

## **Differentiation of a Hyperthermophilic Archaeon *Pyrococcus* sp. strain Pikanate 5017, by Arbitrarily Primed PCR**

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### **Abstract**

*Pyrococcus* and *Thermococcus* are hyperthermophilic archaea in the order Thermococcales. Both genera are strictly anaerobes, gaining energy by fermentation of peptide and several carbohydrates at optimal temperature above 70 °C. A *Pyrococcus* sp. strain Pikanate 5017 (PK 5017) was recently isolated from a hot spring in Northern Thailand. The strain PK 5017 is a valuable source of numerous genes encoding thermostable enzymes. Growth kinetics determined at various temperatures (75-105 °C) indicates that strain PK5017 is a fast growing archaeon. An arbitrarily primed PCR (AP-PCR) technique was successfully applied to differentiate the genome sequences of six members of Thermococcales. Two single primers, ARB-1f (5' ATGAG GACT GAAA CCATT 3') and ARB-2f (5' GTAAA ACGA CGGC CAGT 3'), are effective in producing polymorphisms of the PCR products at 3-10 ng of DNAs. The unique AP-PCR fingerprints distinguish the strain PK5017 from *P. furiosus*, *P. horikoshii*, *P. abyssi*, *T. litoralis*, and *T. celer*. The results indicate a clear distinction of genome sequences among *Pyrococcus* and *Thermococcus* genera.

**Key Words:** Thermococcales; *Pyrococcus*; *Thermococcus*; fingerprint; hyperthermophile; hot spring

## Introduction

Arbitrarily primed PCR (AP-PCR) is a PCR based technique employing a single primer to amplify PCR products, representing fingerprints of genomes of organisms (Welsh and McClelland, 1990; Welsh et al., 1991; Keller et al., 1995). The technique has a number of applications in epidemiology, ecology, taxonomy, and organism identification. The technique is flexible because nucleotide sequences and lengths of the primers can be arbitrarily chosen and performed without prior sequence information. An oligonucleotide sequence of 5' GTAAA ACGA CGGC CAGT 3' was shown to be effective in distinguishing a marine hyperthermophilic archaeon, *Thermococcus alcaliphilus*, from its relatives (Keller et al., 1995).

Hyperthermophiles in the order Thermococcales are anaerobic, heterotrophic Archaea belonging to Euryarchaeota (Woese et al., 1990). *Pyrococcus* and *Thermococcus* are two closely related genera of hyperthermophilic archaea in the order Thermococcales. Both grow anaerobically by fermenting peptides and carbohydrates at optimal temperatures above 70 °C (Adams, 1994; Stetter, 1996). *Pyrococcus* species grow optimally above 90 °C. However, *Thermococcus* species grow optimally below 90 °C (Barbier et al., 1999; González et al., 1999). Both genera have highly homologous *16S rRNA* gene sequences, and can be distinguished using their optimal growth temperatures, physiological characteristics, and DNA properties. The typical ecological systems for the habitats of both genera include deep and shallow hydrothermal vents, geothermal subterranean and hot springs (Stetter, 1993; Zillig et al., 1983; Pikuta et al., 2007). Most members of Thermococcales are marine isolates and require approximately 3% (w/v) of NaCl for their optimal growth, including *Thermococcus*

*celer* (Zillig et al., 1983), *T. litoralis* (Neuner et al., 1990), *T. profundus* (Kobayashi et al., 1994), *T. chitonophagus* (Huber et al., 1995), *T. thio-reducens* (Pikuta et al., 2007), *Pyrococcus furiosus* (Fiala and Stetter, 1986), *P. woesei* (Zillig et al., 1987), *P. abyssi* (Erauso et al., 1993), *P. horikoshii* (González et al., 1998), and *P. glycovorans* (Barbier et al., 1999). However, freshwater species have been restricted to the genus *Thermococcus*, including *T. zilligii* (Ronimus et al., 1997), *T. waiotapuensis* (González et al., 1999), and *T. sibiricus* (Miroshnichenko et al., 2001). Among the marine *Pyrococcus* species, *P. furiosus* and *P. woesei* are two closely related species which were isolated from a shallow hot vent of Vulcano Island, Italy. The latter species was renamed as *P. furiosus* subsp. *woesei*. Both *P. furiosus* and *P. woesei* have identical *16S rRNA* gene operon, highly similar genome sequences, and several copies of the homologous insertion sequence of elements in many identical loci of their genomes (Kanoksilapatham et al., 2004). *P. abyssi*, *P. horikoshii*, and *P. glycovorans* were isolated from deep-sea hydrothermal vents of the North Fiji Basin in the South West Pacific Ocean, the Okinawa Trough in the North East Pacific Ocean, and the East Pacific Rise, respectively. A *Pyrococcus* sp. strain HT3 grew optimally at 80-85 °C, pH 7.5 and 1.5% NaCl was isolated from a hot spring in Northeast Algeria (Kecha et al., 2007). However, the optimal growth temperature reported is in the range of *Thermococcus* (below 90 °C). *Pyrococcus* sp. strain Pikanate 5017 was isolated from a fresh water hot spring in Northern Thailand (unpublished data).

This study has the objectives of 1) comparing various genome sequences of the members in the Order Thermococcales by using an AP-PCR technique with two arbitrarily single primers; and 2) characterizing growth kinetics on the temperature range of the strain Pikanate 5017.

## Materials and Methods

### Organisms

*Pyrococcus* sp. strain Pikanate 5017 was recently isolated from Pong Dueat Hot Spring (unpublished data). Microorganisms used in this study were *P. abyssi* (strain GE5; Erauso et al. 1993), *Pyrococcus furiosus* (DSM3638), *P. horikoshii* (DSM12428), *Thermococcus celer* (DSM2476), and *T. litoralis* (DSM5473).

### Cultivation

Medium is composed of (per liter) 24 g NaCl, 4 g Na<sub>2</sub>SO<sub>4</sub>, 0.7 g KCl, 0.2 g NaHCO<sub>3</sub>, 0.1 g KBr, 0.03 g H<sub>3</sub>BO<sub>3</sub>, 10.8 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.5 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.025 g SrCl<sub>2</sub>·6H<sub>2</sub>O, 5 g tryptone, 1 g yeast extract, 1 ml resazurin solution (0.2 g l<sup>-1</sup>), 3 ml Na<sub>2</sub>S·9H<sub>2</sub>O solution [25% (w/v), pH 7], and 5–10 g elemental sulfur. Cultures were inoculated in serum bottles containing 100 ml of the medium, and incubated anaerobically at appropriate temperatures (95 °C for *Pyrococcus*, and 85 °C for *Thermococcus*).

### Phylogenetic study

The *16S rRNA* gene sequence (total of 1330 bp long) from strain PK 5017 (GenBank accession number FJ793195 and HQ223090) and reference species (see below) were aligned by Neighbor joining algorithms using the program Clustal W2-Multiple Sequences Alignment (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The GenBank accession numbers of the *16S rRNA* sequences from the reference species used in this study are as follows: *Pyrococcus furiosus* (U20163), *P. horikoshii* (D87344), *P. abyssi* (L19921), *P. glycovorans* strain AL585 (AY099168), *Thermococcus litoralis* (AY099180), *T. celer* (M21529), *T. fumicolans* (Z70250), *T. hydrothermalis* (Z70244), *T. profundus* (Z75233), *T. sibiricus* (AJ238992), *T. zilligii* (U76534), and *Palaeococcus ferrophilus* (AB019239). The last number belongs to the sequence of the genus in the order Thermococcales (Takai, et al., 2000). An unrooted phylogenetic tree was constructed by setting a bootstrap value of 1000, and the *16S rRNA* sequence of *Sulfolobus solfataricus* (GenBank

accession number D26490) was employed as out-group.

### Determination of growth parameters

Cultures were grown in 100 ml serum bottles, and samples (1 ml) were drawn at one-hour time interval between 0–6 h. Cell numbers were counted using the MPN method. Specific growth rates ( $\mu$ ) were estimated based on the regression line obtained from plotting  $\ln N$  versus  $t$ :  $N$  is the number of cells ml<sup>-1</sup> and  $t$  is the incubation time (h). At least four data points were used for the regression analyses during the exponential phase of growth.

### Genomic DNA preparation

DNA was extracted from 300 ml of overnight cultures following the method described by Charbonnier and Forterre (1995). Briefly, cell pellets were suspended in 400  $\mu$ l TNE buffer (10 mM Tris, pH 8.0; 100 mM NaCl; 1 mM EDTA) in a microcentrifuge tube. Fifty microliters of 20% N-lauroyl sarcosine and 100  $\mu$ l of 10% SDS were added. After inverted mixing, 100  $\mu$ l of 10 mg/ml Proteinase K solution was added and incubated at 50 °C for a few hours (or overnight). Proteins were precipitated twice by equal volume (750  $\mu$ l) of TE-saturated phenol. The aqueous (top layer) phase was separated by centrifugation at 5,000x g for 10 minutes at room temperature, and was subsequently extracted twice using an equal volume (750  $\mu$ l) of chloroform/isoamyl alcohol (24:1) for approximately 10 minutes. The upper phase was collected by centrifugation (5,000x g for 10 minutes) followed by precipitation of genomic DNA using cold absolute ethanol (-20 °C) in a refrigerator. DNA pellet was collected and washed at least three times at room temperature using 70 % (v/v) ethanol. The dried DNA pellet was dissolved in 50–100  $\mu$ l of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA). RNAs in the sample were digested using DNase-free RNase (10 mg/ml in TE buffer) at 37 °C for at least one hour. The DNA solution was then subjected to ethanol precipitation one more time. DNA solution was stored at 4 °C. DNA concentration (ng/ $\mu$ l) was

determined using a spectrophotometric method.

#### Arbitrarily-Primed PCR Reactions

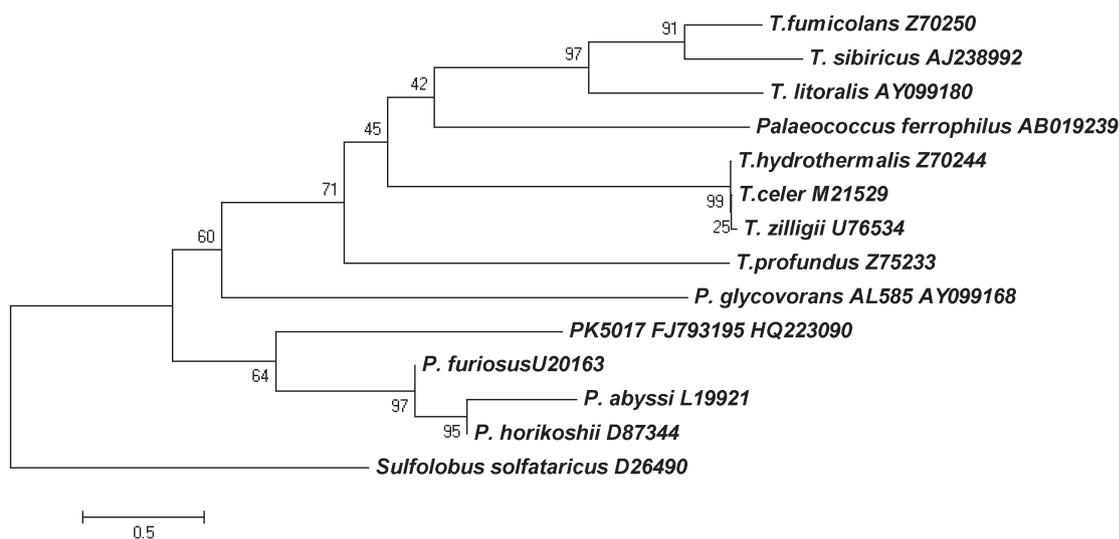
Synthetic oligonucleotide primers, named in this study as “ARB-1f” and “ARB-2f” were from Pacific Science Co., Ltd., Singapore. The nucleotide sequence of the ARB-1f primer (5' ATG AGG ACT GAA ACC ATT 3') was chosen because it was likely to bind to the 5' end of a highly homologous *transposase-integrase* gene family, which is present in multiple copies in the genomes of *P. furiosus* (Kanoksilapatham et al., 2004). The nucleotide sequence of the ARB-2f primer (5' GTA AAA CGA CGG CCA GT 3') was described elsewhere (Keller et al., 1995). A 50 µl reaction volume contained genomic DNAs (3-10 ng), 10 x Taq buffer (5 µl), a primer (20 pmol), dNTP (10 nmol), 50 mM MgCl<sub>2</sub> (1.5 µl), and Taq DNA polymerase (2.5 units). PCR was performed essentially as described by Welsh and McClelland (1990). Briefly, to produce long DNA templates, two cycles of a reaction condition of 94°C for 5 min, 40°C for 5 min, and 72°C for 5 min were conducted. Then, the polymorphic DNA templates obtained were amplified, using 40 cycles of a standard PCR reaction conditions (94 °C for 1 min, 40 °C for 1 min, and 72 °C for 2 min). The

polymerization was completed at 72 °C for 10 min. The reactions were stored at 4 °C. The amplified products were separated using a conventional 1% agarose gel electrophoresis.

#### Results

##### *Pyrococcus* sp. strain Pikanate 5017

*Pyrococcus* sp. strain Pikanate 5017 (PK5017) was isolated from a sediment sample collected from a geyser type hot spring located at Huai Nam Dang National Park (19° 7' 19" N, 98° 56' 37" E), Chiang Mai Province, Thailand (unpublished data). Strain PK5017 is a strict anaerobe growing on peptides as sole carbon and energy sources (see medium compositions in Materials and Methods). *16S rRNA* sequences of the strain PK 5017 (GenBank Accession Numbers FJ793195 and HQ223090) were determined and deposited to the NCBI public database. Phylogenetic analysis reveals that PK 5017, *P. abyssi*, *P. horikoshii* and *P. furiosus* are closely related species (Figure 1). In contrast, *P. glycovorans*, *Palaeococcus ferrophilus* (a member in the order Thermococcales) and the other *Thermococcus* species are grouped together.

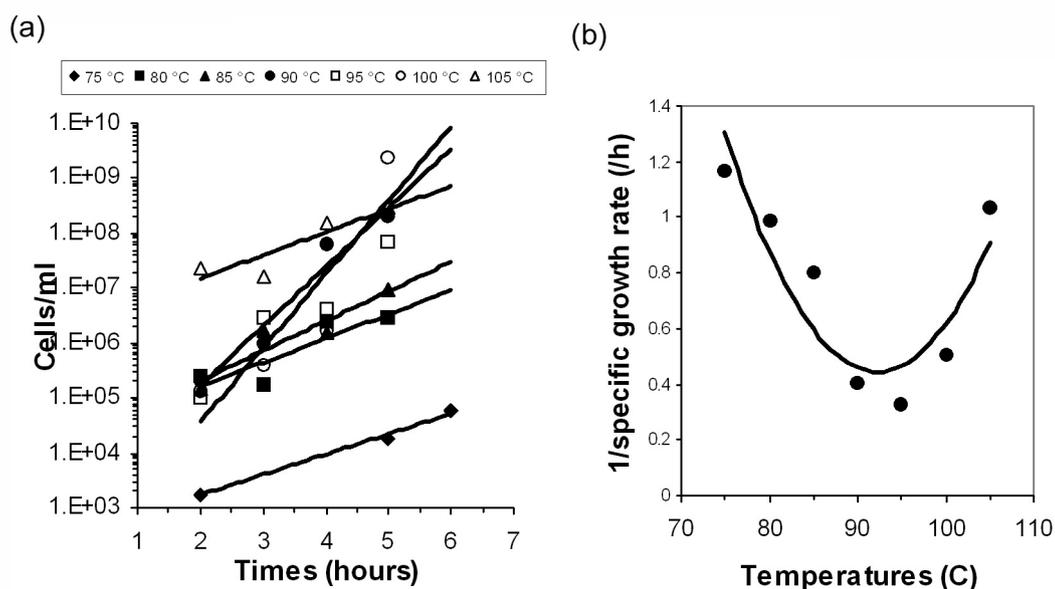


**Figure 1** Phylogenetic tree of PK 5017 and some members of Thermococcales. PK 5017 is classified in the *Pyrococcus* lineages.

### Growth kinetics

Growth kinetics of strain PK 5017 is assayed in exponential phase of growth (Figures 2a and 2b). Figures 2a and 2b indicates that an optimal temperature for growth is 95 °C, with a  $\mu_{\max}$  value of 2.67 h<sup>-1</sup> ( $R^2 = 0.90$ ). Growth rate at 105 °C is approximately 2.5 times slower, with an estimated doubling time of 40 min ( $R^2 = 0.92$ ). Minimal

growth temperature is predicted to be lower than 75 °C, because a positive  $\mu_{\max}$  value of 0.85 h<sup>-1</sup> ( $R^2 = 0.99$ ) is measured. Specific growth rate constants calculated at 80, 85, 90, 100 and 105 °C are 0.89 h<sup>-1</sup> ( $R^2=0.87$ ), 1.25 h<sup>-1</sup> ( $R^2=0.87$ ), 2.48 h<sup>-1</sup> ( $R^2 = 0.95$ ), 2.48 h<sup>-1</sup> ( $R^2 = 0.95$ ), 1.78 h<sup>-1</sup> ( $R^2=0.66$ ) and 1.03 h<sup>-1</sup> ( $R^2=0.92$ ), respectively.



**Figure 2** Growth kinetics of *Pyrococcus* sp. strain Pikanate 5017. Cell count was performed using MPN method (see also Materials and Methods). (a) Specific growth rates (h<sup>-1</sup>) were estimated based on the regression lines obtained from plotting log cells ml<sup>-1</sup> (y axis) versus incubation time in hours (x axis). Optimal growth temperature is 95 °C ( $\mu_{\max} = 2.67$  h<sup>-1</sup>;  $R^2 = 0.90$ ). Maximal temperature is 105 °C ( $\mu_{\max} = 1.03$  h<sup>-1</sup>;  $R^2 = 0.92$ ). Symbols: solid diamond represents exponential growth at 75 °C; solid square represents exponential growth at 80 °C; solid triangle represents exponential growth at 85 °C; solid circle represents exponential growth at 90 °C; blank square represents exponential growth at 95 °C; blank circle represents exponential growth at 100 °C; blank triangle represents exponential growth at 105 °C. (b) Reciprocal values of the specific growth rate (in Figure 2a) were plotted versus temperatures. An optimal growth temperature range of 90-95 °C is predicted.

Because optimal temperature for growth of the strain PK 5017 is above 90 °C (Figures 2a and 2b), *16S rRNA* sequence (GenBank Accession Number FJ793195) is highly related to sequence belonging to species in the order Thermococcales, the strain PK 5017 was classified to be a member of the genus *Pyrococcus* (Figure 1). However, comparisons of genome sequences (an essential parameter for species differentiation) have never been described.

#### Reliabilities of AP-PCR Technique

In order to differentiate genome sequences, two single primers were arbitrarily chosen and named in this study as “ARB-1f” and “ARB-2f” (see nucleotide sequences in Materials and Methods). Initially, reliability of the primers was tested on varied genomic DNA concentrations of 3-10 ng. A concentration of each single primer was fixed at 20 pmol in a 50 µl reaction volume. Results are shown in Figure 3. Figure 3a reveals consistent patterns obtained from DNAs of *Pyrococcus furiosus* (lanes 1 and 2), and strain PK5017 (lanes 3 and 4) performed at concentration of 5 and 10 ng, respectively. Similarly, a distinct pattern was obtained when the template DNA (3, 5 and 10 ng) of *P. abyssi* (lanes 1-3 in Figure 3b) was employed. The results imply that the single ARB-1f primer is reliable in generating PCR profiles regardless of the template concentrations tested. Comparisons among the patterns reveal unique polymorphisms. However, a common band with an approximate size of 700 bp-long (indicated by an arrow in Figure 3a) was observed on the templates of *P. furiosus* and strain PK5017, indicating sequence similarity in a region(s) of these archaea.

Polymorphisms of AP-PCR products generated using the ARB-2f primer are shown in Figures 3c - 3e. As expected, the results reveal three consistent patterns of strain PK5017 (Figure 3c), *P. furiosus* (Figure 3d), and *P. abyssi* (Figure 3e), respectively, suggesting reproducibility of the technique regardless of concentrations and sequence of the primer tested. In addition, clearly different

patterns obtained using the ARB-2f confirms different genome sequences among the three hyperthermophilic archaea.

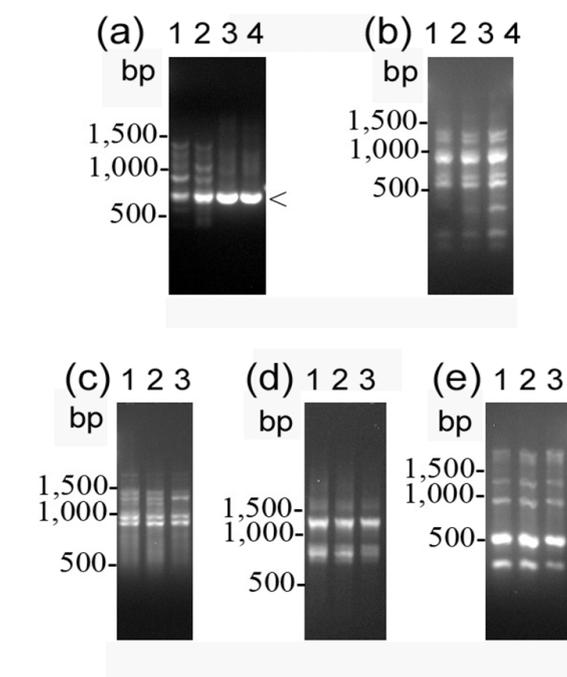
#### Genomic Fingerprints of Thermococcales

In order to compare genomic fingerprints, AP-PCR reactions were conducted using either the ARB-1f or ARB-2f primer with 5 ng of templates. Results are shown in Figure 4. Figure 4a reveals different AP-PCR patterns generated by the ARB-1f primer. The patterns obtained are varied in band numbers and sizes, indicating different genome sequences in several regions. The profiles of the *Pyrococcus abyssi* (lane 3), *P. horikoshii* (lane 4), and *Thermococcus celer* (lane 6) are unique, and contain no prominent band in common. However, the band sized of 700 bp-long mentioned in previous section (Figure 3a) was also observed in the pattern of *Thermococcus litoralis* (lane 5), indicating sequence similarity of *T. litoralis* to the strain PK 5017 (lane 1) and *P. furiosus* (lane 2).

The results shown in Figure 4b confirm unique profiles when the ARB-2f was employed. In Figure 4b, the patterns of the strain PK 5017 (lane 1) and *P. furiosus* (lane 2) are not identical. However, at least 5 bands (indicated by arrows in Figure 4b), were commonly observed in both species. The band patterns of *P. abyssi* (lane 3), *P. horrikoshii* (lane 4), *T. celer* (lane 5), and *T. litoralis* (lane 6) are clearly distinguishable from the strain PK5017.

#### Discussion

Hyperthermophiles have adapted themselves to thrive in habitats with extremely high temperature, and are important sources of highly thermostable enzymes (Stetter et al., 1993). Hyperthermophilic archaea in the order Thermococcales have been isolated from hydrothermal vents mostly in marine solfatara. *Pyrococcus* sp. strain Pikanate 5017 (PK5017) was first isolated from a terrestrial and sulfide rich hot spring in Northern Thailand (unpublished data). Growth parameters determined between the temperature range of 75-105 °C (Figure

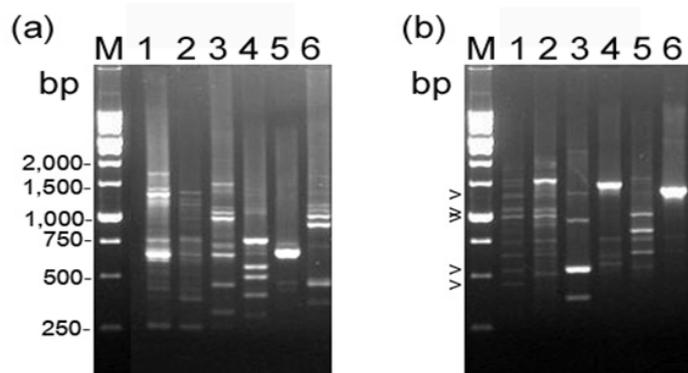


**Figure 3** Reproducibility of AP-PCR technique at varied template concentrations. (a) DNAs were amplified using the ARB-1f primer (5' ATG AGG ACT GAA ACC ATT 3'), templates of *P. furiosus* (lanes 1 and 2), and strain PK5017 (lanes 3 and 4). Lanes 1 and 3 represent 5 ng of the templates. Lanes 2 and 4 represent 10 ng of the templates. (b) DNA was amplified from a template of *P. abyssi* using ARB-1f primer. Lanes 1-3 represent 3, 5 and 10 ng of the template concentrations, respectively. (c) DNA was amplified from a template of strain PK5017 using ARB-2f primer (5' GTA AAA CGA CGG CCA GT 3'). Lanes 1-3 represent 3, 5 and 10 ng of the template concentrations, respectively. (d) DNA was amplified from a template of *P. furiosus* using ARB-2f primer. Lanes 1-3 represent 3, 5 and 10 ng of the template concentrations, respectively. (e) DNA was amplified from a template of *P. abyssi* using ARB-2f primer. Lanes 1-3 represent 3, 5 and 10 ng of the template concentrations, respectively. Sizes of markers (bp) are indicated on the left.

2) indicate a fast growing hyperthermophile calculated to be less than 20 min per generation ( $\mu_{\max} = 2.67 \text{ h}^{-1}$ ;  $R^2 = 0.90$ ) at optimal conditions (95 °C, 2.4% NaCl, pH 7.2). Because its optimal temperature for growth is above 90 °C, the strain PK5017 is classified to be a member of the genus *Pyrococcus* (optimal temperature of *Thermococcus* is less than 90 °C; Barbier et al., 1999; González et al., 1999). It is observed that the  $\mu_{\max}$  value (2.48  $\text{h}^{-1}$ ;  $R^2 = 0.95$ ) determined at 90 °C is near the value obtained at 95 °C. This temperature (90 °C) is close to temperature of the sediments measured *in situ*

and was the isolation temperature.

The *16S rRNA* partial sequence of the strain PK 5017 (GenBank Accession Numbers FJ793195 and HQ223090) are very similar (96-100 %) to the *16S rRNA* gene sequences of most Thermococcales (data not shown). Phylogenetic analysis grouped the strain PK 5017 with *Pyrococcus* (Figure 1). However, the *16S rRNA* gene sequences do not allow the accurate differentiation of species and strains within the genera *Pyrococcus* and *Thermococcus*. An AP-PCR technique is successfully applied to explore genomic fingerprints, one of the essential



**Figure 4** Genomic fingerprints of Thermococcales at 5 ng of genomic DNAs. (a). AP-PCR polymorphisms were generated using the ARB-1f primer (5' ATG AGG ACT GAA ACC ATT 3'). Lanes 1-6 represent templates of *Pyrococcus* sp. strain PK5017, *P. furiosus*, *P. abyssi*, *P. horikoshii*, *Thermococcus litoralis*, and *T. celer*, respectively. (b). AP-PCR polymorphisms were generated using ARB-2f primer (5' GTA AAA CGA CGG CCA GT 3'). M represents size markers. Lanes 1-6 represent templates from *Pyrococcus* sp. strain PK5017, *Pyrococcus furiosus*, *P. abyssi*, *P. horikoshii*, *Thermococcus celer*, and *T. litoralis*, respectively. The distinguishable patterns suggest different genome sequences.

DNA parameters, of six representative members of the Archaea within the Thermococcales. Reliability is disclosed at a genomic DNA concentration range of 3-10 ng (Figures 3). The bands sized of 700 bp-long (compare lanes 1 and 2 with lanes 3 and 4 in Figure 3a), and several bands (compare lane 1 with lane 2 in Figure 4a) observed in common between the strain PK5017 and *P. furiosus*, suggesting that the strain PK5017, compared with the others, is closely related to *P. furiosus*. The unique AP-PCR fingerprints (Figures 4a and 4b) distinguish the strain PK 5017 from *P. furiosus* DSM3638, *P. horikoshii* strain OT3, *P. abyssi* strain GE5, *T. litoralis*, and *T. celer*. The results generated from the use of AP-PCR technique, unlike *16S rRNA* gene sequence analysis, indicate a clear distinction of species and strains belonging to the genera *Pyrococcus* and *Thermococcus*.

## Conclusions

Simplicity and flexibility of the AP-PCR technique is clearly demonstrated in this paper because the technique requires no prior sequence information and can be conducted in a single step with slight modification of a standard PCR reaction. Reliability is disclosed at a DNA concentration range of 3-10 ng (Figures 3). Two arbitrarily primers named in this study as "ARB-1f" (5' ATG AGG ACT GAA ACC ATT 3') and "ARB-2f" (5' GTA AAA CGA CGG CCA GT 3') were successfully employed to compare the genome sequences of the hyperthermophilic *Pyrococcus* sp. strain Pikanate 5017 with those of the closely related species. The growth parameters determined in this study help cell mass optimization and thermostable enzyme production. The strain PK 5017 can be considered as a source of numerous genes, encoding extremely heat stable enzymes. Clearly different fingerprints suggest that strain PK5017 is a distinct species within the genus *Pyrococcus*.

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