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Community phylogenetic diversity of cyanobacterial mats associated with geothermal springs along a tropical intertidal gradient

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Abstract The 16S rRNA gene-defined bacterial diversity of tropical intertidal geothermal vents subject to varying degrees of seawater inundation was investigated. Shannon–Weaver diversity estimates of clone library-derived sequences revealed that the hottest pools located above the mean high-water mark that did not experience seawater inundation were most diverse, followed by those that were permanently submerged below the mean low-water mark. Pools located in the intertidal were the least biodiverse, and this is attributed to the fluctuating conditions caused by periodic seawater inundation rather than physicochemical conditions per se. Phylogenetic analysis revealed that a ubiquitous *Oscillatoria*-like phylotype accounted for 83% of clones. *Synechococcus*-like phylotypes were also encountered at each location, whilst others belonging to the *Chroococcales*, *Oscillatoriales*, and other non-phototrophic bacteria occurred only at specific locations along the gradient. All cyanobacterial phylotypes displayed highest phylogenetic affinity to terrestrial thermophilic counterparts rather than marine taxa.

Keywords Cyanobacteria · Geothermal springs · Hot springs · Intertidal · Thermophiles

The biodiversity of thermophilic cyanobacteria in terrestrial geothermal locations is fairly well documented, where they occur as part of microbial mat communities at temperatures up to ~65°C, and in comparatively simple biofilms at higher temperatures up to 75°C (Ward

et al. 1998). In marine environments, most research on geothermal environments has focused on deep-sea hydrothermal vents, where chemolithotrophic communities occur well below the photic zone. Conversely, very little is known about the species composition of shallow marine geothermal habitats that fall within the photic zone and so are capable of supporting thermophilic cyanobacteria. Sediments of Mediterranean subtidal geothermal springs did not support cyanobacteria (Dando et al. 1998), although planktonic cyanobacteria and other phototrophs were recorded (Lucila et al. 1996). Only one study of intertidal springs is known, where *Chlorogloeopsis*-like, *Fischerella*-like, and *Pseudanabaena*-like phylotypes were recovered from mats in Iceland that experienced tidal inundation (Hobel et al. 2005).

The intertidal geothermal vents at Bicol Pasacau, The Philippines (N13°29.416' and E123°04.634') comprise many pools that are subject to varying degrees of seawater inundation during a lunar tidal cycle. Pools that were either permanently submerged (below mean low-water mark, MLWM) (lower pools), inundated or awash during high tide (middle pools), or never inundated (above mean high-water mark, MHW) (upper pools) by seawater were sampled at three intervals along a gradient of interacting environmental stress, namely temperature, pH, salinity and dissolved sulphide (Table 1).

Epilithic microbial mats occurred in the mouths of vents but did not extend to surrounding rock surfaces on the shore. All mats appeared morphologically similar, with *Oscillatoria* filaments forming a matrix in which unicellular cyanobacteria were infrequently embedded. However, since it is known that morphologically similar filamentous (Jing et al. 2005) and unicellular (Ferris et al. 1996) cyanobacterial morphotypes in thermophilic mats can conceal considerable genetic diversity, we performed an assessment of community molecular diversity. Community DNA was isolated from mats recovered from a single pool at each tidal height (three pools in total) by phenol–chloroform extraction and ethanol precipitation. Triplicate samples from each pool

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Table 1 Physicochemical parameters and diversity estimates for geothermal springs along a tidal gradient

Parameter ^a	Lower ^b	Middle ^b	Upper
Temperature (°C)	28–47	37–54	56 ± 0
pH	6.3–8.0	6.5–8.0	6.3 ± 0
Salinity (‰)	18–30	15–30	14 ± 0
Nitrate (mg/l)	0.02–0.2	0.02–0.2	0.02 ± 0
Phosphate (mg/l)	8.0–12.5	8.0–12.5	7.00.5
Hydrogen sulphide (mg/l)	0	0–1.76	1.5 ± 0.4
Number of clones screened	39	55	50
Total number of phylotypes (cyanobacterial phylotypes ^c)	14 (14)	9 (6)	19 (15)
Coverage (%)	67	85	64
Shannon–Weaver diversity (<i>H'</i>)	1.06	0.54	1.29

^a All measurements were made in the field during low and high tide in a single day. Temperature was measured using an alcohol thermometer; pH was measured using an Orion digital pH meter with automatic temperature compensation (Thermo Electron Corp., Cheshire, UK); Salinity was measured using a refractometer (Atago, Tokyo, Japan); All dissolved ions were measured titrimetrically using portable test kits (Hach, CO, USA)

^b Range of values illustrate fluctuation with ebb and flow of water in lower pools and between low and high tide for middle pools. Values for upper pools are the mean of three replicates

^c Includes four phylotypes not included in phylogenetic trees due to short-sequence length

were PCR-amplified using previously described profiles (Schafer and Muyzer 2001) and the following primers specific to cyanobacteria: Forward, 5'-GGGGAA-TYTTCCGCAATGGG; Reverse, 5'-GACTACWGG-GGTATCTAATCCCWTT (Nubel et al. 1997), and universal bacterial primers: Forward, 5'-CCTACGG-GAGGCAGCAG; Reverse, 5'-CCGTCAATTCMTT-GAGTTT (Schafer and Muyzer 2001). PCR amplicons for each pool were combined and cyanobacterial and universal bacterial clone libraries constructed for each combined pool amplicon (pDrive, Qiagen, Crawley, UK). Sequencing of 144 clones was carried out using an automated DNA Sequencer (ABI 3730 Genetic analyzer, Applied Biosystems, Foster City, CA, USA). For the purpose of this study, phylotypes were determined to be distinct if they displayed less than 99% sequence similarity. Phylogenetic relationships of cyanobacteria were determined using maximum likelihood analysis, with robustness of tree topology supported by bootstrap and Bayesian posterior probability analysis (PAUP* V4.0b8). All sequences have been submitted to the NCBI GenBank database under accession numbers DQ74653-DQ74688.

A total of 40 phylotypes were identified, 33 of which belonged to the cyanobacteria. Only cyanobacterial phylotypes were recovered from the cyanobacterial clone libraries, whilst the universal bacterial clone libraries also detected other bacterial phylotypes. The number of phylotypes recovered from cyanobacterial clone libraries increased progressively from upper < middle < lower pools, whilst bacterial clone libraries displayed the opposite trend. This illustrates the importance of primer-specificity in influencing molecular diversity studies and the need for multiple primer approaches as used in this study. Both primer sets identified common phylotypes but several of the rarely encountered phylotypes were recovered using one primer set only.

The number of phylotypes recovered was greatest in the hottest upper pools that experience no seawater

inundation, and roughly matched levels of diversity encountered in terrestrial geothermal springs (Ward et al. 1998). After applying a correction factor for coverage (reflecting the estimated percentage of the community recovered in clone libraries) at each location, the permanently submerged lower pools were ~25% less biodiverse, whilst the middle pools which experience fluctuating conditions displayed a ~50% reduction in diversity (Table 1). Shannon–Weaver diversity estimates followed a similar pattern (Table 1), indicating a trend for loss in diversity of upper > lower > middle pools. These data reflect the greater challenges in this intertidal location due to periodic dilution of geothermal water by seawater. Clearly, thermal stress is not a limiting factor since the hottest pools were most biodiverse, similarly UV and dessication stress can be ruled out since they would also be greatest in these pools. No animal life was observed around vents and grazing activity is highly unlikely in these pools given the temperatures recorded. We therefore conclude that it is the daily fluctuations in physicochemical water properties that are responsible for reduced diversity. The pattern of diversity created by the interaction of geothermal and seawater along the intertidal gradient is therefore significantly different to that in normal intertidal locations, where a near-linear pattern of reduction in archaeal, bacterial, and cyanobacterial diversity occurs with increasing height in the intertidal, presumably due to desiccation and UV stress (Rothrock and Garcia-Pichel 2005).

A single *Oscillatoria*-like phylotype was ubiquitous to all pools sampled and accounted for 83% of all sequences recovered. This probably indicates the occurrence of a cyanobacterium that is capable of tolerating the fluctuating environmental stresses experienced along this thermal and tidal gradient. All other phylotypes were recovered only once. Several other oscillatory and chroococcalean cyanobacterial phylotypes occurred in a clearly zoned manner along the transect, and this probably reflects differential tolerances to environmental

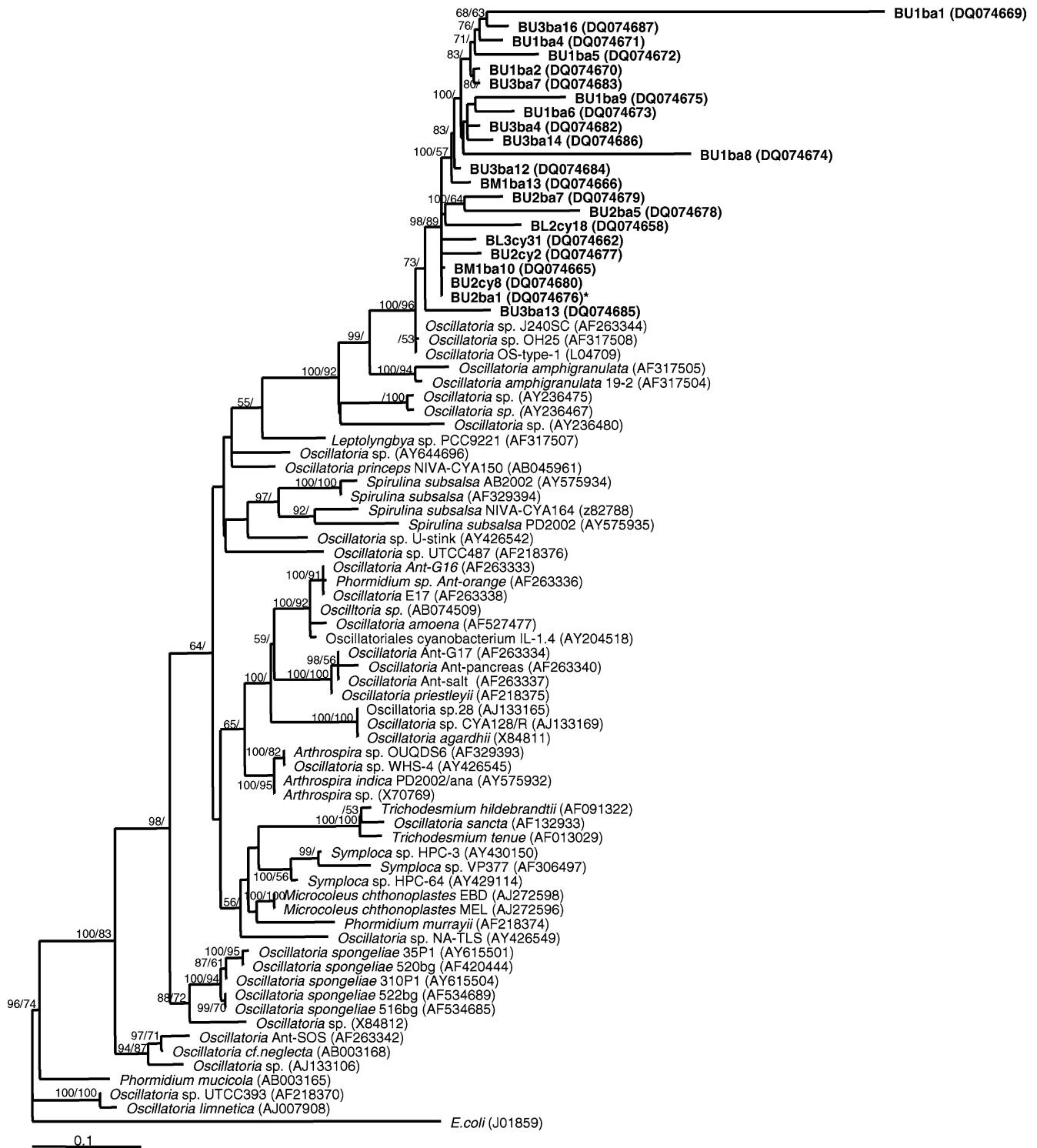


Fig. 1 Phylogenetic relationships among the *Oscillatoriales* (cyanobacteria) based upon Maximum Likelihood analysis of partial 16S rRNA gene sequence data (*E. coli* positions 359–781). Sequence codes with a prefix BL are derived from subtidal mats, BM from intertidal mats and BU from mats above the MHW. *indicates the most commonly retrieved phylotype, which

accounted for 105/144 clones. The sequence Tree topology is supported by Bayesian posterior probabilities (*first number*) and bootstrap values for 1000 replications (*second number*), shown for branches supported by more than 50% of the trees. Scale bar represents 0.1 nucleotide changes per position

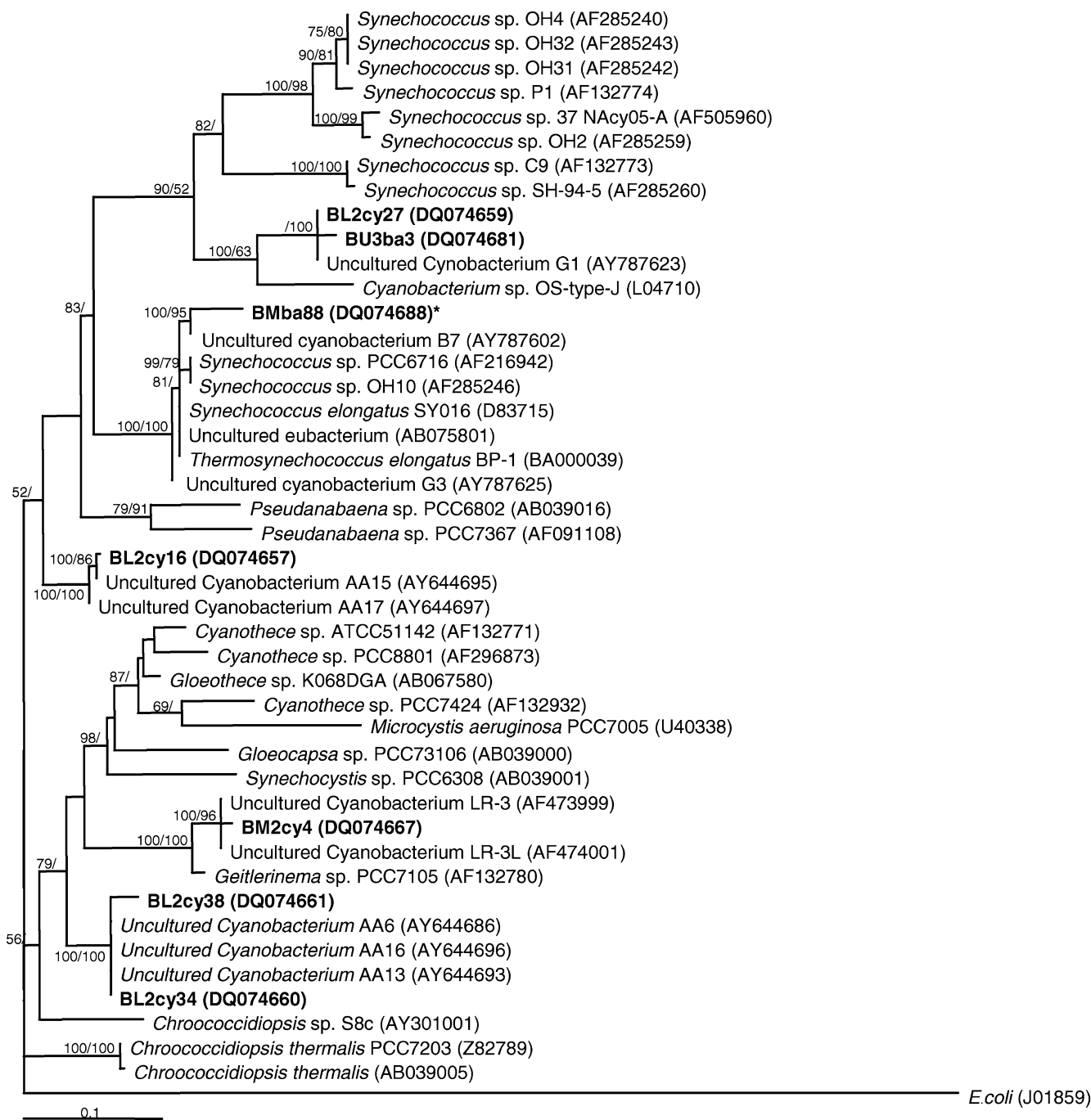


Fig. 2 Phylogenetic relationships among the *Chroococcales* (cyanobacteria) based upon Maximum Likelihood analysis of partial 16S rRNA gene sequence data (*E. coli* positions 359–781). Sequence codes with a prefix BL are derived from subtidal mats, BM from intertidal mats and BU from mats above the MHW. *indicates a sequence derived from enrichment culture in BG11 medium. A *Synechococcus*-like phylotype recovered from clone

libraries of Middle pools yielded only a short (272 bp) sequence and is not included in this analysis. Tree topology is supported by Bayesian posterior probabilities (first number) and bootstrap values for 1000 replications (second number), shown for branches supported by more than 50% of the trees. Scale bar represents 0.1 nucleotide changes per position

stress. Other bacterial phylotypes were relatively uncommon. A single thermophilic phylotype was recovered from upper pools (DQ74655), with highest similarity to *Thermus yunnanensis* from terrestrial geothermal springs (AY557603). Other sequences from upper and middle pools (DQ74653, DQ74656, DQ74663–DQ74664, and DQ74668) shared high similarity to non-thermophilic uncultured marine bacteria. Non-cyanobacterial sequences were not recovered from lower pools.

Phylogenetic analysis revealed that the common *Oscillatoria*-like phylotype resolved into a relatively diverse clade along with several other closely related phylotypes from this study (Fig. 1). These were most closely related to other thermophilic *Oscillatoria* taxa and shared only distant affiliation with marine species. A separate group within this clade represented only by sequences from the hottest upper pools was also delineated with high support (Fig. 1). These may represent obligately thermophilic phylotypes, as they were recovered only from pools close to the recorded maximum temperature for *Oscillatoria* (Castenholz 2000). All pools also supported *Synechococcus*-like phylotypes (Fig. 2). Those derived from clone libraries affiliated most closely with low-temperature adapted phylotypes from terrestrial geothermal springs in North America. The sequence obtained from enrichment culture at 45°C in BG11 medium (Castenholz 1988) affiliated with the C1 *Synechococcus* lineage, which mainly comprises sequences from terrestrial springs in Japan. The middle pools supported a *Geitlerinema*-like phylotype (Fig. 2), closely affiliated with coral-associated cyanobacteria but not common to geothermal locations. Several *Chroococcidiopsis*-like phylotypes (Fig. 2) occurred in the lower pools only. Their phylogenetic affiliation with xerophilic terrestrial species suggests these phylotypes do not represent obligate marine taxa, and so they may reflect mesophilic and/or poorly sulphide-tolerant taxa. That no diazotrophic phylotypes were encountered in any of the pools suggests that sufficient combined nitrogen occurs in this habitat.

In conclusion, we have shown that thermophilic cyanobacterial assemblages occur within intertidal geothermal habitats, with diversity dependant upon the level of fluctuation within a specific niche rather than

physicochemical conditions per se. This result in an altered gradient of biodiversity when compared to non-geothermally influenced shores.

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References

- Castenholz RW (1988) Culturing methods for cyanobacteria. *Methods Enzymol* 167:68–92
- Castenholz RW (2000) Cyanobacteria in geothermal habitats. In: Whitton BA, Potts M (eds) *The ecology of cyanobacteria*. Kluwer Academic Publishers, Dordrecht, pp 37–59
- Dando PR, Thomm M, Arab H, Brehmer M, Hooper LE, Jochimsen B, Schesner H, Stohr R, Miguel J-C, Fowler SW (1998) Microbiology of shallow hydrothermal sites off Paleohori Bay, Milos (Hellenic Volcanic Arc). *Cah Biol Mar* 39:369–372
- Ferris MJ, Ruff-Roberts AL, Kopczynski ED, Bateson MM, Ward DM (1996) Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a single hot spring microbial mat habitat. *Appl Environ Microbiol* 62:1045–1050
- Hobel CFV, Martiensson VT, Hreggvidson GO, Kristjansson JK (2005) Investigation of the microbial ecology of intertidal hot springs by using diversity analysis of 16S rRNA and chitinase genes. *Appl Environ Microbiol* 71:2771–2776
- Jing H, Aitchison JC, Lacap DC, Peerapornpisal Y, Sompong U, Pointing SB (2005) Community phylogenetic analysis of moderately thermophilic cyanobacterial mats from China, the Philippines and Thailand. *Extremophiles* (in press) (DOI: 10.1007/s00792-005-0456-1)
- Lucila M, Acosta Pomar C, Giuffrè G (1996) Pico-, nano- and microplankton communities in hydrothermal marine coastal environments of the Eolian islands (Panarea and Vulcano) in the Mediterranean sea. *J Plankton Res* 18:715–730
- Nubel U, Garcia-Pichel F, Muyzer G (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl Environ Microbiol* 63:3327–3332
- Rothrock MJ, Garcia-Pichel F (2005) Microbial diversity of benthic mats along a tidal desiccation gradient. *Environ Microbiol* (in press) (doi:10.1111/j.1462-2920.2004.0078.x)
- Schafer H, Muyzer G (2001) Denaturing gradient gel electrophoresis in marine microbial ecology. *Methods Microbiol* 30:425–468
- Ward DM, Ferris MJ, Nold SC, Bateson MM (1998) A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiol Mol Biol Rev* 62:1353–1370