

16S Ribosomal RNA-Based Analysis of Thermophilic Bacteria in Gedongsongo Hot Spring

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Denaturing gradient gel electrophoresis was used to identify the bacterial community at the Gedongsongo (WGS-2) hot spring. The bacterial samples were obtained from both culture dependent and independent strategies. Partial 16S rRNA genes were amplified by a set of primers to produce at around 400 bp fragments, including the highly variable V9 region of the 16S rRNA genes. The DGGE profiles showed that there were a few distinct bands, namely G1-G3, and G8-G12, which represent the predominant bacteria in natural habitat and the medium. Further analysis of these bands showed that most of them, except for G7, have a high homology to the 16S rRNA gene sequences of *Thermus* sp. As for G7, the highest homology was shown to unculturable bacteria. In addition to the distinct bands in DGGE, there were other three thin bands, namely G4, G5, and G6, which possibly represent non dominant microorganisms in the natural habitat, but could grow on GS-A medium. Further analysis of these bands showed that G6 has 80% similarity to the 16S rRNA of *Burkholderia* sp., while G4 and G5 have a high homology to each other but only contained 10-15% homology to the sequences of 16S rRNA from unculturable microorganisms. The phylogenetic analyses of the last organisms showed that there was branching from *Burkholderia*. From all the data obtained it was suggested that the WGS-2 hot spring was predominantly occupied by the genus *Thermus*. In addition, there were a few novel microorganisms found in the hot spring.

Key words: thermophiles, 16S rRNA, DGGE

One of the challenges in modern microbial ecology is how to effectively and accurately assess microbial diversity. A common thread described in numerous published studies concerning microbial diversity is that, in most environments, only 0.1 to 1.0% of bacteria detected by direct microscopic enumeration can be recovered on even the most general laboratory media (Staley and Konopka 1985). As a result, microbial ecologists generally are of the opinion that the vast majority of microbial diversity remains uncharacterized due to this gap between cultivable and direct estimation of microbial biomass and diversity. This concern has spurred on the development of molecular approaches for studying microbial communities, usually based on analysis of nucleic acids directly extracted from environmental samples (Pace *et al.* 1986). The application of molecular phylogenetic to study natural microbial ecosystems without the traditional requirement for cultivation has resulted in the discovery of many unexpected evolutionary lineages; members of some of these lineages are only distantly related to known organisms but are sufficiently abundant that likely to have impact on the chemistry of biosphere (Pace 1997).

The classical approach to determine the microbial diversity in a natural or artificial ecosystems starts with culturing of the microorganisms in a sample. Culture-based approaches to isolate microorganisms from any natural environment do not provide comprehensive information on the composition of microbial communities (Bull and Hardman 1991). This technique also fails to determine the majority of microorganisms in nature, which typically not cultivated using standard techniques (Amann *et al.* 1995). It has been

realized that only a minor fraction of the microorganisms (1-5%) are amenable to standard culturing techniques (Schmidt *et al.* 1991; Liesack and Stackebrandt 1992; Fuhrman *et al.* 1993). Due to this difference between cultivable and *in situ* diversity, it is often difficult to assess the significance of cultured members in resident microbial communities. In order to overcome the limitations associated with cultural approaches, a molecular alternative has been developed. The development of techniques for the analysis of 16S rRNA sequences in natural samples has enhanced our ability to detect and identify bacteria in nature (Pace *et al.* 1986). This involves DNA extraction of community directly from water, soil or sediments followed by PCR amplification and then sequencing of 16S rRNA genes, which are known to be one of the established phylogenetic markers (Woese 1987). Such approaches have been successfully applied for hot spring (Ferris *et al.* 2003), compost (Ueda *et al.* 2001), marine bacteria-plankton (Fuhrman *et al.* 1993), soil (Nakatsu *et al.* 2000) and as well as hydrothermal environment (Moyer *et al.* 1995) samples.

Muyzer *et al.* (1993) presented a molecular approach for analyzing the genetic diversity of complex microbial populations called denaturing gradient gel electrophoresis (DGGE) at the first time. Separation fragments in DGGE based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants. This technique is now routinely used in many microbiological laboratories as a tool to compare the diversity of microbial communities and to monitor population dynamics (Muyzer 1999). This approach has now been variously combined with group-specific amplification (Heuer *et al.* 1997), membrane transfer and hybridization (Stephen *et al.* 1998), band excision

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and sequence analysis (Kowalchuk *et al.* 1997; McCaig *et al.* 1999), and with culturing methods (Watanabe *et al.* 1998). In this report we will describe the bacterial community inhabiting a hot spring at Gedongsongo, Ungaran, Central Java.

MATERIALS AND METHODS

Sampling. Sampling was carried out on one of the hot spring, namely WGS-2, at Gedongsongo that located along the southern flank of Ungaran volcano, Central Java (110°20'23.4"E; 07°12'08.5"S; and the altitude 1400 m). This hot spring has temperature at 70.2 °C and a pH 5.85.

For the filtration procedure, the water sample was kept in a sterile plastic container and brought to the laboratory immediately within 2 hours. Afterwards, cells were harvested by filtration of 1 L volumes of spring water gently through 0.2 µm Millipore filters. Filtrates containing bacterial communities were stored at -20 °C until DNA was extracted. The cultivation procedure was carried out using two minimal media. The GS-A medium contains 0.15% (w/v) tryptone and 0.15% (w/v) yeast extract, while the GS-B medium contains 0.5% (w/v) peptone, 0.25% (w/v) yeast extract, and 0.5% (w/v) NaCl. Both media, which used natural hot spring water, were incubated at 60 °C without shaking for 24 hours.

DNA Extraction. Cells from spring water that had been collected on a membrane filter were harvested by putting the filter in a sterile Erlenmeyer that contained 10 ml sterile distilled water. The Erlenmeyer was then shaken gently and the membrane surface was scrubbed aseptically using Ose needle for a couple times until almost all of the cells were suspended in water. Each microbial sample was pelleted by centrifugation at 8000 × g for 15 min. DNA was extracted using slight modification of the Klijn *et al.* (1991) method. The pellet was suspended in 200 µl of 10 mM Tris HCl buffer (pH 8.0) containing 8 mg ml⁻¹ of lysozyme and incubated at 37 °C for 1 h. The cells were lysed by adding 200 µl lysis buffer containing 2% (w/v) SDS, 0.8 mg ml⁻¹ proteinase K and 200 mM EDTA pH 8.0. The lysis process was carried out at 50 °C for 30 min. The purification step was performed by adding 200 µl of chloroform:isoamyl-alcohol (24:1), vortexed and then centrifugated at 12,000 × g, for 30 sec. The upper solution was moved to a clean tube. This step was done 3x. Subsequently, the DNA was precipitated by adding 60 µl of 3 M sodium acetate and 1 ml of 96% (v/v) ethanol (stored at -20 °C). After centrifugation at 12,000 × g for 15 min, the DNA pellet was washed with 70% (v/v) ethanol and finally dissolved in 50 µl of TE buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA).

Amplification of 16S rRNA Gene Fragments. Partial 16S rRNA genes was amplified as described previously by Ferris *et al.* (1996). A set primer (P1 and P2) was used to amplify the gene. One primer (P1: 5' ATGGCTGTCGTCAGCT 3') complements on the conserved region among the *Bacteria* domain of *Escherichia coli* 16S rRNA at positions 1055 to 1070. The other primer (P2: 5' CGCCCGCCGC GCCCGCGCC CGGCCCGCCG CCCCCGCCCC ACGGGCGGTGTGTAC 3') is based on a universal conserved region of the *E. coli* 16S rRNA at positions 1392 to 1406, with the addition of 40-bases of GC clamp. The specificity of this primers is imparted by

the underlined regions. PCRs were performed by using cloned *Pfu* DNA polymerase according to the instructions provided by the manufacturer (Promega). The temperature cycle for the PCR was 1 min of denaturation at 94 °C, 1 min of annealing, and 1 min of primer extension at 72 °C. During an initial touchdown cycle, the annealing temperature was continually decreased from 53 to 43 °C in intervals of 1 °C per cycle; 20 additional annealing cycles were done at 43 °C. The final extension at 72 °C was performed for 10 min.

Denaturing Gradient Gel Electrophoresis. All reagents and techniques were performed as described by Ferris *et al.* (1996) with small modification. Acrylamide gels (8%) were prepared and run with 0.5x TAE buffer (0.04 M Tris base, 0.02 M sodium acetate, and 1.0 mM EDTA; pH adjusted to 7.4). An Ingeny Phor U gel electrophoresis unit was used with glass plates (16 by 18 cm), 1.0-mm spacers, and 1-cm-wide loading wells. A 15 liter aquarium served as the lower buffer chamber. DGGE gels contained a 35 to 60% (w/v) gradient of urea and formamide (UF) solution increasing in the direction of electrophoresis. A 100% UF solution is defined as 40% (v/v) formamide plus 7.0 M urea. DGGE was conducted at 60 °C, firstly at 20V for 10 min and then at a constant voltage of 200 V for 3 hours. The gel was stained using a silver staining (Bassam *et al.* 1991).

Re-PCR of DGGE Bands. The bands (from the fresh gel) were cut and added 50 µl sterile deionized water and allowed DNA to passively diffuse into the water at 4 °C overnight and then 5 µl of eluted fraction was used as template DNA in a PCR (Ferris *et al.* 1996; Nakatsu *et al.* 2000). For the gels that keeps in dry form and storage for a couple months, these dry gels were placed in vial, added water and heated at boiling water for 5 minute. After that, the gels were punched using the end of tips and allowed to passively diffuse into the water at 37 °C overnight.

Sequence Analysis. All confirmed DGGE bands were subjected to double-stranded DNA sequencing. Sequencing were carried out in an ABI PrismR 3100 Genetic Analyzer (Applied Biosystem) by the Macrogen Sequencing Service (Korea). The sequencing reaction was performed with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem) using forward primer (P1). The sequencing results were subjected to analyzing and comparing to the GenBank (NCBI) using the BLAST N program (Atschul *et al.* 1997). The phylogenetic tree was constructed by ClustalW methods from MegAlign program of DNA STAR.

RESULTS

Chromosomal DNA and 16S rRNA Gene Fragments.

The cells from both filtration and cultivation were lysed to extract their total chromosomal DNA. According to examination on ethidium bromide-stained agarose gels, both cultivated and filtered cells were successfully extracted from the chromosomal DNA (data not shown). Total chromosomal DNA from both filtration and cultivation methods were used as templates to amplify partial 16S rRNA gene using primers P1-P2. These primers should amplify DNA fragment with the size 392 bp (including GC clamp). The PCR products of GS-A, GS-B, and GS-F cells were examined on a ethidium bromide-

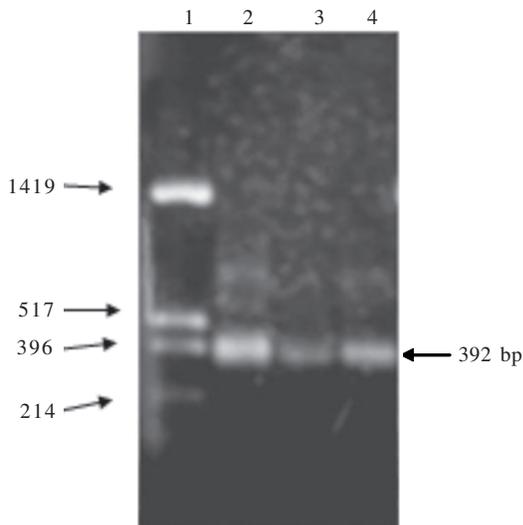


Figure 1 Electrophoregram of PCR product. Lane 1 shows plasmid DNA pUC19 digested with *Hinf*I; lane 2, 3, and 4 show PCR products from GS-A, GS-B and GS-F DNA respectively.

stained agarose gel, and the results showed a single band of the expected size as expected (Figure 1).

Profiles of 16S rRNA Gene Fragments. The profiles of the bacterial community of the WGS-2 hot spring from filtration and cultivation were shown in Figure 2. In order to get the finest profile of the bands, it has been carried out several electrophoresis procedures using different range of gradients. The best DGGE profile that is showed in this report was obtained using gels containing a 35 to 60% (w/v) gradient of urea and formamide. The DGGE patterns showed that there were three distinct bands that appear in each well (GS-F, GS-A, and GS-B). The other three weak bands (G4, G5, and G6) appeared in the GS-A well (Figure 2). The fragment DNA from G1-G12 bands was recovered from the DGGE gel and reamplified using primers P1 and P3. The sequence of P3 is similar to that of P2 but without the GC clamp (5' CACGGGCGGTGTGTAC 3'). The re-PCR amplicons appeared as single bands with the size as expected (data not shown). DNA fragments on DGGE could be stored as dry gels and were stable until a few months for reamplification (Figure 3).

DNA Sequences and Homology of 16S rRNA Gene Fragments. All of the re-PCR results (G1 to G12) have been sequenced and analyzed. Those sequences have been compared with another 16S rRNA gene sequence of domain *Bacteria* using the program BLASTN program at NCBI website. Most of the bands (G1, G2, G3, G8, G9, G10, G11, and G12) except for G7, showed high homology to each other and were closely related to 16S rRNA gene of *Thermus* species (Figure 4). However, homological study of the last four bands (G4, G5, G6, and G7) revealed some unique sequences that closely related to uncultured bacteria (Table 1, 2, 3, and 4). The nucleotide sequences of G4 and G5 are very similar to each other. Of 300 bp that has been compared around 15% of these sequence have match with 138 and 109 sequences of 16S rRNA gene to G4 and G5 respectively, at the downstream of the gene fragment. The sequence of G6 has 80% similarity to that of *Burkholderia* sp. CCBAU23014 (Table 3). The 300 bp of G7 sequence has only a 10% match

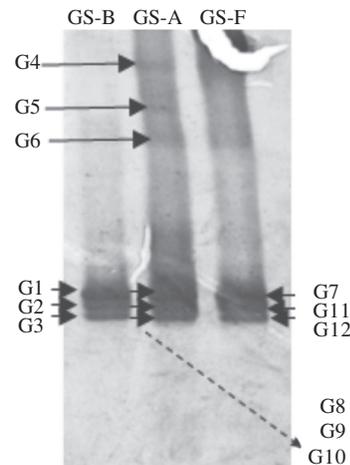


Figure 2 DGGE patterns of PCR-amplified 16S rDNA fragments. Well 1, 2, and 3 are 16S rDNA fragment from GS-B, GS-A, and GS-F respectively, while G1-G12 are bands position on DGGE.

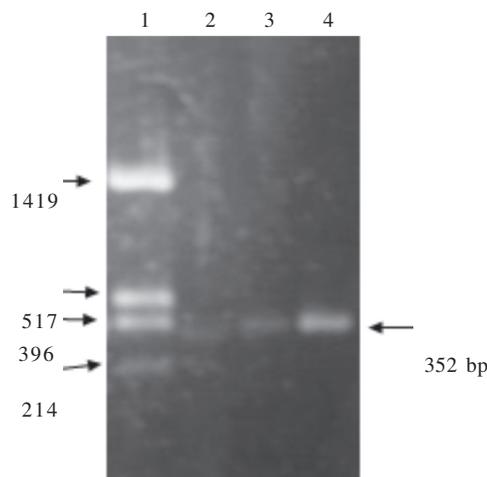


Figure 3 Electrophoregram of re-amplified DGGE bands (without GC clamp) using modified method of different age of dry gel. Lane 1 shows plasmid DNA pUC19 digested with *Hinf*I; lane 2 from gel age 3 months that stored in water and keep in -20 °C until 10 months, lane 3 from gel age 10 months and lane 4 showed fragment DNA from gel age 3 months.

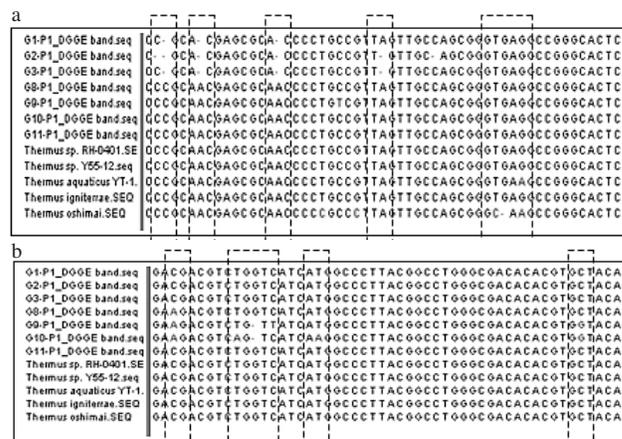


Figure 4 Comparison analysis of the sequence of G1, G2, G3, G8, G9, G10, G11, and G12 with some *Thermus* sp. using MegAlign Program. The difference are shown by dashed line box. a. The upstream part of 300 nucleotides. There are some deletion of G1, G2, and G3 that differ to other sequences. b. The downstream part of 300 nucleotides. There are some substitution of G8, G9, and G10 that differ to other sequences.

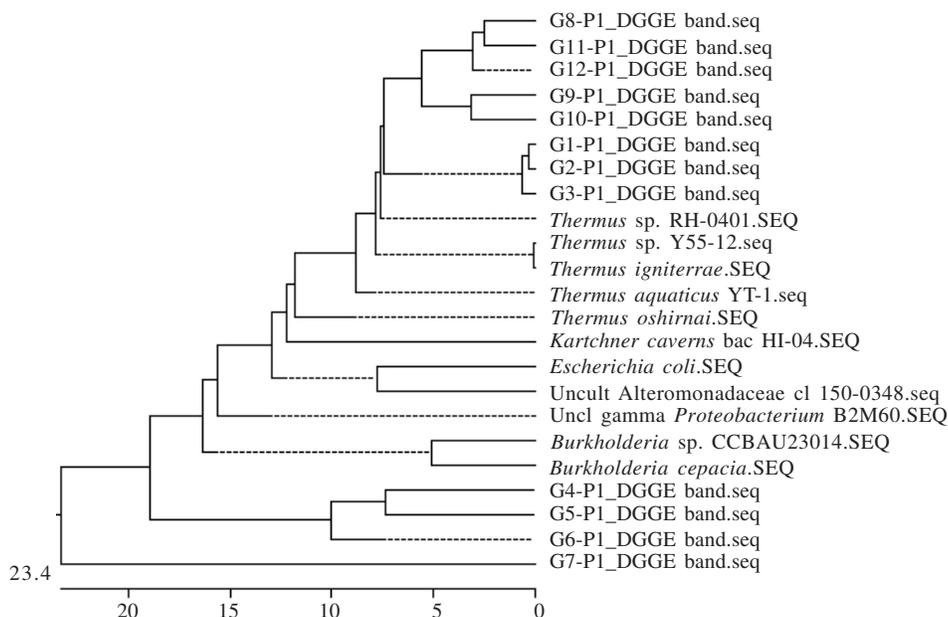


Figure 5 Phylogenetic tree of DGGE bands. The bands of G1, G2, G3, G8, G9, G10, G11, and G12 are closely related to *Thermus* sp. while G4, G5, G6, and G7 making some new branch.

Table 1 The comparison study of G4 sequence. About 15% of 300 bp at downstream position of G4 sequence have a consistency match with about 138 sequences of 16S rRNA gene of mostly uncultured bacteria. This table only shows ten of the highest homology sequence

Accession number	Sequence name	Score (Bits)
AF223299	Uncultured gamma <i>Proteobacterium</i> B2M60 16S ribosomal RNA gene, partial sequence	95.6
DQ419338	Uncultured gamma <i>Proteobacterium</i> clone ST9-55D 16S ribosomal RNA gene, partial sequence	95.6
AB167222	<i>Acinetobacter</i> sp. c61 gene for 16S rRNA, partial sequence	95.6
DQ341037	Uncultured Desulfohalobiaceae bacterium clone 2.88M_24B 16S ribosomal RNA gene, partial sequence	95.6
AF005092	<i>Clostridium</i> sp. SP3 16S ribosomal RNA gene, partial sequence	95.6
DQ458039	Uncultured bacterium clone DX49 16S ribosomal RNA gene, partial sequence	89.7
DQ458033	Uncultured bacterium clone DX63 16S ribosomal RNA gene, partial sequence	89.7
DQ458000	Uncultured bacterium clone DX53 16S ribosomal RNA gene, partial sequence	89.7
AF391977	Uncultured thermal soil bacterium clone YNPFFP50 16S ribosomal RNA gene, partial sequence	89.7
AY511742	Uncultured bacterium clone GC113.16 16S ribosomal RNA gene, partial sequence	89.7

with some 16S rRNA gene sequences from uncultured bacteria (101 related sequences), all of them showed a consistent match to upstream portion of the G7 sequence.

DISCUSSION

The microbial community was performed using culture-dependent and culture-independent methods. The culture-dependent method used enrichment media GS-A and GS-B. Both of them are not specific media. The use of these media was design to pick various kinds of microbes.

Table 2 The comparison study of G5 sequence. About 15% of 300 bp at downstream position of G5 sequence have a consistency match with about 109 sequences of 16S rRNA gene of mostly uncultured bacteria. This table only shows ten of the highest homology sequence

Accession number	Sequence name	Score (Bits)
AF223299	Uncultured gamma <i>Proteobacterium</i> B2M60 16S ribosomal RNA gene, partial sequence	93.7
AF005092	<i>Clostridium</i> sp. SP3 16S ribosomal RNA gene, partial sequence	93.7
DQ458039	Uncultured bacterium clone DX49 16S ribosomal RNA gene, partial sequence	89.7
DQ458033	Uncultured bacterium clone DX63 16S ribosomal RNA gene, partial sequence	89.7
DQ458000	Uncultured bacterium clone DX53 16S ribosomal RNA gene, partial sequence	89.7
DQ463208	Uncultured bacterium clone tIs3-13 16S ribosomal RNA gene, partial sequence	89.7
DQ463183	Uncultured bacterium clone tIs1-5 16S ribosomal RNA gene, partial sequence	89.7
DQ419338	Uncultured gamma <i>Proteobacterium</i> clone ST9-55D 16S ribosomal RNA gene, partial sequence	89.7
AB167222	<i>Acinetobacter</i> sp. c61 gene for 16S rRNA, partial sequence	89.7
AJ863184	Uncultured bacterium partial 16S rRNA gene, clone 20BSU24	89.7

The DGGE profile shown in Figure 2 revealed that three distinct bands from each well are aligning to each other and possibly mean that these bands represent the same and predominant bacteria that survive in this spring. However, the other three bands (G4, G5, and G6) in the GS-A well do not appear in GS-F, possibly due to fact that these bands represent microorganism that is not dominant in the spring but has high grown rate on GS-A medium.

From the results of BLASTN program analysis on 300 bp of each band, three distinct DGGE bands from each well, except for G7, are closely related to *Thermus* sp., the highest similarity of these sequences belongs to *Thermus* RH 0401 (accession number: AY731822). The analysis showed that

Table 3 The comparison study of G6 sequence. This sequence has matches to 163 sequences of 16S rRNA gene. The 300 bp of G6 sequence has the highest similarity (at around 80%) to the *Burkholderia* sp. CCBAU23014

Accession number	Sequence name	Score (Bits)
AY839565	<i>Burkholderia</i> sp. CCBAU23014 16S ribosomal RNA gene, partial sequence	131
AY839129	Uncultured bacterium clone PB 357-359/17 16S ribosomal RNA gene, partial sequence	119
AF223299	Uncultured gamma proteobacterium B2M60 16S ribosomal RNA gene, partial sequence	111
AF005092	<i>Clostridium</i> sp. SP3 16S ribosomal RNA gene, partial sequence	111
DQ228373	uncultured bacterium clone BG.d4 16S ribosomal RNA gene, partial sequence	109
AY842149	Endophyte bacterium SS14 16S ribosomal RNA gene, partial sequence	109
AY839125	Uncultured bacterium clone PB 357-359/25 16S ribosomal RNA gene, partial sequence	109
AY839234	<i>Pseudomonas aurantiaca</i> strain YC4963 16S ribosomal RNA gene, partial sequence	109
AY271791	<i>P. aurantiaca</i> VKM B-816T 16S ribosomal RNA gene, partial sequence	109
DQ444991	<i>Pseudomonas</i> sp. Eur1 9.41 16S ribosomal RNA gene, partial sequence	109

these bands have high similarity (97-99%) to at least 100 sequences of 16S rRNA genes of both culturable and unculturable of *Thermus* sp.

The comparison of G1-G3 and G8-G12 sequences toward some *Thermus* sp. sequences using MegAlign Program revealed some differences from each sequences (Figure 4). In spite of different position at the DGGE and there are some different nucleotides (substitution), the three bands from each well i.e.: G1-G2-G3, G8-G9-10, and G11-G12 seemed to represent the same species. A possible reason for the formation of more than one band from one species is that the universal primers amplified more than one operon (Schmalenberger *et al.* 2001). It is well known that several bacterial species contain more than one 16S rRNA gene in their genomes (Klappenbach *et al.* 2001). The heterogeneity of 16S rRNA gene between multiple copies within one species hampers pattern analysis (McCaig *et al.* 2001), and can confuse the interpretation of diversity from sequences retrieved from banding patterns.

Although there is single or more differences on base sequence of these DGGE bands to each other, all of them showed the same highest similarity with *Thermus* RH 0401. This evidence could not bring into the conclusion whether these DGGE bands sequence lead to the same species or not, but it could not be opposed that these bands represent *Thermus* species. The phylogenetic study that constructed using ClustalW method revealed that the sequence of eight distinct bands are close to *Thermus* group. This evidence could be seen in phylogenetic tree (Figure 5). These data suggested that this hot spring was possibly occupied by *Thermus* predominantly. In addition, data from both culture media are also dominated by these species.

The analysis G6 sequence revealed similarity to at least 163 sequences of 16S rRNA gene both cultivable and

Table 4 The comparison study of G7 sequence. About 10% of 300 bp at upstream position of G7 sequence have a consistency match with about 101 sequences of 16S rRNA gene of mostly uncultured bacteria. This table only shows ten of the highest homology sequence

Accession number	Sequence name	Score (Bits)
AY868092	Uncultured gamma <i>Proteobacterium</i> clone I50-0430 16S ribosomal RNA gene, partial sequence	71.9
DQ219828	Uncultured bacterium isolate DGGE band 5 16S ribosomal RNA gene, partial sequence	69.9
AY048188	Uncultured <i>Xanthomonas</i> sp. 16S ribosomal RNA gene, partial sequence	69.9
AY048191	Uncultured <i>Xanthomonas</i> sp. clone PR-VFA-19 16S ribosomal RNA gene, partial sequence	69.9
AY869139	Uncultured Alteromonadaceae bacterium clone I50-0558 16S ribosomal RNA gene, partial sequence	69.9
AY869123	Uncultured Alteromonadaceae bacterium clone I50-0411 16S ribosomal RNA gene, partial sequence	69.9
AY869663	Uncultured Alteromonadales bacterium clone I3K-0547 16S ribosomal RNA gene, partial sequence	67.9
AY869137	Uncultured Alteromonadaceae bacterium clone I50-0531 16S ribosomal RNA gene, partial sequence	67.9
AY869136	Uncultured Alteromonadaceae bacterium clone I50-0520 16S ribosomal RNA gene, partial sequence	67.9
AY869134	Uncultured Alteromonadaceae bacterium clone I50-0489 16S ribosomal RNA gene, partial sequence	67.9

uncultivable bacteria. This sequence has the highest similarity (80%) to *Burkholderia* sp. CCBAU23014 16S ribosomal RNA gene (accession number: AY839565.1) (Table 3). Actually, *Burkholderia* is a diverse genus with diverse species that live in diverse ecological niches. The molecular and physiological background of this diversity and adaptability are largely unknown. Multiple biotechnologically interesting strains belong to as yet uncharacterized taxa (Coenye and Vandamme 2003).

The other bands (G4, G5, G7) have a low similarity (about 10-15%) to some 16S rRNA gene sequences of unculturable bacteria. However, these sequences showed at consistent position with some 16S rRNA genes (Table 1, 2, and 4). Even though the similarity of these bands is only at around 10-15%, but all of them are similar to 16S rRNA genes and none of them represent another gene sequences (138 sequences for G4, 109 sequences for G5, and 101 sequences for G7). This data suggests that the G4, G5, and G7 sequences are part of the 16S rRNA gene.

From the result of the phylogenetic tree (Figure 5), the G4, G5, G6, and G7 made separate branch in the tree. The reason for the forming separated branch might due to some unique bases that unreaveled in in other 16S rRNA gene that compiled in BLAST. These unique bases include some substitutions along the sequence and the glaring difference of the insertion and deletion of the bases at the middle position of the sequences (the relative position of the *E. coli* at position 1250-1280). Surprisingly, although G6 has the highest similarity with the *Burkholderia* sp. CCBAU23014, this sequence did not share the same branch as some *Burkholderia* sp. in the tree. It suggested that G6

did not belong to 16S rRNA of *Burkholderia* species. This is supported by the analysis result which only showed similarity to *Burkholderia* sp. CCBAU23014 and not to another *Burkholderia*. In fact, the BLAST analysis of G6, as shown in Table 3 was moderately similar to *Pseudomonas* sp. Based on the phylogenetic tree and comparison study profile, the last four bands (G4, G5, G6, and G7) are highly recommended as 16S rRNA genes sequence from novel species. The far branch at phylogenetic tree for these four bands suggest that these bands representing not only a new species but possibly a new genus or family. These sequence analyses of rRNA genes from natural microbial communities have identified a broad diversity of previously unknown microorganisms. Currently, about half of the >70,000 rRNA sequences in the public databases represent uncultured microorganisms (Cole *et al.* 2003). More than one-third of the 40 to 50 main relatedness group, natural divisions, of the domain *Bacteria* are known only from detection of rRNA gene sequences and have no described cultivated representatives. These division-level clades with no cultured representatives, typically known from only limited numbers of rRNA sequences, have been termed candidate divisions to reflect the limited documentation that describes them (Hugenholtz *et al.* 1998).

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