The phylogenetic diversity of *Thermus* and *Meiothermus* from microbial mats of an Australian subsurface aquifer runoff channel

Mark D. Spanevello, Bharat K.C. Patel *

*Microbial Discovery Research Unit, School of Biomolecular and Biomedical Sciences, Faculty of Science, Griffith University, Brisbane, Qld. 4111, Australia*

Received 25 May 2004; accepted 30 May 2004

First published online 17 June 2004

**Abstract**

Spectral analysis of the cell free extracts of four mat samples colonizing a Great Artesian Bain (GAB) aquifer bore runoff channel suggested that *Thermus* was present in the 75 °C grey mat, *Meiothermus* was present in the 66 °C red mat, a mixed population of *Meiothermus/Thermus* and photosynthetic microbes were present in the 57 °C green mat and photosynthetic microbes were present in the 52 °C brown mat. Enumeration studies indicated that *Thermus* dominated the grey mats and *Meiothermus* dominated the red mat but both were absent in samples collected at the bore source (89 °C) and below the bore source (88 °C). Culture-dependent studies followed by 16S rRNA gene sequence analysis indicated that 13 of the 14 *Thermus* isolates clustered closely with each other and to *T. igniterrae*, with the remaining strain clustering with *Thermus* strain SRI-96. The two *Meiothermus* isolates were closely related to *Meiothermus ruber*. A culture-independent study with 367 16S rRNA gene clones concurred that *Thermus* dominated the grey mat, but to a lesser extent in the red mat and the green mats and its complete absence in the brown mat. Of the four *Thermus* phylogroups identified one phylogroup dominated the cloned library and was related to the cluster represented by *T. scotoductus*. The second most dominant phylogroup was related to the cluster represented by *T. igniterrae* and the third and fourth phylogroups, which were the least dominant, were related to *Thermus* strain SRI-248 and *T. oshimai* respectively. *Meiothermus* was only represented in the 16S rRNA gene libraries of the red, green and brown mats and formed two phylogroups, of which the most dominant was associated with the red mat and phylogenetically related to *M. ruber* while the second phylogroup was found only in the green mat gene library and was related to *M. cerberus*.

© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

**Keywords:** *Thermus*; *Meiothermus*; Phylogeny; Pigment; Microbial mats; Diversity; Great Artesian Basin

1. Introduction

*Thermus* and *Meiothermus* are genera of strictly aerobic thermophilic heterotrophs which were first isolated from neutral and alkaline hot springs of Yellowstone National Park, USA and Kamchatka, Russia respectively. Members of the genus *Thermus* are non-pigmented to pale or brightly yellow pigmented and have an optimum temperature for growth between 65 and 75 °C. Some *Thermus* species display halotolerance, with marine isolates being more halotolerant than their terrestrial counterparts [1–4] but in general, there is very little inter- and intra-phenotypic variation amongst *Thermus* isolates. Consequently, eight distinct species of *Thermus*, namely *T. aquaticus*, *T. brockianus*, *T. oshimai*, *T. filiformis*, *T. thermophilus*, *T. scotoductus*, *T. igniterrae* and *T. antranikianus* have been described on the basis of DNA–DNA homology studies [5]. *Meiothermus* species possess pale red to bright red pigmentation, with the exception of *M. chilarophilus* which has a pale yellow pigmentation, and grow optimally at temperatures
between 50 and 60 °C. Early chemotaxonomic and
umerical taxonomic studies had placed *Meiothermus*
species as members of the genus *Thermus* [6] but sub-
sequent phylogenetic and DNA-DNA hybridization
studies showed that *Thermus* and *Meiothermus* were
closely related but phylogenetically distinct [7,8]. The
four species of *Meiothermus*, *M. chilarophilus*, *M. cer-
berus*, *M. silvanus* and *M. ruber* have been described on
the basis of their physiology, fatty acid composition and
DNA homology.

*Thermus* and *Meiothermus* have so far been isolated
from terrestrial and shallow marine hot springs of USA
[1], Iceland [9], New Mexico [10], deep sea hydrothermal
vents [11], New Zealand [12], Japan [13,14], Russia [15],
shallow marine hot springs in Portugal [3,16] and from
the subterranean aquifer, the Great Artesian Basin
(GAB) of Australia [17,18]. *Thermus* species have also
been isolated from man-made environments such as
composts [19] and hot water systems [20].

Members of the genera *Thermus* and *Meiothermus* are
generally found in neutral to slightly alkaline natural
aquatic environments where temperatures range be-
tween 50 and 85 °C. Therefore, it has been suggested
that their isolation from environments that fall outside
these parameters is due to their seeding from areas
where suitable growth conditions exist. *Thermus* and
*Meiothermus* are also frequently associated with pho-
tosynthetic and chemolithotrophic prokaryotes. It is
thought that such associations provide continuous low
concentrations of organic compounds that is beneficial
for their growth as exposure to high organic concen-
trations are inhibitory [21,22].

*Thermus* ecology and diversity studies have so far
been conducted extensively with thermal waters associ-
ated with volcanic activity. We present in this report our
findings from culturable and culture-independent studies
on *Thermus* and *Meiothermus* from the GAB, a
subsurface, non-volcanically heated thermal aquifer of
Australia. The GAB is the largest subsurface aquifer in
the world occupying over 1.7 million km², which is 22%
of the Australian continent (Fig. 1). Though the Basin
was formed between 100 and 250 million years ago, the
age of the water is estimated to be 2 million years. The
Basin consists of alternating layers of water-bearing
permeable sandstone aquifers and non-water-bearing
impermeable mudstones and siltstones. The thicknesses
of these sequences vary from thin 100 m at the aquifer
margin to 3000 m in the deeper parts of the Basin. The
aquifers are recharged by rainfall infiltrating into
the sandstone along the north-east margin of the Basin.
The aquifers flow at the rate of between 1 and 5 m per
year in the south–west direction and to the north–west
and north in the northern section of the Basin. The
driving force behind this flow is the difference in eleva-
tion between the recharge and natural discharge regions
in which approximately 600 mound spring complexes

2. Materials and methods

2.1. Site and sample collection

Several samples were collected from the New Lorne
bore (registered bore number 17263) which is situated
near Blackall (24°54′48″ S, 145°08′18″ E), approximately
1000 km northwest of Brisbane, Queensland,
Australia (Fig. 1). The bore has a depth of 1613 m, the
flow rate of the water at the bore source is approxi-
mately 8 l s⁻¹ and the water temperature at the source
is 89 °C. The source water falls into a small concrete pool
approximately 1 m³ in volume immediately below the
bore and flows to form a runoff channel in which a
temperature gradient from 88 °C to ambient is formed
as the waters cool. Approximately 10 g of the four dis-
tinctively colored, macroscopic mat communities that
develop in the runoff channel at specific temperatures
were collected. These include a grey mat community
that develops at 75 °C (sample referred to as Y), a red mat
community that develops at 66 °C (sample referred to as
R), a green mat community that develops at 57 °C
(sample referred to as G), and a brown mat community
that develops at 52 °C (sample referred to as B). A fifth
sample consisting of water was collected at the bore
source and the sixth sample consisting of water and
sediments was collected from the bottom of the concrete
pool, below the outflow source. All samples were col-
lected in 50 ml screw cap tubes, transported to the lab-
atory at Griffith University at ambient temperatures
and stored at 4 °C until used.
2.2. Microscopy and pigment analysis

Microscopic examination of the samples was performed as described [17,18]. Carotenoids and pigments from cell free supernatants of approximately 200 mg microbial mat samples or pure cultures that had been sonicated on ice (three bursts of 10 s each at full power) using a Soniprep 150 attached with a fine probe (MSE, USA) or had been extracted in acetone were analysed by scanning from 400 to 900 nm in a Cintara 20 double-beam UV–visible Spectrophotometer (GBC, Australia).

2.3. Media, enrichment, isolation identification and most probable number

Medium D broth was used for enrichment and isolation and prepared as described [1,23]. Five millilitre of the mat community samples were disrupted by sonication on

Fig. 1. The GAB of Australia is the world’s largest artesian aquifer occupying 22% of the Australian continent and extends into four of the seven states including Queensland, South Australia, South Australia and the Northern Territory. The direction of water flow (---), recharge areas along the Basin margin (shaded areas) and, natural discharge which occurs in the form of springs (●) with a number of springs in the same vicinity forming a super spring (<> ) are indicated. The New Lorne bore (registered bore number 17,263) which is situated near Blackall (24°54′48″ S, 145°08′18″ E), approximately 1000 km northwest of Brisbane, Queensland, Australia is shown (★).
ice (three bursts of 10 s each at full power) using a Soni-prep 150 attached with a fine probe (MSE, USA). One millilitre of the sonicate, source water sample and sample collected below the source were inoculated into 9 ml of medium D and a 10-fold serial dilution to $10^{-5}$ in medium D prepared. Enrichments were initiated by incubation of the 10-fold serial dilutions at 55 and 70 °C for up to 72 h. Pure isolates were obtained by streaking positive enrichment cultures from a randomly selected range of 10-fold serial dilutions on to medium D plates amended with 2% agar, followed by incubation at the enrichment temperatures. Single, well-separated distinct colonies were picked and subcultured in medium D. This procedure was repeated at least twice before the culture was considered pure. Pure cultures obtained were stored in a 50:50 (v/v) medium D-glycerol mix at −20 °C.

Identification of *Thermus* and *Meiothermus* was confirmed by the presence of yellow and red colored colonies which developed at 70 and 55 °C respectively. Acetone cell free extracts of these colonies showed characteristic carotenoid spectra of a peak at 455 nm and a shoulder at 480 nm typical for members of *Thermus* and a spectrum of a peak at 480 nm and a shoulder at 435 nm which typifies *Meiothermus*. Electron microscopy of thin sections of cells showed a typical annelid cell envelope structure. Most Probable Numbers (MPN) were estimated from the highest 10-fold dilution of the mat sample from which *Thermus* and *Meiothermus* were identified.

### 2.4. DNA extraction

DNA was extracted from pure cultures and environmental samples following a modification of Marmur’s method [24]. Cell lysis was observed by phase-contrast microscopy and by checking for increase in viscosity. Ten to thirty millilitre of a late log phase cell culture (24–48 h old) or 1 ml of microbial mat cell homogenate were harvested by centrifugation, the pellet resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) containing lysozyme (0.75 mg ml$^{-1}$) and achromopeptidase (0.75 mg ml$^{-1}$). After incubation at 37 °C for 1 h, SDS and Proteinase K were added to a final concentration of 0.5% and 0.1 mg ml$^{-1}$ respectively. After incubation for a further 1 h at 50 °C, NaCl and CTAB were added to final concentrations of 0.7 M and 1% respectively, the mixture vortexed and incubated at 65 °C for 10 min. RNase A was added to a final concentration of 200 μg ml$^{-1}$ and incubated for 30 min at room temperature. DNA was extracted with an equal volume of chloroform:iso-amylo-alcohol (24:1 v/v), followed by an extraction with phenol:chloroform:iso-amylo-alcohol (25:24:1 v/v). DNA was precipitated with isopropanol and pelleted by centrifugation. The DNA pellet was washed with 70% ethanol, dried and resuspended in TE buffer. The integrity of the DNA was checked by agarose gel electrophoresis [25].

### 2.5. 16S rRNA gene amplification, cloning and sequencing

The Polymerase chain reaction (PCR) was used to amplify the 16S rRNA genes from the DNA of pure cultures or the environmental communities as previously described [26,27]. The ensuing PCR products were purified using QiaQuick® PCR Purification Spin Columns as per manufacturer’s instructions (Qiagen, Germany).

Purified PCR products obtained from the six environmental samples were ligated into the pGEM®-T Easy Vector according to the manufacturer’s instructions (Promega Corporation, USA). One microlitre of the ligation was mixed with a 50 μl of freshly prepared competent *Escherichia coli* XL-10 cells in a BioRad Gene Pulser® cuvette with an electrode gap of 0.2 cm and electroporated using a BioRad Gene Pulser® II at 1.75 kV, 25 μF capacitance and 200 Ω resistance with time constants ranging from 4.3 to 4.8 s. Following electroporation the cells were diluted 20-fold in SOC medium (tryptone 2 g, yeast extract 0.2 g, 1 M NaCl 1 ml, 1 M KCl 0.25 ml, 1 M MgCl$_2$·6H$_2$O, 1 M MgSO$_4$·7H$_2$O, 1 ml, 1 M Glucose 2 ml, deionised water to 100 ml), incubated for 90 min at 37 °C with shaking and the culture plated on Luria–Bertani (LB) agar plates (tryptone 10 g, yeast extract 5 g, NaCl 5 g, agar 15 g, deionised water to 1 l) supplemented with 100 μg/ml ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Positive clones were picked after overnight incubation at 37 °C using blue/white colour selection. These cultures were subsequently regrown in LB broth containing 100 μg ml$^{-1}$ ampicillin overnight at 37 °C with shaking and used for plasmid DNA purification using Qiagen’s QIAprep® Miniprep columns following the manufacturer’s instructions.

The purity and concentrations of PCR products and plasmids were checked using agarose gel electrophoresis using routine methods [25].

Sequence reactions were prepared using 4 μl of ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Mix and the recommended protocols for PCR products and purified plasmids. The sequence products were purified using ABI’s recommended ethanol precipitation for BigDye™ Terminators. Electrophoresis was performed on an Applied Biosystems 377 DNA sequencer with 96-lane upgrade.

Raw sequence data were imported into the sequence editor BioEdit v5.0.9 [28], base calling examined and a contiguous consensus sequence generated. Using blastn against the GenBank database [29,30], the nearest relatives of these consensus sequences were identified. The consensus sequences were automatically aligned using the RDP’s *Sequence Aligner* program [31] and subse-
the five environmental samples were estimated by Thermus Spectral analysis and the concentration (cfu/g wet weight of sediment or microbial mat) of Table 1 3.2. Studies on culturable Thermus morphological diversity and density. whereas, as expected, the mat samples had a very high source and the sediment sample revealed a low mor-

amination of the water sample collected at the bore cell-free mat supernatants (Table 1). Microscopic ex-

difference was reflected in the spectral analysis of the indicated that all four mats had distinct colors and this channel of the New Lorne Bore. Visual inspection in-

with a distinct temperature, were observed in the runoff spatially separate from each other and each associated below the source. However, four mats, which were

3. Results 3.1. Microscopy and examination of mat pigments Microbial mats were absent at the bore source or below the source. However, four mats, which were spatially separate from each other and each associated with a distinct temperature, were observed in the runoff channel of the New Lorne Bore. Visual inspection indicated that all four mats had distinct colors and this difference was reflected in the spectral analysis of the cell-free mat supernatants (Table 1). Microscopic examination of the water sample collected at the bore source and the sediment sample revealed a low morphological diversity with very few cells being present, whereas, as expected, the mat samples had a very high morphological diversity and density.

3.2. Studies on culturable Thermus and Meiothermus The total numbers of Thermus and Meiothermus in the five environmental samples were estimated by checking for the presence of typical yellow and/or red colored colonies on agar plates that had been streaked from positive serial dilution enrichment cultures and the results of these studies are presented in Table 1. Thermus species were absent from the sediment sample but were cultured from all the four microbial mat samples. Their numbers in the grey and red mats were approximately 100–1000 times higher than the numbers in the green and brown mats. Thermus strains were isolated from the grey mat sample but not from the red, green or brown mat samples at an incubation temperature of 55 °C, but the numbers were approximately 10–100 times less than when incubated at 70 °C. Meiothermus was found to be present only in the red mats incubated at 55 °C but their numbers were approximately 10 times less than the numbers of Thermus in the same mat.

A total of 59 colonies were isolated from the serial dilutions of the four microbial mat samples incubated at 55 and 70 °C for three days. Fourteen isolates were randomly selected and identified as Thermus (Y70-05, Y70-06, Y70-07, R70-05, R70-06, G70-05, G70-06, G70-07, G70-08, B70-04, B70-05, Y55-07, Y55-08 and Y55-09) and a further two as Meiothermus (R55-10 and R55-11). The identification of Thermus and Meiother-

mus was confirmed by the presence of yellow and red colored pigmented colonies after incubation at 70 and 55 °C respectively. Spectral analysis of acetone extracted pigments from these colonies showed a peak at 455 nm and a shoulder at 480 nm typical for members of Thermus and a spectrum with a peak at 480 nm and a shoulder at 435 nm which typified Meiothermus (Table 1). Phase contrast microscopy showed distinctive thin cells (3–100 μm × 0.5 μm) which lacked spores and electron microscopy of thin sections showed annelid cell envelope structures, all of which are typical characteristics of members of the genus Thermus and Meiother-

mus. The remaining 43 isolates produced opaque white

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Absorbance pattern (nm) and presumptive identification</th>
<th>Thermus cultured at 70 °C</th>
<th>55 °C</th>
<th>Meiothermus cultured at 55 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source water sample (89 °C)</td>
<td>Not performed</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bore pool sediment (88 °C)</td>
<td>Not performed</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grey mat (75 °C)</td>
<td>Peak at 455 and shoulder at 480; Thermus species</td>
<td>1 × 10^7</td>
<td>1 × 10^6</td>
<td>0</td>
</tr>
<tr>
<td>Red mat (66 °C)</td>
<td>Peak at 480 and shoulder at 435; Meiothermus species. Trace 662 (chlorophyll a) cyanobacteria.</td>
<td>2.5 × 10^7</td>
<td>0</td>
<td>2.5 × 10^6</td>
</tr>
<tr>
<td>Green mat (57 °C)</td>
<td>[432, 455, 480 Thermus/Meiothermus]; [630 (phycocyanin) 662 (chlorophyll a) cyanobacteria]; [745 (bacteriochlorophyll c) 805 (bacteriochlorophyll a) Chloroflexus species]</td>
<td>8 × 10^7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brown mat (52 °C)</td>
<td>[630 (phycocyanin)]; [662 (chlorophyll a) cyanobacteria]; [745 (bacteriochlorophyll c) 805 (bacteriochlorophyll a) Chloroflexus species]</td>
<td>7 × 10^7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
colonies containing sporulating cells whose diameter was >1 μm suggesting that these isolates were most likely to be members of the genus *Bacillus*, and hence were not studied any further.

16S rRNA gene sequences of 13 of the 14 *Thermus* isolates were found to be related to the cluster A represented by *Thermus igniterrae* species (similarity 100%) and the sequence of the additional isolate B70-05 was...
closely related to cluster D represented by *T. scotoductus* (similarity of 99.1%) (Fig. 2). The 16S rRNA gene sequences of isolates R55-10 and R55-11 were closely related to *M. ruber* (similarity of 100%) (Fig. 3). These results support the earlier tentative identification of the isolates that had been based on cellular and colony characteristics.

### 3.3. Culture-independent studies

A total of 367 16S rRNA gene clones were generated from the DNA of the samples. Of these, 46 clones were from the sediment sample collected below the bore source, 72 clones from the grey mat, 68 clones from the red mat, 88 clones from the green mat and 93 clones from the brown mat. The remaining 36 clones represented other phyla (Table 2).

![Phylogenetic analysis of *Meiothermus*-related prokaryotes from the GAB.](image)

**Fig. 3.** Phylogenetic analysis of *Meiothermus*-related prokaryotes from the GAB. The dendrogram was created using nine database sequences, two GAB isolate sequences, and four GAB clone sequences. The unambiguous masked data set included 1410 bp. Sequences were obtained from the RDP-II and Genbank. Accession numbers are in brackets. *D. radiodurans* DSM 20539\(^T\) (Y11332), *M. ruber* strain Loginova 21 ATCC 35948\(^T\) (Z15059), *M. rosaceus* strain RH99-01 (AF312766), *M. silvanus* strain V1-R2 DSM 9946\(^T\) (X84211), *M. chliarophilus* strain ALT-8 DSM 9957\(^T\) (X84212), *M. ruber* strain 16105 (Y13596), *M. cerberus* strain GY-1 DSM 11376\(^T\) (Y13594) and *M. rosaceus* strain RH9901 (AF312766, China).

**“”** indicates that the strain has not yet been taxonomically validated.

#### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phylogroups represented by (^\text{a})</th>
<th>T. scotoductus</th>
<th>T. igniterrae</th>
<th>T. oshimae</th>
<th>Strain SRI-248</th>
<th>M. ruber</th>
<th>M. cerberus</th>
<th>Other phyla (^\text{a})</th>
<th>Chimera (^\text{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grey mat (75 (^\circ)C)</td>
<td>Isolates (^\text{b})</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>% clones</td>
<td>59</td>
<td>14</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>16</td>
<td>9</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Clones (^\text{c})</td>
<td>Y03</td>
<td>Y10, Y88, Y90</td>
<td>Y71</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Red mat (66 (^\circ)C)</td>
<td>Isolates</td>
<td>R70-05, R70-06</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>R55-10, R55-11</td>
<td>47</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>% clones</td>
<td>4</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>R03</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Clones</td>
<td>R58</td>
<td>R75</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Green mat (57 (^\circ)C)</td>
<td>Isolates</td>
<td>–</td>
<td>G70-05 to -08</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>% clones</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>72</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Clones</td>
<td>–</td>
<td>–</td>
<td>G24</td>
<td>–</td>
<td>G21</td>
<td>G34</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Brown mat (52 (^\circ)C)</td>
<td>Isolates</td>
<td>B70-05</td>
<td>B70-04</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>34</td>
<td>45</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>% clones</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Clones</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>– B01</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>– indicates zero clones or isolates identified.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) The source water sample (89 \(^\circ\)C) and sediment sample (88 \(^\circ\)C) did not contain culturable or culture-independent representatives of *Thermus* and *Meiothermus*.

\(^{\text{b}}\) Isolates or clones related to the phylogroups represented by *T. brockianus*, *T. aquaticus*, *T. antranikianus*, *T. filiformis*, *T. thermophilus*, *M. silvanus* and *M. chliarophilus* were not found.

\(^{\text{c}}\) Data includes all other phyla except *Thermales*.

\(^{\text{d}}\) The scheme used for naming the isolates is as follows: The alphabet indicates the mat color code (Y = grey mat, R = red mat, G = green mat and B = brown mat), the temperature of incubation (70 or 55 \(^\circ\)C) followed by the isolate number.

\(^{\text{e}}\) The clones are designated by the mat color followed by the clone number.
were from the brown colored mat. All 367 clones were partially sequenced (≈1000 nucleotides) and the sequences from each of the mat having a similarity >98% were regarded as phylogenetically identical and grouped as a phylgroup. A representative from each phylgroup was selected and completely sequenced. A significant fraction of the 367 clones were found to represent Thermus (17% of the library) and Meiothermus (22% of the library) with the remaining clones were either chimeras or were related to other phyla (Table 2).

Collectively, four distinct Thermus phylgroups were present in the library clones. Of these, the phylgroup related to T. scotoductus (63%) was the most dominant, followed by T. igniterrae related phylgroup (17%) with Thermus strain SRI-248 and T. oshima related phylgroups being represented to a much lower extent (1% and 2% respectively). When sequences from clones of individual mat samples were compared, Thermus was found to dominate the grey mat (75% of the total library), followed by the red mat (7% of the library) and the green mat (1% of the library) but Thermus were absent from the brown mat and the sediment sample. In the grey mat library, T. scotoductus dominated over T. igniterrae whereas in the red mat T. scotoductus was marginally dominated by T. igniterrae.

Collectively, two distinct phylgroups of Meiothermus, namely M. ruber and M. cerberus, were present in the mats. When individual mat samples were compared, Meiothermus species was found to dominate the red mat (47%), followed by the brown mat (34%) and then by the green mat (6%). The red mat was dominated exclusively by the M. ruber cluster, the brown mat exclusively by M. cerberus and the green mat by a mixture of M. ruber and M. cerberus in which M. ruber dominated by a factor of 2.

4. Discussion

We have previously reported that Thermus species are widely represented in the waters of the subsurface non-volcanically heated GAB of Australia [18] and that novel species may exist [17]. We present in this report detailed culture-dependent and culture-independent studies undertaken on a bore runoff channel in which temperature gradient associated microbial mats develop.

4.1. The GAB bore environment, microbial mats and their pigments

Mats which develop around the edges of volcanic hot springs where the temperatures are conducive for their growth have a gelatinous consistency [22,37]. The grey and red microbial mats observed in the GAB New Lorne Bore runoff channel have a consistency that resembles streamers and therefore are different to those found in volcanic hot spring environments. However, the GAB green and brown mats have a gelatinous consistency and resemble their volcanic counterparts. Acetone extracts and sonicated cell-free extracts of the mats showed that the grey mat had a typical spectrum for members of the genus Thermus whereas that of the red mat had a profile similar to that of Meiothermus species. The green mat had a mixed profile indicative of the presence of Thermus/Meiothermus, Chloroflexus species and cyanobacteria whereas that of the brown mat was indicative for the presence of Chloroflexus species and cyanobacteria (Table 1).

4.2. Culture-dependent and culture-independent diversity of Thermus and Meiothermus

Thermus species were isolated from the four microbial mats developing at temperatures between 52 and 75 °C, but not from the 89 °C water sample or the 88 °C sediment sample taken directly below the bore source. Thermus species were the most dominant in mats growing at temperatures between 66 and 75 °C with concentrations 100–1000 times greater than those mats developing at 52 or 57 °C. These results also concur with our culture-independent studies in which far higher numbers of clones of Thermus were found in the grey and red mats than in the green and brown mats. Our present studies therefore confirm previous findings which had shown that Thermus could be cultured from volcanic hot springs with temperatures between 52 and 75 °C [9] but not from hot springs with temperatures greater than 85 °C [38,39]. Both the culture-dependent studies carried out at 70 °C and culture-independent studies indicated that the highest counts of Thermus were from the grey mat samples. Thermus were also only detected from the grey mat samples but not from the cooler mat samples in enrichments incubated at 55 °C suggesting that high numbers were present in the grey mat presumably to active growth at 75 °C. These results suggest that 75 °C is the preferred habitat for Thermus in this GAB environment. Previous studies have also shown that Meiothermus can be isolated from volcanic hot springs with temperatures less than 70 °C and it was suggested that they were adapted to a lower temperature mode of life than Thermus species [5]. The isolation of strains R55-10 and R55-11 from the red mat at 66 °C and the detection of Meiothermus in the 16S rRNA gene clone libraries from the red, green and brown mats with temperatures less than 66 °C, but not from the 75 °C grey mat, provides further evidence for their ecological limitation to temperatures at or below 66 °C. The data presented here confirms that temperature limits the growth of Thermus and Meiothermus in thermally heated environments such as the thermal subsurface derived waters of the GAB and is somewhat similar to that observed for volcanic hot springs.
The phylogenetic diversity of *Thermus* and *Meiothermus* members in the clone library was higher than that obtained through culture-based studies. Loss of prokaryotic diversity through enrichment is well documented [40,41]. More specifically, Saul et al., [12] demonstrated that enrichment processes yield a dominant *Thermus* phylotype. Their results showed that very minor differences in 16S rRNA sequence signified a phenotypic diversity in the natural environment that is not revealed during the enrichment process due to the selection of dominant strains. In our studies, although *T. scotoductus* phylotypes were detected in the grey and red mat clone libraries (59% and 4% respectively), the enrichment culture was dominated by *T. igniterrae* isolates, which only accounted for 14% and 3% of the clones of each respective library. *T. igniterrae* was also isolated from the green and brown mat communities, but was not detected in their respective clone libraries. Therefore, data from both the culture and the clone libraries studies suggest that the populations of *T. igniterrae* occurs at much higher concentrations in the high temperature grey and red mat communities, than the low temperature green and brown mat communities. *M. ruber* and *M. cerberus* phylotypes dominated the red mat clone library (47% of clones) and the brown mat clone library (34% of clones) respectively. However, only strains of *M. ruber* could be isolated from the red mat community. Although the majority of clones in the red mat clone library were *M. ruber*, cell numbers indicate that the population of *M. ruber* is 10 times less than the population of *T. igniterrae* in the same sample. This also confirms other reports of biases being introduced in both culture based, and culture-independent techniques when analysing environmental communities. We were unable to demonstrate the presence of all three phylotypes of *Thermus* found in the clone library studies and only a single dominant phylotype was cultured. This was also the case for *Meiothermus*, which were detected in three of the five clone libraries, but only one phylotype could be cultured. However, it should be noted that culturing at 55 °C proved to be difficult as plates were invariably overgrown with *Bacillus* making the task of detecting *Meiothermus* very difficult and this could be the probable cause of the failure to detect other *Meiothermus* phylotypes.

4.3. The natural habitat of Thermus and Meiothermus species-transient surface dwellers or dwellers of the deep subsurface

Members of *Thermus* and *Meiothermus* were not detected in the New Lorne Bore source waters samples or water collected directly below the bore source by culture-dependent and culture-independent methods. This could lead one to suggest that they are not deep dwelling resident flora but are merely surface dwelling opportu-nistic microbes, which have the ability to colonize the runoff channel. Therefore the reason for their absence from bore source waters. However, our previous findings based on plasmid profiling [18], DNA probe techniques [17] and 16S rRNA gene library construction and sequencing [18,42], showed that *Thermus* and *Meiothermus* were present in thermal waters collected from the low temperature bore source waters (52–72 °C), as well as from the runoff channels suggesting that the members of these genera were indigenous residents of the deep subsurface GAB environment. These bores were chosen for our earlier work as they approximated the growth temperature optima for these genera but our present studies have been conducted with a high temperature bore (89 °C), whose temperature lies on the outer fringes of the growth temperature range [9,22,43]. It is known that GAB is a complex hydrogeochemical environment in which temperatures can vary from 30 to 100 °C [44]. There is also evidence of the presence of *Thermus* in other subsurface environments but this proof comes from culture-independent rather than culture-dependent studies. *Thermus* clone H21.73.1 (cluster F, Fig. 2) was identified in a 16S rRNA gene library prepared from DNA extracted from the biomass of a 188 m paleosol ([45]. We suggest that *Thermus* and *Meiothermus* could thrive in the cooler parts of the GAB environment from where they are subsequently transported and seeded on to the surface via bores to colonize and form mats in appropriate niches of the runoff channel. It is possible that we overlooked their presence in the samples collected at the bore source and below the source due to their low numbers. Concentrating the cells from these samples may need to be done in future.

4.4. Biogeography and chemical adaptation

Ecologically, the hot spring ecosystem can be considered as isolated islands, which provide barriers for the dispersal of microbes. The sporadic distribution and discontinuous nature of these islands throughout the world have provided a unique opportunity for studying the speciation of *Thermus* species. Pulsed-field gel electrophoretic studies of six species of *Thermus* has revealed a closely related gene organisation structure in isolates of the same species, especially if they were isolated from the same thermophilic environment [46]. It has been shown that there was a distinct correlation between the pH and temperature of the environment and phylogenetic clusters observed [43]. *Thermus* phylogeny based on 16S rRNA gene sequence analysis also displays geographic clustering [10,21]. Our culture-independent studies reported here indicated that the majority of 16S rRNA sequences from the four cloned microbial mat libraries had a very restrictive phylogenetic relationship to most of the other geographical relatives. They were closely related to the *T. igniterrae*
and Thermus strain SRI-248 clusters which are solely represented by Icelandic isolates and the T. scotoductus and T. oshimai clusters which are represented by the visibly endemic Thermus species isolated from Japan, South Africa, New Mexico, Taiwan and Portugal but not to, for example, T. brockianus and T. aquaticus, which have only been found in Yellowstone National Park or to the T. filiformis cluster which has so far been reported only from New Zealand. The clustering of a majority of the Thermus isolates and Thermus clones to the Icelandic Thermus clusters represented by T. igniiterreus and Thermus strain SRI-248 is not due to a bias due to lack of available data as other well studied thermal environments such as Yellowstone National Park and New Zealand thermal environments were also included in our phylogenetic studies. We suggest that this may be a result of similar geochemical attributes of the Iceland and the GAB thermal environments as both are alkaline and have very low sulfide concentrations [9,47]. Icelandic Thermus and Meiothermus strains have a much greater diversity than their New Zealand or Yellowstone National Park counterparts. However, our studies have focused on a single site and therefore may not yet represent fully the Thermus and Meiothermus diversity present in the GAB.

The GAB Meiothermus 16S rRNA gene sequences were predominantly related to M. ruber and M. cerberus. So far, the M. ruber cluster contains representatives from China, Russia and Iceland whereas the M. cerberus cluster has so far only been restricted to members from Iceland and Yellowstone National Park. The other two species of M. chlorophilus and M. silvanus have only been reported from Iceland and none of the GAB isolates are represented in these clusters. In general, Meiothermus distribution studies have not been exhaustive. A number of strains have been isolated from volcanic hot springs including some from New Zealand but detailed studies have not been conducted (Patel, unpublished results). Given the lack of available data, no firm conclusions can be drawn on the endemism or geographical clustering of Meiothermus.

In conclusion, we have demonstrated by spectral analysis, culture-dependent enumeration, isolation and phylogeny procedures and culture-independent phylogeny methods that the grey mat (75 °C) is dominated by Thermus and the red mat (66 °C) by Meiothermus making these mat environments unique and distinct from all other microbial mat environments reported to date.

Acknowledgements

The Department of Natural Resources and the staff at Terrick Terrick Stud are acknowledged for their help and assistance on site. This work was funded by an Australian Research Council project grant.

References
