegina depressa 642 symbiont
Heterostegina depressa 643 symbiont
Heterostegina depressa 838 symbiont
Heterostegina depressa 1577 symbiont
Fragilaria ulna AJ866993
Fragilaria crotonensis AF525662
Fragilariforma virescens AJ535137
Synedra hyperborea AY485464
Synedra sp. P527 AJ535138
Tabularia tabulata AY216907
Cyclophora tenuis AJ535142
Asterionella japonica AY485463
Asterionellopsis kariana Y10568
Rhaphoneis belgica X77703
Delphineis sp. CCMP1095 AY485465
Talaroneis posidoniae AY216905
Staurosira construens AF525659
Nanofrustulum shiloi AF525658
Diatoma hyemalis AB085829
Diatoma tenue AJ535143
Rhabdonema sp. LM-2002 AF525660
Licmophora abbreviata AY633760
Licmophora gracilis AY633758
Licmophora juergensii AY633759
Concoluta convoluta symbiont AY345013
Licmophora communis AY633756
Licmophora flabellata AY633757
Achnanthes minutissima AJ866992
Pauliella taeniata AY485528
Encyonema triangulatum AJ535157
Amphora capitellata AJ535158
Pleurosigma intermedium AY485489
Gyrosigma limosum AY485516
Navicula diserta AJ535159
Surirella fastuosa AJ535161
Nitzschia communis AJ867014
Peridinium foliaceum symbiont Y10567
Nitzschia sigma AJ867279
Peridinium balticum symbiont Y10566
Bacillaria paxillifer M87325
Psammodictyon panduriforme AY485485
Eunotia pectinalis AJ535146
Thalassiosira eccentrica X85396
Papiliocellulus elegans X85388
Outgroup
FRAGILARIOPHYCEAE
BACILLARIOPHYCEAE

Planoperculina, and *O. complanata*) build a monophyletic cluster, although it is weakly supported (PP of 0.71 and ML BV of 58%; Fig. 1). However, one deep-water symbiont sequence (*Cycloclypeus carpenteri*_6) branches with *Thalassionema* and some shallow water operculinoids (Fig. 1). Diatom sequences obtained from nummulitids living in shallow water zones down to 65 m (*Heterostegina*, *Nummulites*, *Operculinella*, *O. elegans*, and *O. ammonoides*) build three weakly supported clusters and some sequences branch separately (Fig. 1). Shallow-water diatom sequences could be considered as a paraphyletic group of lineages from which deep-water symbionts evolved.

Spliceosomal introns of various lengths have been found in all diatom sequences obtained from *H. depressa*. The inserts are characterized by three conserved regions, the donor site, acceptor site and branch site (Bhattacharya et al., 2000). Our study brings the first evidence for occurrence of spliceosomal introns in endosymbiotic diatoms. However, this is not a unique case within Bacillariophyta. Medlin et al. (1996) first observed three unusual insertions in the SSU rDNA of the centric diatom *Cymatosira belgica* X85387, but at the time the authors did not recognize them as spliceosomal introns. We performed a screening of other available diatom SSU rDNA sequences, which revealed that spliceosomal introns can also be found in a few other published sequences, although none of these introns are annotated as such in the sequence descriptions. Three spliceosomal introns can be found in the SSU rDNA sequence of the centric diatom *Extubocellulus spinifer* AY485504, two in the case of *Amphora coffeaeformis* AY485498 (Bacillariophyceae), and one in the case of *Nanofrustulum shiloi* AY485505 (Fragilariophyceae) (Table 3).

Spliceosomal introns have previously been detected in several eukaryotic protein-coding genes and in the SSU and LSU rDNA sequences of some fungi. The occurrence of these introns seems to be especially widespread in ribosomal genes of Ascomycetes, a group that consists of lichen forming and free living members (Bhattacharya et al., 2000, 2003).

See figure on previous page.

Figure 1. Bayesian phylogenetic tree (GTR + G + I model) of pennate diatoms showing the phylogenetic position of the 30 SSU rDNA sequences of nummulitid symbionts we obtained. Numbers at nodes indicate (from left to right) the posterior probabilities deduced from 10,000 sampled trees, the maximum likelihood bootstrap values after 100 replicates, and the minimum evolution bootstrap values after 10,000 replicates; groupings supported by posterior probabilities under 0.5 were collapsed and are shown as polytomies. All branches are drawn to scale. The tree was rooted with two sequences from centric diatoms.

To date, we cannot answer the question why the group of intron bearing diatom symbionts is restricted to *Heterostegina*, but it is an interesting fact that spliceosomal introns have been found in the ribosomal genes of two unrelated groups comprising important symbiotic representatives, the Ascomycetes and the diatoms. More work will be necessary to clarify these questions and get more information about spliceosomal introns in symbiotic and non symbiotic diatoms.

Diatom endosymbionts have been previously described from two dinoflagellate species, *Peridinium foliaceum* and *Peridinium balticum* (Chesnik et al., 1997). A diatom endosymbiont has also been reported from the metazoan *Convoluta convoluta* (GenBank entry AY345013). The symbiont sequences of *Peridinium* cluster with Bacillariophyceae, whereas the sequence obtained from *Convoluta* branches with *Licmophora*, which belongs to Fragilariophyceae (Fig. 1). Our results show that diatom endosymbionts in nummulitid Foraminifera are different from symbionts found in other protozoan or metazoan species.

Future work will concentrate on molecular aspects of diatom endosymbionts in the remaining foraminiferal families Amphisteginidae, Calcarinidae and Alveolinidae. This will increase our knowledge about phylogeny, systematics and biogeographical distribution of diatom endosymbionts in Foraminifera.

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