Community structure of free-floating filamentous cyanobacterial mats from the Wonder Lake geothermal springs in the Philippines

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Abstract: Cyanobacterial mats were characterized from pools of 45–60 °C in near-neutral pH, low-sulphide geothermal springs in the Philippines. Mat structure did not vary with temperature. All mats possessed highly ordered layers of airspaces at both the macroscopic and microscopic level, and these appear to be an adaptation to a free-floating growth habit. Upper mat layers supported biomass with elevated carotenoid:chlorophyll *a* ratios and an as yet uncharacterized waxy layer on the dorsal surface. Microscopic examination revealed mats comprised a single *Fischerella* morphotype, with abundant heterocysts throughout mats at all temperatures. Molecular analysis of mat community structure only partly matched morphological identification. All samples supported greater 16S rDNA-defined diversity than morphology suggested, with a progressive loss in the number of genotypes with increasing temperature. *Fischerella*-like sequences were recovered from mats occurring at all temperatures, but some mats also yielded *Oscillatoria*-like sequences, although corresponding phenotypes were not observed. Phylogenetic analysis revealed that *Fischerella*-like sequences were most closely affiliated with *Fischerella major* and the *Oscillatoria*-like sequences with *Oscillatoria amphigranulata*.

Key words: cyanobacteria, *Fischerella*, geothermal springs, microbial mats, *Oscillatoria*.

Résumé : Nous avons caractérisé des tapis des cyanobactéries provenant de mares de sources géothermiques des Philippines à 45–60 °C, d'un pH quasi-neutre et faibles en sulfures. La structure des tapis n'a pas varié avec la température. Tous les tapis renfermaient des couches hautement structurées d'espaces aériens tant au niveau macroscopique que microscopiques et celles-ci semblaient être une adaptation à la croissance par flottaison. Les couches supérieures des tapis contenaient une biomasse comportant un rapport caroténoïde:chlorophylle *a* élevé et une couche cireuse encore non caractérisée sur la surface dorsale. Un examen microscopique a révélé que les tapis renfermaient un seul morphotype de *Fischerella* avec de nombreux hétérokystes au travers des tapis à toutes les températures. L'analyse moléculaire de la structure de la communauté des tapis n'a que partiellement concordé avec l'identification morphologique. Tous les échantillons renfermaient une diversité définie par l'ADNr 16S supérieure à ce que la morphologie suggérait, avec une perte progressive du nombre de génotypes à mesure que la température augmentait. Des séquences semblables à *Fischerella* ont été prélevées des tapis ayant évolué à toutes les températures mais certains tapis ont également généré des séquences semblables à *Oscillatoria* bien qu'aucun phénotype correspondant ne fut observé. L'analyse phylogénétique a révélé que les séquences semblables à *Fischerella* se rapprochaient davantage de *Fischerella major* et que les séquences semblables à *Oscillatoria* s'apparentaient à *Oscillatoria amphigranulata*.

Mots clés : cyanobactéries, *Fischerella*, sources géothermiques, tapis microbiens, *Oscillatoria*.

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Introduction

Geothermal springs are extreme aquatic environments presenting thermal and chemical challenges to life. Nonetheless, springs of neutral to alkaline pH support prokaryotic microbial mats that have photoautotrophic production and varying community structures (Ward and Castenholz 2000).

The species composition and community structure of thermophilic mats was first described for 2 geothermal spring systems in The Great Basin and Yellowstone National Park, USA, in the pioneering studies by Brock (1978), Castenholz (1973), Ward and co-workers (1987, 1989). Morphological examinations revealed several epilithic mat types with the filamentous cyanobacteria *Calothrix*,

Mastigocladus (Fischerella), Oscillatoria, and *Phormidium*, variously occurring as the dominant photosynthetic organism, from channels of geothermally heated water at temperatures below ~60 °C. To date, there have been few molecular investigations of such filamentous mat communities, although several *Oscillatoria* sequences have been recovered from hot springs in Italy, Japan, and New Zealand (Papke et al. 2003). In addition, some moderately thermophilic *Synechococcus* mats in Yellowstone National Park have also been shown to support filamentous genotypes (Norris et al. 2002). The unicellular morphospecies *Synechococcus* cf. *lividus* is the only cyanobacterial morphotype encountered at temperatures above 64 °C (Ward and Castenholz 2000). Most recent research has focused on resolving community structure within such *Synechococcus* mats, largely from selected geothermal springs within the Great Basin and Yellowstone sites, using molecular techniques (Ferris et al. 1996a, 1996b; Ward et al. 1997). Such mats were found to vary little in terms of 16S rDNA-defined diversity both seasonally and upon recovery from disturbance (Ferris and Ward 1997; Ferris et al. 1997; Norris et al. 2002). Very little difference in 16S rDNA-defined diversity of *Synechococcus*-like sequences was identified between those studied in geothermal springs up to 100 km apart in Yellowstone (Ward et al. 1998).

Comparatively, few studies have recorded geothermal spring mat biodiversity in other locations with approaches generally using microscopy and enrichment culture to observe taxa. *Synechococcus* and *Oscillatoria* appear to be commonly encountered in Asian (Arif 1997; Castenholz 1996; Panyoo and Peerapornpisal 2001), European (Pentecost 1995; Pentecost et al. 1997), and New Zealand (Castenholz 1976) geothermal springs. One recent study identified significantly different 16S rDNA-defined lineages of *Synechococcus*- and *Oscillatoria*-like sequences between north American, European, Japanese, and New Zealand geothermal springs (Papke et al. 2003). Since no correlation between aqueous geochemistry and diversity was identified, diversity was attributed, at least in part, to genetic drift resulting from the isolated nature of geothermal habitats. This highlights the need to study diversity in geothermal springs across environmental gradients over a wide biogeographical area, particularly the tropics that have not been well studied. In this study, both microscopic observation and molecular methods were used to characterize unusual filamentous mats from geothermal springs in the Philippines, yielding new data on thermophilic mat structure and environmental 16S rDNA-defined thermophilic genotypes.

Materials and methods

Sample collection

Twenty-seven mat samples were collected using a hierarchical random sampling design from 3 separate pools 0.5 m to 8 m in depth in the Wonder Lake geothermal springs, Laguna, Philippines. The 3 pools were characterized as follows: the low-temperature (45 °C) pool was between 44.2–45.4 °C, pH 6.93–7.17, and had a 30 µmol/L H₂S content; the mid-temperature (53 °C) pool was between 52.5–54 °C, pH 6.98–7, and had 30 µmol/L H₂S; and the high-temperature (60 °C)

pool was between 59.6–60.5 °C, pH 6.81–6.83, and had 30 µmol/L H₂S. Mat samples (2 cm × 2 cm and varying thickness) were aseptically cut from selected mats using a scalpel and stored in sterile glass bottleson ice in the dark when in the field and then at 4 °C in the laboratory until processed (~1 week).

Water chemistry

Temperature and pH were recorded using a thermometer and digital pH meter with automatic temperature compensation (Orion, Boston, Mass.). The hydrogen sulphide content of water was assessed by methylene blue titration using a HS-WR field test kit (Hach, Loveland, Co.) according to the manufacturers instructions.

Microscopy

All measurements and photographs were taken using either an Olympus SZH10 stereomicroscope or BX50 compound microscope with differential interference contrast optics, both fitted with an Olympus DP11 digital camera.

Pigment analysis

The absorption spectra of pigments were measured spectrophotometrically (Spectronic Genesys 5; Thermo Electron Corp., Madison, Wis.) after extraction of biomass overnight in 10 volumes of absolute methanol.
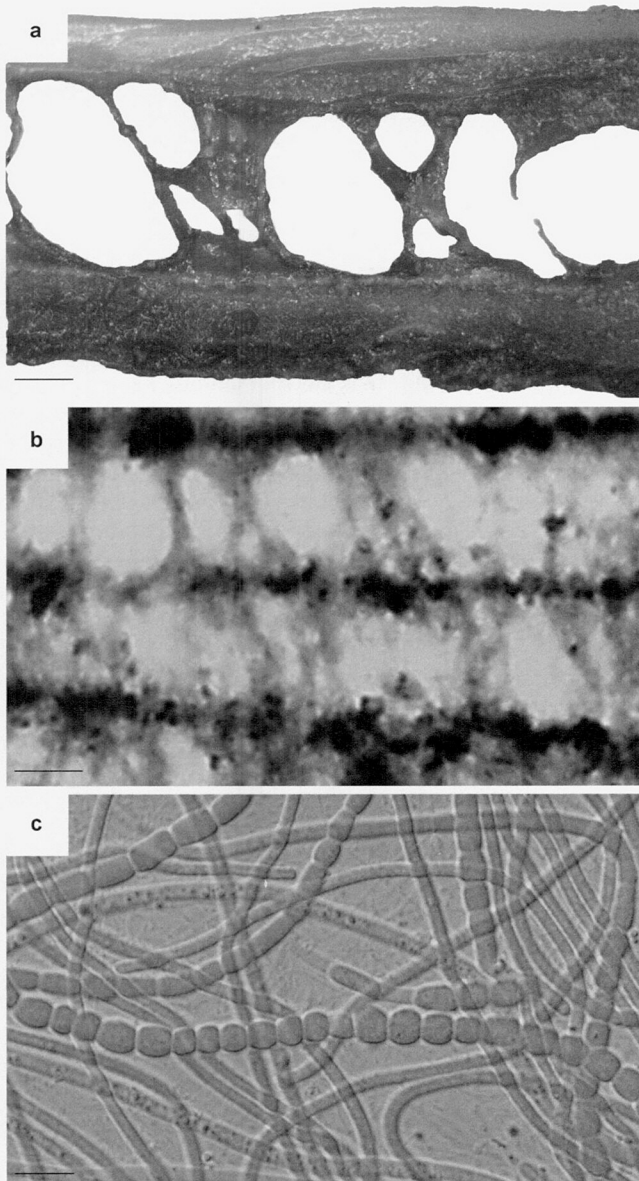
Polymerase chain reaction and denaturing gel gradient electrophoresis

DNA extraction followed a previously described cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle and Doyle 1987 with the following modifications: RNAse A and proteinase K incubations were included in the lysis step (final concentration 200 µg/mL); and phenol – chloroform – isoamyl alcohol (25:24:1) extractions were carried out at 60 °C to maximize recovery from thick-walled cyanobacteria. 16S rRNA genes were amplified by polymerase chain reaction (PCR) using cyanobacterial-specific primers CYA359F (GGGGAATYTTCCGCAATGGG) and CYA781R (GACTACWGGGGTATCTAATCCCWTT) (Nübel et al. 1997) with a 40-bp GC clamp added to the forward primer and the following profile: 35 cycles of 1 min at 94 °C, 50 s at 52 °C, 1 min at 72 °C; with an initial denaturation step of 3 min and a final extension step of 10 min. The PCR-reaction mixture contained 1.5 mmol/L MgCl₂, 0.2 mmol/L of each deoxynucleoside triphosphate (dNTP), 0.3 µmol/L of each primer, and 1.0 U of *Taq* DNA polymerase. We confirmed specificity of the cyanobacteria-specific PCR primers across a broad taxonomic range of 20 filamentous reference taxa from The University of Hong Kong Culture Collection (cyanobacteria). PCR products were separated by denaturing gel gradient electrophoresis (DGGE) as previously described (Myers et al. 1988). A 15%–45% urea–formamide denaturant gradient was used in a 7% acrylamide gel, run at 150 V for 5 h in 1× Tris – acetic acid – EDTA (TAE) buffer (pH 8) at 60 °C (DGGE-2001; C.B.S. Scientific Co., Del Mar, Calif.).

Sequence analysis

Bands were excised, soaked overnight in Tris–EDTA (TE) buffer (pH 8) at 4 °C, re-amplified, and purified (Amersham

Fig. 1. Structure of cyanobacterial mats from Wonder Lake geothermal springs. (*a*) Section through microbial mat revealing distinct macroscopic airspaces, bar represents 10 mm; (*b*) section through microbial mat revealing microscopic airspaces, bar represents 50 μm; and (*c*) primary trichomes and heterocystous cells of *Fischerella* within mat, bar represents 10 μm.

Fig. 2. Denaturing gel gradient electrophoresis analysis of 16S rDNA-defined cyanobacterial community structure in Wonder Lake cyanobacterial mats from (*a*) 45 °C, (*b*) 53 °C, and (*c*) 60 °C geothermal pools, with 3 replicates (1–3) for each.
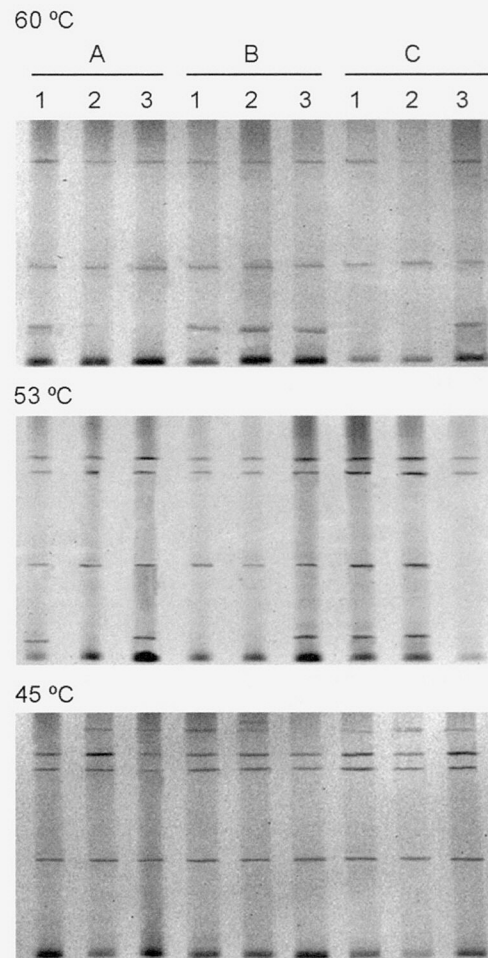
bilities (Rannala and Yang 1996) and bootstrap values (100 replications) were calculated and are shown for branches supported by more than 50% of the trees.

Nucleotide sequence accession numbers

All nucleotide sequences obtained during the course of this investigation have been deposited in GenBank under accession Nos. AY236467–AY236480.

GFX Gel Band Purification Kit; Amersham Biosciences, Buckinghamshire, UK) prior to automated sequencing (ABI Prism 377; Applied Biosystems, Foster City, Calif.). Bands that could not be sequenced directly were cloned (pDrive Cloning Vector (PCR Cloning Kit); Qiagen Inc., Valencia, Calif.) before successful sequencing. Approximate phylogenetic affiliations were determined by BLAST searches of the NCBI GenBank database. Multiple alignments were then created with reference to selected GenBank sequences using BioEdit v. 5.0.9 (Hall 1999). Maximum likelihood analysis using PAUP* v. 4.0b8 (Swofford 2001) was used to illustrate the relationship of partial 16S rRNA gene sequences with representative cyanobacteria. Bayesian posterior proba-

Results

Filamentous mats were oxic throughout, with a thin upper yellow layer of biomass overlying a thicker lower green layer (Fig. 1*a*). The upper layer had a carotenoid:chlorophyll *a* ratio of ~1:1, whereas in the lower layers it was ~1:2. The dorsal surface was covered in a thin, non-cellular waxy layer. Microscopic analysis revealed an identical morphotype composition throughout all mats from 45 to 60 °C. Of great interest, there was the occurrence of macroscopic near-spherical airspaces arranged in a honeycomb-like pattern throughout the green layer of all mats (Fig. 1*a*). These airspaces had an estimated average volume of 7.43 mm^3 (assuming spherical shape). Further

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Fig. 3. Phylogenetic relationships among Wonder Lake cyanobacterial mat sequences based upon Maximum Likelihood analysis of partial 16S rRNA gene sequence data. The tree is supported by bootstrap values for 100 replications (first number) and Bayesian posterior probabilities (second number), shown for branches supported by more than 50% of the trees. Scale bar represents 0.1 nucleotide changes per position. Sequences derived from this study are shown with the prefix WL. Numbers after sequence codes denote temperature from which sequence was obtained.

interest lies in the presence of highly ordered horizontal layers of smaller near-spherical airspaces visible at the microscopic level between apparent "growth-lines" of debris and cellular material (Fig. 1b). These smaller airspaces had an estimated average volume of 153 μm^3 (assuming spherical shape) and were not bordered by a membrane but rather by densely packed cellular filaments.

Microscopic analysis revealed mats to comprise filamentous *Fischerella*-like cells (Fig. 1c) with numerous heterocystous cells and primary trichomes visible. No other morphotypes of either filamentous or unicellular cyanobacteria were observed. Since morphology of the filamentous mats was identical from pools of significantly different temperature, we investigated 16S rDNA-defined diversity using DGGE to establish any genetic diversity between mats within and between pools (Fig. 2). In all cases, template, PCR, and DGGE conditions were optimized and the number of bands obtained in all cases was comparable with that of other studies on thermophilic microbial mats. Six band migration classes were identified in samples from the low-temperature pool (45 °C), 5 from the mid-temperature pool (53 °C), and 4 in the high-temperature pool (60 °C) (Fig 2). Sequences from DGGE bands all displayed high similarity (>90%) to *Fischerella* or *Oscillatoria* sequences as revealed by BLAST searches of the NCBI GenBank database.

Phylogenetic analysis revealed sequences resolved into 2 distinct groups; one with the closest affiliation to *Fischerella* spp. and the other to *Oscillatoria* spp. (Fig. 3). The *Fischerella*-like sequences formed a closely related group with highest affinity to *Fisherellla major* from Japan and an uncultured *Fischerella* sequence from an Iceland hot spring. The resolution of the genus, however, is not supported by high bootstrap or Bayesian values. No patterns separating sequences obtained from different temperature pools were evident, although a single sequence from the lowest temperature pool branched on its own within the *Fischerella* lineage. The *Oscillatoria*-like sequences affiliated most closely with thermophilic *Oscillatoria amphigranulata* sequences also recovered from geothermal springs, and with one exception, they form what is probably a new lineage with moderate support.

Discussion

The thermophilic mats described in this paper are interesting for 2 main reasons. The first reason being for the documentation of the free-floating growth form and apparent adaptations to this in terms of airspaces at the macroscopic and microscopic level. An additional adaptation to environment may lie in the carotenoid-rich layer of biomass and in the unidentified waxy layer in dorsal mat layers. Sec-

ondly, the sequences obtained contribute to knowledge about the diversity of thermophilic cyanobacteria, notably for this *Fischerella* mat type, which has not been previously investigated at the community molecular level. The recovery of *Oscillatoria*-like sequences, which are known from several studies of other geothermal regions, contributes data that may also be useful in further understanding the phylogeography of thermophilic filamentous cyanobacteria.

The structural organization of the Wonder Lake mats is of great interest. It is clear that the filamentous nature of component species facilitates the creation of the macroscopic and microscopic airspaces within mats, and these airspaces appear to be maintained without a membranous surrounding. Such organization would probably not be possible in mats comprising only unicellular species. The method of formation for these airspaces is not clear at present. It is reasonable, however, to assume that they function to provide buoyancy. A free-floating lifestyle may confer some advantage over epilithic growth in tropical geothermal springs such as Wonder Lake, where large seasonal fluctuations in water level and turbidity due to monsoon rains (pools at Wonder Lake become several metres deeper and very turbid during the rainy season) may compromise the photosynthetic efficiency of attached mats as they become more deeply submerged, but floating mats can maintain their position on the water surface. Since the Wonder Lake mats were also oxic throughout, it is possible that the airspaces fulfill a role in maintaining aeration throughout mats. This may explain why they do not support anaerobic layers commonly reported for unicellular thermophilic mat communities. It would be interesting to establish the method of formation and gaseous composition of these airspaces in situ through future study and to establish why such gases are not consumed by heterotrophic activity.

The high carotenoid levels in the upper layers of mats probably reflect a response to high UV exposure. Elevated carotenoid:chlorophyll *a* (and phycobilin) ratios are a well-known response to high UV irradiance (Castenholz and Garcia-Pichel 2000), and since the UV-screening ability of carotenoids is minimal, it is assumed that an indirect role in UV-protection occurs by carotenoids acting as quenching agents for radicals and other reactive species. Elevated carotenoid levels are also known to occur as a result of a deficiency in nitrogen (Collier and Grossman 1992), although this seems unlikely considering the diazotrophic nature of *Fischerella*. The nature of the as yet unidentified waxy surface layer of mats is unknown. Further work is necessary to elucidate its origin, composition, and function (if any) in UV-protection. Other mat-forming thermophilic cyanobacteria taxa including *Calothrix* and *Pleurocapsa* produce UV-screening sheath pigments such as scytonemin (Garcia-
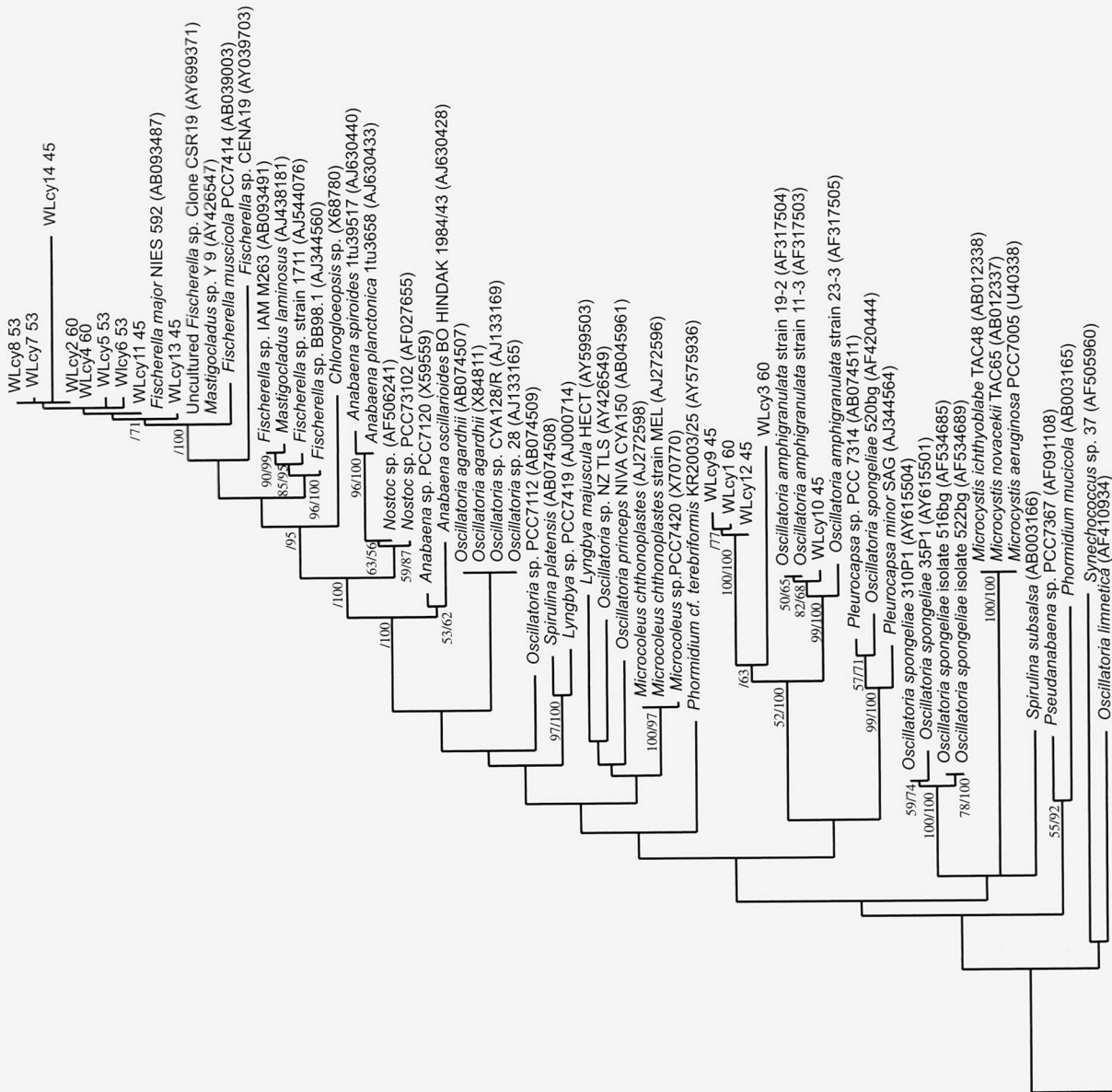
Pichel and Castenholz 1991) and mycosporine-like compounds (Garcia-Pichel et al. 1993), although *Fischerella* (as *Mastigocladus* cf. laminosus) is not known to produce such compounds (Ward and Castenholz 2000).

The discrepancy between morphological and molecular data regarding mat composition is interesting. *Oscillatoria* morphotypes were not observed by microscopy, however, since the primary trichomes of *Fischerella* can appear similar, it is possible that if they were present in low numbers that they were not identified. It is also possible that all genotypes belong to the *Fischerella* morphotype. There have been other cases of 16S rDNA sequences not matching observed cyanobacterial morphotypes, even in axenic culture (Nadeau et al. 2001; de la Torre et al. 2003), suggesting some phenotypic plasticity may occur among certain cyanobacteria. In the context of this study, it is believed that the *Oscillatoria*-like sequences probably do originate from *Oscillatoria* species since the genotypes are highly similar to those of known thermophilic members of this genus. Successful cultivation of *Oscillatoria* from these mats and sequencing of axenic isolates would confirm this.

All sequences obtained in this study were novel compared with other studies targeting this region of the 16S rRNA gene, and thus expand our knowledge of genetic diversity in geothermal habitats. There was no apparent grouping of genotypes according to temperature despite apparent temperature niches among thermophilic *Synechococcus* genotypes being documented (Ferris et al. 1996b). Phylogenetic analyses revealed that all sequences grouped closely with those from other geothermal habitats, but distinct from mesophilic counterparts. Relatively few sequences have been published for *Fischerella*, and so our study helps to expand our phylogenetic knowledge of this genus, although the resolution provided using the partial gene sequence in this study does not allow high bootstrap support for tree topology. Targeting the complete 16S rDNA may help in resolving relationships better. The *Oscillatoria*-like sequences are of interest since they resolve into a distinct group with moderate support, which probably reflects a distinct phylogeographic lineage. The concept of phylogeographic lineages has been demonstrated for thermophilic *Synechococcus* and is attributed to isolation between geothermal habitats (Papke et al. 2003). Whether such lineages exist for *Fischerella* is currently unclear.

In terms of extrapolating these data to the ecology of Wonder Lake mats, the presence of abundant heterocystous cells in *Fischerella*-like morphotypes throughout indicates nitrogen fixation occurs in Wonder Lake mats at temperatures up to 60 °C, similar to the previously recorded maximum temperature of 58 °C for growth of this genus (Ward and Castenholz 2000). In geothermal springs rich in combined nitrogen, *Fischerella* is out competed by other non-nitrogen fixing taxa (Ward and Castenholz 2000), suggesting Wonder Lake is a nitrogen-poor habitat. The *Oscillatoria*-like sequences from this study shared greatest similarity to thermophilic *Oscillatoria amphigranulata* and *Oscillatoria* spp. from New Zealand that are phenotypically deficient in nitrogen assimilation (Miller and Castenholz 2001). If the *Oscillatoria*-like sequences from Wonder Lake also possess such phenotypes, an interesting point is raised since these cells must co-exist with the diazotrophic *Fischerella*. *Oscillatoria* sequences have been recovered from geothermal

springs of up to 64 °C (Papke et al. 2003) and this genus is known to tolerate high sulphide levels of up to 3 mmol/L (Ward and Castenholz 2000), so the Wonder Lake pools are a likely habitat for thermophilic members of the genus.

Acknowledgements

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