CHAPTER 3.0: METHODOLOGY

3.1 Materials

3.1.1 Chemicals, apparatus, and instruments

Table 3.1 shows all chemicals, apparatus, and instruments used. All the glassware, tubes, and tips were steam-sterilized at 121 ℃ for 20 minutes in an autoclave before use.

Table 3.1: Chemicals, apparatus, and instruments used in this study

Chemical, apparatus, and instrument	Manufacturer
100 % Glycerol	BDH Laboratories Supplies, UK
40 % Acrylamide Bis	Bio-Rad Laboratories Inc., USA
50 % Ammonium Persulphate (APS)	Bio-Rad Laboratories Inc., USA
50 ml centrifuge tubes	Greiner Bio-one, Germany
Agarose powder	Promega, USA
Autoclave	Tomy, Japan
Biophotometer	Eppendorf, Germany
Centrifuge machine	Eppendorf, Germany
D-Code Universal Mutation Detection System	Bio-Rad Laboratories Inc., USA
dNTP	Promega, USA
EDTA	Invitrogen, USA
Electrophoresis gel tank	Bio-Rad Laboratories, Inc., USA
Ethanol	System Chemar, Malaysia
Ethidium bromide	Promega, USA
Formamide	Merck, Germany
Freezer (-20 ℃)	Anders Brondum A/S, Denmark
Glassware	Pyrex, UK
Gloves	Savegard, Malaysia
Goldbalance weighing machine	Mettler Intsrument, Switzerland
Gradient Delivery System	Bio-Rad Laboratories Inc., USA
Laminar flow chamber	Esco Micro Pte. Ltd., Singapore
Microcentrifuge tubes	Greiner Bio-one, Germany
Microwave oven	Nova, USA
Mini shaker	Ika Works Inc., USA
PCR tubes	Greiner Bio-one, Germany
pH meter	Eutech Instrument-Oaklon Instrument, Singapore
Pipettes	Orange Scientific, Belgium
Power pack	Bio-Rad Laboratories Inc., USA
Salinity kit (Mi306)	Martini Instrument, Romania
SYBRGold nucleic acid gel stain	Invitrogen, USA
Taq DNA polymerase	Invitrogen, USA

Table 3.1, continued

TEMED	Bio-Rad Laboratories Inc., USA
Thermal cycler	Bio-Rad Laboratories Inc., USA
Tips	Greiner Bio-one, Germany
Tris	Vivantis, Malaysia
Urea	Sigma Chemical Corp., USA
UV transilluminator	Syngene Bio Imaging, UK
Water bath	Memmert, Germany

3.1.2 Solutions for agarose gel electrophoresis

6X Bromophenol Blue (BPB) loading dye

6X Bromophenol Blue (BPB) loading dye [0.15 % (w/v) BPB, 9 % (w/v) Ficoll 400, 40 % (v/v) glycerol] was prepared and stored at 4 ℃:

BPB	15g
Ficoll 400	0.9 g
Glycerol, $C_3H_5(OH)_3$	4 ml
Sterile distilled water top up to	10 ml

50X Tris-acetic (TAE) buffer

A total volume of 1 L 50X TAE buffer, pH 8.0 [10 mM Tris, 10 mM glacial acetic acid, 1 M EDTA] was prepared:

Tris-base, $C_4H_{11}NO_3$	242 g
Glacial Acetic Acid, $CH_3.COOH$	57.1 ml
EDTA $2H_2O$ ·NAOH	37.2 g
Sterile distilled water top up to	1 L

The 50X TAE stock solution was stored at room temperature and used as soon as possible to avoid precipitation. The 50X TAE was subjected to dilution of 1: 50 (v/v) to give 1X TAE buffer (10 mM Tris-Acetic, 1 mM EDTA, pH 8.0) for routine Agarose Gel Electrophoresis (AGE).

Ethidium Bromide (EtBr) solution

The Ethidium Bromide (EtBr) solution was prepared and stored in a covered container in the dark:

Ethidium Bromide (10 mg/ml)	100 ml
1X TAE buffer	500 ml

3.2 Methods

3.2.1 Soil and sediment collection sites

The Arctic soils and sediments (Figure 3.1) were collected from a total of eighteen sites in Ny-Ålesund, Norway during the summer of 2006. Table 3.2 shows the description and the site locations (GPS). The soil and sediment samples were collected in sterile sampling bottles and were kept at -20 ℃ until further use (Figure 3.2).

Table 3.2: The description of collection sites for Arctic soils and sediments

Sample	Collection site	Sample description	Location (GPS)
1	Harbour	Beach soil	78 °55.647N; 011 °56.242E
3	Rail track	Terrestrial soil	78 °55.530N; 011 °56.160E
5	Runnel	Terrestrial soil	-
8	Storvatnet	Inland lake bank sediment	78 °55.375N; 011 °53.004E
9	Dry tundra	Terrestrial soil	78 °55.376N; 011 °52.876E
17	Tundra	Terrestrial soil	78 °54.846N; 011 °59.023E
24	Top layer (2 to 4 cm) of sediment core from 10 m depth	Marine sediment	-
25	Middle layer (4 to 6 cm) of sediment core from 10 m depth	Marine sediment	-
26	Lower layer (6 to 8 cm) of sediment core from 10 m depth	Marine sediment	-
28	Top layer (2 to 4 cm) of sediment core from 20 m depth	Marine sediment	-
29	Middle layer (4 to 6 cm) of sediment core from 20 m depth	Marine sediment	-
30	Lower layer (6 to 8 cm) of sediment core from 20 m depth	Marine sediment	-
31	Below glacier	Periglacier soil	78 °54.419N; 011 °57.545E
32	Beside glacier	Periglacier soil	-
33	Above glacier	Periglacier soil	78 °54.405N; 011 °57.148E
34	Old mine mound	Terrestrial soil	78 °54.428N; 011 °57.420E
35	Freshwater	Inland lake bank sediment	78 °54.699N; 011 °52.527E
39	Melt lake	Melt lake sediment	78 °54.995N; 011 °51.538E

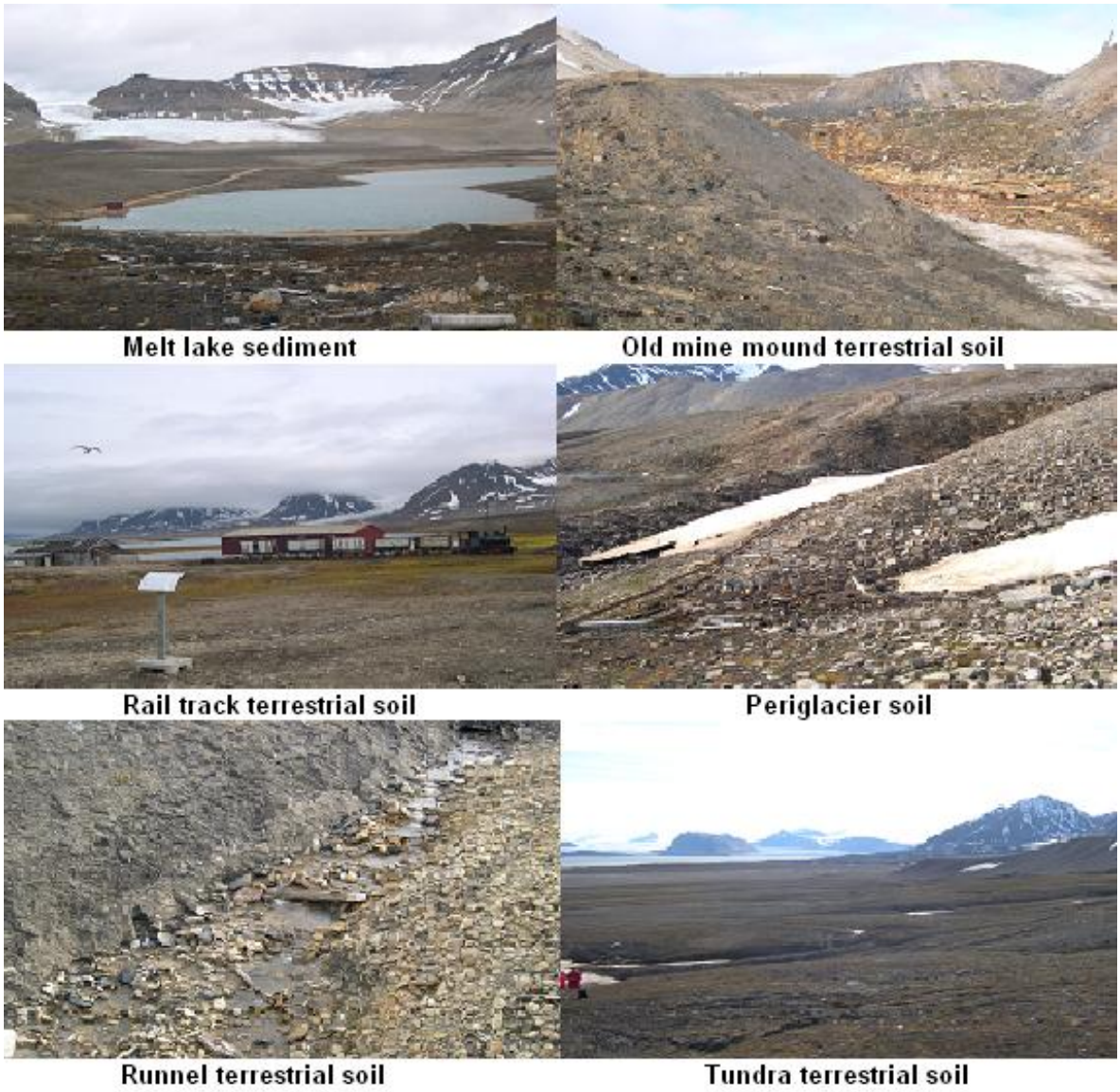


Figure 3.1: Arctic soils and sediments collected from a few studied sites (Photo credit: Irene K. P. Tan).

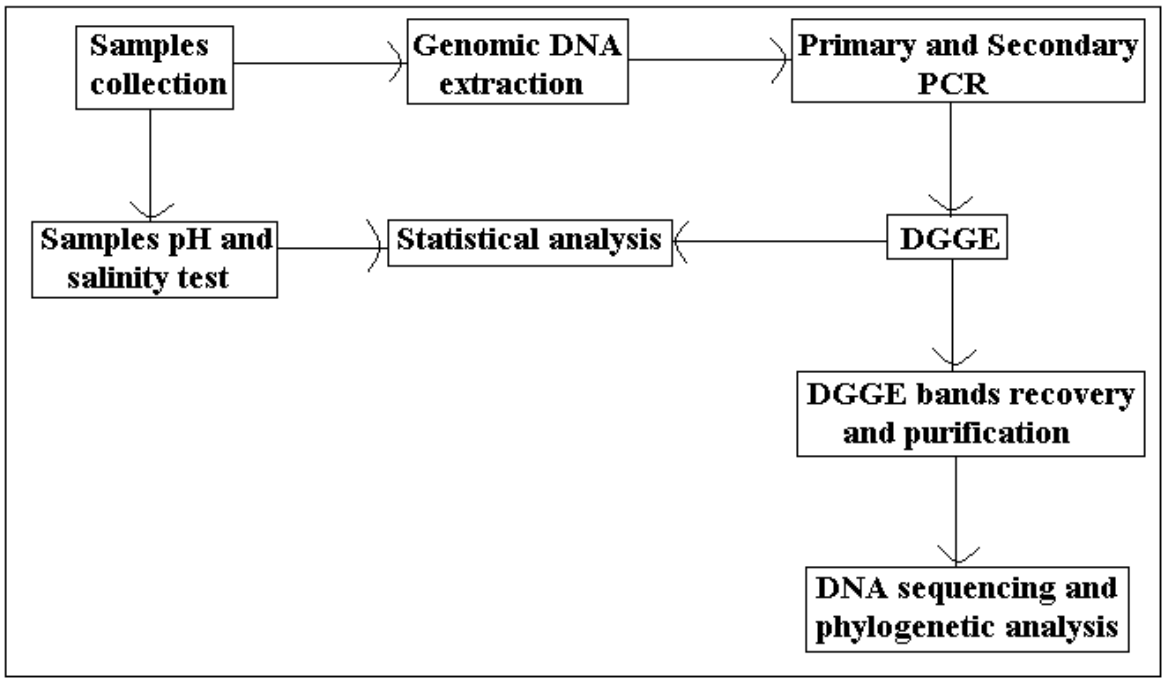


Figure 3.2: Flow chart of experiments carried out in this study.

3.2.2 Chemical analysis of soil and sediment samples

3.2.2.1 pH of soil and sediment samples

The pH of soils and sediments was measured by a pH meter (Eutech Instrument-Oaklon Instrument, Malaysia), in 1:5 (w/v) suspensions of each sample in sterile distilled water (Kastovska *et al*., 2007). The pH reading of the sample was taken twice to avoid reading error.

3.2.2.2 Salinity of soil and sediment samples

The salinity of soils and sediments was measured as electrical conductivity (EC), in 1:5 (w/v) suspensions of each sample in sterile distilled water (Chong *et al*., 2009b), using a conductivity meter (Martini Instrument, Romania).

3.2.3 Extraction of genomic DNA from soil and sediment samples

The genomic DNA from soil and sediment samples were extracted using Ultra Clean™ Soil DNA Isolation Kit (MoBio Inc., CA) based on the manufacturer's protocol (Figure 3.3). The yield and the purity of extracted genomic DNA were measured as UV absorbance at 230 nm, 260 nm, and 280 nm, using a biophotometer (Eppendorf, Germany).

An experiment was carried out with one of the samples (sample 17) with the dilution of the extracted DNA and another without. This was conducted to compare the differences in the DNA yield and purity, and to check the need to dilute DNA template during amplification of 16S rRNA gene fragment from sample DNA by polymerase chain reaction (Section 3.2.6.1).

MO BIO Laboratories, Inc.

Alternative Protocol (For maximum yields)

Please wear gloves at all times

1. To the 2ml Bead Solution tubes provided, add 0.25 - 1gm of soil sample. (For larger sample sizes up to 10 grams, try using our Mega Prep Kit, catalog number 12900-10. For amounts of sample to process see Hints and Troubleshooting Guide).
2. Gently vortex to mix.
3. **Check Solution S1.** If Solution S1 is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60µl of Solution S1 and invert several times or vortex briefly.
5. Add 200µl of Solution IRS (Inhibitor Removal Solution). Only required if DNA is to be used for PCR.
6. Secure bead tubes horizontally using the Mo Bio Vortex Adapter tube holder for the vortex (cat.13000-V1. Call 1-800-606-6246 for information) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes. (See alternative lysis method for less DNA shearing).
7. Make sure the 2ml tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds. **CAUTION:** Be sure not to exceed 10,000 x g or tubes may break.
8. Transfer the supernatant to a clean microcentrifuge tube (provided).
9. **Note**: With 0.25gm of soil and depending upon soil type, expect between 400 to 450µl of supernatant. Supernatant may still contain some soil particles.
10. Add 250µl of Solution S2 and vortex for 5 sec. Incubate 4°C for 5 min.
11. Centrifuge the tubes for 1 minute at 10,000 x *g*.
12. Avoiding the pellet, transfer entire volume of supernatant to a clean microcentrifuge tube (provided).
13. Add 1.3ml of Solution S3 to the supernatant (careful, volume touches rim of tube) and vortex for 5 seconds.
14. Load approximately 700µl onto a spin filter and centrifuge at 10,000 x *g* for 1 minute. Discard the flow through, add the remaining supernatant to the spin filter, and centrifuge at 10,000 x *g* for 1 minute. Repeat until all supernatant has passed through the spin filter. **Note**: A total of three loads for each sample processed is required.
15. Add 300µl of Solution S4 and centrifuge for 30 seconds at 10,000 x *g*.
16. Discard the flow through.
17. Centrifuge again for 1 minute.
18. Carefully place spin filter in a new clean tube (provided). Avoid splashing any Solution S4 onto the spin filter.
19. Add 50µl of Solution S5 to the center of the white filter membrane.
20. Centrifuge for 30 seconds.
21. Discard the spin filter. DNA in the tube is now application ready. No further steps are required.

We recommend storing DNA frozen (-20°C). Solution S5 contains no EDTA.

Thank you for choosing the UltraClean Soil DNA Isolation Kit.

Version 03222005

Figure 3.3: The manufacturer's protocol of genomic DNA extraction from soil and sediment samples (MoBio Inc., CA).

3.2.4 Amplification of 16S rRNA gene fragment from soils and sediments DNA

3.2.4.1 Primary Polymerase Chain Reaction (PCR)

Primary PCR was conducted to amplify the bacterial 16S rRNA gene fragment from the extracted genomic DNA of the samples. The 16S rDNA universal primers: the forward primer (27F, 5'-AGA GTT TGA TCM[a] TGG CTC AG-3', [a] Mixed bases nomenclature, M = A + C) and the reverse primer (1492R, 5'-GGT TAC CTT GTT ACG ACT T-3') (Lane, 1991; Webster *et al*., 2003; Newberry *et al*., 2004), which were synthesized by First BASE Laboratories Sdn. Bhd., Malaysia were used to amplify the bacterial 16S rRNA gene fragment (1500 bp). The PCR reactions were performed in a tube using Thermal Cycle (Bio-Rad Laboratories Inc., CA). For each reaction, a total volume of 25 µl reaction mixture containing 1 µl DNA template (10X dilution of extracted DNA), 0.5 µM of each primer, 0.26 mM of each dNTP, 1X PCR buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl] and 0.75 U *Taq* DNA Polymerase (Invitrogen, USA) was used. The preparations of PCR (Table 3.3) were conducted under aseptic conditions in a laminar flow chamber. PCR conditions (Webster *et al*., 2003) were as follows: initial denaturation at 95 ℃ for 2 minutes, followed by 30 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 52.5 ℃ for 30 seconds, and extension at 72 ℃ for 1 minute and 30 seconds. The final extension was initiated at 72 ℃ for 10 minutes. The amplicons were then analysed by agarose gel electrophoresis.

Table 3.3: Reaction mixture for primary PCR

Materials for PCR reaction mixture	**Volume used**	**Final concentration**
Magnesium Chloride	0.75 µl	50 mM
10X PCR buffer	2.5 µl	1X
27F(forward primer)	2 µl	0.5 µM
1492R (reverse primer)	2 µl	0.5 µM
dNTP mix	0.65 µl	0.26 mM
Taq DNA polymerase	0.15 µl	0.75 U
DNA template (extracted genomic DNA)	1 µl	-
Sterile distilled water	15.95 µl	-
Total volume	25 µl	-

3.2.4.2 Secondary Polymerase Chain Reaction (PCR)

Secondary PCR with GC-rich forward primer (341 F-GC) was conducted to amplify a portion of the 16S rRNA gene fragments obtained from the primary PCR with a GC-clamp. The GC-rich forward primer was used to stabilize the migration fragments during DGGE, in order to prevent complete strand separation and to produce sharp bands on DGGE profile (Sheffield *et al*., 1989; Kirk *et al*., 2004). The secondary PCR amplicons were able to separate into several bands during Denaturing Gradient Gel Electrophoresis (DGGE).

The secondary PCR primers: the forward primer (341F-GC, 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3') and the reverse primer (907R, 5'-CCG TCA ATT CCT TTG AGT TT-3') which were synthesized by First BASE Laboratories Sdn. Bhd., Malaysia would generate a 643 bp fragment (Powel *et al*., 2003). For each reaction, a total volume of 50 µl reaction mixture containing 2 µl DNA template (50X dilution of primary product), 0.5 µM of each primer,

0.1 mM of each dNTP, 1X PCR buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl] and 2.5 U *Taq* DNA Polymerase (Invitrogen, USA) was used. The preparations of PCR (Table 3.4) were conducted under aseptic conditions in a laminar flow chamber. PCR conditions (Powel *et al*., 2003) were as follows: initial denaturation at 94 ℃ for 5 minutes, followed by 10 cycles of denaturation at 94 ℃ for 1 minute, annealing at 65 ℃ for 1 minute, and extension at 72 ℃ for 3 minutes; continued by 20 cycles of denaturation at 94 ℃ for 1 minute, annealing at 55 ℃ for 1 minute, and extension at 72 ℃ for 2 minutes. The final extension was initiated at 72 ℃ for 45 minutes. The amplicons were then analyzed by agarose gel electrophoresis.

Table 3.4: Reaction mixture for secondary PCR

Materials for PCR reaction mixture	**Volume used**	**Final concentration**
Magnesium Chloride	1.5 µl	50 mM
10X PCR buffer	5 µl	1X
27F(forward primer)	5 µl	0.5 µM
1492R (reverse primer)	5 µl	0.5 µM
dNTP mix	0.5 µl	0.1 mM
Taq DNA polymerase	0.5 µl	2.5 U
DNA template (primary PCR product)	2 µl	-
Autoclaved distilled water	30.5 µl	-
Total volume	50 µl	-

3.3.5 Agarose Gel Electrophoresis (AGE)

AGE was used to analyze the products of genomic DNA extraction, primary PCR, secondary PCR, PCR without GC-clamp, and gel purification. It was used to confirm the presence of the expected products. 5 µl of product was mixed well with 2 µl of 6X gel loading dye before being loaded into a well of the agarose gel (1 % w/v in 1X TAE buffer). AGE was carried out in 1X TAE buffer at 110 V for 45 minutes. The gels were stained with Ethidium Bromide and visualized under a UV transilluminator (Syngene Bio Imaging, UK). A 100bp DNA ladder purchased from Vivantis, Malaysia or Norgen, Canada (Figure 3.4) was used to estimate the DNA yield and the size of the products.

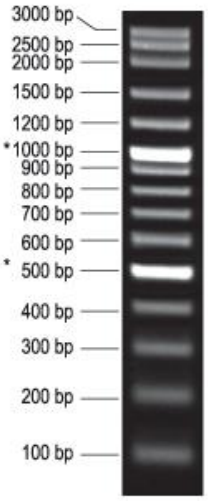
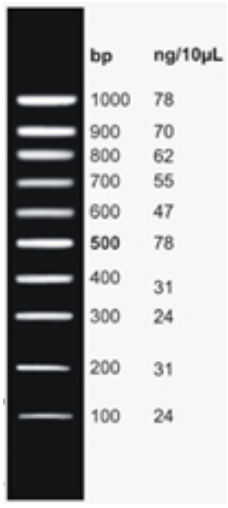
VC 100 bp Plus DNA Ladder (Vivantis)	**PCR Sizer 100 bp DNA Ladder (Norgen)**
	
100 bp DNA ladder separated by agarose gel 1.0 % (w/v) in 1X TAE stained with Ethidium Bromide.	100 bp DNA ladder separated by agarose gel 1.0 % (w/v) in 1X TAE stained with Ethidium Bromide.

Figure 3.4: Description of molecular sizes of purchased DNA ladder.

3.3.6 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

3.3.6.1 DGGE analysis on amplified 16S rRNA gene fragments from DNA sample with and without dilution

DGGE was carried out on 16S rRNA gene fragments amplified from a sample after 10-fold dilution and without dilution of DNA extracts during PCR. This was aimed to compare the influences of DNA dilution on DGGE banding pattern, and to determine whether or not the dilution of DNA template during PCR is necessary for a clearer DGGE profile.

3.3.6.2 DGGE analysis on amplified 16S rRNA gene fragments from samples DNA

DGGE was carried out on 16S rRNA gene fragments amplified from samples by PCR to compare the bacterial diversity between samples (Schafer and Muyzer, 2001). The PCR amplicons were separated using 6 % (w/v) polyacrylamide gels with a denaturant gradient between 35 % and 60 % (100 % denaturant contains 7 M urea and 40 % formamide). The DGGE gel solutions were prepared (Table 3.5) and poured with the aid of 30 ml volume Gradient Delivery System (Bio-Rad Laboratories Inc., CA) into a gel mixer (Bio-Rad Laboratories Inc., CA). The polyacrylamide gels were let stand to polymerize for 3 hours and were placed into a D-Code Universal Mutation Detection System (Bio-Rad Laboratories Inc., CA) and pre-run at 80 V with the temperature of 60 ℃ for 30 minutes in 1X TAE buffer (Powell *et al*., 2003; Powell *et al*., 2004).

A mixture containing 45 µl of PCR amplicon of each sample and 10 µl of 6X Bromophenol blue (BPB) loading dye was loaded into the wells of the polyacrylamide gel. Excised bands with distinct melting position from previous DGGE runs, which were amplified by PCR, were used as markers for DGGE profile alignments. The markers generated from previous DGGE runs have distinct positions on the DGGE profiles, and

thus can be used to normalize the banding pattern of samples in different DGGE gels. The DGGE was then run at 80 V with the temperature of 60 ℃ for 15 hours (Powell *et al*., 2003; Powell *et al*., 2004).

The polyacrylamide gels were then stained with 1:10,000 (v/v) SYBRGold nucleic acid gel stain in the dark for 1 hour and the rinsed with distilled water. The gels were viewed under UV transilluminator (Syngene Bio Imaging, UK) and the captured gel images were further statistically analyzed using Primer 6 multivariate data analysis package (Plymouth Marine Laboratory, UK).

The well defined bands from the DGGE gels were excised to re-amplify and purify for sequencing purposes (Details in Section 3.2.8, 3.2.9 and 3.2.10).

Table 3.5: Preparation of DGGE gel solution for a polyacrylamide gel with a denaturant gradient between 35 % and 60 %

Components of DGGE gel solution	**35 % gel solution**	**60 % gel solution**	**Cap solution**
Autoclaved distilled water (ml)	8.7	6.2	3.6
40 % Acrylamide Bis (ml)	2.3	2.3	1.3
50X TAE buffer (ml)	0.3	0.3	0.1
7 M Urea (g)	2.2	3.7	-
40 % Formamide (ml)	2.1	3.6	-
100 % Glycerol (ml)	0.3	0.3	-
10 % Ammonium Persulphate (µl)	300	300	100
TEMED (µl)	15	15	5

3.3.7 Statistical analysis of DGGE profiles

3.3.7.1 Comparison of bacterial diversity between the samples

The DGGE profiles were transformed into a presence/absence binary matrix using Quantity-One™ image analysis software (BioRad Laboratories Inc., USA). Markers that re-amplified from previous DGGE runs were used to normalize the banding pattern of different DGGE gels. The transformed DGGE profiles were then used for several statistical analyses that are available in the Primer 6 multivariate data analysis package (Plymouth Marine Laboratory, UK).

Non-metric Multidimensional Scaling (nMDS) plots and Hierarchical cluster analyses were used to compare the similarity of the bacterial communities among the samples. These analyses were generated by resemblance matrix based on Bray-Curtis ordination (Bray and Curtis, 1957):

$$S_{jk} = 100 \left\{ 1 - \frac{\sum_i^p |y_{ij} - y_{ik}|}{\sum_i^p (y_{ij} + y_{ik})} \right\}$$

Bray-Curtis ordination calculated the similarity between two samples, where value 100 was showed in two identical samples while value 0 was showed in two samples that have no species (DGGE band) in common (Clarke and Gorley, 2006). nMDS plots was used for the integration of complex data sets (DGGE banding patterns) into new mathematical variables which can be displayed into a few-dimension simple scheme (Ter Braak *et al.*, 1995; Clarke and Gorley, 2006). Hierarchical cluster analyses clustered samples that have similar DGGE banding patterns together (Ibekwe *et al.*, 2001; Yang *et al.*, 2001; Boon *et al.*, 2002), and the similarity of bacterial communities between samples can be assessed rapidly.

Shannon's Diversity Index (*H′*) (Shannon and Weaver, 1971) inferred from DGGE presence/absence binary matrix data was used to compare the dominant bacterial diversity (species richness) in each of the studied samples. *H′* was calculated using the equation:

$$H' = - \sum Pi \ln (Pi)$$

where *Pi* is the proportion of the *i*th species in each sample (Gafan *et al*., 2005; Clarke and Gorley, 2006; Barrett *et al*., 2006).

3.3.7.2 Correlation between bacterial diversity and environmental variables

The Biota and Environment matching (BEST) procedure (Clarke and Gorley, 2006) from Primer 6 multivariate data analysis package (Plymouth Marine Laboratory, UK) was used to correlate the measured environmental variables (pH and electrical conductivity) and the bacterial composition as showed in DGGE profiles (Chong *et al*., 2009b). The correlation was calculated according to Spearman rank order correlation (Kendall, 1970). The correlation measured was the agreement between resemblance matrices of biotic and abiotic by matching the elements. The equation of Spearman rank order correlation was as follows:

$$\rho_s = 1 - \frac{6}{N(N^2 - 1)} \sum_{i=1}^{N} \frac{(r_i - s_i)^2}{r_i + s_i}$$

Where r_i and s_i are the unravelled elements and $N = n\,(n - 1)/2$ (where n is the number of samples)

3.3.8 Recovery of well-defined DGGE bands by PCR amplifications

The well-defined bands in the DGGE were excised using a sterile scalpel blade, and were incubated overnight in sterile distilled water at 4 ℃ prior to re-amplification using PCR primers without GC-clamp (341F, 5'-CCT ACG GGA GGC AGC AG-3') and (907R, 5'-CCG TCA ATT CCT TTG AGT TT-3'), which were synthesized by First BASE Laboratories Sdn. Bhd., Malaysia. For each reaction, a total volume of 100 µl reaction mixture containing 10 µl of the suspension as DNA template, 0.5 µM of each primer, 0.12 mM of each dNTP, 1X PCR buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl] and 3 U *Taq* DNA Polymerase (Invitrogen, USA) was used. The preparations of PCR reaction (Table 3.6) were conducted under aseptic conditions in a laminar flow chamber. PCR conditions referred to Powell *et al*. (2003) with some modifications were as follows: initial denaturation at 94 ℃ for 3 minutes, followed by 30 cycles of denaturation at 93 ℃ for 30 seconds, annealing at 56 ℃ for 30 seconds, and extension at 72 ℃ for 1 minute. The final extension was initiated at 72 ℃ for 10 minutes. The amplicons then were analyzed by agarose gel electrophoresis.

Table 3.6: Reaction mixture for PCR without GC-clamp

Materials for PCR reaction mixture	**Volume used**	**Final concentration**
Magnesium Chloride	3 µl	50 mM
10X PCR buffer	10 µl	1X
27F(forward primer)	10 µl	0.5 µM
1492R (reverse primer)	10 µl	0.5 µM
dNTP mix	1.2 µl	0.12 mM
Taq DNA polymerase	0.6 µl	3 U
DNA template (DGGE band supernatant)	10 µl	-
Autoclaved distilled water	55.2 µl	-
Total volume	100 µl	-

3.3.9 Purification of amplified DGGE bands

The products from PCR without GC-clamp were then purified by using Gel DNA Recovery Kit (Vivantis, Malaysia). The purification steps were based on manufacturer's protocol (Figure 3.5).

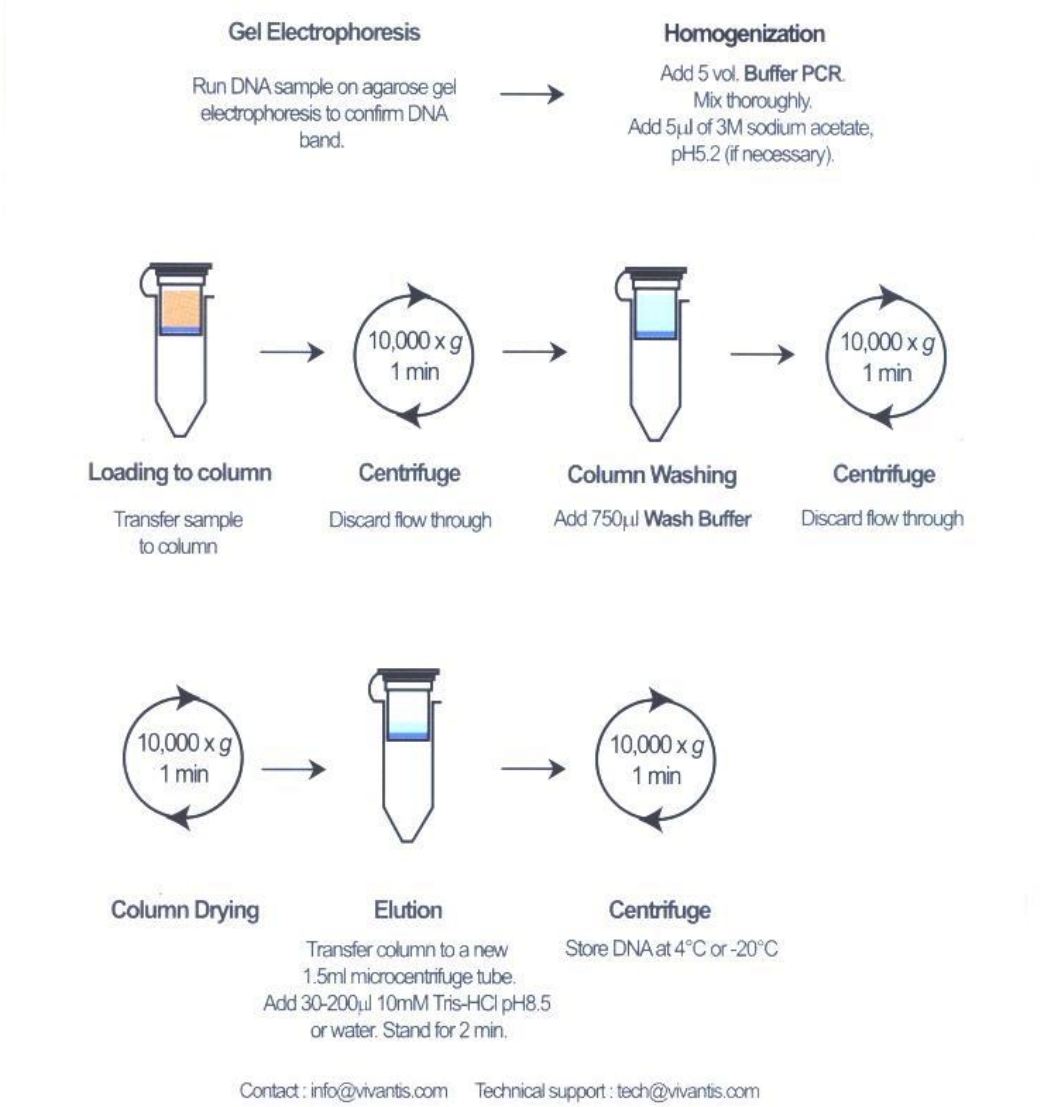


Figure 3.5: The manufacturer's protocol of purification of amplified DGGE bands.

3.3.10 DNA nucleotide sequence analysis and phylogenetic analysis

DNA sequencing for purified DGGE bands was carried out by Macrogen Inc., Seoul, Korea. The sequences were compared to NCBI nucleotide database by Basic Local Aligned Search Tool (BLAST) (Altschul *et al*., 1990) to obtain the closest match. The sequences were then aligned using Clustal W, and a Neighbor-Joining (NJ) tree (Saitou and Nei, 1987) was constructed on the aligned sequences using the Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 (Tamura *et al*., 2007). Taxonomic classification of each sequence was identified based on Ribosomal Database Project 10 classifier (Wang *et al*., 2007) and the confident threshold was set at 80%.