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Application of rDNA-PCR Amplification and DGGE Fingerprinting for Detection of Microbial Diversity in a Malaysian Crude Oil

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Two culture-independent methods, namely ribosomal DNA libraries and denaturing gradient gel electrophoresis (DGGE), were adopted to examine the microbial community of a Malaysian light crude oil. In this study, both 16S and 18S rDNAs were PCR-amplified from bulk DNA of crude oil samples, cloned, and sequenced. Analyses of restriction fragment length polymorphism (RFLP) and phylogenetics clustered the 16S and 18S rDNA sequences into seven and six groups, respectively. The ribosomal DNA sequences obtained showed sequence similarity between 90 to 100% to those available in the GenBank database. The closest relatives documented for the 16S rDNAs include member species of *Thermoincola* and *Rhodopseudomonas*, whereas the closest fungal relatives include *Acremonium*, *Ceriporiopsis*, *Xeromyces*, *Lecythophora*, and *Candida*. Others were affiliated to uncultured bacteria and uncultured ascomycete. The 16S rDNA library demonstrated predomination by a single uncultured bacterial type by >80% relative abundance. The predomination was confirmed by DGGE analysis.

Keywords: 16S rDNA, 18S rDNA, crude oil, microbial diversity, DGGE

Petroleum crude oil consists primarily of alkanes, aromatics, and asphaltenes [10]. Over the years, the microbiological diversity in relation to petrochemicals has been vastly studied, including bacteria, yeast, filamentous fungi, algae, and protozoa [10, 27]. Traditional approaches through culture-dependent methods have resulted in considerable understanding on the various petrochemical microorganisms and their mode of actions [29]. Some commonly reported hydrocarbon- and petrochemical-degrading strains include members of *Mycobacterium*, *Rhodococcus*, *Pseudomonas*, *Alcaligenes*, *Nocardioides*, *Acinetobacter*, *Arthrobacter*, *Bacillus* [7, 10, 30], and fungal strain *Phanerochaete chrysosporium* [8].

Despite the abundant works done using the traditional culture-dependent methods, the data obtained may not represent the real microbial community in the natural habitats, since only a tiny fraction of the environment's microbes can be cultivated in the laboratory condition [4]. The recent advancements in molecular biology have developed new methods that enable scientists to analyze not only the cultured microorganisms but also the uncultured ones. These methods among others include direct PCR amplification of microbial 16S or 18S rDNA and the denaturing gradient gel electrophoresis (DGGE). The specified methods have been successfully and widely used to analyze the microbial communities from soil, water, extreme environments, *etc*. [1, 11, 13, 22, 27, 31].

In the current study, we extended two culture-independent approaches, namely the ribosomal DNA libraries and denatured gradient gel electrophoresis (DGGE), to directly analyze the microbial diversity in the concentrated Tapis crude oil of Malaysia. Tapis is an international reference for the light Far East crude oil, which contains less than 0.2% sulfur in weight with 44° API [32]. It represents the major crude oil type produced in Malaysia, mainly in the petrochemical zone of Kertih, Terengganu. In this paper, we provide important information on the microbial availability in a concentration crude oil, supported by previous reports on the occurrences of aerobic [5] and anaerobic [2, 24] biodegradation of the Earth's petroleum resources. Early investigation on microbial availability in crude oil may assist in determining the quality of crude oil, as well as the potential impact on subsequent petrochemical processing and the environment [25].

Analysis of the 16S and 18S rDNAs Libraries Constructed from the Crude Oil Bulk DNA Samples

Samples of the Malaysian Tapis crude oil were obtained from the Petronas Penapisan Kertih, Terengganu, Malaysia,

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in sterile 1-l amber sampling bottles. Crude oil samples were processed immediately for DNA extraction. Bulk DNAs were extracted from four crude oil samples in triplicates using the PowerSoil DNA kit as instructed by the manufacturer (Mo Bio Laboratory, Carlsbad, U.S.A.). The bulk DNAs were further purified according to the sodium acetate/EtOH precipitation protocol (http://golab.unl.edu/protocols/PCR-EtOH.html). The additional purification steps were crucial, as PCR amplification from the bulk DNAs was otherwise inhibited.

Community rDNAs were PCR-amplified in reaction mixtures containing (as final concentrations) 1× PCR reaction buffer, 2.0 mM $MgCl_2$, 200 mM concentration of each deoxynucleoside triphosphate, 250 nM concentration of each forward and reverse primer, and 2 U of *Taq* polymerase per 100-µl PCR reaction (Fermentas, Vilnius, Lithuania). Prokaryotic 16S rDNAs were PCR-amplified with bacterial-specific universal forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') [18] in

Table 1. Taxonomic affiliation and abundance of 16S and 18S rDNA sequence types as defined by RFLP analysis.

RFLP pattern/ sequence type	Number of clones (% abundance)	Representative sequence	% Identity	Phylogenetic group	Description of closest relative in GenBank database (accession number, name, and source)
Bacteria					
I	249 (83)	MOB64, MOB65, MOB72, MOB73, MOB121, MOB145, MOB176, MOB185, MOB189, MOB204, MOB223, MOB226, MOB238, MOB292, MOB295, MOB300	90	Uncultured	DQ223203, uncultured bacterium clone EV221H2111601SAH29 (subsurface water of Kalahari Shield, South Africa)
II	4 (1.3)	MOB17	93	α-Proteobacteria	AY428572, *Rhodopseudomonas julia* DSM 11549 (sulfur spring, Russia)
III	3 (1)	MOB164	98	Uncultured	AY162944, uncultured bacterium clone ZB-Z11 (Zabuye Lake in Tibet, China)
IV	4 (1.3)	MOB190	95	Uncultured	DQ125650, uncultured bacterium clone AKAU370 (uranium-contaminated soil, U.S.A.)
V	9 (3)	MOB237	98	Firmicutes	AY603000, *Thermoincola carboxydiphila* 2204 (hot spring of the Lake Baikal area, Russia)
VI	5 (1.7)	MOB219	98	Firmicutes	AY631277, *Thermoincola ferriacetica* Z-0001 (Russia)
VII	26 (8.6)	MOB97, MOB178, MOB186	99	Uncultured	DQ424920, uncultured bacterium clone D96 (Thermophilic Microbial Fuel Cell, Korea)
Fungi					
I	34 (11.7)	MOF138	99	Ascomycota	AJ496247, *Lecythophora mutabilis* CBS157.44T (river water, Germany)
II	123 (42.4)	MOF163, MOF200	100	Ascomycota	AB108787, *Acremonium*-like hyphomycete KR21-2 (Japan)
III	39 (13.4)	MOF203	98	Ascomycota	AB024049, *Xeromyces bisporus* CBS236.71 (Japan)
IV	9 (3.1)	MOF3, MOF149	95	Ascomycota	AB018143, *Candida floricola* JCM9439 (Dandelion flower, Japan)
V	37 (12.8)	MOF104	99	Ascomycota	AY934712, uncultured ascomycete isolate F4_M13F_Platte2 (gut mucosa, Germany)
VI	48 (16.6)	MOF12, MOF20, MOF51	99	Basidiomycota	AF334906, *Ceriporiopsis subvermispora* strain FPL90031 (USA)

a Bio-Rad MyCycler thermal cycler (Bio-Rad, Hercules, U.S.A.) at 95°C for 5 min (for initial denaturation), followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then by a final extension period of 20 min at 72°C. PCR products were resolved on a 1.0% LE agarose gel (Cambrex Bio Science Rockland, East Rutherford, U.S.A.), excised, purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and eluted in 10 mM Tris buffer (pH 8.0). The purified PCR products were ligated into pGEM-T Vector System I according to the manufacturer's instructions (Promega, Madison, U.S.A.), and transformed into DH5α competent cells. Accordingly, we constructed a 16S rDNA library consisting of 300 clones, each carrying rDNA insert of approximately 1,500 bp in size. RFLP analysis involving double-digestion with HaeIII and HinfI (New England Biolabs, Hertfordshire, England) clustered the cloned 16S rDNA into seven patterns (Table 1). The digested fragments were resolved on 3.0% NuSieve 3:1 agarose gels (Cambrex Bio Science Rockland, East Rutherford, U.S.A.) and visualized *via* ethidium bromide staining and UV illumination. The results revealed a predominant pattern (Type I), which represents 83% (249 clones) of the total library clones. Sequencing reactions were then carried out for plasmid clones that represent the different RFLP patterns using the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Piscataway, U.S.A.). Sequencing primers including the vector primers (pUC/M13) and bacterial-specific small-subunit (SSU) rDNA internal primer Seq1 (5'-CAG CAG CCG CGG TAA TAC-3') were used to obtain nearly complete 16S rRNA gene sequences. Sequencing was carried out commercially. The rRNA gene sequences obtained were subjected to a National Center for Biotechnology Information (NCBI) BLAST search [3] to identify their closest relatives. The sequences were checked for possible chimeras by using the CHIMERA_CHECK program at the Ribosomal Database Project Web site (http://rdp8.cme.msu.edu). The nucleotide sequences of the 16S rDNA clones were deposited in the GenBank database under the accession numbers EF095416-EF095440. The 16S rRNA gene sequences of related taxa were obtained from the GenBank database and aligned with the library sequences with the multiple alignment tool ClustalW [28] in the Mega 3.1 [16] program. The evolutionary distances were estimated with the Kimura two-parameter model [14], and the phylogenetic trees were constructed using the neighbor-joining method based on bootstrap values of 1,000 replications to assign confidence levels to the nodes in the trees. Sequencing analysis of the nearly complete 16S rRNA gene sequences of the predominating pattern showed highest sequence homology (90%) to the uncultured bacterium clone EV221H2111601SAH29 (GenBank Accession No. DQ223203) isolated from the subsurface water of the Kalahari Shield, South Africa. The second most abundant pattern

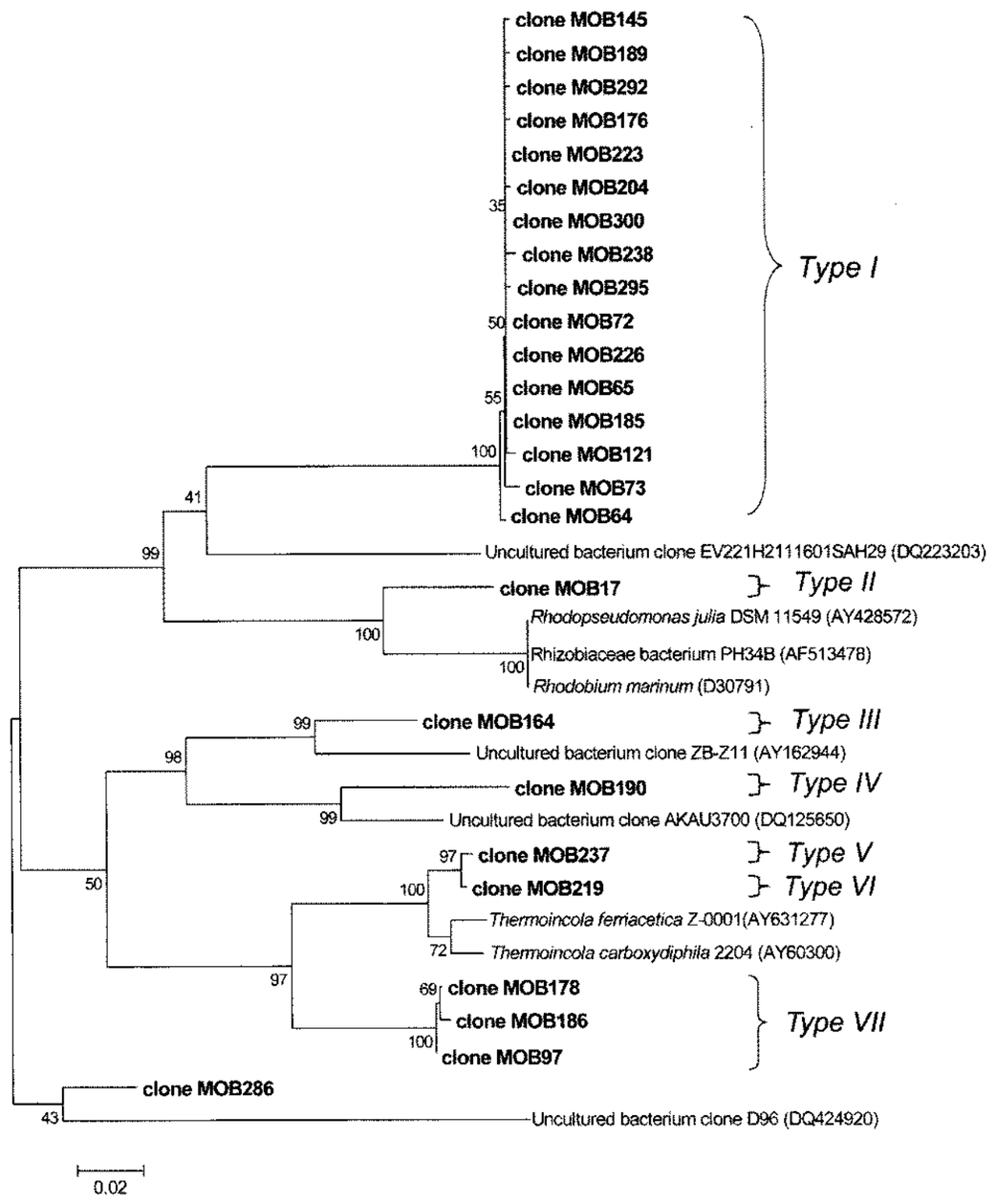

Fig. 1. Neighbor-joining tree based on complete 16S rRNA gene sequences showing the phylogenetic positions of representative crude oil clones' sequences.
Titles in bold font are sequences obtained in this study. Accession numbers of the sequences retrieved from the databases are indicated in the parentheses. Bootstrap values are shown for each node that had ≥50% support in a bootstrap analysis of 1,000 replicates. The evolutionary distance was estimated based on the Kimura 2-parameter nucleotide substitution model. The scale bar indicates 0.02 substitution per site.

(Type VII) as represented by 26 clones (8.6% abundance) was most closely related (99% sequence homology) to the uncultured bacterium clone D96 (GenBank Accession No. DQ424920) originated from a thermophilic mediatorless microbial fuel cell [11]. Other patterns were represented by less than 5% of the library clones, respectively (Table 1). A phylogenetic tree was constructed based on nearly complete 16S rDNA sequences (Fig. 1).

Fungal 18S rDNAs were PCR-amplified with forward primer FUN18S1 (5'-CCA TGC ATG TCT AAG TWT AA-3') and reverse primer FUN18S2 (5'-GCT GGC ACC AGA CTT GCC CTC C-3') [19] at 95°C for 5 min (for initial denaturation), followed by 30 cycles at 95°C for 30 s, 46°C for 30 s, and 72°C for 1 min, and then by a final extension period of 20 min at 72°C. The primers targeted the divergent region of the community fungal 18S ribosomal sequence of approximately 550 bp in size. RFLP analysis of 290 clones *via* double-digestion with HaeIII and MspI (New England Biolabs, Hertfordshire, England) produced six different restriction patterns. Sequencing and bioinformatics

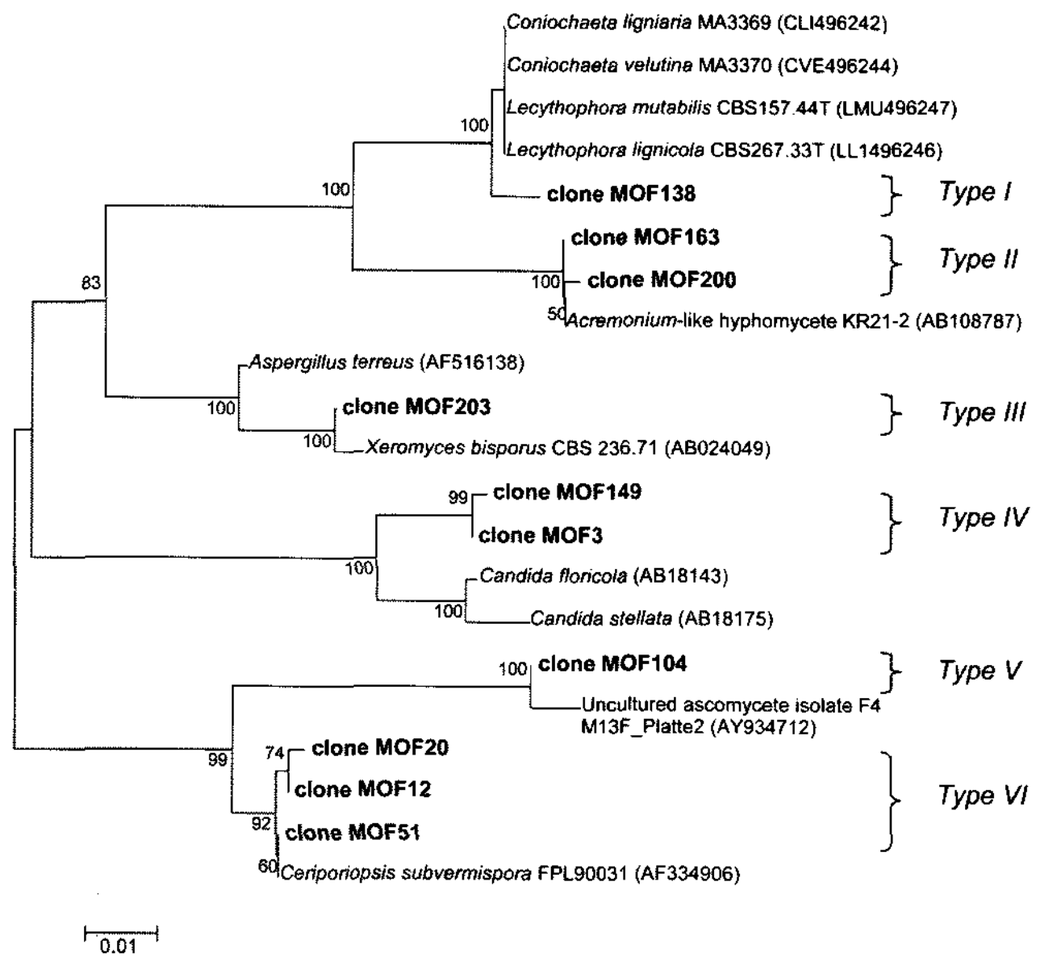

Fig. 2. Neighbor-joining tree based on 18S rDNA sequences (~550 bp) showing the phylogenetic positions of representative crude oil clones' sequences.

Titles in bold font are sequences obtained in this study. Accession numbers of the sequences retrieved from the databases are indicated in the parentheses. Bootstrap values are shown for each node that had ≥50% support in a bootstrap analysis of 1,000 replicates. The evolutionary distance was estimated based on the Kimura 2-parameter nucleotide substitution model. The scale bar indicates 0.01 substitution per site.

analysis were performed as described above. The sequences of the 18S rDNA clones were deposited in the GenBank database under the accession numbers EF095441-EF095450. Nearly all RFLP patterns showed sequence similarity greater than 97% to known sequences in the GenBank database, except for one pattern that showed higher divergence (95% sequence homology) to its closest counterpart (Table 1). Generally, the library was predominated by the Type II RFLP pattern with 42.4% relative abundance (123 clones). The representative sequences showed 100% sequence similarity to *Acremonium*-like hyphomycete KR21-2 (GenBank Accession No. AB108787), capable of enzymatic formation of manganese oxides. Other sequence types were closely related to *Ceriporiopsis subvermispora*, *Xeromyces bisporus*, uncultured ascomycete, *Lecythophora mutabilis*, and *Candida floricola* (Table 1). Phylogenetic relationships were as shown in Fig. 2.

Generally, based on the microbial community structure shown in Figs. 1 and 2, the microbial community conforms to the crude oil habitat studied. The constructed ribosomal DNA libraries in this study consist of sequences similar to specific phylogenetic groups of *Thermoincola* and *Rhodopseudomonas* based on the 16S rDNA sequences, and *Candida*, *Ceriporiopsis*, *Acremonium*, *Lecythophora*, and *Xeromyces* based on the 18S rDNA sequences. Most of these sequences were closely related to previously reported microorganisms originating from hostile environments, among which *Candida*, *Acremonium*, and *Lecythophora* were reported from extreme habitats with low pH and high heavy metal content [18]. Several rDNA sequences were related to hydrocarbon-degrading characteristics: for example, member species of *Rhodopseudomonas* were reported to be able to degrade benzoate, *Ceriporiopsis* were found able to degrade anthracene, and *Candida* were reported to be able to degrade long-chain n-alkane, kerosene, diesel oil, and crude oil [9, 12, 15, 23]. Whereas most (87%) of the fungal sequence types were correlatively matched to known fungi sequences in the GenBank database, 94% of the 16S rDNA clones corresponded to uncultured bacteria (Table 1). A predomination of 83% by a single 16S rDNA RFLP pattern was most intriguing. The dominant sequence showed 90% sequence homology to its closest counterpart, the uncultured bacterium clone EV221H2111601SAH29, indicating possible novelty of this dominant population. Sequence similarities ranging from 93 to 99% (Table 1) of other RFLP patterns to their closest relations further suggest genotypic novelty in the studied community, indicating a big challenge in the recovery of specialized crude oil microorganisms. The microbial diversities obtained were in contrast to the study by Sánchez *et al.* [25] where both the bacterial and fungal diversities were predominated by culturable strains of *Bacillus* and *Aspergillus,* respectively.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

DGGE-PCR was performed on community 16S rDNA targeting the V3 region using primers 341F (with GC-clamp) 5'-*CGCCCGCCGCGCGCGGCGGGCGGGGCG-GGGGCACGGGGGGG*GACGGGGGGCCTACGGGAG-GCAGCAG-3' and 534R 5'-ATTACCGCGGCTGCTGG-3' [20]. Reaction conditions for the amplification were 95°C for 7 min (for initial denaturation), followed by 19 cycles of touchdown protocol at 95°C for 30 s, 65°C (↓0.5°C per cycle) for 30 s and 72°C for 45 s, then 20 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 20 min. DGGE analysis of the V3 fragment was performed in a DCode Universal Mutation Detection System (BioRad, Hercules, U.S.A.) apparatus. A parallel gel was prepared with a linear denaturing gradient of urea and formamide ranging from 30% to 60% on a 10% acrylamide gel. Gel electrophoresis was carried out at a constant voltage of 130 V for 5 h at 60°C, after which the gel was stained in an ethidium bromide solution (0.25 μg/ml) and documented with the ChemiGenius Gel Documentation System (Syngene, Frederick, U.S.A.). The DGGE gel revealed distinctive predomination by an intense band, as shown in Fig. 3. The DNA band of interest was excised from the acrylamide gel and eluted overnight in 10 mM Tris buffer (pH 8.0) at 4°C. The recovered DNA was PCR-amplified with the same primer pair without GC-clamp

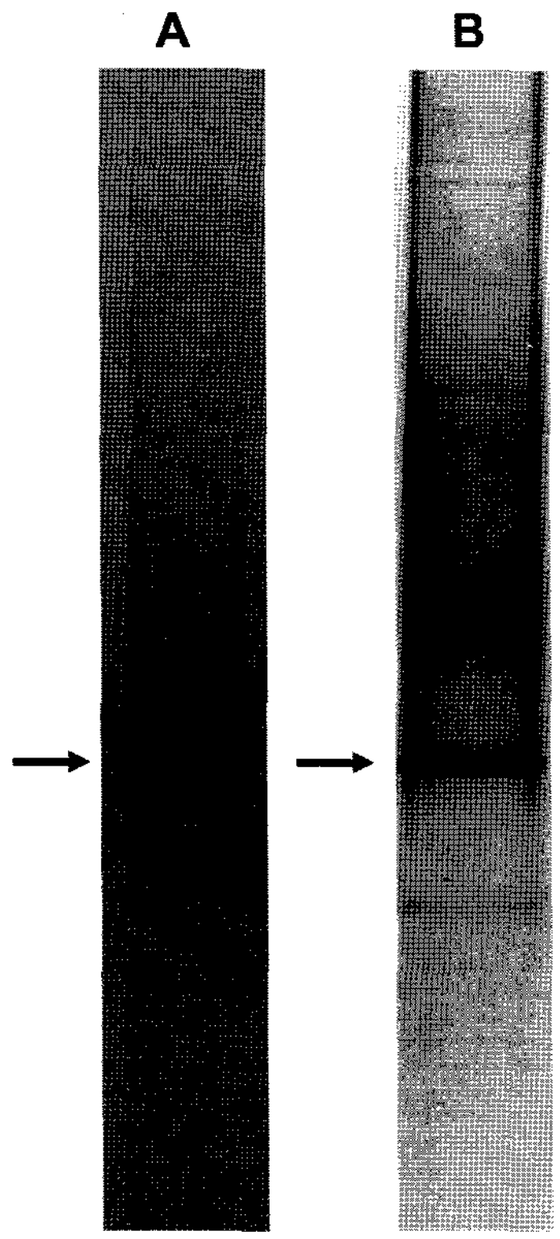

Fig. 3. Negative image of DGGE gel with the PCR-amplified V3 segment of community 16S rDNA genes of crude oil samples.
DNA bands indicated by arrows represent the predominating population. Lanes A and B demonstrate consistency of the predomination in replicate samples.

(95°C, 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 20 min), and then cloned and sequenced as described before. Sequencing and bioinformatics analysis revealed 100% sequence homology of the gel-recovered nucleotide sequence to the predominating 16S rDNA sequence representing RFLP pattern Type I (Table 1) in the respective clone library, and thus verified the amazingly high predomination by the single bacterial type as described earlier of the 16S rDNA library. The sizes of the ribosomal DNA libraries evaluated were sufficient with reference to the agreeable results produced by the DGGE analysis. In this study, community DGGE analysis was shown to be an easy and quick fingerprinting technique to uncover and profile the unknown microbial diversity.

To summarize, we uncovered a microbial community in the Malaysian Tapis crude oil through culture-independent approaches. Overall, the microbial diversity observed was small, with most of the sequence types linked to hostile conditions, a characteristic shared by the studied habitat, including hydrocarbon- or oil-related, anaerobic, and thermophilic environments. The small community variability was expected, as there is no external microbial input, for instance from the environmental soil and water. The community structure was in correlation with previous findings, whereby crude oil contains toxic components that could reduce microbial diversity [1, 6, 26], but stimulate the metabolic activity and abundance of tolerant microbes [21]. The definition of the microbial community also implied that they might be able to utilize crude oil. Although the significance of the microbial presence, in particular the predominating population(s), is not known at the moment, it has definitely pointed to some worth-looking-into potentials in the crude oil environments.

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