Chapter 2: Materials and methods

2.1. Sample collection

Sampling of nematodes needs to be done on untreated soil and roots of banana trees. Here, nematodes were extracted from randomly chosen banana trees in University of Malaya, Kuala Lumpur. Three cavities (around 30cm deep) were made around a banana tree and untreated soil and roots were collected from the cavities by spade. The untreated soil and banana roots collected were kept in secured and labeled plastic bags. Nematode extraction from untreated soil and root was carried out in Malaysia Agriculture Research and Development Institute (MARDI) in Serdang, Selangor.

2.2. Nematode extraction from soil and root

2.2.1. Separation of roots from soil

The banana roots have to be separated from the soil before proceeding to nematode extraction. The untreated soil and roots mixture was poured on a plastic cover and the roots were picked and placed into a labeled 200ml cup (Figure 2.1). The remaining untreated soil was mixed well before being scooped into another labeled 200ml cup (Figure 2.2).

Figure 2.1: Untreated roots

Figure 2.2: Untreated soil

2.2.2. Nematode extraction from soil samples

First, a sieve was placed on a plate and followed by placing a two-ply dampened tissue paper to cover the inside surface of the sieve (Figure 2.3). The untreated soil was mixed well with tap water and then poured into the Oostenbrik funnel (Figure 2.4). The mixture was left until the water pressure reaches to 9 and then immediately the stopper was removed. The soil mixture moves through the sieve then surrounding of 2mm sieve was washed with tap water. The sample was collected in 2mm sieve was poured into sieve which prepared earlier (Figure 2.5). The sieves were covered by a plate to prevent of water evaporation and left overnight.

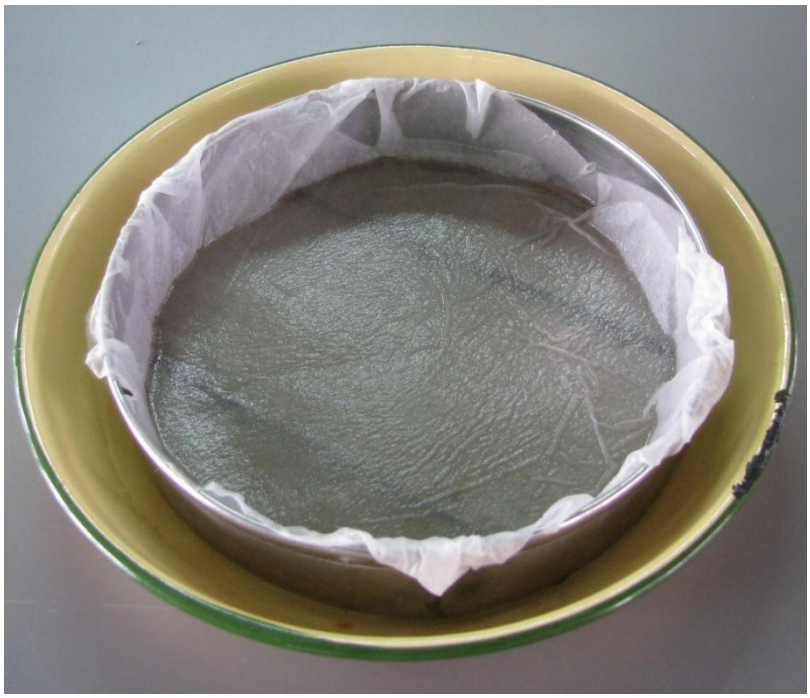

Figure 2.3: Sieve layered with damp tissues

Figure 2.4: Oostenbrink funnel

Figure 2.5: Sieves with untreated soil

2.2.3. Nematode extraction from the root samples

The banana roots were placed into a labeled 200ml cup and washed with tap water. After that, 5g of the root samples were ground using kitchen blender with a small quantity of dH_2O. The mixtures were poured into the sieves and left for overnight (Figure 2.6).

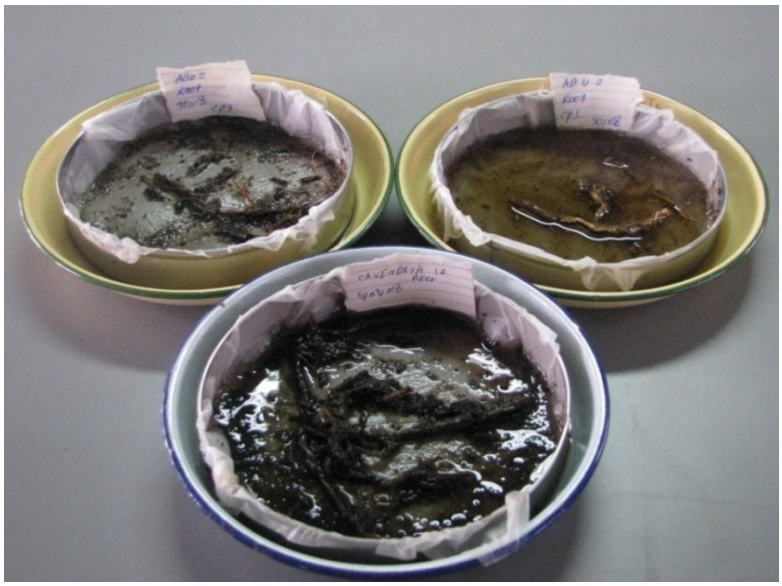

Figure 2.6: Sieve containing ground banana roots

2.2.4. Morphological identification of nematode

Morphological identification of the nematodes was carried out by Mr. Zaidun Bilal Mat from MARDI, Selangor with the aid of a light microscope. Sterile 1.5ml microcentrifuge tubes were filled with 30µl dH_2O and an individual identified nematode was placed into each tube and labeled accordingly.

2.3. DNA extraction

In this study, 3 different DNA extraction methods were used. First method is a conventional protocol adapted from Madania *et al*., 2005. DNA extraction was also carried out using G-spin™ Genomic DNA Extraction kit and QIAamp DNA Min kit for DNA following the manufacturers' guidelines.

2.3.1. Modified Madania *et al*., 2005 method.

In each sterile 1.5ml microcentrifuge tube containing a single nematode and 30µl of dH_2O, 0.6mg/ml Proteinase K (Vivantis, Malaysia) and 1× Mg-free Thermophilic DNA polymerase reaction buffer (Promega, USA) was added to the sample. Then dH_2O was added to the sample until the final volume of 132µl and sample was incubated at 65°C for either seven hours or overnight in a waterbath. After the incubation is completed, the sample was incubated further at 95°C for 15 minutes before storing it at -20°C.

2.3.2. Modified G-spin ^{TM} Genomic DNA Extraction kit

In this method, a single nematode in a sterile 1.5ml microcentrifuge tube was first snap-frozen in liquid nitrogen before 400µl of G buffer was added to the tube. Sample was homogenized using a mechanical homogenizer. Next, the sample was incubated in a waterbath set at 70°C overnight. The mixture was then mixed by pipetting for several times. This step is a semi-homogenization step that can denature proteins as well as facilitate cell lysates to pass efficiently through the filter column. Next, 400µl of binding buffer was added to the mixture and it was mixed gently, followed by a centrifugation step at 13,000 rpm for five minutes at room temperature. A spin column was placed on a provided 2ml collection and the mixture was pipetted to spin column and centrifuged at 13,000 rpm for another minute at room temperature. The flow-through was discarded and the spin column was placed back into the same collection tube. The spin column was centrifuged again at 13,000 rpm for one minute at room temperature to remove all traces of cell lysates. The flow-through was completely removed and the spin column was placed back into the same collection tube. In the next step, 500µl of washing buffer was pipetted into the spin column and centrifuged at 13,000 rpm for one minute at room temperature. The flow-through was discarded and spin column was returned to the same collection tube before repeating centrifugation step to dry the filter membrane. Later, the spin column was placed into a new sterile 1.5ml microcentrifuge tube. Finally 100µl of elution buffer was dropped to the center of the filter membrane and left to stand for one minute at room temperature before being centrifuged for one minute at 13,000 rpm. The DNA sample was kept at 4°C overnight and then transferred to -20°C the next day.

2.3.3. Modified QIAamp DNA Mini kit

20µl Proteinase K was added to the sterile 1.5ml microcentrifuge tube containing a single nematode and was mixed by vortexing. The sample was incubated in a waterbath set at 56°C with agitation at 220 rpm overnight to completely lyse the cells. After the overnight incubation, the sample was briefly centrifuged at 13,000 rpm to remove droplets from inside the lid. 0.4mg of RNAse A (Sigma-Aldrich, USA) was added to the mixture, mixed by vortexing for 15 seconds and left for two minutes at room temperature. The sample was briefly centrifuged at maximum speed to remove droplets from inside the lid. Next, 200µl buffer AL was added to the sample, mixed by vortexing for 15 seconds and incubated at 70°C for 10 minutes. It was necessary for buffer AL and the sample to be mixed thoroughly to yield a homogeneous solution. Then the sample was again centrifuged at maximum speed to remove drops from inside the lid. In the next step, 200µl of absolute ethanol (Merck, Germany) was added to the sample and mixed by vortexing for 15 seconds to yield a homogeneous solution. The sample was briefly centrifuged at 13,000 rpm at room temperature to remove drops from inside the lid. A QIAamp spin column was placed into a provided 2ml collection tube before the mixture was carefully transferred into the spin column without wetting the rim. The spin column was then capped to avoid aerosol formation during centrifugation. Centrifugation was done at 8,000 rpm for one minute at room temperature. The 2ml collection tube containing the filtrate was discarded as QIAamp spin column was placed into a new clean 2ml collection tube. 500µl of buffer AW1 was carefully added into the sample without wetting the rim, capped and centrifuged at 8,000 rpm for another minute at room temperature. This washing step was repeated using buffer AW2 and centrifuged at 14,000 rpm for three minutes at room temperature. The QIAamp

spin column was placed into a new 2ml collection tube and centrifugation step was repeated at 14,000 rpm for one minute at room temperature. The reason for this was to eliminate any risk of buffer AW2 carryover due to improper handling of the column. Subsequently, QIAamp spin column was placed into a sterile 1.5ml microcentrifuge tube and 50µl dH_2O was carefully added to the center of spin column membrane. The tube was left to stand at room temperature for one minute before the sample was centrifuged at 8,000 rpm for another minute at room temperature. In order to increase the final DNA yield, the same eluent was applied to the membrane again, left at room temperature for five minutes and then spun down as previously described. The DNA sample was kept overnight at 4°C then transferred to -20°C on the following day.

2.4. Qualitative and quantitative analysis

Qualitative and quantitative measurement of DNA was done using a spectrophotometer (Eppendorf, Germany). This technique is based on measuring the amount of light that was absorbed by the solution placed in a Quartz cuvette. The sample was diluted 50× with dH_2O to the final volume of 50µl. The wavelengths used were 260nm and 280nm. A DNA sample is considered pure when its value falls in a range of between $OD_{260/280}$ 1.8 to 2.0. OD $_{260/280}$ reading of less than 1.8 or more than 2.0 indicates that the DNA sample may be contaminated with protein.

2.5. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is used to copy specific region of DNA fragment and amplifying it in an exponential manner. A basic PCR reaction requires a set of reagents including DNA template, forward and reverse primers, *Taq* DNA polymerase, PCR buffer solution, $MgCl_2$ solution, dNTP mixture and dH_2O. Polymerase chain reaction consists of several amplification cycles with each cycle is consist of three main steps; DNA template denaturation, primer annealing and primer extension. Fallas-Kaplan primer pair (Fallas *et al*., 1996; Kaplan *et al*., 2000) was used to amplify the ITS1 and ITS2 region of the DNA. The primers used in this study were 18S forward primer 5'TTGATTACGTCCCTGCCCTTT3' (Fallas *et al*., 1996) and 28S reverse primer 5'ATATGCTTAAGTTCAGCGGGT3' (Kaplan *et al*., 2000). PCR was carried out in a sterile 0.2ml tube to the total reaction volume of 25µl. One PCR reaction consists of 1× AmpliBuffer A (EURx, Poland), 1.5mM $MgCl_2$ (EURx, Poland), 200µM dNTP (Fermentas, Canada), 0.5mM of each forward and reveres primers (BioNEER, Korea), 1U of *Taq* DNA polymerase (EURx, Poland), 100ng DNA sample and topped up with dH_2O to the final volume of 25µl.

Thermal cycling of PCR was performed using PTC-100^{TM} Programmable Thermal Control (MJ Research, USA). PCR was carried out with initial denaturation step at 94°C for three minutes, followed by 35 cycle of denaturation at 94°C for one minute, annealing at 55°C for one minute , extension at 72°C for one minute followed by the final elongation step at 72°C for five minutes.

2.6. PCR product analysis

2.6.1. Agarose gel electrophoresis

The amount of agarose and TBE to be used depends on the concentration of the gel required for analysis. Agarose gel of 1% concentration is normally adequate to analyse the PCR products. To prepare a 1% gel, 0.45g agarose powder (Promega, USA) was dissolved by heat in 45ml of 1× Tris-Borate EDTA solution (TBE) buffer using a microwave and was cooled down under running tap water. One microlitre of Ethidium bromide (EtBr) (10mg/ml) was added into the mixture and mixed before the mixture was poured into gel caster set that has been prepared earlier. The gel was left to solidify at room temperature for around 30 minutes. Once harden, the gel was placed into an electrophoresis tank filled with 1× TBE buffer and proceeded to sample loading.

2.6.2. Sample loading

5µl of each PCR product was mixed by pipetting with 6× loading dye at 5:1 ratio on a parafilm strip before transferring them into their respective wells. At the end, 2.5µl of 100bp DNA ladder (Seegene, USA) was loaded into one of the wells. Electrophoresis was carried out at 120V for approximately 25 minutes. DNA is negatively charged due to the phosphate backbone of DNA chain. So it would migrate towards the positive electrode through the agarose gel matrix, separated according to fragment size. Upon completion of electrophoresis, gel was viewed under the UV light using Alpha Imager 2200 UV transilluminator (Alpha Innotech Corporation, USA). Under UV exposure, EtBr molecules

that have bound to DNA molecule will start to fluoresce, indicating the presence of DNA as well as its fragment size that can be determined by comparison to the DNA ladder.

2.7. Purification of PCR products by gel extraction.

In this study, PCR products were purified through gel extraction, using QIAquick Gel Extraction kit (Qiagen, Germany). QIAquick gel extraction kit allows extraction and purification of DNA of 70bp to 10kb in size from standard or low-melting agarose gels in TAE or TBE buffer. Agarose gel was prepared according to the method described in section 2.6.1. PCR products were pooled together in a tube and were added with 6× loading dye to the ration of 5:1. Then the whole sample mixture was loaded into a gel well. Electrophoresis was carried out at 110V for 50 minutes. After electrophoresis is completed, the gel was viewed under the UV light and the DNA bands of desired size was excised using a clean scalpel. Excised gel pieces were placed into weighed and labeled sterile 1.5ml microcentrifuge tube.

Three volume of buffer QG was added to one volume of gel. Then the sample was incubated on a heat block (Gemmy industrial copr, Taiwan R.O.C.) at 50°C for 10 minutes with occasional vortexing. Buffer QG is yellow in color which is indicative of pH $\leq$ 7.5 and which is the optimal pH for DNA binding. 10µl of 3M sodium acetate (pH 5.0) was added to the sample mixture if there was any color change during incubation. Next, 100µl of isopropanol (Systerm, Malaysia) was added to each 100mg of gel weight and was mixed well. A QIAquick spin column was placed into a provided 2ml collection tube before 750µl of sample mixture was pipetted into QIAquick spin column. The column was centrifuged at

13,000 rpm for one minute at room temperature. Filtrate collected was discarded and QIAquick column was placed back into the same collection tube. After that, 500µl of buffer QG was transferred into the column and centrifuged at 13,000 rpm for one minute at room temperature. In this step all traces of agarose was removed. 750µl of buffer PE (ethanol-diluted to the ratio of 1:5) was added to QIAquick column and centrifuged at 13,000 rpm for one minute at room temperature. After discarding the flow-through, the QIAquick column was placed back into the same collection tube and the centrifugation step was repeated again any to remove residual ethanol from the sample. Next the QIAquick column was transferred into a new sterile 1.5ml microcentrifuge tube and finally, 30µl-50µl of buffer EB or dH_2O was dropped into the center of QIAquick membrane to elute the DNA. Then the column was left to stand for one minute before it was centrifuged at 13,000 rpm for one minute at room temperature.

To check the presence of DNA from elution was mixed with 6× loading dye and 30% glycerol at 5:1:1 ratio on a parafilm strip by pipetting. The mixture was loaded into 1% agarose gel and electrophoresis was set at 120V for 30 minutes. Then gel was viewed under UV light by Alpha Imager 2200 UV transilluminator (Alpha Innotech Corporation, USA). The sample is kept at 4°C for overnight and later stored at -20°C.

2.8. Cloning

2.8.1. Luria Bertani (LB) agar plate

For preparation of 200 mililitre of LB agar media, 7g of LB agar powder (Pronadisa, Spain) was mixed with 200ml of sterile dH_2O using a magnetic stirrer until the powder is completely dissolved. Then the mixture was autoclaved at 121°C, 100 kPa for 15 minutes. After the sterilization step is completed, the mixture was cooled down under running tap water. Next, 50µg/ml of Ampicillin (Sigma, USA), 0.5mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermentas, Canada) and 0.08mg/ml of X-gal (Vivantis, Malaysia) was added to the mixture. It was mixed well before 20ml of the liquid agar was poured into each plate. All the plates were left to solidify for about 2 hours and then stored at 4°C.

2.8.2. Luria Bertani (LB) broth medium

For preparation of LB broth medium, 8g of LB broth powder base (Pronadisa, Spain) was mixed with 400ml of sterile dH_2O using a magnetic stirrer until all the powder was completely dissolved. After that 10ml of the mixture was aliquoted into each universal bottle and autoclaved at 121ºC, 100 kPa for 15 minutes. After autoclaving, the bottles were kept at room temperature for future use.

2.8.3. Preparation of competent cells

2µl of *Escherichia coli* strain JM109 bacterial stock was cultured in 10ml LB broth at 37°C, with 220 rpm agitation for overnight. The next day, 1ml of the overnight culture was subcultured into a new 10ml LB broth aliquot at 37°C with 220 rpm agitation in for approximately 10 minutes until cell density reaches OD_{600} value of 0.5. Once the desired cell density was reached, the subculture was transferred into sterile 15ml Falcon tube and left in ice for 30 minutes. After that the sample was centrifuged at 3,000 rpm for five minutes at 4°C. Next, the supernatant was decanted before 5ml of RF1 solution was added. The pellet formed was resuspended by gentle inversion on ice. Then the suspension was incubated in ice for 20 minutes and was centrifuged at 3,000 rpm for 15 minutes at 4°C. After decanting the supernatant, the pellet was resuspended with 2ml of RF2 by gentle inversion on ice. 100µl of the competent cell suspension was aliquoted into sterile 1.5ml microcentrifuge tubes and were snap-frozen in liquid nitrogen for a few seconds before being stored in -80°C for future use.

2.8.4. DNA ligation

DNA cloning was done using pGEM®-T Easy ®Vector Systems (Promega, USA). The ligation reaction was set up to the final volume of 10µl in a sterile 0.5ml tube on ice. The 3µl of gel-purified DNA sample was mixed with 5µl of 2× Rapid Ligation Buffer, 1µl of 50ng of pGEM®-T Easy Vector and lastly 1µl of 3U T4 DNA ligase. The mixture was mixed by pipetting very slowly and kept in 4°C overnight.

2.8.5. Transformation by heat shock method

The overnight ligation mixture was centrifuged at 13,000 rpm for 15 seconds to collect the content at the bottom of the tube. Then 2.5µl of ligation product was pipetted into a pre-chilled sterile 1.5ml microcentrifuge tube. After that, 100µl of competent cell were added into sterile 1.5ml microcentrifuge tube that contains the ligation reaction and mixed together gently before the tube was placed in ice for 20 minutes. The tube is then placed in 42°C waterbath for 45-50 seconds and immediately returned to ice for two minutes. Subsequently, 900µl of sterile LB broth media was added into the tube and followed by incubation at 37°C for two hours with 150 rpm agitation. After the incubation is done, the culture was centrifuged at 1,000 rpm for 10 minutes to concentrate the cells. 500µl of the supernatant was discarded and the pellet formed was homogenized through gentle pipetting. 300µl of mixture was transferred onto an agar plate supplemented with LB/Ampicillin/IPTG/X-Gal and was spread evenly using a sterilized glass rod spreader. The plate was then incubated at 37°C for overnight bacterial culture.

2.8.6. Colonies screening

Overnight culture produced blue and white bacterial colonies. Selection was made for white color colonies, as it is indicative of successful recombination of DNA insert in the vector. A new LB/Ampicillin/IPTG/X-Gal agar plate was divided into 6 x 6 grids to form a mini library and a set of sterile 0.5ml microcentrifuge tubes were filled with 30µl of dH_2O and labeled with names corresponding to the clone library. The white colonies were picked using a sterile wire loop and briefly touched on the surface of the library plate before the

rest of colony was transferred into a sterile 0.5ml microcentrifuge tube that was prepared earlier. The mixture was placed in heat block at 99°C for 10 minutes. At this temperature cells were lysed and DNA was released. This DNA preparation was then subjected to PCR.

2.8.7. Colony PCR

PCR for colony screening was done as described in section 2.5 with several modifications. PCR was carried out in final reaction volume of 25µl in sterile 0.2ml tubes. Every reaction contains 1× of AmpliBuffer B (EURx, Poland), 1.5mM of $MgCl_2$ (EURx, Poland), 200µM of dNTP (Fermentas, Canada), 0.5mM of M13 forward primer (5' GTAAAACGACGGCCAGT 3') and M13 reverse primer (5' GGAAACAGCTATGACCATG 3') (BIO BASIC INC, Canada), 1U of *Taq* Polymerase (EURx, Poland), 100ng of DNA sample and sufficient dH_2O to make up the final volume of 25µl. Thermal cycling of PCR was performed on PTC-100^{TM} Programmable Thermal control (MJ Research, USA). The PCR reactions undergo initial denaturation step at 95.0°C for five minutes, followed by 35 cycle of denaturation at 95°C for one minute, primer annealing at 60°C for two minutes, primer extension at 72°C for two minutes and thirty seconds before finishes off with the final elongation step at 72°C for five minutes.

The PCR products were subjected to agarose gel electrophoresis right after and then viewed under the UV light using Alpha Imager 2200 UV transilluminator (Alpha Innotech Corporation, USA). The colonies with the accurate insertion size were identified and marked.

2.8.8. Plasmid extraction

Colonies that had the correct inserts were transferred from the mini library into LB broths media supplemented with 50µg/ml of Ampicillin (Sigma, USA) using sterile wire loop. The colonies were cultured at 37ºC water bath with 215 rpm agitation for 16 hours. After the incubation time, the cultures was poured into sterile 15ml Falcon tubes and centrifuged at 6,000 rpm for 15 minutes at room temperature. The supernatant was discarded before 200µl of solution I was added to each sample and vortexed vigorously to completely homogenize the pellets. Then the mixture was transferred completely into a sterile 1.5ml microcentrifuge tube and 200µl of solution II was added to the sample, mixing gently at room temperature for four minutes. After that, 200µl of solution III was added into the mixture and left at 0ºC for 15 minutes with occasional inversion. Next, the mixture was centrifuged at 13,000 rpm for 15 minutes at room temperature. The supernatant was transferred to a new sterile 1.5ml microcentrifuge and 0.25mg/ml RNAse A (Sigma-Aldrich, USA) was added to it before the sample was incubated at 37ºC for three hours.

Upon completion, one volume of phenol (Sigma, USA) was added into solution, mixed by vigorous vortexing and then centrifuged at 13,000 rpm for three minutes at room temperature. The aqueous upper layer was aspirated and transferred into a new sterile 1.5ml microcentrifuge tube. This is followed by the addition of one volume of chloroform (Merck, Germany) into the sample, again mixed by vortexing before it was centrifuged at 13,000 rpm for three minutes at room temperature. The upper layer of supernatant was transferred into a new sterile 1.5ml microcentrifuge tube and then 0.1 volume of 5M Sodium chloride (NaCl) and 2.5 volume of ice-cold isopropanol were added into the sample. The mixture was mixed very slowly and placed in ice for 20 minutes incubation.

The mixture was centrifuged at 13,000 rpm for 15 minutes at room temperature after the incubation was completed. The supernatant was completely discarded and the tube was inverted on a piece of paper towel to dry. Next, one mililitre of ice-cold 70% ethanol (Merck, Germany) was added into the sample and centrifuged at 13,000 rpm for five minutes at room temperature. Again, the supernatant was completely removed from the pellet and the DNA pellet was further dried using a vacuum-drier (Heto lab equipment, Denmark) for 10 minutes. Finally, 30µl of dH_2O was added to dissolve the pellet and kept at 4ºC overnight.

The next day, the sample was quantitatively measured using the spectrophotometer (Eppendorf, Germany) as described in section 2.4. The best reading for concentration was higher than 0.300µg/µl with $purity_{260/280}$ value between 1.7-1.9. The samples with suitable quality and quantitative were subjected to restriction enzyme digestion.

2.8.9. Restriction enzyme digestion

Restriction enzyme digestion was carried out in 20µl of reaction volume in a sterile 1.5ml microcentrifuge tube. For a single reaction, 1× of Buffer H (Promega, USA), 0.1mg/ml of BSA (Promega, USA), 5U of *Eco*RI (Promega, USA) and 5µg of DNA was mixed together and incubated at 37ºC for three hours. After the incubation, the mixture was placed for 10 minutes in a heat block set at 65ºC to deactivate leftover enzyme in the reaction mixture. The presence of insert DNA was by assessed by observing the digested product on agarose gel. The plasmids that have correct insert size were diluted to 300ng was sent for DNA sequencing.

2.9. Sequencing analysis

DNA sequencing result was analysed using Chromas V2.33 (Copyright © 2003-2008 Technelysium Pty Ltd). DNA sequence was checked for homology by using Blast program by National Center for Biotechnology Information (NCBI) that is available online. All the edited DNA sequences were aligned using MEGA4 V4.0 sequence alignment program (Tamura *et al*., 2007) before proceeding to phylogenetic tree construction.