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REPORT

One-year survey of a single Micronesian reef reveals extraordinarily rich diversity of *Symbiodinium* types in soritid foraminifera

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Abstract Recent molecular studies of symbiotic dinoflagellates (genus *Symbiodinium*) from a wide array of invertebrate hosts have revealed exceptional fine-scale symbiont diversity whose distribution among hosts, regions and environments exhibits significant biogeographic, ecological and evolutionary patterns. Here, similar molecular approaches using the internal transcribed spacer-2 (ITS-2) region were applied to investigate cryptic diversity in *Symbiodinium* inhabiting soritid foraminifera. Approximately 1,000 soritid specimens were collected and examined during a 12-month period over a 40 m depth gradient from a single reef in Guam, Micronesia. Out of 61 ITS-2 types distinguished, 46 were novel. Most types found are specific for soritid hosts, except for three types (C1, C15 and C19) that are common in metazoan hosts. The distribution of these symbionts was compared with the phylotype of their foraminiferal hosts, based on soritid small subunit ribosomal DNA sequences, and three new phylotypes of soritid hosts were identified based on these sequences. Phylogenetic analyses of 645 host-symbiont pairings revealed that most *Symbiodinium* types associated specifically with a particular foraminiferal host genus or species, and that the genetic diversity of these symbiont types was positively correlated with the genetic diversity found within each of the three host genera. Compared to previous molecular studies of *Symbiodinium* from other locations worldwide, the diversity reported here is exceptional and suggests that Micronesian coral reefs are home to a remarkably large *Symbiodinium* assemblage.

Keywords Molecular diversity · ITS-2 rDNA · Soritinae · *Symbiodinium* · Symbiosis

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Introduction

Coral reef ecosystems are home to a large array of protist and invertebrate phyla, including representatives of the Foraminifera, Ciliata, Porifera, Cnidaria and Mollusca, which live in symbiosis with dinoflagellates belonging to the genus *Symbiodinium* (Trench 1993; Rowan 1998; Pawlowski et al. 2001; Lobban et al. 2002). Extensive phylogenetic investigations, based primarily on the analyses of small subunit (SSU) and large subunit (LSU) nuclear ribosomal (nrDNA) genes, have led to the current recognition of eight distinctive groups or sub-generic lineages of the genus *Symbiodinium*, referred to as clades A, B, C, D, E, F, G and H (reviewed in Coffroth and Santos 2005; Stat et al. 2006). This classification scheme and phylogenetic reconstruction were recently confirmed by the analysis of plastid

genes coding for the ribosomal LSU 23S Domain V (Santos et al. 2002; Pochon et al. 2006).

While *Symbiodinium* clades A, B, C and D are mostly associated with metazoan hosts, remarkable symbiont diversity, including representatives in clades C, D, F, G and H, has been found in large benthic foraminifera belonging to the sub-family Soritinae (Pochon et al. 2001, 2004, 2006). At higher taxonomic levels, these associations exhibit symbiont specificity, as evidenced by the association of soritids with entire *Symbiodinium* lineages (clades F and H) or sub-lineages (in clades C, D and G). Although no evidence for sensu stricto co-evolution between the soritids and their symbionts exists, a high degree of host–symbiont specificity has also been observed at lower taxonomic levels, not only at the sub-familial (Soritinae) level, but also within each of the three soritid genera (*Sorites*, *Amphisorus*, and *Marginopora*) (Garcia-Cuetos et al. 2006).

Although SSU and LSU rDNA-based markers have been extensively applied to deciphering appreciable levels of ecological and physiological variations in the genus *Symbiodinium* (Rowan and Knowlton 1995; Baker et al. 1997; Rowan et al. 1997; Belda-Baillie et al. 1999; Carlos et al. 1999; Karako-Lampert et al. 2004; Chen et al. 2005), the recent use of highly variable markers such as polymorphic microsatellites (Santos et al. 2004; Magalon et al. 2006), the plastid-coding *psbA* minicircle (Barbrook et al. 2006) and nrDNA Internal transcribed spacer regions (LaJeunesse 2001; 2002; Rodriguez-Lanetty 2003; van Oppen et al. 2001, 2005a, b) have revealed additional phylogenetic structure within each *Symbiodinium* clade. General knowledge of *Symbiodinium* diversity, specificity and distribution has been greatly increased by several surveys of *Symbiodinium* ITS-2 diversity within entire reef invertebrate communities, comprising diverse species of hard corals, soft corals, gorgonians, anemones, zoanthids, corallimorphs, tridacnid clams and nudibranchs (LaJeunesse 2002, 2005; LaJeunesse et al. 2003, 2004a, b). In each locality, the genetic examination of 18 to 74 different host genera uncovered between 20 and 32 *Symbiodinium* types, respectively (LaJeunesse et al. 2003, 2004a, b). In total, approximately 200 distinct types, as defined by ITS-2 sequences within *Symbiodinium* clades A–D, can be currently recovered from GenBank, more than half of which are members of clade C.

Here, denaturing gradient gel electrophoresis (DGGE) was used to investigate diversity in *Symbiodinium* ITS-2 from soritid foraminifera living over a 40 m depth gradient on a single reef in Guam. Additionally, the SSU rDNA marker was used to examine the diversity of soritid hosts. This study, based on >1,000 *Symbiodinium* samples isolated from the three soritid genera, revealed an unexpectedly rich assemblage of symbionts and their hosts. The diversity of *Symbiodinium* types discovered during this survey is synthesized with those identified in previous studies.

Materials and methods

Sampling

Soritid foraminifera were collected monthly between September 2002 and September 2003, at "Gun Beach" (13°32′N; 144°47′E), on the island of Guam, Micronesia (Fig. 1). Within this locality, four sampling sites were selected corresponding to different sampling depths: the reef flat (>1 m), higher reef slope (10 m), mid reef slope (20 m), and lower reef slope (40 m). Soritid foraminifera were abundant at all sites, and were found attached to various substrates such as coral rubble, boulders, rocks, encrusting algae and macroalgae.

Because soritid representatives in the genus *Sorites* prefer phytal substrata while those in *Amphisorus* and *Marginopora* are usually more abundant on rocks, two collecting strategies were followed monthly at each of the four sites. First, following line transects ~40–50 m long, rocks and dead coral debris were collected to fill up a mesh bag to a total volume of 0.02 m^3. Second, macroalgae (mainly *Halimeda* sp.) were collected to fill up plastic containers to a total volume of 1 L. The bag and the containers were then placed in a cooler with seawater to avoid desiccation during transport. Once at the laboratory, collected samples were carefully examined and all living soritids were removed from the rocks and algae, one by one, with tweezers. In total, 1,010 specimens were selected for total DNA

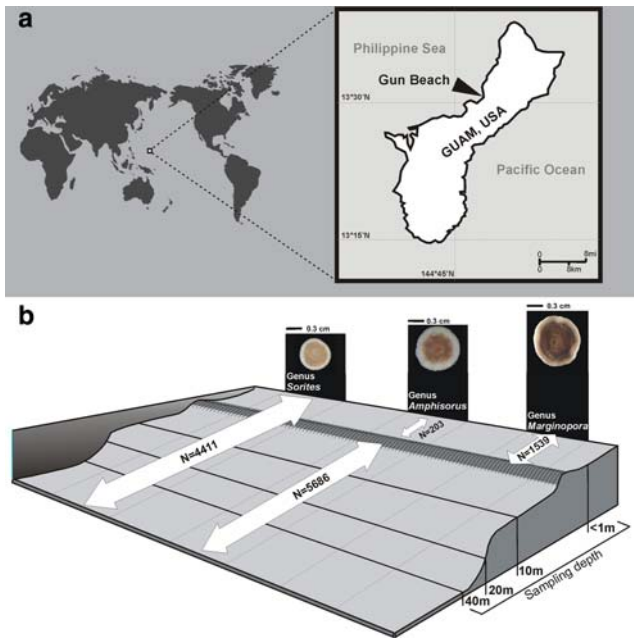

Fig. 1 **a** Map of Guam (Micronesia), showing the location of the Gun Beach sampling site. **b** Spatial zonation and abundance of the three soritid genera *Sorites*, *Amphisorus*, and *Marginopora* collected each month at Gun Beach between September 2002 and August 2003 in four different site (shallow, 10, 20, and 40 m depth)

extractions, after being morphologically identified to the genus level (*Sorites*, *Amphisorus* and *Marginopora*), and diametrically measured by means of an ocular micrometer.

DNA extraction, PCR, sequencing, and DGGE analyses

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing for both soritid hosts (partial SSU rDNA) and their symbionts (ITS-2 and partial LSU rDNA) were performed following Garcia-Cuetos et al. (2006) and Pochon et al. (2001), respectively. Amplified products from 20 soritid specimens (see "Results") were ligated into pGEM-T vector system (Promega) and cloned into XL-2 ultracompetent cells (Stratagene).

The *Symbiodinium* ITS-2 amplicons were analysed by DGGE using a CBS Scientific system (Del Mar, CA). PCRs were run following the "touchdown" protocol of LaJeunesse (2002) and using the internal primers itsD (5′ GTG AAT TGC AGA ACT CCG TG-3′) and the GC-clamped primer "ITS2clamp2" (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCT TAC TTA TAT GCT TAA ATT CAG CGG GT-3′). PCR products were verified by electrophoresis on agarose gels (1.5% agarose in 40 mM Tris-acetate, 1 mM EDTA). Samples containing successfully amplified PCR products were subsequently run on denaturing gradient gels (45–80% formamide, 8% polyacrylamide denaturing gradient; 100% consisting of 7 M urea and 40% deionized formamide) for approximately 12 h at 150 volts. Each DGGE band or type was referenced and periodically submitted for sequencing verification to control for methodological artifacts and intragenomic variation. Direct sequences of these controls were obtained either after PCR amplification of the referenced sample, or after gel excision and PCR re-amplification following LaJeunesse (2002). This gel excision method was also employed when multiple ITS-2 amplicons were detected simultaneously. For example, sequences were obtained whenever co-occuring patterns involving the same dominant band with different associated fingerprints were encountered.

Phylogenetic analyses

Two different phylogenetic approaches were applied to investigate relationships within the soritid foraminifera and their symbionts. First, ITS-2 rDNA types of *Symbiodinium* were manually aligned using the BioEdit version 5.0.9 sequence alignment software (Hall 1999). Intraspecific relationships within *Symbiodinium* clades C, D, F, G and H were determined by statistical parsimony using the program TCS version 1.18 (Clement et al. 2000). Networks within each clade were delineated with 95% certainty, the gaps being treated as a fifth state. The same approach and settings were also applied to all foraminiferal SSU rDNA sequences obtained in the genus *Sorites*, to test for clade delimitations.

Secondly, partial SSU rDNA sequences of soritid foraminifera were aligned using Clustal X (Thompson et al. 1997) and then improved manually using BioEdit (version 5.0.9). Modeltest, implemented in the PAUP* version 4.0.b10 software (Swofford 2002), identified the general time reversible (GTR) model (Lanave et al. 1984) as the best model for the analyses, taking into account a proportion of invariant sites (I) and a gamma distribution shape parameter (γ). Using these settings, a tree was reconstructed with the PhyML software (Guidon and Gascuel 2003) using the maximum likelihood (ML) method (Felsenstein 1981). The reliability of internal branches was assessed using the non-parametric bootstrap method with 100 replicates. A Bayesian tree estimation method was also employed using the program MrBayes, version 3.1 (Huelsenbeck and Ronquist 2001). One out of every ten trees was sampled for 10^6 generations with kappa and DNA substitution parameters estimated during the search. After excluding the first sampled trees, categorized as the "burn-in period", a consensus tree was constructed using MrBayes.

Statistical analyses

Total inertia tests of the correspondence analyses (COA) module implemented in the program ADE-4 (Thioulouse et al. 1997) were applied to verify if the high diversity of *Symbiodinium* types observed in the foraminiferal genus *Sorites* were randomly or specifically distributed within this genus. Three taxonomic levels in both *Sorites* spp. and *Symbiodinium* spp. were compared two by two: the level of types, the level of networks obtained with the program TCS, and the level of clade (i.e. clades C, F, G, H for *Symbiodinium* and clades Sor I–Sor XII for the host *Sorites*).

The Fisher exact test (Zar 1999) was performed using the R environment (Ihaka and Gentleman 1996) to statistically verify the specificity of the *Symbiodinium* types detected in the sub-clades Amp I and Amp IV of the host *Amphisorus*.

Results

Symbiodinium identifications

The 1,010 specimens selected for DNA analysis represented the isolation of approximately ten soritid specimens per genus, per site, per month. Given the observed depth zonation (Fig. 1b), with the genus *Sorites* abundant at all sites, the genus *Amphisorus* common at deeper sites (10–40 m) and the genus *Marginopora* restricted to the shallow site only, the total numbers of isolated specimens per genus throughout this survey were 477, 413, and 120,

respectively. Of these, 461, 399, and 115 samples provided positive PCR amplications and successful DGGE profiles of *Symbiodinium* ITS-2 rDNA diversity. Representative DGGE profiles obtained during this survey ($N = 975$) are shown in Fig. 2.

The observed profiles were mainly characterized by a single distinctive band, corresponding to a specific fingerprint of the ITS-2 rDNA region (Fig. 2a–e). These single bands or types were sometimes accompanied by faint background bands, resulting either from the presence of paralogous loci or from the creation of heteroduplexes during the PCR amplification process (see LaJeunesse 2002). Each new band was excised from the gel, sequenced, and compared with the ITS-2 rDNA database. Sequences from each excised band received an alphanumeric name based on the taxonomy of LaJeunesse (e.g., 2002, 2005), starting with a letter corresponding to the *Symbiodinium* clade and followed by a number referring to the within clade diversity. A lower case letter was added to the types belonging to a given TCS network (see next section), and which were identified as a minor or derived type (i.e., rarely detected in foraminiferal populations during the survey).

Sometimes, mixed *Symbiodinium* genotypes were detected within a single host specimen (Fig. 2f–g). These co-occurring patterns were only found in the genera *Sorites* and *Amphisorus*. However, the genus *Marginopora* was mostly found with the symbiont type D1.1 ($N = 87$) in clade D1 (sensu Pochon et al. 2006), which could not be assessed using DGGE due to its unusual base pair composition as well as its longer sequence length (Fig. 3a); hence, the presence of closely related co-occurring clade D genotypes in *M. vertebralis* cannot be completely excluded. As shown in Table 1, the number of samples detected with co-occurring symbiont types in *Sorites* sp. and *Amphisorus* sp., represented approximately 10% ($N = 46$) and 15% ($N = 67$) of investigated samples, excluding mixed patterns involving potential paralogs (see symbiont types with asterisks in Table 2). Some co-occurring types belonged to the same *Symbiodinium* clade (see Fig. 2f), while other co-occurring types belonged to two or occasionally three *Symbiodinium* clades (Fig. 2g). Most incidences of mixed genotypes observed during this survey were found in the smallest hosts, i.e., juveniles with a diameter of 0.1–2.0 mm (Table 1).

In total, 61 *Symbiodinium* ITS-2 types were identified during this survey. Their host distribution, abundance and corresponding number of sequences obtained for type verification, as well as the sequences size and GenBank accession numbers are shown in Table 2. The diversity of *Symbiodinium* types retrieved from previous studies is presented in Table 3.

The occurrence of *Symbiodinium* types in the genera *Sorites*, *Amphisorus*, and *Marginopora*, are plotted in Fig. 4. As many as 43 different *Symbiodinium* types were detected in *Sorites*, of which 36 associated specifically with this genus (see Table 2). C91 was by far the most common *Symbiodinium* type in *Sorites* (Fig. 4a), occurring in 184 specimens. Ten additional types belonging mainly to *Symbiodinium* sub-clades F4 and F5 (sensu Pochon et al. 2006) were the next most common *Symbiodinium* types, occurring in 10–31 specimens. The remaining 32 types were found at low frequency (<10 times specimens each). The genus *Amphisorus* harbored 18 different *Symbiodinium* types (Fig. 4b); the most common one being C1 ($N = 140$), a pandemic symbiont type extremely common in cnidarians (LaJeunesse 2005). The second most common type in *Amphisorus* was C91 ($N = 77$), followed by types F3.2, F3.1, C93, and C92 with 65, 61, 18, and 10 counts, respectively. The remaining 13 types identified in this genus were found at low frequencies (≤ 10). In the genus *Marginopora*, only nine types were identified (Fig. 4c): type D1.1 ($N = 87$), type G2 ($N = 19$), and seven other types found at extremely low frequencies (≤ 2).

Symbiodinium type networks

Following the completion of the *Symbiodinium*-DGGE survey, five ITS-2 datasets were compiled for clades C, D1, F, G, and H, including a total of 156 unique ITS-2 types. Each alignment was analyzed by statistical parsimony, which produced 16 ITS-2 networks and 19 single types (Fig. 5).

The most intricate network was produced by *Symbiodinium* clade C (Fig. 5a). This clade included 82 ITS-2 types previously identified by LaJeunesse (2005) in numerous Indo-Pacific metazoan hosts, and ten types specific to soritid foraminifera (Pochon et al. 2004). Since clade C in soritid foraminifera is restricted to the Indo-Pacific (Pochon et al. 2004), only Indo-Pacific non-foraminiferal ITS-2 types were considered here. A complete network, including all known clade C types, was also analysed and produced congruent results (data not shown). The great majority of clade C types detected in soritid foraminifera cluster in a specific region of the network: between the ancestral type C3 and the derived type C15 which is very common throughout Indo-Pacific corals in the genus *Porites* and from which a number of specific or endemic types have evolved (LaJeunesse 2005).

The highest diversity was found within *Symbiodinium* clade F (Fig. 5b), in accordance with previous analyses of ITS-1 sequences (Rodriguez-Lanetty 2003). This clade contained 43 ITS-2 types specific to soritid foraminifera, of which 32 were characterized in Guam (this study), and 11 were recovered from other localities (Table 3). The parsimony criterion resolved 11 networks and four single types (Fig. 5b), including the complete diversity of types detected to date in soritid symbionts from sub-clades F2, F3, F4, and

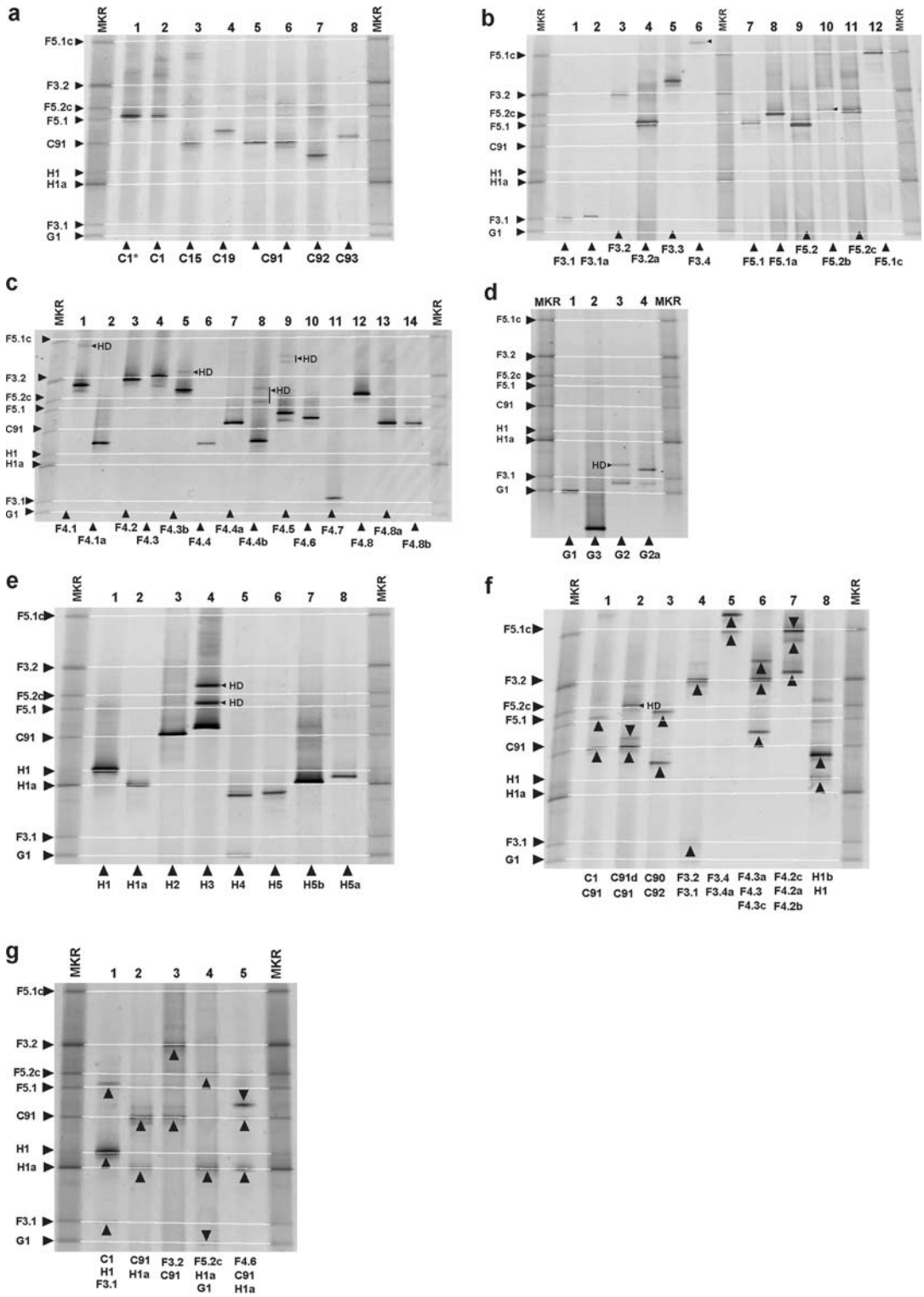

Fig. 2 *Symbiodinium* types negative images of PCR-DGGE profiles obtained after analyses of ITS-2 rDNA gene from the 975 soritid hosts analyzed during the survey. **a** Single ITS-2 types belonging to the *Symbiodinium* clades C. Type C1* (*lane 1*) correspond to the C1 reference type sensu LaJeunesse (2005). **b**, **c** Single ITS-2 fingerprints belonging to the *Symbiodinium* sub-clades F3, F5, and F4, respectively. **d**, **e** Single ITS-2 types belonging to clades G and H, respectively. **f**, **g** Multiple ITS-2 patterns containing profiles of more than one symbiont type simultaneously. **f** Co-occurring patterns of types belonging to the same *Symbiodinium* clade. **g** Co-occurring patterns of types belonging to different *Symbiodinium* clades. Standards (*MKR*) in both sides of the gels are pooled PCR-DGGE amplifications from the nine types indicated on the left side of the gels. HD refers to the presence of heteroduplexes

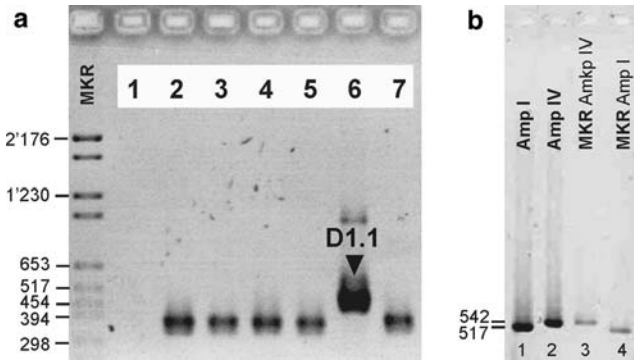

Fig. 3 **a** Negative image of a PCR gel showing the unique size fragment (444 bp; *lane 6*) of the *Symbiodinium* D1.1 type. Other PCR fragments (*lanes* 2–5, 7) correspond to the size obtained with all other types. **b** Negative image of a PCR gel showing the visible difference in size fragments between the soritid species *Amphisorus kudakajimaensis* (phylotypes Amp I) and *Amphisorus hemprichii* (Amp IV). Markers of reference (MKR) were placed at each side of the PCR gels to identify the 263 and 136 soritids belonging to Amp I and Amp IV, respectively

Table 1 Number of mixed symbiont genotypes co-occurring in single hosts and identified by DGGE and DNA sequence analyses during the soritids survey

Host genus	Total[a]	Mixed[b]	Same clade[c]	Different clade[d]	Juveniles[e] (%)
Sorites	461	46	13	33	67.39
Amphisorus	399	67	54	13	77.61
Marginopora	115	0	0	0	0

[a] Total number of specimens which symbionts were analysed by DGGE fingerprinting

[b] Number of specimens observed with mixed patterns of *Symbiodinium* types

[c] Number of mixed patterns involving the same *Symbiodinium* clade

[d] Number of mixed patterns involving different *Symbiodinium* clades

[e] Percentage of soritid juveniles (0.1–2 mm in diameter size) harboring more than one symbiont type simultaneously

F5. Sub-clades F4 and F5 are the most diversified in this clade, possessing 21 and 11 types organized in eight and two networks, respectively. The F2 symbionts previously documented in the Jamaican coral *Meandrina meandrites* (LaJeunesse 2001) corresponded to type F5.1, while those reported in *Alveopora japonica* (Rodriguez-Lanetty et al. 2003) could not be assessed since ITS-2 sequences are not available in GenBank. Compared to clades C and F, the diversity of remaining clades G, H, and D1 was much lower (Fig. 5c–e). *Symbiodinium* clade G and H contained, respectively, 5 and 11 ITS-2 types that are highly specific to soritid foraminifera and mostly collected in Guam. Subclade D1 included two sequences: one sequence called D1.1 (D1 in Garcia-Cuetos et al. 2006) and another sequence named D1.2 and obtained from the PSP1-05 *Symbiodinium* sample originally isolated from the sponge *Haliclona koremella* (see Carlos et al. 1999).

Soritid diversity

In order to assess the degree of specificity between the soritid foraminifera and their symbionts collected during this survey, the SSU rDNA sequences of 169 selected soritid foraminifera were obtained and compared with 24 previously published (Garcia-Cuetos et al. 2006) sequences representing the diversity of all soritid morphospecies. A total of 16 different SSU phylotypes were found during this survey (Fig. 6). This number comprised 13 out of the 22 formerly described phylotypes obtained from the soritids collected in nine localities worldwide (Garcia-Cuetos et al. 2006). In addition, three new phylotypes Sor I.a, Sor I.b, and Sor I.c, represented by 29 sequences, were described. Of the 169 samples analyzed, 149 produced high-quality direct sequences; the remaining 20 sequences were obtained by cloning. Of these 20 clones, 14 produced unique sequence signatures, while six revealed the presence of paralogous copies, all within the soritid phylotype Sor I.c. GenBank accession numbers, as well as the geographic source of all soritid sequences are found in Table 4.

Host–symbiont specificity

An accurate examination of host–symbiont relationships between the many *Symbiodinium* types and the 16 soritid SSU phylotypes identified during this survey requires that the molecular identification of hosts and symbionts be as exhaustive as possible. This endeavor was successfully completed for the 399 *Amphisorus* sp. and 115 *M. vertebralis* specimens, but only partially achieved for the 461 *Sorites* sp. individuals. In the genus *Amphisorus*, the sequences of *A. kudakajimaensis* (Amp I) were 25 bp shorter than the sequences of *A. hemprichii* (Amp IV), allowing their separation on a PCR gel (Fig. 3b). Using this approach, the 399 *Amphisorus* specimens were amplified, of which 263 and 136 individuals belonging to Amp I and Amp IV were identified, respectively. In the species *M. vertebralis*, only one phylotype (Mar I) is present on the Island of Guam, as evidenced by the 12 sequences produced in this study (Table 4) and 13 sequences from previous work (Garcia-Cuetos et al. 2006). Consequently, all 115 specimens analyzed in this study were attributed to the phylotype Mar I. Finally, due to time and cost considerations, 131 of the 461 *Sorites* specimens were randomly selected and sequenced according to the *Symbiodinium* type they contained, to ensure balanced sampling according to relative *Symbiodinium* abundance (see scatters in Fig. 4a).

The proportion of *Symbiodinium* types falling into the soritid phylotypes Amp I, Amp IV, Mar I, and Sor I–Sor XII,

Table 2 Diversity of *Symbiodinium* ITS-2 types obtained in soritid foraminifera during the Guam DGGE survey (brightest diagnostic bands)

Type name	Soritid host	Type abundance	Number of sequences	Sequence length	GenBank accession
C1	a	140	17	283	AM748551
C15	a	1	1	283	AM748552
C19	a	2	2	282	AM748553
C90	a	1	1	285	AM748554
C91	s, a, m	262	53	283	AM748555
C91a[a]	s	2	2	283	AM748556
C91b[a]	s	1	1	283	AM748557
C91c[a]	s	1	1	283	AM748558
C91d[a]	s	3	3	283	AM748559
C92	s, a	40	15	283	AM748560
C92a[a]	s	1	1	283	AM748561
C93	a	18	6	283	AM748562
C93a[a]	s	1	1	283	AM748563
D1.1	m	87	14	444	AM748564
F3.1	a	61	8	320	AM748565
F3.1a	a	1	1	320	AM748566
F3.2	s, a	66	14	315	AM748567
F3.2a	m	2	2	315	AM748568
F3.3	s, m	2	2	311	AM748569
F3.4	s	2	2	311	AM748570
F3.4a[a]	s	1	1	311	AM748571
F4.1	s	5	3	297	AM748572
F4.1a	s	2	2	297	AM748573
F4.2	s	6	4	297	AM748574
F4.2a[a]	s	1	1	296	AM748575
F4.2b[a]	s	1	1	296	AM748576
F4.2c[a]	s	1	1	297	AM748577
F4.3	s, a, m	13	6	298	AM748578
F4.3a[a]	s	1	1	298	AM748579
F4.3b	s	6	2	298	AM748580
F4.3c[a]	s	1	1	298	AM748581
F4.4	s, a	23	6	298	AM748582
F4.4a	s	14	5	297	AM748583
F4.4b	s	1	1	295	AM748584
F4.5	S	2	2	300	AM748585
F4.6	s, a	17	6	297	AM748586
F4.7	s	2	2	299	AM748587
F4.8	s	3	2	300	AM748588
F4.8a	s, a	22	8	300	AM748589
F4.8b	S	1	1	300	AM748590
F5.1a	s, a	25	6	310	AM748591
F5.1	s, a	32	14	310	AM748592
F5.1c	s	3	2	310	AM748593
F5.2	s	31	8	310	AM748594
F5.2b	s	2	1	310	AM748595
F5.2c	s	10	2	310	AM748596
G1	s, m	3	1	333	AM748597
G2	m	19	9	332	AM748598

Table 2 continued

Type name	Soritid host	Type abundance	Number of sequences	Sequence length	GenBank accession
G2a	m	1	1	332	AM748599
G3	s, m	2	1	333	AM748600
G4	s	1	1	335	AM748601
H1	s, a	10	9	326	AM748602
H1a	s	9	6	326	AM748603
H1b	s	3	1	326	AM748604
H2	s, a	8	6	320	AM748605
H3	s	1	1	317	AM748606
H4	s	1	1	325	AM748607
H4a	s	1	1	325	AM748608
H5	s	1	1	319	AM748609
H5b	s	1	1	322	AM748610
H5a	s	1	1	319	AM748611

s, *Sorites*; a, *Amphisorus*; m, *Marginopora*

[a] Types detected only as a secondary or tertiary band in co-occuring DGGE patterns (intragenomic variants)

Table 3 Diversity of *Symbiodinium* ITS 2 types retrieved from previous studies (Pawlowski et al. 2001; Pochon et al. 2001, 2004, 2006)

Type name	Soritid host	Sampling locality	Number of sequences	GenBank sequence accession numbers
C1	s, a	liz	3	AJ621537–AJ621539
C3	m	liz	4	AJ621533–AJ621536
C19	m	liz	2	AJ291515
C15	s, m	liz	3	AJ291516; AJ621540–AJ621541
C90	s	pp	12	AJ620934–AJ620945
C92	a	e	1	AJ291514
D1.2	HK[a]	p	1	AM748617
F2b	s	e	6	AJ291521–524; AJ830912; AJ830914
F2	s	s	1	AJ830908
F2a	s	s	1	AJ830911
F3.4b[b]	s	r	1	AM748616
F4.1b	s	f	2	AJ621146–AJ621147
F4.8c	s	pc, f	12	AJ621135–145; AJ291527
F5.1[b]	a	liz	1	AM748615
F5.1b	a	m	1	AJ291535
F5.2	a	e, r	2	AJ291532; AJ308898
F5.2a[b]	m	r	1	AM748614
F5.2b[b]	s	liz	1	AM748613
F5.2c	m	r	4	AJ872077
F5.2d	a	e	2	AJ291530–AJ291531
F5.2e	a	e	1	AJ291533
F5.2f	s	e, s	2	AJ291512; AJ291534
G1	m	g	1	AJ291538
G2	m	g	1	AJ291539
H1	s	f, liz	6	AJ291513; AJ621152–155; AJ621157
H1a[b]	m	liz	1	AM748612
H5	s	pc, pp, f	8	AJ621129–134; AJ621148–149
H5a	s	f	3	AJ621150–151; AJ621156
H6	s	g	1	AJ291520

s, *Sorites*; a, *Amphisorus*; m, *Marginopora*; e, Elat, Israel; f, Florida Keys; g, Guam; m, Maldives; liz, Lizard Island, Eastern Australia; p, Palau; pc, Bocas del toro, Panama Caribbean; pp, Isla Taboga, Panama Pacific; r, Reunion Island; s, Safaga, Egypt

[a] *Symbiodinium* ITS-2 type obtained from the sponge *Haliclona koremella* (Carlos et al. 1999)

[b] Type detected in our unpublished data base

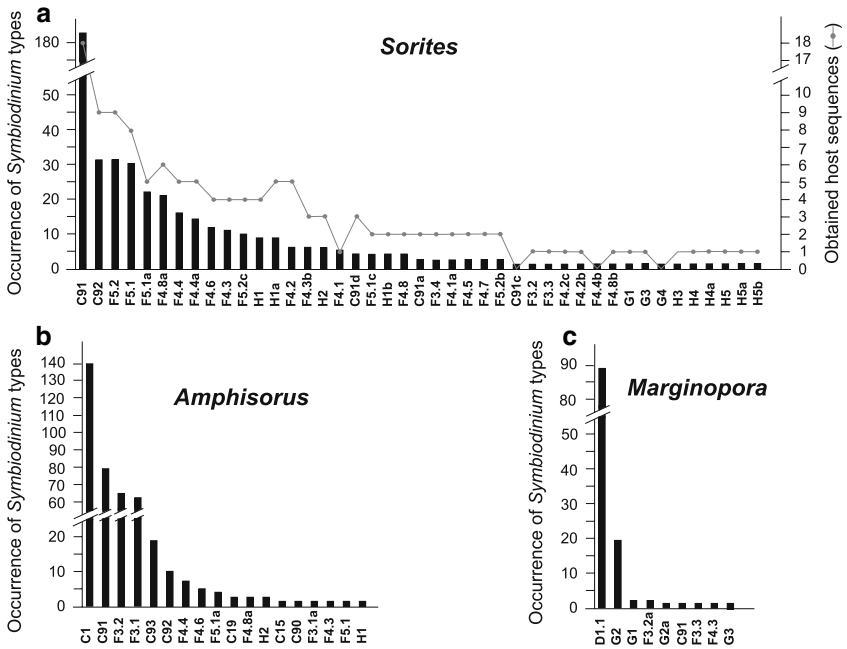

Fig. 4 Histograms showing the prevalence of each *Symbiodinium* type (brightest diagnostic bands) observed during the survey in (**a**) the soritid host genus *Sorites*, (**b**) genus *Amphisorus*, and (**c**) genus *Marginopora*. The *grey scatter* in Fig. 2a corresponds to the number of SSU rDNA sequences obtained from soritid specimens in the genus *Sorites*, approximately following the respective abundance of each *Symbiodinium* types identified in these hosts (see also Table 4)

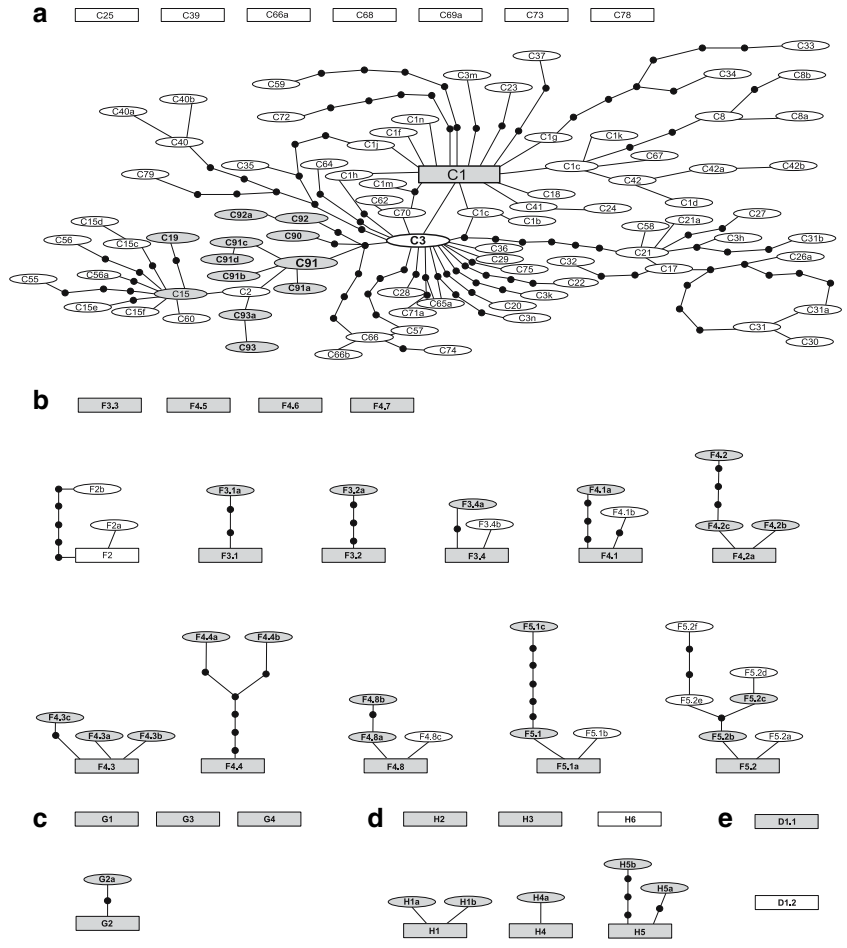

Fig. 5 **a**–**e** ITS-2 rDNA type networks from *Symbiodinium* dinoflagellates in clades C, F, G, H, and D1, respectively. The root for each network (estimated by the algorithm) is represented as a rectangle. Each *line* in the networks represents a single base pair change. The *black dots* between some *lines* represent hypothetical intermediate symbionts. *Loops* indicate a lack of statistical power to discern between two possible connections in the network, and might suggest potential sexual recombination events. The *grey* types highlight the foraminiferal symbiont types detected during the Guam 1-year survey. The *colorless* types in clade C represent the diversity of types previously identified in numerous Indo-Pacific metazoan hosts (LaJeunesse 2005), while those in clades F and H represent additional type diversity collected in soritid foraminiferans from previous studies (see Table 3)

are shown in the pie charts of Fig. 6. In each pie chart, only the types present in >5% of the total number of samples are represented. Highly specific host–symbiont association patterns were evident in the genera *Amphisorus* and *Marginopora*. The species *A. kudakajimaensis* (Amp I) was specifically associated with *Symbiodinium* types in clade C

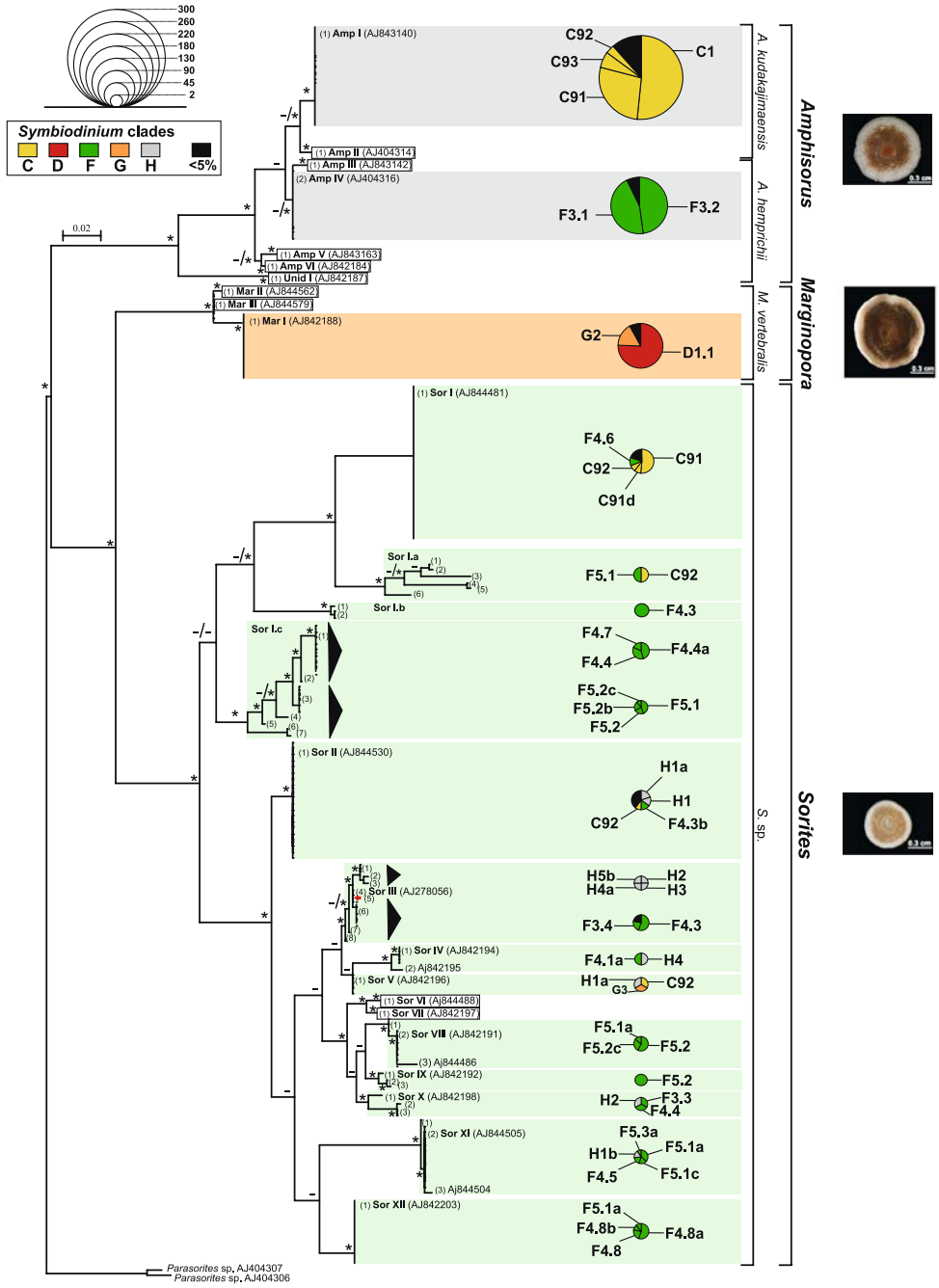

Fig. 6 Maximum likelihood phylogenetic reconstruction of Soritinae inferred from 193 partial small subunit rDNA sequences, including 169 sequences obtained during this survey and 24 previously published sequences used as references (see phylotypes names followed by the GenBank accession numbers) for the delimitations of the 22 soritid phylotypes described in Garcia-Cuetos et al. (2006). *Symbols* at nodes correspond to bootstrap values in ML and posterior probabilities in MrBayes analyses, respectively, with a *dash* (-) and an *asterisk* (*) representing values lower and higher than 70% (ML) and 0.8 (MrBayes) of support. *Numbers* in brackets in each soritid phylotype correspond to the specific SSU types detailed in Table 4. *Left* pie charts represent the proportions of the *Symbiodinium* types (brightest diagnostic bands) detected in each soritid phylotypes, with the diameters of the pie charts proportional to the number of soritid hosts analysed (see *circular inset scale*). The *colours* in the *left* pie charts correspond to the membership of each types to their respective *Symbiodinium* clade (see legend), the *black colour* corresponding to the types detected in less than five percent of the total number of samples analysed in each soritid phylotype

(C1, C91, C92 and C93), while *A. hemprichii* (Amp IV) associated specifically with types F3.2 and F3.1. The Fischer exact test applied to the 263 (Amp I) and 136 (Amp IV) *Symbiodinium* types was highly significant ($P < 2.2\ e^{-16}$), rejecting unambiguously the independence of these associations. In *M. vertebralis*, the soritid phylotype Mar I was the unique host for the *Symbiodinium* types D1.1 and G2. Finally, in the highly divergent genus *Sorites*,

Table 4 List of soritid phylotypes and corresponding SSU rDNA type(s) shown in Fig. 6, the number of newly obtained sequences in each soritid phylotype, the sampling locality, and sequence GenBank accession numbers are indicated

Soritid species	Soritid phylotypes	Soritid type[a]	Sequences[b]	Locality	GenBank[c]
A. kudakajimaensis	Amp I	(1)	14	g	AJ843140
A. kudakajimaensis	Amp II	(1)	–	j	AJ404314
A. hemprichii	Amp III	(1)	–	e	AJ843142
A. hemprichii	AmpIV	(2)	12	g	AJ404316
A. hemprichii	Amp V	(1)	–	liz	AJ843163
A. hemprichii	Amp VI	(1)	–	p	AJ842184
A. hemprichii	Unid I	(1)	–	e	AJ842187
M. vertebralis	Mar I	(1)	12	g	AJ842188
M. vertebralis	Mar II	(1)	–	liz	AJ844562
M. vertebralis	Mar III	(1)	–	liz	AJ844579
Sorites sp.	Sor I	(1)	34	g	AJ844481
Sorites sp.	Sor I.a	(1)	1	g	AM748618
		(2)	1	g	AM748619
		(3)	1	g	AM748620
		(4)	1	g	AM748621
		(5)	1	g	AM748622
		(6)	1	g	AM748623
Sorites sp.	Sor I.b	(1)	1	g	AM748624
		(2)	2	g	AM748625
Sorites sp.	Sor I.c.	(1)	10	g	AM748626
		(2)	1	g	AM748627
		(3)	5	g	AM748628
		(4)	1	g	AM748629
		(5)	1	g	AM748630
		(6)	1	g	AM748631
		(7)	1	g	AM748632
Sorites sp.	Sor II	(1)	20	g	AJ844530
Sorites sp.	Sor III	(1)	2	g	AM748633
		(2)	1	g	AM748634
		(3)	1	g	AM748635
		(4)	–	f	AJ278056
		(5)	1	g	AM748636
		(6)	5	g	AM748637
		(7)	1	g	AM748638
		(8)	1	g	AM748639
Sorites sp.	Sor IV	(1)	–	f	AJ842194
		(2)	–	f	AJ842195
Sorites sp.	Sor V	(1)	3	g	AJ842196
Sorites sp.	Sor VI	(1)	–	s	AJ844488
Sorites sp.	Sor VII	(1)	–	e	AJ842197
Sorites sp.	Sor VIII	(1)	2	g	AM748640
		(2)	5	g, m, liz	AJ842191
Sorites sp.	Sor IX	(1)	–	s, e	AJ842192
		(2)	1	g	AM748641
		(3)	1	g	AM748642
Sorites sp.	Sor X	(1)	–	f, r	AJ842198
		(2)	1	g	AM748643
		(3)	2	g	AM748644
Sorites sp.	Sor XI	(1)	1	g	AM748645
		(2)	10	g, liz	AJ844505
		(3)	–	liz	AJ844504
Sorites sp.	Sor XII	(1)	10	g, pc	AJ844517

e Elat, Israel; *f* Florida Keys; *g* Guam; *j* Okinawa, Japan; *m* Maldives; *liz* Lizard Island, Eastern Australia; *p* Perth, Western Australia; *pc* Bocas del Toro, Panama Caribbean; *pp* Isla Taboga, Panama Pacific; *r* Reunion Island; *s* Safaga, Egypt

[a] Soritid types identified in each soritid phylotypes shown in Fig. 4

[b] Number of sequences obtained in Guam during this study

[c] GenBank sequence accession numbers

the patterns of host-symbiont specificity were less evident (see "Discussion"). However, overall host–symbiont specificity in the genus *Sorites* was significant (Table 5). Total inertia tests on nine datasets combining three different classification levels (type, TCS networks, and phylotypes or clades) for *Sorites* spp. and their symbionts, showed that significant host–symbiont structure exists in all comparisons, except for the "*Sorites* type" versus "*Symbiodinium* clade" comparison ($P > 0.05$). It is particularly striking that the *Symbiodinium* type C91, commonly detected in *A. kudakajimaensis*, was only found in a single phylotype of *Sorites* (Sor I).

Discussion

By identifying 61 symbiont types in only three soritid host genera, this study revealed the most diverse *Symbiodinium* assemblage sampled to date from a single reef. Compared to previous studies (and unpublished data) from several locations in the Red Sea, Indo-Pacific and Caribbean, Guam is the only locality where seven out of the eight currently described *Symbiodinium* lineages have been documented (Pochon et al. 2001; Pochon and Pawlowski 2006). Moreover, none of several surveys of *Symbiodinium* ITS-2 types performed over entire reef invertebrate communities worldwide have reported as many types as the present study. For example, surveys of 77 different species of hosts (in 40 genera) from two Caribbean reef systems revealed the existence of 28 different *Symbiodinium* types in clades A–D (LaJeunesse 2002). Based on this finding, and unpublished data, it was estimated that as many as 40 distinctive types populate invertebrate hosts in the Caribbean. In the Pacific Ocean, the investigations of 40 and 74 host genera in southern and central Great Barrier Reef (GBR), uncovered 23 and 32 *Symbiodinium* types, respectively (LaJeunesse et al. 2003, 2004b). Finally, 20 genetically distinct symbiont types were detected in the host communities from both Hawaii (18 host genera) and Okinawa (31 host genera) (LaJeunesse et al. 2004a, b).

The soritid–*Symbiodinium* diversity described here represents up to three times the diversity of symbiont types previously reported in surveys of reef invertebrates. Such high diversity might be a consequence of the following three factors: first, the collection of >1,000 soritid hosts over a 40 m depth gradient on one reef over the course of an entire year, represents the most targeted and exhaustive sampling effort ever undertaken for any group of *Symbiodinium*-bearing hosts. Second, asexual reproduction in soritid foraminifera, i.e., the process of host division by multiple fission during which symbionts are vertically transmitted from mother cells to offspring, might be a dominant feature in these organisms and is held responsible for the high diversity and specificity observed in soritid–*Symbiodinium* associations (Garcia-Cuetos et al. 2006). Third, Micronesian waters might contain one of the most biologically diverse *Symbiodinium* communities on Earth (Pochon 2006), in agreement with some marine biodiversity surveys of the Marianas Islands showing that Guam has the greatest record of marine diversity (protists and metazoans) for any area of comparable size (Pauley 2003). Our general knowledge of *Symbiodinium* populations, such as the recent finding of high diversity and host specificity of symbiont types from Hawaiian invertebrate host communities (LaJeunesse et al. 2004a), would benefit from additional fine-scale surveys of *Symbiodinium* types in metazoan host communities from Guam.

To better understand the factors underlying the unusual soritid symbiont diversity in Guam, some aspects of *Symbiodinium* diversity merit further discussion. These include: (1) the accuracy of ITS-2 as a marker of *Symbiodinium* species diversity; (2) the specificity of the relationship between foraminifera and their hosts, and (3) intraspecific symbiont diversity in soritid foraminifera. Detailed analyses of spatio-temporal patterns of symbiont distribution in relation to the life cycle of the soritid hosts surveyed in this study are in progress.

Taxonomic meaning of ITS-2 types

Our general knowledge of the diversity of *Symbiodinium* types is based on hundreds of ITS rDNA genotypes described recently (Rodriguez-Lanetty 2003; LaJeunesse 2005; van Oppen et al. 2005a, b). However, an important, yet unresolved, issue is what constitutes a species in

Table 5 Tests of total inertia (COA) between soritids in the genus *Sorites* and their *Symbiodinium* dinoflagellates at the levels of types, TCS networks, and lineages

	Symbiodinium types ($N = 43$)	*Symbiodinium* TCS networks ($N = 21$)	*Symbiodinium* clade ($N = 4$)
Sorites types ($N = 39$)	$P = 0.0014$	$P = 0.00015$	$P = 0.1390$*
Sorites TCS networks ($N = 20$)	$P = 0.0000$	$P = 0.00000$	$P = 0.0121$
Sorites phylotype ($N = 13$)	$P = 0.0000$	$P = 0.00000$	$P = 0.0000$

* $P > 0.05$

Symbiodinium, and how accurately ITS-2 sequence divergence equates to functional diversity in these organisms. The findings of LaJeunesse (2001), that *Symbiodinium* species with formal descriptions are strongly supported by differences in ITS rDNA sequences, created the paradigm that ITS signatures or types approximate the species level in this genus. While there is now ample evidence that each *Symbiodinium* clade is composed of a diverse group of organisms or types exhibiting distinctive host taxonomic, geographic, and/or environmental distribution (LaJeunesse 2002, 2005; Ulstrup and van Oppen 2003; LaJeunesse et al. 2004a, b; Pochon et al. 2004; Rodriguez-Lanetty et al. 2004), considering each of the hundreds of described ITS-2 types as individual species is questionable at this time. Because intragenomic variation at rDNA loci is very common in the ITS regions (van Herwerden et al. 1999; Harris and Crandall 2000; Santos et al. 2003; Wörheide et al. 2004), treating the many types with single base pair differences as different species is likely to overestimate actual species diversity. Sequence analysis of cloned ITS PCR products of *Symbiodinium* reveals many distinct sequence types (van Oppen et al. 2005a; Reimer et al. 2006), most of which are intragenomic variants occurring at relatively low copy number (van Oppen and Gates 2006). Similarly, 12 *Symbiodinium* types identified in the present study are likely to represent intragenomic variants rather than distinct symbiont types (see Table 2). For example, type C91d was never observed as a single DGGE fingerprint, but co-occurred frequently as a minor band with type C91 (Fig. 2f, line 2). Other probable variants, such as for instance F3.4a, F4.3a, and F4.2a where detected only once in association with one to two genetically closely related types, respectively (Fig. 2f, lines 5–7).

Furthermore, visual interpretation of ITS-2 DGGE gel patterns can be sometimes misleading due to identical melting point temperature of different ITS-2 fragments (van Oppen and Gates 2006; Apprill and Gates 2006). In the present study, special emphasis was placed on the verification of DGGE signatures by sequencing 277 uncertain gel patterns. As many as 17 of these patterns (6%) were misidentified, reflecting the apparent limitation of the DGGE methodology to resolve migration differences between a number of *Symbiodinium* types. For example, types C91 and C15 were visually indistinguishable (Fig. 2a, lanes 3 and 5). However, C15 was detected only once in a total of 54 sequences of control, suggesting that this type is either the result of sequencing errors, or present at a very low frequency in soritid populations. Other DGGE patterns, such as those produced by types F4.1a, F4.4, and F4.4b (Fig. 2c, lanes 2, 6, and 8) and types F4.4a, F4.8a, and F4.8b (Fig. 2c, lanes 7, 13, and 14), were extremely similar and needed to be interpreted with caution. Misinterpretation of DGGE profiles is likely to increase when comparing *Symbiodinium* types from different sampling localities. For example, *Symbiodinium* types F2 and F2a from the Red Sea (Fig. 5b; Table 3) produced identical banding patterns to the types C93 and F5.1 from Guam, respectively (data not shown). Consequently, reference DGGE profiles obtained from one locality may be misleading when applied to interpreting profiles from other localities. This indicates that DGGE-based *Symbiodinium* identification should always be verified by routine sequencing and/or cloning, and that reference profiles should be made from samples obtained from the same location as the samples being analyzed.

Soritid diversity and specificity

The present study reinforces several previous hypotheses concerning diversity and specificity in soritid–*Symbiodinium* relationships. It is quite remarkable that soritid diversity on a single reef should be so high, with up to 16 phylotypes in three genera. The most striking soritid diversity was found in the genus *Sorites*, represented in Guam by 13 phylotypes. This included almost all described phylotypes, except for Sor VI and Sor VII which seem to be endemic to the Red Sea (Garcia-Cuetos et al. 2006). The same pattern of diversity is apparent in the *Symbiodinium* diversity found in the three soritid genera, with 43, 18, and 9 symbiont types detected in the genera *Sorites*, *Amphisorus*, and *Marginopora*, respectively. This strongly suggests that the biodiversity of both soritid hosts and symbionts are positively associated. Together, these observations support the idea that soritid foraminifera, at one time or another, must have been flexible enough to accept unrelated *Symbiodinium* from neighboring metazoan hosts (Lee 2001). At the same time, however, *Symbiodinium* assemblages in soritids appear to have evolved in such a way that several highly specific host–symbiont relationships became evolutionarily stable, limiting further exchange between soritids and metazoan hosts in contemporary coral reef ecosystems. This process could be compared to allopatric speciation with the host considered as the environment where reproductive isolation and evolution takes place.

Different degrees of host–symbiont specificity are also clearly evident in soritid species. Of a total of 73 ITS-2 *Symbiodinium* types detected to date in soritid foraminiferans worldwide (Fig. 5), only three types (C1, C15, and C19) have been found in other metazoans. In Guam, these three types were found exclusively in the soritid species *A. kudakajimaensis* (Fig. 6). In fact, *A. kudakajimaensis* appears to favor a number of generalist symbiont types, as shown by the prevalence of pandemic host-generalist type C1 in this host species, as well as a high number of specimens containing C91, C92, F4.4, F4.6, and F5.1a, which are among the most common *Symbiodinium* types in the

genus *Sorites*. However, the overall specificity between *Symbiodinium* types and soritid species was statistically supported (see "Results"). *A. kudakajimaensis* and *A. hemprichii* were extremely well discriminated, and seem to have preferentially adapted towards C and F3 *Symbiodinium* types, respectively. On the other hand, the shallow-water dwelling *M. vertebralis* associated specifically with types D1.1 and G2, both belonging to *Symbiodinium* lineages that show high stress tolerance and/or opportunistic abilities (Baker et al. 2004; Rowan 2004; van Oppen et al. 2005a, b).

In the highly divergent genus *Sorites*, patterns of host–symbiont specificity were much more diffuse (Fig. 6) than in *Amphisorus* and *Marginopora*. For example, the soritid phylotype Sor II appeared to be the most flexible, possessing up to eight unrelated symbiont types, six of which fall under the 5% limit of the pie chart (data not shown). Similarly, Sor III and Sor XI were found in association with six and four unrelated *Symbiodinium* types, respectively. In contrast, some *Symbiodinium* types, such as C92 and F5.1a, were detected in four and three different soritid phylotypes, respectively. However, total inertia tests were statistically significant in most comparisons (Table 5), suggesting that the majority of symbiont types identified in the genus *Sorites* were not randomly distributed between phylotypes, and supporting the existence of a diverse but structured population of holobionts. It is highly probable that the use of SSU rDNA sequences are inappropriate for deciphering the true diversity of soritid lineages, especially in the genus *Sorites*, and that the use of a more variable molecule will uncover even greater host–symbiont structure and specificity.

Intraspecific symbiont diversity in soritid foraminifera

An increasing number of studies have reported the presence of multiple *Symbiodinium* genotypes co-occurring within the same host species (reviewed in Baker 2003; Coffroth and Santos 2005). This intraspecific diversity can involve symbionts belonging to different *Symbiodinium* clades (Rowan and Knowlton 1995; LaJeunesse and Trench 2000) or different types of symbionts belonging to the same clade (van Oppen et al. 2001; Diekmann et al. 2003; Coffroth and Santos 2005). Even though the effects of this coexistence are not yet well understood, it has been suggested that this diversity provides considerable physiological flexibility for the host in question, allowing it to respond to changes in environmental conditions (Rowan et al. 1997; Baker 2003). An alternative view considers multiple symbiotic partnerships within single coral hosts to be exceptions rather than the norm (Goulet 2006), and this debate continues to be a subject of considerable controversy (Baker and Romanski 2007; Goulet 2007).

In this study, a number of *Sorites* (10%) and *Amphisorus* (15%) specimens were recorded with co-occurring genotypes, regardless of the sampling time or depth (Table 2). While some mixed genotypes corresponded to symbiont types belonging to the same *Symbiodinium* clade, other cases revealed the simultaneous presence of two or three different clades. Such patterns of symbiont distribution are usually interpreted as representing a community of different *Symbiodinium* strains.

Given that *Symbiodinium* types occurring at less than 5–10% of the total symbiont population will not be detected by DGGE (Thornhill et al. 2006), it may also be possible that considerable cryptic symbiont diversity exists at undetectable levels (Baker 2003; Mieog et al. 2007). Interestingly, the majority of mixed genotypes observed during this survey were usually harbored by the smallest hosts, i.e., those with a diameter of 0.1–2 mm (Table 2), suggesting that: (1) juvenile foraminifera may be better able to switch or shuffle heterogeneous symbiont communities than adults; and/or (2) that the early ontogeny of these forams is characterized by symbiont diversity, which is reduced as the juveniles grow and their symbiont communities become "optimized" for the prevailing environmental conditions (Coffroth et al. 2006; Little et al. 2004).

These observations, together with the remarkable host–symbiont community structure reported here, suggest that a considerable range of flexibility/specificity exists within and between the soritid lineages. The subtle balance between specificity and flexibility observed in soritid–*Symbiodinium* associations may be a key element in the continued evolutionary success of these protists in coral reef ecosystems worldwide, and emphasizes the unusual symbiotic complexity of these organisms.

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