

MARKERS OF DNA APPLICABLE FOR GENETIC MAPPING OF JATROPHA GENOME

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ABSTRACT

Jatropha (Jatropha curcas) is an oil producing crop that can be used as supplements or substitutes of diesel fuel to replace fossil-based energy. This crop has a relatively long life cycle, and therefore, a new alternative breeding method is necessary to expedite breeding program. The purpose of this study was to find out DNA marker technologies that can be used to map the genome of *Jatropha*, and later can be used to label the important agronomic characters of this plant. DNA markers tested in this study included Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Target Region Amplification Polymorphism (TRAP), and Sequence Related Amplification Polymorphism (SRAP). Studies were initiated by finding the best method for isolating *Jatropha* genomic DNA. Initial studies to obtain molecular markers applicable for this crop resulted in a total of 43 polymorphic RAPD bands that can be used for DNA fingerprinting studies as well as be useful for genetic mapping purposes. However, the number of markers available was extremely lower than the minimum target of at least 250 polymorphic markers necessary for a minimum coverage of the *Jatropha* genome. The remaining markers can be obtained from other marker methods such as AFLP, TRAP, and SRAP and based on this study the latter types of markers demonstrated more polymorphism level than that of RAPD.

Kata Kunci : DNA marker, genetic mapping, *Jatropha curcas*, genome.

ABSTRAK

Marka DNA yang dapat digunakan untuk konstruksi peta genetik dari genom jarak pagar. Jarak pagar merupakan tanaman penghasil minyak nabati yang dapat digunakan sebagai pencampur atau pengganti bahan bakar minyak berbasis fosil. Siklus hidup tanaman ini relatif panjang sehingga diperlukan terobosan teknologi baru untuk mempercepat program pemuliaannya. Tujuan dari penelitian ini untuk mempelajari aplikasi teknologi marka DNA yang dapat digunakan untuk memetakan genom jarak pagar yang nantinya dapat digunakan untuk melabel karakter agronomi penting tanaman ini. Marka DNA yang diuji pada penelitian ini termasuk Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Target Region Amplification Polymorphism (TRAP), dan Sequence Related Amplification Polymorphism (SRAP). Penelitian diawali dengan upaya pencarian metode isolasi DNA yang tepat untuk jarak pagar. Hasil penelitian awal dalam mencari marka DNA yang cocok untuk tanaman ini telah menghasilkan 43 pita RAPD polimorfik yang selain dapat digunakan sebagai sidik jari DNA juga dapat dimanfaatkan untuk membuat peta genetik. Namun jumlah marka yang diperoleh masih jauh dari target minimal yaitu 250 marka DNA polimorfik untuk kegiatan pemetaan genetik. Kekurangan jumlah marka dapat dilengkapi dengan marka-marka lain dari metode AFLP, TRAP, dan SRAP yang sejauh ini terlihat lebih polimorfik dari RAPD.

Keywords : Marka DNA, analisis genetik, jarak pagar, genom.

INTRODUCTION

Jatropha (Jatropha curcas) is a multi-purpose tree crop. Originated from the South American countries, this crop has spread to other tropical countries including Indonesia. *Jatropha* seeds are rich in oil and pure oil extracted from the seeds can be chemically modified to biodiesel or used

directly as fuel for certain diesel engines (Heller, 2002). This attractive feature caused the rapid introduction of *J. curcas* in various rural programs, especially during the peak increase of oil prices as it may contribute to rural development through income generation and increasing efficiency of rural agricultural system. However, *J. curcas* productivity can vary greatly and a lot of

profitability claims were made without well-founded proof nor reliable sources of information. Being an uncultivated wild species, there is insufficient knowledge regarding the environmental and the genetic factors that influence *Jatropha* oilseed production. This information is crucial to the success of any *J. curcas* development programs, along with appropriate knowledge on what varieties of *J. curcas* need to be chosen and under which environmental and agronomic conditions those varieties must be cultivated.

The main constraint for *Jatropha* cultivation is the lack of new varieties with local or broad adaptation to many Indonesian regions. The genetic information of this crop is also limited. A Malaysian consortium recently announced the completion of *J. curcas* genome sequencing, however they never made the sequence available to the general public (<http://www.syntheticgenomics.com/media/press/52108.html>). *Jatropha* is also classified as perennial crop and started to produce fruits after 6 to 8 months old (Hartati, 2008), which makes breeding process for this species a relatively time-consuming endeavor. A new technological breakthrough is required to expedite the breeding process for this species.

One of the strategies is by exploiting DNA marker technology. DNA markers are very diverse and polymorphic, therefore the construction reasonably dense genetic maps on any crop species can be achieved with relative ease. From these genetic maps, chromosome segments carrying important agronomic characters can be identified by mapping their proximity to superior qualitative traits and quantitative trait loci. With such knowledge, breeding process can be made more precise because unnecessary chromosomal segments that normally cause linkage drag can be selected against. DNA markers can also be selected as soon as sufficient quantity of DNA can be extracted from the individual plants. This means that selection can be performed at the seedling stage, minimizing cost and environmental variability that typically complicate classical breeding programs.

DNA is also universally found in most living organisms, with the exception of some RNA viruses, which means that DNA marker technology

developed for a certain species are often applicable in other species. This is especially true for markers that do not rely on sequence data. Various DNA markers have been developed, which are based on DNA hybridization, amplification using polymerase chain reaction, and DNA restriction digests used in combination with the previous two techniques. Choosing DNA markers for a breeding program will depend on the amount of funding available, availability of expertise and equipment for such markers, and the amount of sequence data that can be accessed.

In this study we studied DNA markers that are applicable for *J. curcas*, a commodity whose genome sequences is not yet publicly available. The markers under investigation were RAPD, AFLP, SRAP, and TRAP. Randomly amplified polymorphic DNA (RAPD) uses a single, arbitrarily short oligonucleotide primer (10 nucleotides in length) to amplify genome segments flanked by two complementary binding sites in inverted orientation (Williams *et al.*, 1990). RAPD markers are observed as the presence and the absence of amplified bands in a particular genotype and therefore act as dominant markers. Amplified fragment-length polymorphism (AFLP) markers are based on PCR used in combination with restriction digests. A prior knowledge about the genome sequence is not required to develop this marker. Genomic DNAs are first digested with two different restriction endonucleases (one is a six-base cutter and the other is a four-base cutter; e.g., *EcoRI* and *MseII*). Adaptors are then used to tag the restriction fragments and as the annealing point for universal primers in PCR reactions. A two-to three-selected based extension is attached to the primers and acts as selective binding of the primers on the target DNA templates (Vos *et al.*, 1995). As in RAPD, AFLP bands are mostly dominant.

A more recent variant of PCR-based marker technology is called sequence-related amplified polymorphism (SRAP). It uses a very similar concept as in RAPD, but the primers for SRAP are longer (around 18 bases) and designed to anneal to sequences around the coding regions in the genome (Li and Quiros, 2001). The longer primer length increases the specificity and repeatability, which makes it more reliable than RAPD. SRAP was also reported to produce some

codominant bands, which could be as much as 20% of total bands produced in each primer combination. SRAP was later modified to targeted region amplified polymorphism (TRAP), which replace one of the primer with a primer sequence specific to a gene or chromosome region (Hu and Vick, 2003). This modification was performed because in some organisms, more and more sequence data were becoming available, even though the complete genome sequence will probably take a while to complete. Thus TRAP can potentially be used to map the location of genes of interest, as long as the sequence data for that particular gene is available. This modification has been shown to work in mapping sequences specific to the telomeric region (Hu, 2006).

As more sequence data are slowly accumulating, more specific markers can be designed in the future. We plan to develop microsatellite markers, also known as *simple sequence repeat* (SSR), as this type of marker is very specific to certain chromosomal regions, reliable, and very consistent. This marker as shown by RFLP is co-dominant, in which the detected marker can differentiate between heterozygous plants from the ones that are homozygous (Akayya *et al.*, 1995, Smith *et al.*, 1997, Tasma *et al.*, 2001).

The objective of this study was to obtain types of DNA markers applicable for genetic mapping of the *J. curcas* genome. The genetic map then will be used for dissecting agronomically important traits such as yield, oil content, and environmental stresses tolerance.

MATERIALS AND METHODS

DNA isolation

As *J. curcas* leaves are rich in latex, isolating DNA from such type of plants is different from the ones having no latex. We found that the method developed by Murray and Thompson (1980), which was normally used in our lab to isolate DNA from other species, could not produce satisfactory DNA yield. The best DNA quality was obtained from young leaves using a modification of methods described by Michiels *et al.* (2003). As a DNA source, we found that there were no significant differences in DNA yield and DNA

quality between the first and second most developed leaf tissues. We subsequently used the first most developed leaf tissues for latter DNA isolation. The method for isolating DNA was as follows: The *J. curcas* young leaf samples were ground to fine powder using liquid nitrogen in a mortar and a pestle. The leaf powders were then transferred into a 2- ml eppendorf tube until it filled a third of the tube volumes. Six hundred μ l of 60°C-prewarmed CTAB extraction buffer (100 mM Tris pH 8, 1,4 M Na Cl, 20 mM EDTA pH 8, 0.2 % (v/v) beta mercaptoethanol, 2% Polyvinylpyrrolidone (PVP), 2% CTAB) were added into the tube and mixed well. The solution was then incubated at 60°C for 1 h and the tubes were inverted once in 15 minutes. The tubes were filled with 600 μ l chloroform:isoamyl alcohol (24:1) and were mixed well by vortexing. The samples were later cetrifuged for 5 min at 2,500 rpm and the upper phase of the solution was transferred into a new 2-ml eppendorf tube. The chloroform:isoamyl alcohol (24:1) extraction of the samples then was repeated two more times until the upper phase of the solution is clear. The upper phase of the solution was transferred into a 1.5 mL microtube before adding 400 μ l isopropanol. The tubes were then incubated at room temperature overnight. The next day the DNA was collected by centrifugation at 5,000 rpm for 10 min. The collected DNA was washed using a washing solution (10 mM ammonium acetate and 70% ethanol). The DNA was collected by centrifugation as above. The washing step was repeated one more time. The collected clean DNA was then dried overnight before diluting it using 100 μ l TE buffer (10 mM Tris pH 8, 1 mM EDTA). DNA concentration was measured using a spectrophotometer (Bio Rad, California, USA). DNA was then diluted to make a concentration of 10 ng/ μ L.

Randomly Amplified Polymorphic DNA (RAPD)

DNA of 50 *J. curcas* provenances including one provenance of *J. integerrima* were used as templates in the RAPD reaction. A 10- μ L PCR reaction was used. The PCR reaction consisted of 20 ng DNA, 0.4 mM dNTP, 4.0 mM MgCl₂, 1.0 μ M primer, and one unit of *Taq* DNA polymerase Core System from (Promega, Wisconsin, USA).

PCR reaction was conducted in an MJ Research 96-well PCR machine (MJ Research, New Jersey, USA) as follows: Preheating at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C 1 min, primer annealing at 32°C 3 min, and primer extension at 72°C 2 min. The reaction was closed by DNA extension at 72°C for 10 min. The PCR products then were run in electrophoresis using 1.5% agarose at 70 volt for 2 h. The gel was stained with ethidium bromide and was visualized under UV light using a Chemidoc (Bio Rad, California, USA). Each RAPD primer reaction was repeated twice to obtain the consistent RAPD bands.

Amplified Fragment Length Polymorphism (AFLP)

AFLP detection was conducted using the AFLP System I kit (Invitrogen, California, USA) in 16 *J. curcas* genotypes showing differential oil contents. Template DNA preparation and PCR reaction was conducted by following the protocols provided in the kit (Invitrogen, California, USA). PCR products were loaded into SDS PAGE and were visualized using silver staining method as described in standard SDS-PAGE protocol. For testing the consistency appearance of DNA bands resulted by each DNA samples, PCR reactions for all template DNA samples were conducted twice. Number of polymorphic bands resulted were then compiled to determine the best compatible primer combinations that can be used for DNA markers.

Sequence Related Amplification Polymorphism (SRAP)

SRAP is unique in terms that they are able to make codominant markers (i.e. the heterozygote genotypes can be recognized from the homozygous ones). Primers used in this study were designed according to the primer designs listed in Li and Quiros (2001) and Li *et al.* (2003). The primers were synthesized by Invitrogen (Invitrogen, California, USA). PCR reaction was performed in a total volume of 20 ul, and started with a denaturation step at 94°C for 2 minutes. This was followed by five cycles of 94°C for 1 minute, 35°C for 1 minute, and 72°C for 1 minute. The next 35 cycles began with 94°C for 1 minute, followed by 50°C for 1 minute, and 72°C for 1 minute. The PCR product was either visualized on

1.5% agarose gel stained with ethidium bromide, or 4% polyacrylamide gel with silver staining.

Target Region Amplification Polymorphism (TRAP)

Development of TRAP marker in *J. curcas* was done by following the method of Hu & Vick (2003). The specific primers were designed from genes controlling seed oil content. One of them was *Diacylglycerol acyltransferase* (DGAT), which was patented as a gene that can improve seed oil content in transgenic plants (Nykiforuk *et al.*, 2003). The other gene was *Glycerol-3-phosphate acyltransferase* (G3PAT), which was shown to increase seed oil content by as much as 21% when introduced into the genome of Arabidopsis (Jain *et al.*, 2000). The primers designed from these two genes are shown in Table 1. These primers were then combined with a SRAP primer, under a PCR condition specified for SRAP above.

Table 1. TRAP primers designed from DGAT and G3PAT sequences

Table 1. Primer marka TRAP yang dirancang dari sekuen gen DGAT dan gen G3PAT

Primer Name	Sequence
DGI2-F	5'-AGAGTCCGCTTAGCTCTGATG-3'
DGI2-R	5'-GGTTGAACAGACCTGCATGA-3'
DGI3-F	5'-AACAGCAGGCTTATCATTGAA-3'
DGI3-R	5'-CCCCGTTTTAATTAACCAACC-3'
DGI4-F	5'-CGGGGTTTTGGTTTAGTTCA-3'
DGI4-R	5'-AGATAGGCGCAAGAGAGAA-3'
DGI5-F	5'-TCTTGCCGCTATCTAGTTGA-3'
DGI5-R	5'-AACTGCTGTTGTGGTAATGAGC-3'
G3PI-F	5'-AGGCAACTCCTCGAGAAAGG-3'
G3PI-R	5'-TGTGCCTTCAGGACAGATCA-3'
G3PE-F	5'-CGGAAAACCTGGAAACCTCT-3'
G3PE-R	5'-TTCTCGAGGAGTTGCCTCAT-3'

RESULTS AND DISCUSSION

For the RAPD method, PCR reactions were conducted in 50 jatropha accessions as this study was also used for genetic diversity studies on jatropha accessions maintained by the Indonesia Agency for Agricultural Research and Development. In total, RAPD method used in the 50 accessions produced 43 polymorphic RAPDs.

This means that on average 4.3 polymorphic RAPD bands were created per RAPD primer. Example of the RAPD banding pattern from this study is shown in Figure 1.

The polymorphism level between two *Jatropha* accessions can be lower than the total polymorphism observed in all the accessions studied. For example, the polymorphism level between NTB and JT-5 (two accessions with the highest contrasting oil content) was only 58%. In other words, from the 43 polymorphic RAPD bands produced by RAPD reactions using the 10 primers, only 25 of those bands will be polymorphic if the two accessions are used as parents in a cross to develop a mapping population. Nevertheless, this lower polymorphism level still shows that RAPD is an economically efficient method since on average each primer will be able to produce 2.5 polymorphisms for the two *Jatropha* accessions. In terms of cost, developing a RAPD marker is much cheaper than an SSR marker since SSR markers utilize a pair of primers that are around 20 bp long. Thus SSR is approximately four times as expensive as RAPD, since the cost of

primers is calculated per base and each PCR reaction for SSR will need primers with four times as many bases as that of RAPD. In addition, most SSR only produce one polymorphic marker from each pair of primers in diploid organisms. SSR markers are also more costly to develop and require some knowledge on the genome sequence of the target organism. However, this makes them more specific, reliable, and reproducible, along with another advantage of being codominant. Nevertheless, such specificity does have a drawback; most SSR will only work on the species they are designed for.

The biggest disadvantage of RAPD method is that they are more sensitive to variations during the PCR reaction process and therefore the resulting DNA bands are often inconsistent. This problem can be reduced by maintaining consistency in every PCR reactions and duplicating the same PCR reactions at least twice. Only RAPD bands that consistently appear in each PCR duplications can then be used as markers and for genetic analysis. The bands that are not consistent must be discarded from the analysis.



Figure 1. The banding pattern of PCR product using RAPD 5 primer on 50 *Jatropha* accessions. M = 100 bp DNA ladder; 1-50, fifty *Jatropha* accessions used in the RAPD analysis.

Gambar 1. Pola pita produk PCR menggunakan primer RAPD 5 yang diuji pada 50 aksesori jarak pagar. M = marka 100 bp; 1-50, lima puluh aksesori jarak pagar yang digunakan pada analisis RAPD



Figure 2. Banding pattern produced by AFLP method. Tk is the predigested tomato DNA from the kit, Tp is pure tomato DNA from the kit that was digested along with the two *Jatropha* accession (Jt and Nt). The first four lanes were amplified using different specific primer combination to the last three lanes.

Gambar 2. Pola pita yang dihasilkan oleh metoda AFLP. Tk adalah DNA tomat yang sudah dipotong dengan enzim restriksi (bawaan kit AFLP); Tp adalah DNA tomat murni yang sudah dipotong dengan enzim (bawaan kit AFLP); dan DNA dari dua aksesori jarak pagar (Jt dan Nt). DNA pada empat lajur pertama telah diamplifikasi dengan kombinasi primer spesifik yang berbeda dengan tiga lajur terakhir.

Development of AFLP markers is relatively easy to perform, since a variety of kits are available for various organisms. One of the major drawbacks of AFLP is that the process of DNA template preparation is relatively time-consuming and complex. The use of commercial kit can reduce the optimization process required, at the expense of significantly higher cost per sample. Due to the higher cost, the feasibility study of AFLP marker for *Jatropha* genetic mapping was only performed on two accessions with a high contrast in oil content (JT-5 and NTB).

Figure 2 shows the banding pattern produced by the two accessions, along with two control DNA template provided in the kit. One control is a pure tomato DNA (Tp) that needs to be digested and ligated to the adapter along with the two *Jatropha* accessions, while the other control is the same tomato DNA already digested and ligated to adaptors by the kit supplier (Tk), and thus ready for PCR. It can be seen that the

template preparation was successful since the banding pattern produced by tomato DNA prepared by us is indistinguishable from those produced by the template from the kit. The two *jatropha* accessions produced complex banding pattern, although no polymorphism was apparent in this particular primer combination. More primer combinations need to be investigated to deduce the polymorphism level of AFLP markers in *Jatropha*.

SRAP markers are very similar to SSR in terms of initial cost and methods. The primers are similar in length to SSR, hence their price are also similar. The PCR method and DNA visualization is also similar, but each SRAP primer pairs produce more bands than SSR. The higher number of bands does not always translate to more polymorphism however, since many are monomorphic or too faint to score. The claim that there is a higher number of codominant loci in SRAP could not be verified because we did not have accessions that can be compared with their progeny or parents.

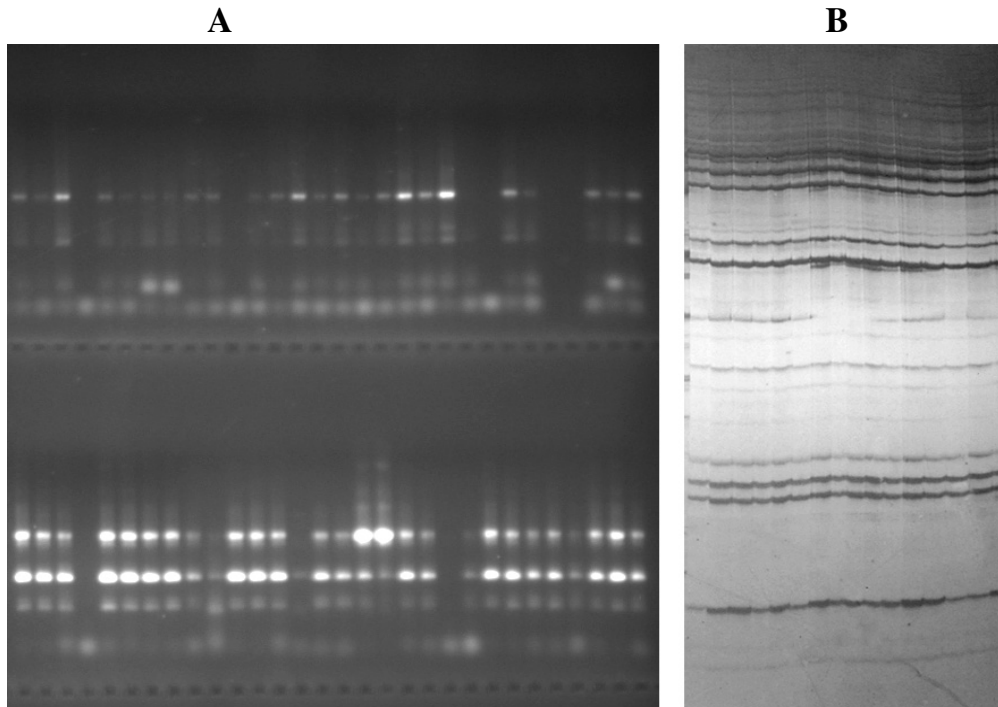


Figure 3. SRAP banding pattern from various *Jatropha* accessions obtained using two different primer combinations when run on agarose gel (dark gel with bright DNA bands) (panel A) compared with the banding pattern on polyacrylamide gel electrophoresis stained with silver nitrate (light gel with dark DNA bands) (panel B).

Gambar 3. Pola pita marka SRAP dari berbagai aksesi jarak pagar yang dihasilkan dari PCR menggunakan dua kombinasi primer yang berbeda ketika di elektroforesis menggunakan gen agarose (wama gel gelap dan warna pita DNA terang) dibandingkan dengan pola pita pada gel poliakrilamid menggunakan pewarnaan silver nitrat (gel warna terang dengan pita warna gelap) (panel B).

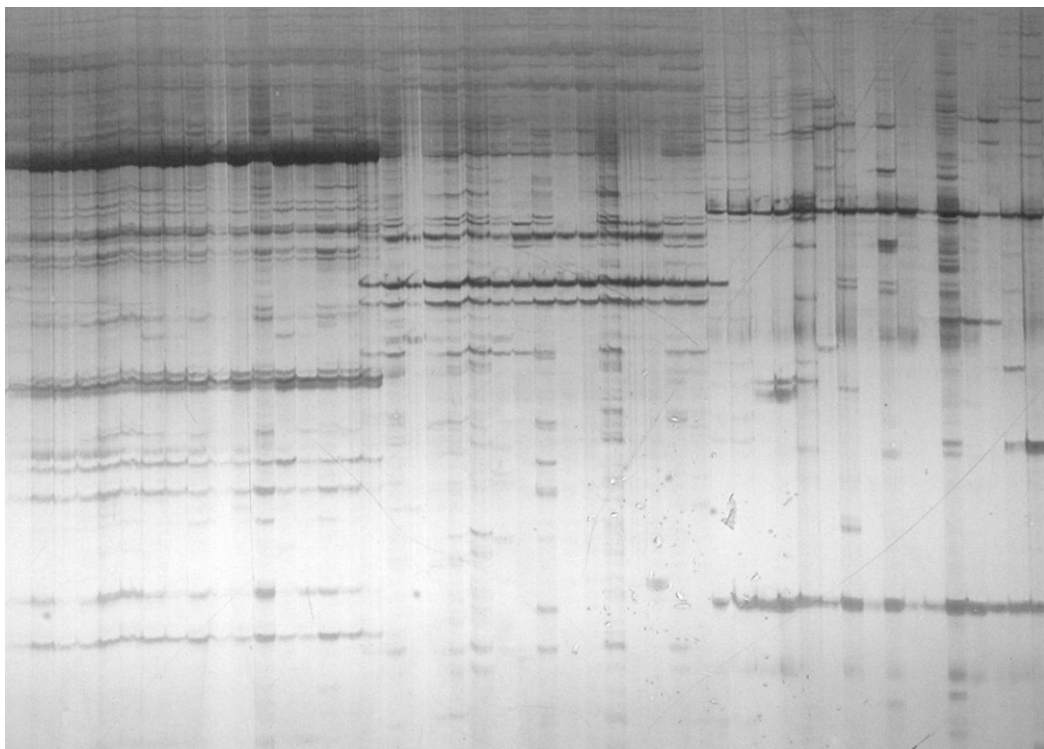


Figure 4. TRAP banding patterns on polyacrylamide gel produced by three primer combinations when amplified on 16 selected *Jatropha curcas* accessions.

Gambar 4. Pola pita marka TRAP hasil elektroforesis pita DNA pada gel poliakrilamid menggunakan pewarnaan silver nitrat dihasilkan dari PCR menggunakan tiga kombinasi primer yang diamplifikasi pada 16 aksesi jarak pagar terpilih.

Figure 3 shows the appearance of DNA bands produced by SRAP. The darker gel shows the polymorphisms in several *Jatropha* accessions using a combination of EM2 and ME2 primers (top) and EM2 and ME8 primers (bottom) when visualized on 1.5% agarose gel. It can be seen that due to the shorter migration path and less sensitive ethidium bromide stain the banding pattern is much less complex compared to the pattern observed on a 4% polyacrylamide gel with a length of 40 cm visualized using silver staining (right). The higher number of observable bands and more complex banding pattern did not produce more scorable polymorphisms in this case, because the majority of bands are monomorphic.

The TRAP method demonstrated that the substitution of one random SRAP primer with a gene-specific primer can still result in DNA bands polymorphism across 16 *Jatropha curcas* accessions that can potentially be used as DNA markers (Figure 4). The number of polymorphic bands resulted by each TRAP primer combination is evidently higher than the ones shown by the RAPD method. Nevertheless, it is possible that some of the polymorphic DNA bands might not be useful as markers since the bands appeared to be the result of non-specific amplifications. Another reason was that the band intensities were relatively low and very likely to be false positive markers.

From this study 43 polymorphic RAPD markers were produced that can be used for genetic diversity analysis, fingerprinting of individual *Jatropha* accessions and constructing a genetic map of *J. curcas* genome. However, the number of RAPD markers obtained from this study is still far from the target of at least 250 polymorphic molecular markers required to construct a modest resolution genetic map of this crop. It will need to be supplemented from other methodologies like SRAP and TRAP as these markers demonstrated more polymorphism level than that shown by RAPD. In the near future we expect to apply these DNA markers to construct a genetic map of *J. curcas* genome and fingerprint the superior breeding lines and accessions from the *J. curcas* collection within the Indonesian Agency for Agricultural Research and Development to accelerate the breeding process for this species using marker assisted selection.

CONCLUSION

RAPD method using 10 primers resulted 43 polymorphic DNA bands. Available commercial kit for AFLP is applicable to produce DNA markers in *Jatropha*. SRAP and TRAP methods resulted in more polymorphism than that shown by RAPD. The RAPD, AFLP, SRAP, and TRAP markers obtained from this study can be used for constructing a genetic map of *J. curcas* genome. The development of other types of DNA markers is underway to achieve the target of obtaining at least 250 scorable polymorphic DNA markers to construct a modest resolution genetic map of *J. curcas* genome.

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