

Development of protocols for genomic library construction of Agarwood (*Aquilaria malaccensis*)

ULFAH J. SIREGAR^{1,✉}, MUHAMMAD IQBAL MAULANA¹, UTUT W. SUHARSONO^{2,†}

¹Department of Silviculture, Faculty of Forestry, Institut Pertanian Bogor. Jl. Lingkar Akademik Kampus IPB Darmaga, Bogor 16680, West Java, Indonesia. Tel. +62- 251-8621677, ✉email: siregaruj@gmail.com

²Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor. Kampus IPB Darmaga, Bogor 16680, West Java, Indonesia. † deceased

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Abstract. Siregar UJ, Maulana MI, Suharsono UW. 2017. Development of protocols for genomic library construction of Agarwood (*Aquilaria malaccensis*). *Biodiversitas* 18: 1150-1158. Agarwood is one of non timber forest products (NTFP) which has high economic value. Diminishing agarwood trees in natural forest due to intensive harvesting has shifted the production paradigm to forest plantation. In order to support agarwood tree improvement, investigation on the agarwood tree genomes and genes involved in agarwood production is highly needed through genomic library construction. This research aimed at establishing cloning protocols for genomic library construction of agarwood tree (*Aquilaria malaccensis*) until sequencing process. Genomic DNA was isolated using DNeasy Plant Mini Kit from Qiagen, then was digested with *Eco*R1, and was ligated to pGem®-T Easy vector system before it was finally transformed and was cloned to *E.coli* strain DH5 α . Confirmation of DNA inserts was done using PCR colony with universal primer of SP6 and T7, plasmid isolation and also PCR plasmid and plasmid DNA digestion used Quick Plasmid Miniprep Kit from ThermoFisher. Cloned *A. malaccensis* DNA fragments were sequenced and BLAST at NCBI site. The cloning successfully obtained five white colonies which contain inserted *A. malaccensis* DNA fragments with the size of 136-225 bp. PCR colony, plasmid isolation, PCR plasmid, and plasmid DNA digestion had confirmed the existence of inserted *A. malaccensis* DNA genome inside the bacteria cells. Sequencing and BLAST showed that DNA inserts from colony number 6, 8, and 9 were not similar with *A. malaccensis* sequence that was recorded in NCBI, indicating that the genomic region in this library construction is different from the genomic DNA sequences of *A. malaccensis* in NCBI.

Keywords: *Aquilaria malaccensis*, BLAST, cloning, genomic library

INTRODUCTION

Agarwood (locally called: gaharu) is one of non-timber forest products (NTFP) which has high economic value. It has been used across Asia, Middle East, and Europe for many purposes such as religious ceremony equipment, cosmetics, perfumes mixture, soaps, traditional medicines and other derived products (Okudera and Ito 2009; Gao et al. 2012; Santoso 2014), which has made the price of good quality agarwood skyrocketing. Traditionally harvested from agarwood trees in natural forest, it is hard now to find it in natural forest due to overharvesting of the trees triggered by ever-increasing price and demand of agarwood has decreased the tree population substantially. Agarwood trees belong to *Thymelaeaceae* family, which consists of several genera such as *Aquilaria*, *Gyrinops*, *Wikstoremia*, *Enkleia*, *Gonystylus*, *Phaleria*, etc. Their rare existence in natural habitat has made the all agarwood-producing species especially from genera *Aquilaria* and *Gyrinops* listed as vulnerable under the International Union for Conservation of Nature (IUCN) Red List (IUCN 1998), also as threatened in Appendix II of CITES (Gao et al. 2012) since 2005. The international trade involving their products is under strict control.

Gao et al. (2012) and Santoso (2014) explained that agarwood is a lump of brownish black resin produced in

agarwood tree due to biotic stress from fungi infection (Tamuli et al. 2008) activity inside the wood tissue. Pathogens infection causes some changes to wood anatomy properties (Siburian et al. 2013; Gao et al. 2012) such as odor, color, deposits in vessel element and the frequency of included phloem as well as enzymatic activity (Tamuli et al. 2008) which shows that infected plants exhibited higher activity of pectinase, cellulose, peroxidase, and polyphenol oxidase. Agarwood resin quality is different according to its tree origin and influenced by the kind of attacked pathogens, as well as duration of pathogens infection. One of the agarwood tree species, which produces best quality agarwood, is *Aquilaria malaccensis*.

The decreasing population of agarwood trees in natural forest has shifted the agarwood production paradigm into tree cultivation or plantation forest with superior trees. One of the mostly cultivated species is *A. malaccensis*. Planting trees, followed by artificial inoculation of fungi onto the trees to induce agarwood formation, however, does not automatically produce desired agarwood. It is because the exact mechanism underlying the agarwood production and the defensive response remain unknown (Okudera and Ito 2009; Gao et al. 2012). This phenomenon has led to an idea of planting superior tree, which is capable of producing good quality of agarwood, by tree improvement program. The first requirement in agarwood tree improvement

program using molecular approach is information on the genomes of agarwood tree and genes involved in agarwood production. Information on genomic DNA could be obtained by constructing a genomic library of agarwood tree. Agarwood tree as one of tropical forest tree species has been known as difficult to be studied molecularly, because of difficulty in isolating good quality of DNA or RNA, as its tissues usually contain many inhibiting chemicals, such as polysaccharides, polyphenols, alkaloids, flavonoids, terpenes and other organic acids.

A genomic library is a group of DNA fragments of particular organism stored in a suitable vector by DNA ligation. Genomic library construction is important to identify genes involved in encoding various phenotypes of a particular organism, molecular markers for genetic diversity analysis (Tnah et al. 2012), developing microsatellite marker (Nunome et al. 2006), which eventually could shorten the tree improvement cycle. Thus, effective and efficient cloning protocol is highly needed to obtain the optimum result of genomic library construction. Although molecular cloning is very basic molecular work, and most laboratories do not have problems conducting the experiment, many others still difficult to develop an optimum protocol (Matsumura 2015). Cloning protocol for genomic library construction conventionally starts from DNA isolation, DNA digestion using restriction enzyme to create DNA fragments, ligation of DNA fragments to a vector, transformation of recombinant vector to bacteria cell, multiplication of bacteria cell into colonies, plasmid vector isolation, and DNA sequencing of insert. DNA sequencing aimed at identifying nucleotide sequence of genomic library isolated and replicated in those bacteria cells. This research's purpose was to develop cloning protocols for genomic library construction of *A. malaccensis*.

MATERIALS AND METHODS

Procedures

Materials and chemicals

Plant materials used in this research were leaves of three months old seedlings of *A. malaccensis* grown in nursery of Forest Research and Development Center, *DNeasy Plant Mini Kit* which was purchased from Qiagen (2012), agarose gel, 50X buffer TAE (*Tris-Acetate-EDTA*) and 1X TAE, gel red, 10X loading dye, DNA lambda 100ng/ul, 1 kbp DNA ladder, nuclease free water (NFW), *EcoR1* restriction enzyme from Promega (2011), *Acetylated BSA*, 10X buffer *EcoR1*, vector pGEM-T Easy, T4 DNA ligase, 2X buffer T4 DNA ligase, bacterium *E. coli* strain DH5 α in a glycerol form, a liquid media LB (Luria Bertani), LB agar, antibiotic ampicillin, IPTG (*Isopropyl β -D-1-thiogalactopyranoside*) 0.1 M, X-gal (*5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside*) 50 mg/mL, CaCl₂ 0.1 M, ice, SOC, primer SP6-F 10mM, primer T7-R 10mM, *Green Go Taq Mix*, *Quick Plasmid Mini Prep Kit* from ThermoFisher (2014). DNA sequence was analyzed using Bioedit software and tool BLAST (Basic Local Alignment Search Tool) onto NCBI (National Center for

Biotechnology Information) (Genbank).

Preparation of leaf samples

The agarwood seedling was three months old, at about 40-50 cm height. The sampled leaves were the young ones, at the bud of the plants, weighing 100 g. The leaves were ground in liquid nitrogen until they turned into powder.

DNA genome isolation

Genomic DNA isolation was done according to kit protocol from *Qiagen DNeasy® plant mini kit* Qiagen (2012). The electrophoresis was then carried out in 1% agarose gel to test the quality of DNA obtained. The concentration of DNA was quantified using UV-Spectrophotometer.

DNA genome and vector digestion using restriction enzyme

The DNA genome and vector pGem®-T Easy from Promega (2010) was digested using *EcoR1* restriction enzyme from Promega (2011). The concentration of DNA for digestion was adjusted to the recommended DNA concentration in the restriction enzyme protocol in order to obtain full digestion of 1 μ g/ μ L *EcoR1* enzyme.

DNA target ligation to vector

Digested genomic DNA was then ligated to vector pGem®-T Easy from Promega (2010) using T4-DNA Ligase from Promega under condition following the protocol from supplier. The result of recombinant vector was verified using 1% agarose gel electrophoresis.

Transformation to bacteria cell and cloning

The cloning process consisted of three steps, namely preparation of competent bacteria cells, transformation, and recombinant bacteria selection. Bacteria used in the cloning process was *E. coli* strain DH5 α . Competent bacteria cell was prepared by spraying 100 μ L stock bacteria in glycerol onto sterilized solid LB agar media, and then, incubating the bacteria culture in an incubator under a temperature of 37°C for overnight. Single colony that appeared was picked up with a sterile toothpick and then dipped in 5 mL liquid LB media in an Erlenmeyer. The colony was incubated in a shaker incubator with the speed of 150 rpm/minute, under 37°C for 16-20 hours. The incubation period followed the recommended period in the protocol since it will affect the effectiveness of the cells to uptake the recombinant plasmid.

Next step was sub-culturing 1 mL from the overnight culture into fresh 10 mL liquid LB media. Subculture was incubated in the shaker incubator under similar condition as above until it reached the Optical Density (OD) value of 0.3-0.4. The OD is critical that it should not pass the recommended OD boundaries. In this experiment, OD value of 0.31 was obtained after incubation for 30 minutes, under temperature of 37°C at 250 rpm/minute. Later, the subculture was divided into several 1.5 mL tubes depending on the amount needed for the transformation.

The divided subculture was centrifuged at 4°C, with the speed of 4000 rpm/minute for 10 minutes. Supernatant was discarded and 495 μ L of 0.1 M CaCl₂ was added, and then,

incubated on ice for 10 minutes. The mixture was then centrifuged at 4°C, with the speed of 4000 rpm/minute for 10 minutes. Supernatant was discarded and another 150 µL of 0.1 M CaCl₂ was added into a pellet and homogenized by pipetting, followed by incubation on ice for 10 minutes. The subculture was divided into several tubes by adding 50 µL each.

Solution containing recombinant vector from ligation mix was then added into the bacterial solution and homogenized by pipetting and incubated on ice for 25 minutes. Later the mixture was subjected to heat shock by dipping the mixture in a water bath at a temperature of 42°C for 45 seconds, and then immediately put back on the ice for 10 minutes. Haris et al. (2005) stated that the best incubation period in ice after the heat shock is 20-30 minutes. The mixture was then moved to room temperature for 5 minutes. After added by 800 µL of SOC, the mixture was placed in at shaker incubator for another 30 minutes to 1 hour of incubation period at a temperature of 37°C and rotation speed of 250 rpm. The mixture was then centrifuged at 4000 rpm, under 4°C, for 10 minutes, after which 150 µL of the shaped supernatant was collected and the rest was discarded. Onto collected supernatant, 10 µL of IPTG and 50 µL of X-gal were added, and the mixture was then spread to a sterilized media containing 100 ppm of ampicillin and was incubated at temperature of 37°C for one night. The recombinant colony that had captured plasmid containing inserted DNA target will change its color to white, while the colony without the insertion would remain blue. White colony was then replicated in the LB agar media with ampicillin for further analysis.

PCR colony

Primers used for PCR colony were SP6-F and T7-R. These primers will amplify the plasmid in the inserted area as far as 200 bp (Haris et al. 2005). If the target DNA is present, a single thick band will appear after the PCR showing the amplified insertion of PCR primers. The PCR colony followed this procedure: the replicated bacteria colony was picked up and dipped into micro-tube filled with 7 µL of ddH₂O or NFW (Nuclease-Free Water) and was incubated in the PCR machine at 95°C for 10 minutes. After that, the mixture was subjected to a reaction mixture as presented in Table 1. The mixture was vortexed and put in PCR machine with reaction conditions as seen in Table 2.

Plasmid DNA isolation

Plasmid DNA isolation was done to extract vector and inserted target DNA, which was replicated inside bacteria cells. The isolation process followed recommended a protocol of *Quick Plasmid Mini Prep Kit* from Thermo Fisher. The isolated recombinant plasmid was then electrophoresed in 1% of agarose gel (w/v) and visualized using Kodak Gel Logic 200.

PCR plasmid using SP-6 and T7 primer

PCR plasmid was performed to ensure that the amplified band in PCR colony was target DNA and not contamination. The procedure and condition were the same as PCR colony.

Table 1. Composition of the PCR colony reaction

Materials	Volume
Denaturated colony	2 µL
Primer SP6-F 10mM	0.25 µL
Primer T7-R 10mM	0.25 µL
Green Go Taq Mix	5 µL
NFW	2.5 µL
Total	10 µL

Table 2. Steps and condition of the PCR colony

Steps	Temp. (°C)	Time (minutes)	Number of cycles
Pre-denaturation	94	10	1
Denaturation	94	1	35
Annealing	52	1	35
Elongation	72	1.5	35
Post elongation	72	5	1

Plasmid DNA digestion using restriction enzyme

Another way to confirm the existence of target DNA inside bacteria plasmid is by cleaving the plasmid using the same enzyme previously used. The protocol was similar to previous DNA genome and vector digestion.

Sequencing target DNA

After confirmation by PCR plasmid and restriction enzyme digestion on the existence of inserted target DNA inside bacteria plasmid, the sequencing was conducted to find out the inserted sequence that was successfully cloned. DNA sequencing was done at the 1st Base DNA Sequencing Laboratory of Genetica Science Co. Malaysia. The sequence was processed using software of Bioedit and further BLAST-ed- (*Basic Local Alignment Search Tool*) to NCBI (*National Center For Biotechnology Information*) to obtain similarity of the nucleotide of target DNA with a particular species sequences.

RESULTS AND DISCUSSION

DNA genome isolation and digestion

Various protocols for genomic DNA isolation from leaves have been published, mainly using CTAB buffer which was originally developed by Doyle and Doyle (1990) with several modifications depending on the plant species (Ginwal and Maurya 2010; Islam et al. 2013; Zhang et al. 2012). The modified CTAB method had been tried before to isolate DNA on *A. malaccensis*, but the result was not satisfying. Liquid nitrogen was used to freeze the leaves and make the grinding process into powder easier. It also prevents action of DNase and RNase which could influence the isolated DNA quality. The result of DNA isolation using *Qiagen DNeasy Plant Mini Kit* showed relatively thick and clean bands (Figure 1) indicating a good quality DNA. The thick and clean DNA band means the DNA is not degraded (Herison et al. 2003). The elution

process was conducted twice to obtain maximum amount of DNA, of which the remaining DNA in a *mini spin column* from the first elution could be extracted in the second elution (Qiagen 2012). The first and second elution used 100 μ L of AE buffer and the result of first and second elution can be seen in Figure 1.

Figure 1.A shows that the DNA band from the first elution (lane A) was much thicker than the second one (B) indicating the amount of DNA in the first elution is higher than the second one. DNA concentration was then measured using UV-Spectrophotometer, and the result was 125ng/ μ L on 1st elution and 25ng/ μ L on the 2nd elution. These concentrations were sufficient for the next DNA digestion process. Purity and the amount of isolated DNA are an important factor influencing the success of following experiments. Contaminants such as polysaccharides, phenols or organic acids could inhibit restriction or PCR reactions (Weising et al. 2005). Figure 1.B shows electrophoresis of DNA which was successfully digested with *Eco*R1 as indicated by clean smear bands on the agarose gel. In this experiment, the DNA was fully digested into fragments with small differences in sizes, so when they were electrophoresed, the DNA fragments will appear in agarose gel lane as a smear. The best DNA digestion was R2 since it had thick and long DNA band because the original DNA (DNA in lane 2) also had good quality.

Digestion using *Eco*R1 gives result in a fragment with overhang tail (or sticky end) which will be complemented by other DNA fragment having a similar tail. The digestion site of *Eco*R1 is 5'GAATTC3' meaning that any site containing such base sequence in the DNA will be cleaved by the enzyme.

Ligation of DNA fragment with vector

Ligation process combines restricted genomic DNA fragments and a plasmid vector which both were cleaved by the same restriction enzyme. Plasmid vector is circular, extrachromosomal double-stranded DNA found in several species of bacteria and functions as genetic exchanges tool with several criteria such as the ability to multiply itself and the DNA fragment inside it in a huge number independently, has some restriction enzyme sites which allow the DNA fragment to be inserted, and has marker gene for recombinant selection (Klug et al. (2010).

Vector used in this research was pGem®-T Easy from Promega. Some benefits of this vector are the size is relatively small enabling insertion of big DNA fragments (approximately up to 10 Kbp), containing ORI (Origin of Replication) site for replication inside *E.coli*, carrying ampicillin resistance gene for selection, and having 19 unique Multiple Cloning Sites (MCS), of which one of the restriction sites is *Eco*R1, as seen in Figure 2.

pGem®-T Easy plasmid vector carries *Amp^r* gene, an ampicillin resistance gene (Figure 2), which is a marker for recombinant plasmid selection. The bacteria plasmid, which does not uptake the plasmid, will not be able to grow in media containing ampicillin because of the antibiotic activity. Only those, which have plasmid vector, will be able to grow and multiply.

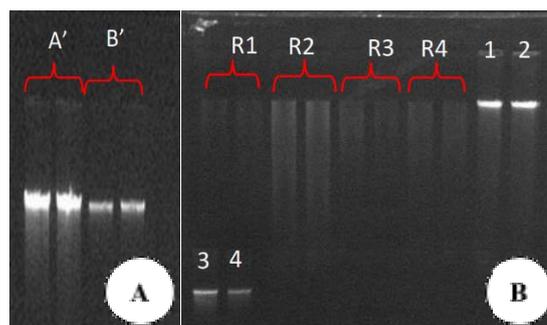


Figure 1. A. Genomic DNA extraction using *Qiagen DNeasy Plant Mini Kit*. A' = first elution, B' = second elution. B. DNA digestion by *Eco*R1. R1 = Digestion of DNA on lane 1, R2 = Digestion of DNA on lane 2, R3 = Digestion of DNA on lane 3, R4 = Digestion of DNA on lane 4

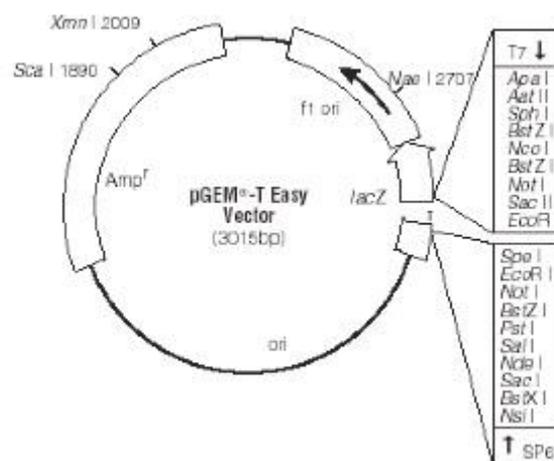


Figure 2. The map of pGem®-T Easy vector from Promega (2010)

The enzyme used for ligation reaction is T4 DNA Ligase which is a ligation enzyme originally purified from a bacteriophage T4 virus (Sambrook and Russel 2001). The enzyme catalyzed the formation of phosphodiester bonding among nucleotides of two DNA strands, e.g DNA target and vector DNA. T4 DNA Ligase efficiently ligates sticky ends of both DNA target and vector DNA created by *Eco*R1 because of the presence of overhangs on both ends of the fragments. Ligation process depends much on the concentration of reactants, catalysts, and other chemicals present in the mixture, only to a lesser degree on temperature and time (Matsumura 2015). The ligation proceeded at a temperature of 4^oC for one night to increase the stability of hydrogen bond that connected the nucleotides chain in ligation process. Ligation result was confirmed by electrophoresis on 1% agarose and the result is given in Figure 3.

Figure 3 shows that ligation had succeeded in generating higher molecular weight than empty vector. The lower molecular weight vector moved faster in electrophoresis and occupied a lower position than the heavier molecule of recombinant plasmid (L) on an agarose gel, confirming that the target DNA has been inserted into vector successfully.

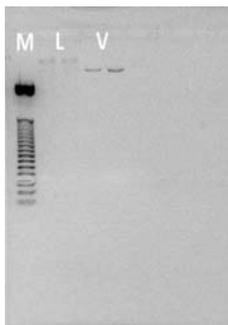


Figure 3. Ligation of genomic DNA fragments and vector DNA. M = Marker 50 bp, L = Ligated genomic DNA fragment and vector, V = Empty vector of pGem®-T Easy

Cloning and recombinant bacteria selection

Gene cloning is started with the process of genomic DNA digestion with a particular restriction enzyme. It is followed by inserting digested DNA fragments to a vector, which is then cultured in a particular host. This process is called shotgun cloning. The bacteria used in this experiment were *E.coli* strain DH5 α , which are the most commonly used bacteria for plasmid replication. The bacteria can be stored for a long time in glycerol and can be cultured quickly when needed (Hutchins 2015). Furthermore, *E.coli* strain DH5 α contains *recA* mutation which eliminates recombination between inserted genes with the host, which could replace the ϕ 80 *IacZ* Δ M15 site with gene encoding β -galactosidase (Hanahan 1983).

The efficiency of bacteria cells to uptake the recombinant plasmids determines the success of a cloning project (Matsumura 2015). Among two transformation methods available, i.e. heat shock and electroporation, the electroporation is considered as better technique, however, not all laboratories have the equipment. In this research, the transformation process utilized heat shock as recommended by Tomley (1996). For the transformation, bacteria cell has to be competent first. Competent cells were prepared by suspending bacteria cells in a solution containing a high concentration of calcium, which would make small holes on the bacteria cell wall. The bacteria used was the colony from an overnight culture which were then sub-cultured until the bacteria solution reach OD₆₀₀ 0.3-0.4 (Zhimming et al. 2005). CaCl₂ solution creates pores in the bacteria cell wall so that the plasmid would be easier to pass through cell wall. Right after the competent cells were ready, the transformation was done by heat shock on them at a temperature of 42°C for 45 seconds and then immediately they were put on ice for 10 minutes. The bacterial cells were then recovered using 800 μ L of SOC and incubated in a shaker incubator at 37°C for 30 minutes, with speed of 250 rpm.

The last stage was the addition of 10 μ L of IPTG and 50 μ L of X-gal into bacterial mixture and it continued by culturing the competent cell in LB (Luria Bertani) medium supplied with ampicillin. The addition of IPTG and X-gal can be done in two ways; first, by mixing them with the transformation solution, and second, by spreading them onto the LB medium. In this experiment, the IPTG and X-

gal were added to the transformation solution, so that they could be utilized more effectively by the bacteria cell. During bacteria cell multiplication, the inserted DNA fragment in the vectors will be automatically multiplied by bacteria cells.

Result of transformation process was an appearance of white and blue colonies on the LB media, of which white colonies consisted of bacteria cells containing recombinant plasmid vector, while the blue colonies were formed by non-recombinant bacteria, which will eventually die. The white color was caused by inactivated *lacZ* gene which was interrupted by foreign DNA insertion and failed to produce an enzyme which converts X-gal into blue color (Suharsono et al. 2009). On the other hand, without insertion in the *lacZ* gene, this gene would be expressed, producing β -galactosidase (Messing and Vieira 1982), which is an enzyme that converts uncolored X-gal into blue color. The insertion of a DNA fragment into one of the restriction site led to the occurrence of a nonfunctional α -peptide and the loss of β -galactosidase activity. Under appropriate plating conditions, the functional α -peptide results in blue plaques; a nonfunctional α -peptide result in colorless plaques (Messing and Vieira 1982).

Figure 4 shows comparison among control positive, control negative, and recombinant bacteria cultures. Control positive (Figure 4.A) was non-recombinant bacteria which were cultured in LB medium to confirm that the bacteria have good quality and can multiply. Control negative (Figure 4.B) was non-recombinant bacteria which were cultured in LB medium added with ampicillin to confirm that the ampicillin worked well, which was indicated by the absence of bacterial growth. Non-recombinant bacteria will not be able to grow in ampicillin contained medium because the cell cannot synthesize an enzyme that destroys ampicillin. Cultures of recombinant bacteria (Figure 4.C) were indicated by the existence of blue and white colonies. Totally, nine white colonies were obtained from this experiment.

Figure 4.C shows the existence of blue and white colonies in the medium supplied with ampicillin. The number of white colonies was much lower than the blue colonies. The white colony was then replicated to multiply the number of white colony for next stages. The replication of white colony was done by stripping each white colony from the original medium and transferring it to new medium containing ampicillin. The new bacteria culture was incubated for one night at 37°C. Figure 5 shows the white colony after replication.

PCR colony

PCR screening was used to confirm the presence of inserted DNA on white colony (Pinar et al. 2001; Suharsono et al. 2009). PCR colony has been proven to be the easiest and most convenient way to amplify target DNA sequence in several yeast cells without any DNA extraction and purification prior to PCR (Mirhendi et al. 2007). This experiment used complementary primers SP6-F and T7-R of which sequences also exist in the plasmid vector. Result of PCR on white and blue colony can be seen in Figure 6.

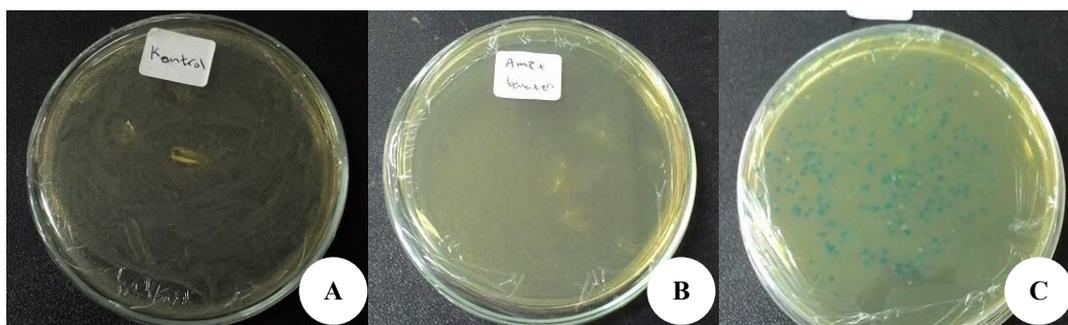


Figure 4. A. Control positive, B. Control negative, C. Recombinant bacteria

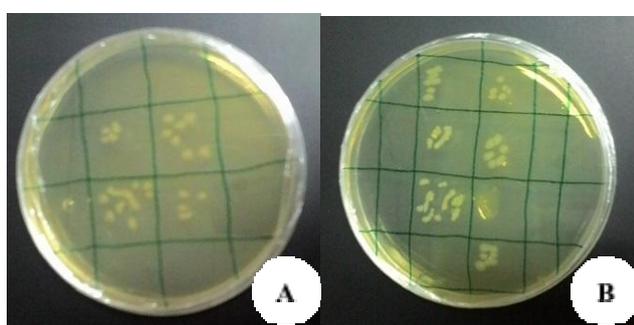


Figure 5. A. Replication of colony number 1 to 4. B. Replication of colony number 5 to 10

Result of PCR colony shows that only colony number 5 to 9 contained inserted DNA, while colony number 1 to 4 did not contain DNA insertion. The gel showed no amplicon band on colony number 1 to 4. Contrasted to the thick and clear amplicon band on colony number 5 to 9. The 1 Kbp DNA ladder, as a marker, indicated that inserted DNA has small size i.e. 136-250 bp. Figure 7.B shows that PCR colony of blue colony forms a thick amplicon band with very small size i.e. around 100 bp because the amplified site was a small region located between SP6 and T7 sequences in the plasmid, without additional DNA insertion.

Plasmid isolation and PCR plasmid

Plasmid isolation procedure refers to *Quick Plasmid Mini Prep Kit* from ThermoFisher. Isolated plasmid was then subjected to PCR using similar complementary primers SP6 and T7 to reconfirm the existence of target DNA. The procedure for PCR plasmid is same as PCR colony procedure. Figure 7 shows the result of plasmid isolation and PCR plasmid.

The result of electrophoresis in Figure 7.A shows the size of isolated plasmid DNA which is about 3000 bp. This size matched the vector size, namely 3015 bp (Promega 2010). Figure 7.A also shows that only colony number 5, 6 and 8 exhibiting DNA bands, while colony number 7 and 9 giving no band but a thin smear only. The absence of DNA bands doesn't necessarily mean that colony number 7 and 9 contain no plasmid DNA, but there are some possibilities

that the concentration of plasmid DNA which was isolated from colony number 7 and 9 was too low or the plasmid was in a shape in which agarose gel cannot detect it. Figure 7.B shows result of PCR plasmid of colony no 5 to 9, which confirms that all colonies including colony number 7 and 9 contain plasmid DNA.

Plasmid DNA digestion with restriction enzyme

Matsumura (2015) suggested that every stage in cloning experiment should be confirmed by electrophoresis in an agarose gel. Before the target DNA was transformed into a bacteria cell, the target DNA was digested with a restriction enzyme. Therefore, if the recombinant plasmid DNA was digested with the same restriction enzyme there should be two single bands appear in electrophoresis, of which consisted of vector band and target DNA fragment (Budiani et al. 2009). To confirm the target DNA's existence, plasmid DNA was digested using *EcoR1*.

Plasmid DNA from colony number 5, 8 and 9 were successfully digested by *EcoR1* (Figure 8) and resulted in the appearance of two bands in agarose gel. The first band is vector DNA with the same size for all colonies. The second lower band is the target DNA, because the molecular weight is smaller (Suharsono et al. 2009). The size of target DNA from colony number 5, 8 and 9 were relatively the same. Colony number 6 and 7 produced neither vector nor target DNA band in electrophoresis. It probably happens because the concentration of plasmid DNA was too low that both DNA vector and target DNA was unseen.

The volume of plasmid DNA used for digestion was 29 μL , which was rather high, because the concentration of plasmid DNA from isolation was very low, about 35 $\text{ng}/\mu\text{L}$, while *EcoR1* requires DNA as much as 1 $\mu\text{g}/\mu\text{L}$ for one reaction (Promega 2011). Therefore, as the plasmid DNA concentration has to reach 1 $\mu\text{g}/\mu\text{L}$, the volume used in digestion mix was 29 μL . To balance the reaction, the amount of *EcoR1*, 10x buffers, and Acetylated BSA used in the reaction mix were also multiplied by two. After that as much as 20 μL DNA mix was used for electrophoresis since the concentration of target DNA from plasmid digestion should be very low, that the amount of DNA has to be increased in order to make the DNA band to emerge in agarose gel.

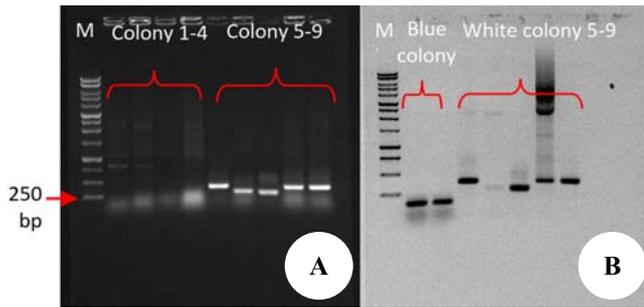


Figure 6. A. PCR colony of white colony no 1-9, B. PCR colony of blue colony and white colony no 5-9. M = 1 kbp DNA ladder

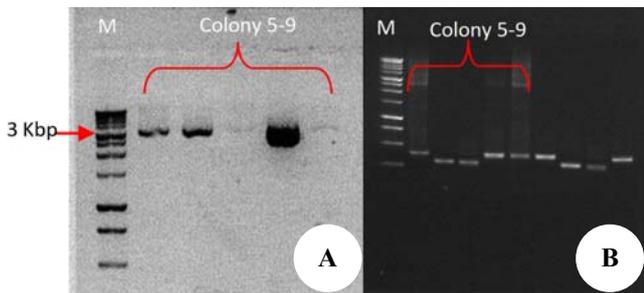


Figure 7. Result of plasmid isolation (A), Result of PCR plasmid (B). M = Marker 1 Kbp DNA ladder

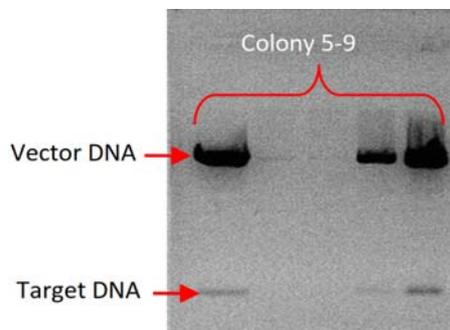


Figure 8. Plasmid DNA digestion using *EcoRI*

Sequencing and sequence data processing

DNA sequencing is the determination and order of the nucleotide in a DNA strand. Sequencing is done to predict the function of sequence and manipulate it molecularly or to perform phylogenetic analysis and identification (Pinar et al. 2001). In this research, DNA sequencing was conducted after the plasmid was successfully isolated. DNA sequences were obtained only from colony number 6, 8 and 9, as colony number 5 and 7 had too low DNA concentration for sequencing process. The DNA concentration needed for sequencing was 100ng/ μ L. Sequencing was conducted from two directions using T7 promoter and SP6 universal primer (Lee et al. 2011). The result showed that approximately 1400 bases were successfully sequenced. The sequence size was rather big

because the utilization of universal primer made the sequenced DNA bigger than target DNA, as it contains some of plasmid DNA sequences flanking the insert (Budiani et al. 2009).

The existence of target DNA sequence could be identified by locating *EcoRI* restriction sequences. The sequence located between two *EcoRI* recognition sites was target DNA sequence, as both genomic DNA and vector were previously digested with *EcoRI*. After the target DNA had been identified, the sequences data were processed by software Bioedit to see the complement of both sequences from two directions. Complement test of target DNA sequences from primer SP6 and T7 was conducted to confirm that the DNA sequences are the same. After complement test, which showed that both DNA sequences were the same, the sequence was then BLASTn-ed (*Basic Local Alignment Search Tool*) to gene bank.

Sequenced DNA target showed that colony number 6 contains the shortest target DNA, while colony number 8 and 9 have exactly the same number of base and sequence. The sizes matched the results of electrophoresis of PCR colony and PCR plasmid on an agarose gel, of which white colonies contained target DNA ranging from about 150 bp to 250 bp. These DNA fragments were multiplied inside bacteria cells. Successfully cloned DNA fragments in bacteria cells have a short strand, probably because the genomic DNA was fully digested, that every *EcoRI* restriction site in the genomic DNA would be cleaved into short fragments. Another possibility was cloning efficiency was low, as it only took small DNA fragments rather than bigger ones, and therefore still needs to be optimized. This experiment proved that molecular cloning is still difficult to be achieved efficiently in our Laboratory.

Target DNA sequences were then analyzed by BLASTing them on NCBI to find similarities of DNA sequence or protein query with sequences present in central data of NCBI. BLASTn program works to compare nucleotide query with a nucleotide sequence in the gene bank database of NCBI. E-value (Expect value) is the parameter indicating the similarity of one sequence with other sequences in database (Lodge et al. 2007). The result of BLAST DNA sequence from colony number 6, 8 and 9 can be seen in Table 4.

The result of BLASTn shows that DNA sequence from colony number 6 has highest similarity to zinc finger protein of *Dasypus novemcinctus* (an animal) with the E-value of 0.003 and 92% similarity. Zinc finger protein has various structures and, along with other protein, has a great role in cellular processes, such as replication, transcription, and translation, signaling, metabolism, cell proliferation, as well as apoptosis. Zinc finger protein functions as an interaction media and the binding of various chemical compounds such as nucleic acid, protein, and other small molecules (Krishna et al. 2003). Due to its very important function, zinc finger protein must be owned by all organisms, and thus it is understandable that zinc finger sequence of *D. novemcinctus* was similar with the one of *A. malaccensis* sequence. Sequences of colony number 8 and 9 have highest similarity to *Populus trichocarpa*'s

Table 3. The nucleotides sequences of target DNA

Colony	DNA Sequence (5'-3')	Number of nucleotides (bp)
6	ACATGTAATCCAAAACCTTAAATTACAATTACATTATGTTTACATTACAAAATATATGTATTGCCTAATA TACTCCAGCTAAACTACTCTTATGGTGGCTTATACTTGTACTCACATAGTCGATCACAATGTGCTC	136
8	AACAGTAAAATTAACCAGAAAAAGCTTTAAATTAATTTCTTAATTTTGAGTAACCAAATATTGAAAAGA AATTTCTAATAAACCCAAAATTAGCATTAAAGAATAATGGACAAAAGTTTATTTCTATGCAATAATCCAAA AGTTTAAACAATTTAACCTATCCTACTTTTCCGTATGAACCTAAGAAATAAAAATAACCTCCAATCA ATTTACTTGCTTCTAATT	225
9	AACAGTAAAATTAACCAGAAAAAGCTTTAAATTAATTTCTTAATTTTGAGTAACCAAATATTGAAAAGA AATTTCTAATAAACCCAAAATTAGCATTAAAGAATAATGGACAAAAGTTTATTTCTATGCAATAATCCAAA AGTTTAAACAATTTAACCTATCCTACTTTTCCGTATGAACCTAAGAAATAAAAATAACCTCCAATCA ATTTACTTGCTTCTAATT	225

Table 4. Result of BLAST white colony sequences to NCBI database

Accession number	Colony	Description	E-Value	Query cover	Identity
XM 012528651.1	6	<i>Dasyptus novemcinctus</i> zinc finger protein 77- like (loc101414147) mRNA	0.003	27%	92%
AC2 151625.1	8	<i>Populus trichocarpa</i> clone pop011-a24, complete sequence	1e-05	66%	71%
AC2 151625.1	9	<i>Populus trichocarpa</i> clone pop011-a24, complete sequence	1e-05	66%	71%

complete sequence with 71% similarity and E-value of 1e-05. Claveri et al. (2003) explained that theoretically E-value \leq e-04, indicates high similarity. The three colonies sequences, however, did not show any similarity with available *A. malaccensis* sequence stored in the gene bank probably because the length of a nucleotide being compared was too small, which was only about 136-225 bp. Short fragment is usually not suitable for BLASTn because it tends to give a high bias in alignment process, making the sequence more similar to other species than that of being expected. There is a possibility that the cloned DNA fragments in this research were originated from different region of *A. malaccensis* genome compared with those registered sequences at NCBI. Two sequences obtained were similar to plant sequences in the database, indicating that the cloned fragments were truly from *A. malaccensis* and not the results of a contamination. Nevertheless, cloning protocol employed in this experiment could be applied for cloning other tropical forest tree genomes in the future.

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