16S rRNA phylogeny of sponge-associated cyanobacteria

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ABSTRACT

Phylogenetic analyses of 16S rDNA sequences of sponge-associated cyanobacteria showed them to be polyphyletic, implying that they derived from multiple independent symbiotic events. Most of the symbiont sequences were affiliated to a group of *Synechococcus* and *Prochlorococcus* species. However, other symbionts were related to different groups, such as the Oscillatoriales.

Although both cyanobacteria and sponges have a very long evolutionary history (1,3), little is known about the identity and phylogeny of cyanobacterial sponge symbionts. For marine sponges symbiosis with cyanobacteria can be obligate or non-obligate according to the sponge species (2). Thus far, all attempts of culturing sponge-associated cyanobacteria have failed (unpublished data, 13). It is thus not known whether sponge-associated cyanobacteria are able to survive outside their host. The most common sponge-associated cyanobacterium was not found in water samples (13, 15). In *Chondrilla australiensis* this cyanobacterium is transmitted vertically (through sponge eggs) (14). Additionally, a study showing co-speciation between *Dysidea* species and their associated cyanobacteria (12) supports the hypothesis that sponges and associated cyanobacteria are coevolving. Few studies sequenced the 16S rRNA gene of sponge-associated cyanobacteria: Diaz (5) identified the first two cyanobacteria from marine sponges, Webb and Maas (16) found that the cyanobacteria inhabiting *Mycale hentscheli* were phylogenetically related to *Cyanobacterium stanieri* and species of *Synechocystis* and *Prochloron*, and Hentschel *et al.* (7) found that 7 sequences from cyanobacteria inhabiting the sponges *Aplysina aerophoba* and *Theonella swinhoei* could be divided into two clades (*Synechococcus/Prochlorococcus* and *Pleurocapsa*). The aim of the present study was to increase our understanding on the diversity of sponge-associated cyanobacteria, and to determine their phylogenetic position.

Sponge samples from 16 species were collected from four locations: The Caribbean (Bahamas, 26º33'N, 77º52'W), Mediterranean (Rapallo, Italy, 44º18'N, 9º12'E), Red Sea (Elat, 31º35'N, 34º54'E), and Western Indian Ocean (Zanzibar, 06º09'S, 39º11'E). Aposymbiotic specimens (specimens that do not contain cyanobacterial symbionts), growing in dark caves or overhangs, were also collected

for two sponge species (*Petrosia ficiformis* and *Xestospongia muta*). Those samples, collected at short distances from symbiotic specimens, were used as negative controls, to ensure that 16S rDNA sequences were derived from true symbionts and not from surface associated cyanobacteria or digested cyanobacteria. Photosynthetic activity inside the living sponge tissue was tested by pulse amplitude modulated fluorometry (Diving PAM, Walz, Germany). Tissue samples (1 cm^3) were rinsed twice in 100% EtOH, and kept in 100% EtOH at 4 ºC. DNA was extracted following the procedure of Bernatzky and Tanksley (4). 16S rDNA was amplified with the primers 361F (5'-GAATTTTCCGCAATGGGC -3') and 1459R (5'- GGTAAYGACTTCGGGCRT -3') (5). 1060-bp fragments were cloned in the PTZ57R/T vector (Fermentas). Twenty clones per individual were amplified using M13 universal primers. The PCR products were digested with restriction enzymes *Apa*I and *Hae*III. One clone was sequenced for each pattern present in more than 10% of the clones. The sequences were deposited in Genbank (accession numbers AY701287-AY701315).

Neither photosynthetic activity, nor amplification of the 16S rRNA gene was obtained for aposymbiotic specimens. On the opposite, photosynthetic activity was recorded for all the other samples. Up to three different cyanobacterial clones were sequenced per individual sponge investigated (with a sequence homology of 90-99.7%). Similarly, Webb and Maas (16) showed four closely related (99.1-99.8%) cyanobacterial clones in the sponge *Mycale hentscheli*. Different cyanobacterial types from the same individual were usually phylogenetically close to each other (<1-2% sequence divergence), but in one case (*Lendenfeldia dendyi*) the symbionts were very divergent: two types were in group 5, while the third type was in group 2, with 10% sequence divergence from the former two (Fig. 1). Closely related cyanobacterial

types should not be the result of Taq-polymerase errors or cloning-bias since only patterns present in more than one clone were sequenced. However, the true cyanobacterial diversity in sponges might be underestimated. Nevertheless our results are in agreement with other studies (13, 16). For example, Usher et al. (13) also identified only one cyanobacterial type in the sponge *Petrosia ficiformis*.

A maximum likelihood (ML, (6)) analysis was done based on 147 taxa and 1396 nucleotides. The sequences were chosen to include: 1- representatives of the cyanobacteria diversity; 2- all sponge-associated cyanobacteria sequences overlapping with the new 16S rDNA sequences; and 3- representatives of the *Synechococcus* and *Prochlorococcus* diversity because sponge symbionts have been suggested to belong to these genera. BLAST searches were also conducted for each sponge sequence in order to include in the analysis the most similar sequences available in the databanks. The GTR model with a gamma distribution (4 categories), and a proportion of invariant site was found to be the most appropriate using Modeltest version 3.06 (9)). The ML tree was reconstructed in an iterative way using PAUP* version 4.0b10 (11). First a heuristic search was conducted using the ML parameters identified by Modeltest; Modeltest topology as starting tree and NNI branch swapping. The topology found at the end of the search was used to identify new parameters. These new parameters and topology were used to conduct a new search till convergence. The best ML parameters found were then used to compute 500 bootstrap replicates starting with a NJ tree and setting the maximum number of tree to one to reduce computation time.

The phylogenetic tree obtained in this study was in agreement with the results of other studies that divided cyanobacteria into seven or eight major lineages (8, 10), except that group 2 (8) was here paraphyletic (Fig. 1). Sponge-associated

cyanobacteria were found to be polyphyletic. They were divided into thirteen lineages spread among various groups of cyanobacteria (Fig. 1 and 2). The major clade of sponge-associated cyanobacteria (37 strains from 18 different sponge species collected from a wide range of geographic regions: Australia, Caribbean, French and Italian Mediterranean coast, Red Sea, Zanzibar) was strongly affiliated with group 6 (*Prochlorococcus*, *Synechococcus* and *Microcystis*, (8)). Three additional sponge-associated cyanobacteria (from *Chondrilla* spp. and *Haliclona* sp.) were also part of group 6, but were closer to free-living *Synechococcus* than to the other sponge symbionts (Fig. 2). A second group of sponge-associated cyanobacteria (consisting of four sequences from *Dysidea herbacea*, *Dysidea granulosa*, *Lendenfeldia dendyi* and *Aplysina gerardogreeni*, originating from Guam, Mexican Pacific and Zanzibar) were affiliated to group 2 (morphologically classified in the order Oscillatoriales). A few other sponge-associated cyanobacteria were affiliated with group 5 (Chroococcales, Oscillatoriales, Pleurocapsales and Prochlorales, (8)), but they did not form a monophyletic group (Fig. 1). One additional cyanobacterium (AF295635) from an Australian sponge was found affiliated to the marine group 7 (8). Finally, a sequence of a cyanobacterium associated with the sponge *Pseudoaxinella flava* appeared unrelated to any major group of cyanobacteria (Fig. 1). The polyphyletic origin of sponge-associated cyanobacteria indicates that they derived from multiple independent symbiotic events, involving several different cyanobacterial types and/or host sponges.

The phylogenetic relationships between the cyanobacterial sequences of group 6 suggest only a partial specificity of cyanobacteria to the host sponges. Two specimens of *Petrosia ficiformis*, one collected in Italy (this study), and another in France (13), were found to be 99% identical, and closely related (BP = 100%). Three

cyanobacterial types from two Bahamian *Xestospongia muta* specimens were strongly affiliated to each other and with that from the congeneric species *Xestospongia proxima* (BP= 100%). Cyanobacteria from two congeneric *Theonella* species (*swinhoei* and *conica*) were affiliated to each other (BP=77%). Finally, six cyanobacteria associated with *Chondrilla australiensis* (13, 15) were closely affiliated (BP = 49%). These results may also support the idea of co-evolution between sponges and their symbionts. However, cyanobacteria associated to *Theonella swinhoei* from Japan and the Red Sea clustered in different groups (Fig. 1 and 2). In this large-scale phylogenetic analysis, sponge-associated cyanobacteria clades deriving from distant geographic location (as the clades in group 2 and 6) are most probably true-symbionts, while the symbiotic nature of sponge cyanobacteria that are closely related to free living species (*e.g*. those in *Pseudoaxinella flava*) remains uncertain.

In the future it will be interesting to examine whether cyanobacterial sponge symbionts from different lineages perform diverse functions in this symbiosis. In addition, reconstruction of sponge molecular phylogeny will enable to test for co-evolution of cyanobacteria and their host sponges.

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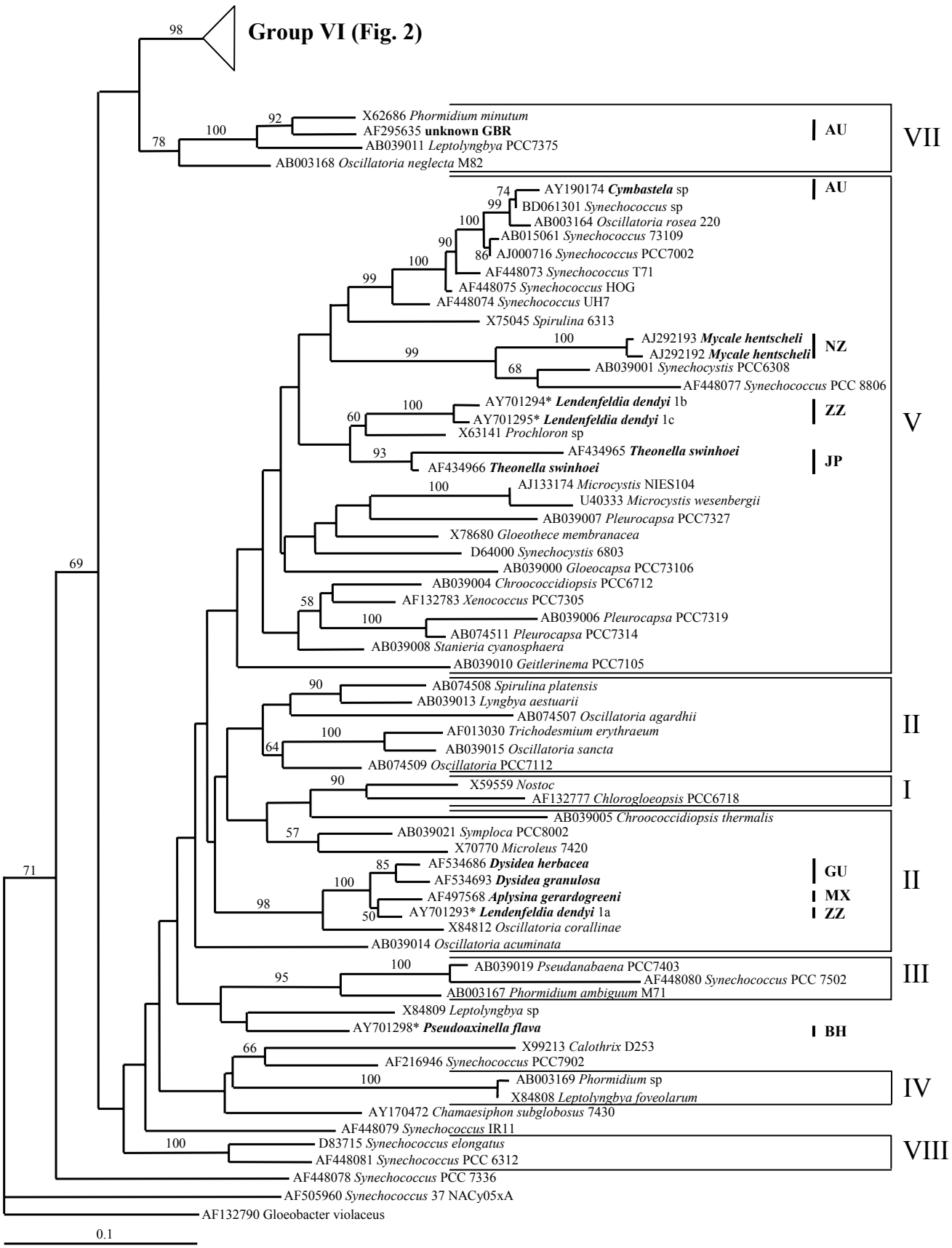
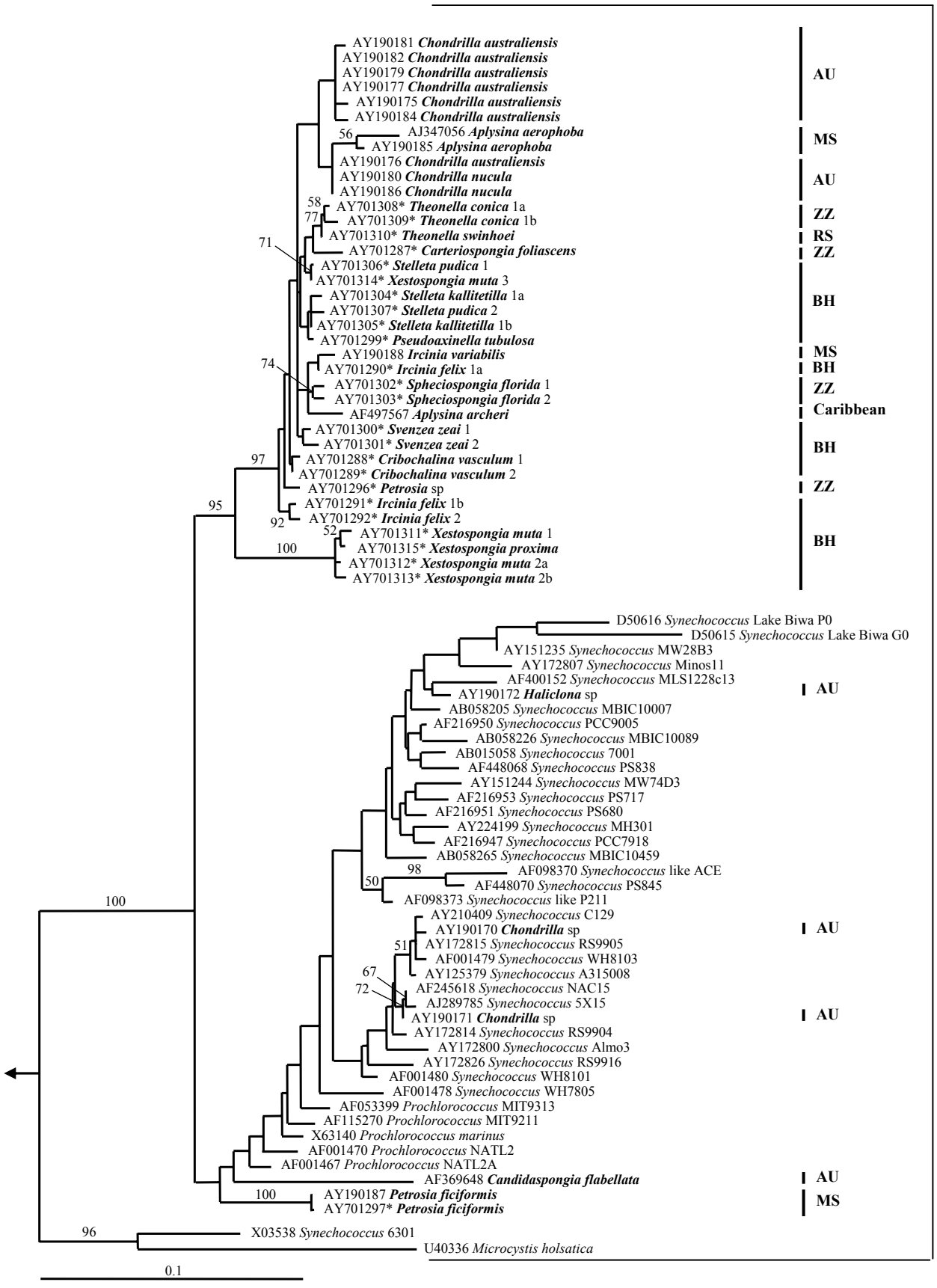
FIGURE LEGENDS

FIG. 1: Maximum Likelihood tree of sponge-associated cyanobacteria based on 16S rRNA. Bootstrap values above 50% are indicated. Sponge-associated cyanobacteria (which were named following their host species) are indicated in bold. The group numbers follow Honda *et al.* (8) and Robertson *et al.* (10). The triangle represents group 6 detailed in Figure 2. The individual identification number is given at the end of the sequence; different letters represent different clones obtained for the same sponge individual. Sequences from this work are indicated by a star after the accession number. The sampling localities of sponge-associated cyanobacteria are indicated by bars. AU-Australia, BH-Bahamas, GU-Guam, JP-Japan, MX-Mexican Pacific, NZ-New Zealand, ZZ-Zanzibar.

FIG. 2: "Group 6" detailed 16S rRNA-based Maximum Likelihood tree of sponge-associated cyanobacteria. Arrow, to outgroup (cf. Fig. 1). Bootstrap values above 50% are indicated. Sponge-associated cyanobacteria (which were named following their host species) are indicated in bold. The individual identification number is given at the end of the sequence; different letters represent different clones obtained for the same sponge individual. Sequences from this work are indicated by a star after the accession number. The sampling localities of sponge-associated cyanobacteria are indicated by bars. AU-Australia, BH-Bahamas, MS-Mediterranean Sea, RS-Red Sea, ZZ-Zanzibar.


Group VI (Fig. 2)
X62686 Phormidium minutum
AF295635 unknown GBR
AB039011 Leptolyngbya PCC7375
AB003168 Oscillatoria neglecta M82
AU
VII
AY190174 Cymbastela sp
BD061301 Synechococcus sp
AB003164 Oscillatoria rosea 220
AB015061 Synechococcus 73109
AJ000716 Synechococcus PCC7002
AF448073 Synechococcus T71
AF448075 Synechococcus HOG
AF448074 Synechococcus UH7
X75045 Spirulina 6313
AU
AJ292193 Mycale hentscheli
AJ292192 Mycale hentscheli
NZ
AB039001 Synechocystis PCC6308
AF448077 Synechococcus PCC 8806
AY701294* Lendenfeldia dendyi 1b
AY701295* Lendenfeldia dendyi 1c
ZZ
X63141 Prochloron sp
AF434965 Theonella swinhoei
AF434966 Theonella swinhoei
JP
V
AJ133174 Microcystis NIES104
U40333 Microcystis wesenbergii
AB039007 Pleurocapsa PCC7327
X78680 Gloeothece membranacea
D64000 Synechocystis 6803
AB039000 Gloeocapsa PCC73106
AB039004 Chroococcidiopsis PCC6712
AF132783 Xenococcus PCC7305
AB039006 Pleurocapsa PCC7319
AB074511 Pleurocapsa PCC7314
AB039008 Stanieria cyanosphaera
AB039010 Geitlerinema PCC7105
AB074508 Spirulina platensis
AB039013 Lyngbya aestuarii
AB074507 Oscillatoria agardhii
AF013030 Trichodesmium erythraeum
AB039015 Oscillatoria sancta
AB074509 Oscillatoria PCC7112
II
X59559 Nostoc
AF132777 Chlorogloeopsis PCC6718
I
AB039005 Chroococcidiopsis thermalis
AB039021 Symploca PCC8002
X70770 Microleus 7420
AF534686 Dysidea herbacea
AF534693 Dysidea granulosa
GU
AF497568 Aplysina gerardogreeni
MX
AY701293* Lendenfeldia dendyi 1a
ZZ
X84812 Oscillatoria corallinae
AB039014 Oscillatoria acuminata
II
AB039019 Pseudanabaena PCC7403
AF448080 Synechococcus PCC 7502
AB003167 Phormidium ambiguum M71
III
X84809 Leptolyngbya sp
AY701298* Pseudoaxinella flava
BH
X99213 Calothrix D253
AF216946 Synechococcus PCC7902
AB003169 Phormidium sp
X84808 Leptolyngbya foveolarum
IV
AY170472 Chamaesiphon subglobosus 7430
AF448079 Synechococcus IR11
D83715 Synechococcus elongatus
AF448081 Synechococcus PCC 6312
VIII
AF448078 Synechococcus PCC 7336
AF505960 Synechococcus 37 NACy05xA
AF132790 Gloeobacter violaceus
0.1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AY190175 Chondrilla australiensis
AY190184 Chondrilla australiensis
AU
56
AJ347056 Aplysina aerophoba
AY190185 Aplysina aerophoba
MS
AY190176 Chondrilla australiensis
AY190180 Chondrilla nucula
AY190186 Chondrilla nucula
AU
58
AY701308* Theonella conica 1a
77
AY701309* Theonella conica 1b
ZZ
AY701310* Theonella swinhoei
RS
71
AY701287* Carteriospongia foliascens
ZZ
AY701306* Stelleta pudica 1
AY701314* Xestospongia muta 3
AY701304* Stelleta kallitetilla 1a
AY701307* Stelleta pudica 2
AY701305* Stelleta kallitetilla 1b
AY701299* Pseudoaxinella tubulosa
BH
AY190188 Ircinia variabilis
MS
74
AY701290* Ircinia felix 1a
BH
AY701302* Spheciospongia florida 1
AY701303* Spheciospongia florida 2
ZZ
AF497567 Aplysina archeri
Caribbean
AY701300* Svenzea zeai 1
AY701301* Svenzea zeai 2
BH
97
AY701288* Cribochalina vasculum 1
AY701289* Cribochalina vasculum 2
AY701296* Petrosia sp
ZZ
95
AY701291* Ircinia felix 1b
92
AY701292* Ircinia felix 2
52
AY701311* Xestospongia muta 1
AY701315* Xestospongia proxima
100
AY701312* Xestospongia muta 2a
AY701313* Xestospongia muta 2b
BH
D50616 Synechococcus Lake Biwa P0
D50615 Synechococcus Lake Biwa G0
AY151235 Synechococcus MW28B3
AY172807 Synechococcus Minos11
AF400152 Synechococcus MLS1228c13
AY190172 Haliclona sp
AU
AB058205 Synechococcus MBIC10007
AF216950 Synechococcus PCC9005
AB058226 Synechococcus MBIC10089
AB015058 Synechococcus 7001
AF448068 Synechococcus PS838
AY151244 Synechococcus MW74D3
AF216953 Synechococcus PS717
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AY224199 Synechococcus MH301
AF216947 Synechococcus PCC7918
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AY125379 Synechococcus A315008
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AY172800 Synechococcus Almo3
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X63140 Prochlorococcus marinus
AF001470 Prochlorococcus NATL2
AF001467 Prochlorococcus NATL2A
AF369648 Candidaspongia flabellata
AU
100
AY190187 Petrosia ficiformis
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