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Molecular Identification of Shallot Progenitors Generated From True Seeds By PCR Based Techniques

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ABSTRACT

Thousand tons of shallot bulbs have been imported into Indonesia from overseas in every year for food spices and seed bulb as well, though this vegetable is considered as main crop that ranked in the first in harvested area and the third in production weight. Shallot breeding program is concentrated to develop an efficient propagation system toward on self-sufficient of seeds and production through 'true seeds'. This system is considered as the best plant materials of shallot to make G_0 that can be regenerated twice (G_1) in the following season without significant degradation of genetic power. The purposes of this research are: to test the ability of five commercial cultivars to produce true seeds, to identify them with molecular markers of SSR and to analyze genetic variability of true seeds produced from self-pollination. The goal of this research is to find out commercial shallot cultivars that feasible to be used as parent stock for producing genetically good true seeds either for inbred or hybrid cultivars. Research was held in Laboratory of biotechnology, Faculty of Agriculture, Brawijaya University, Indonesia, and in farm station from March to November 2010. Five commercial cultivars in Indonesia, i.e., Super Phillip, Bali hijau, Bali kuning, Ampenan and Sumenep were used as materials of research. Two sets of SSR primer linked with yield were used, and then profiled by 0.8 % agarose electrophoresis system after amplification in certain PCR program using these two sets of primer. Digital photograph was used for preparing analysis. The result showed that only Sumenep variety is homozygous, but Ampenan variety can also be considered as candidate to be used as homozygous parent. These two homozygous shallot varieties (Sumenep and Ampenan) can then be used as the parent stocks to produce genetically good true seeds either for inbred or hybrid variety.

Key words: Shallot, true seeds, SSR molecular marker, homozygous parent stock**.**

INTRODUCTION

Small-bulb onion that usually called as shallot (*Allium cepa* var. *ascalonium* Linn.) is more popular than common onion in Indonesia. Though this vegetable ranks the third in production weight, it has been cultivated dominantly in most land area of vegetables. Nevertheless, until recently the total production (around 800 thousand tons a year) has not been covering national demand [1]. Thousand tons of shallot bulbs have been imported from Philippines and Thailand in every year. Severe problem is often arisen by the fact that Indonesia has also imported shallot bulbs from generation (G_1) for covering national need of seeds.

Development of shallot production and breeding strategy was not systematically programmed, whereas the productivity achieved in Indonesia is lower than that in Europe. Bulbs are more familiarly used as planting material compare to the true seed, and using G_1 shallot has several advantages by means to obtain more vigorous plant, early harvest and simply agronomical practices. From cultivating G_1 seeds, farmers usually produce bulbs and sell either for consumption or replanted as G_2, G_3 seeds and so forth in the following seasons. However, as the rule of thumb in shallot cultivation, genetic power degradation of seed is higher as many times as bulb to be regenerated. Beside this, another problem when used regenerated bulb for producing shallot, especially in short day zone like in Indonesia, is physiologically resulting immature bulb due to the short growth life.

True seeds have longer growth life than seed bulb ones, while lower temperature during cultivation will also promote longer their life. In Java Island, shallot which is planted in lowland is usually harvested for only around 70 days after planting (dap), while in highland it can reach more than 120 dap [2]. Consequently, only in a few highland area farmers can produce good enough quality of seed bulb, and this is the reason why Indonesia is always getting insufficient of shallot seed from year to year.

Shallot is the vegetable having little diversity in varieties, whether it is local or high yield varieties. Shallot propagation by using the bulb will cause neither segregation nor variability. Continued cultivation using bulb of a shallot variety as planting

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material can cause the decreasing of yield and its quality due to the accumulation of pest and disease population brought by preceding cultivation. True seeds is the best plant material for making G_0 and can be regenerated twice (G_1) in the following season without significant degradation of genetic power. The only disadvantage, if it is any, of using true seed is that farmers do not familiar yet to grow it. However, since last years several progressive farmers in Indonesia have begun to acknowledge true seeds. In future, using true seed as planting material of shallot will be very prospective and it will be the most possible way toward self sufficient of seed.

Several national cultivars of shallot have been released by Institute of vegetable research station of Indonesia. However, as already mentioned above, they are mainly used for vegetative propagation means. Although producing true seeds have some advantages in seed trade and simply handling and genetic power of bulb formed (generation zero or G_0), no report was found in utilizing such cultivars for producing true seed through sexual propagation in Indonesia. This is important rationale of this research that finding prosperous commercial cultivar(s) having ability to produce efficiently good quality of true seed is a must. One of prerequisite of targeted cultivars is that they must have genetically homozygous so that their progenitors will not be segregated into different individuals and in other word, the true seeds resulted from sexual pollination must be in a uniform true type. On the other hand, the development of molecular identification techniques helps researchers not only to identify genotypes, but also in assessing and exploiting the genetic variability [3]. Traditionally, identification has been based on morphological characters; however the development of new techniques has allowed basing these analyses on DNA information. One of them is the PCR based techniques, i.e., SSR and RAPD. Polymerase Chain Reaction is widely held as one of the most important inventions of the 20th century in molecular biology. Small amounts of the genetic material can now be amplified to be able to identify, manipulate DNA, and detect genetic variation of organisms.

The purposes of this research were to test the ability of five most popular cultivars in Indonesia to produce true seeds, to identify with molecular marker of SSR and to analyze genetic variability of progenies regenerated from true seeds produced from self-pollination of five shallot cultivars. The goal of this research was to find out commercial shallot cultivars that feasible to be used efficiently as parent stock for producing genetically good true seeds either for inbred or hybrid cultivars.

MATERIALS AND METHODS

Research was held in Laboratory of biotechnology, Faculty of Agriculture, Brawijaya University, Indonesia, and experimental farm sited around the university and was conducted from March to November 2010. Five commercial cultivars in Indonesia i.e., Super Phillip, Bali hijau, Bali kuning, Ampenan and Sumenep were used as research materials. Bulbs were induced by artificial vernalization with the exposure in 9^{o}C for two weeks before planting to induce flowering. 20 g high KNO3 content of fertilizer was sown in twice i.e., one and four weeks after planting in each pot. To prevent cross pollination, each cultivars were covered separately with transparent plastic just a day before anthesis. Artificial hand pollination was done to fertilize stigma for three days. Within five to six weeks after pollination, seeds were harvested and dried in ambient temperature for a week. Dried seeds then were grown in sterilized organic media for morphological and molecular examination.

Plant DNA was extracted by non-ionic detergent *cetyltrimethylammonium bromide* (CTAB) method modification [4]. Two sets of primer linked with yield of shallot were used in PCR Reaction program. DNA amplification reactions were performed in a volume of 25 μl containing approximately 10 ng template DNA, 0.2 μm of a single decanucleotide, 200 μm of each dNTP, 2.5 μm of Mg^{2+}, and 1 unit *Taq* DNA polymerase in the buffer provided by the manufacturer of the enzyme (Biotools). The reaction mixture was overlaid with a drop of mineral oil. Amplification was performed in a DNA Thermal Cycler 480 (Perkin Elmer Cetus) programmed as follows : one cycle of 1 minute at 94◦C ; 35 cycles of 45 seconds at 92◦C, 1 minute at 37◦C and 2 minutes at 72◦C, followed by one cycle of 3 minutes at 72◦C. Aliquots of 12 μl of amplification products were loaded onto 1.5% (w/v) agarose gels for electrophoresis in 1xTBE buffer [5], followed by staining in ethidium bromide, then visualized and photographed under UV light. Molecular weights were estimated by reference to a 100 Base-Pair Ladder (Pharmacia).

RESULTS AND DISCUSSION

It was found that the DNA obtained was contaminated by RNA, but this could be overcome by the addition of RNase to the sample solution. Some samples were also browning, and it had to be re- extracted.

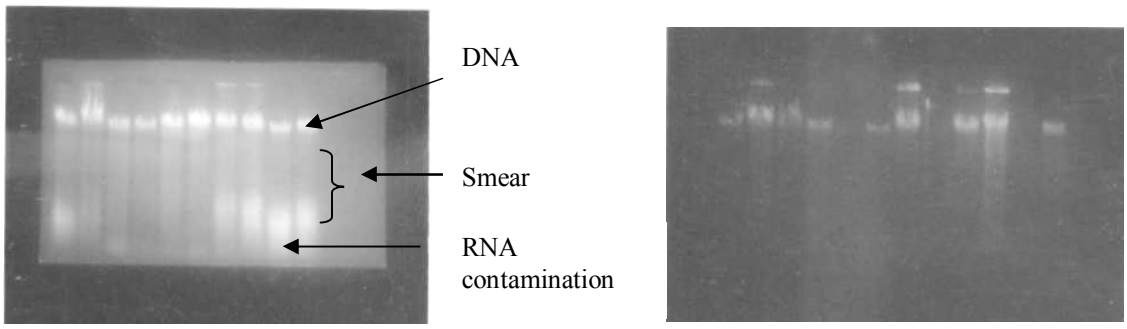

Figure 1. Electrophoresis result of DNA samples contaminated by RNA (a) and after RNase addition in the DNA samples (b)

The quality of the extracted DNA in Figure 1 showed that the good quality of DNA was indicated by the thick line, while the thin line indicated that the purity of DNA was of inferior quality. Lines on the bottom of the electrophoresis indicated that the sample was contaminated by the RNA and the smear between the two lines was probably the degraded DNA (figure 1a). However, after the addition of RNase gave result a better quality of DNA. This was shown in the figure 1(b) which showed no white line after RNase added to the sample.

Table 1. Absorbance ratio of DNA samples of 5 shallot varieties

No.	Shallot Varieties	Absorbancy Ratio of A_{260}/A_{280}	[DNA] (µg/µl)
1	Super Phillip	1.822	1.971
2	Bali Hijau	1.787	1.260
3	Bali Kuning	1.625	0.696
4	Ampenan	1.559	0.568
5	Sumenep	1.657	0.621

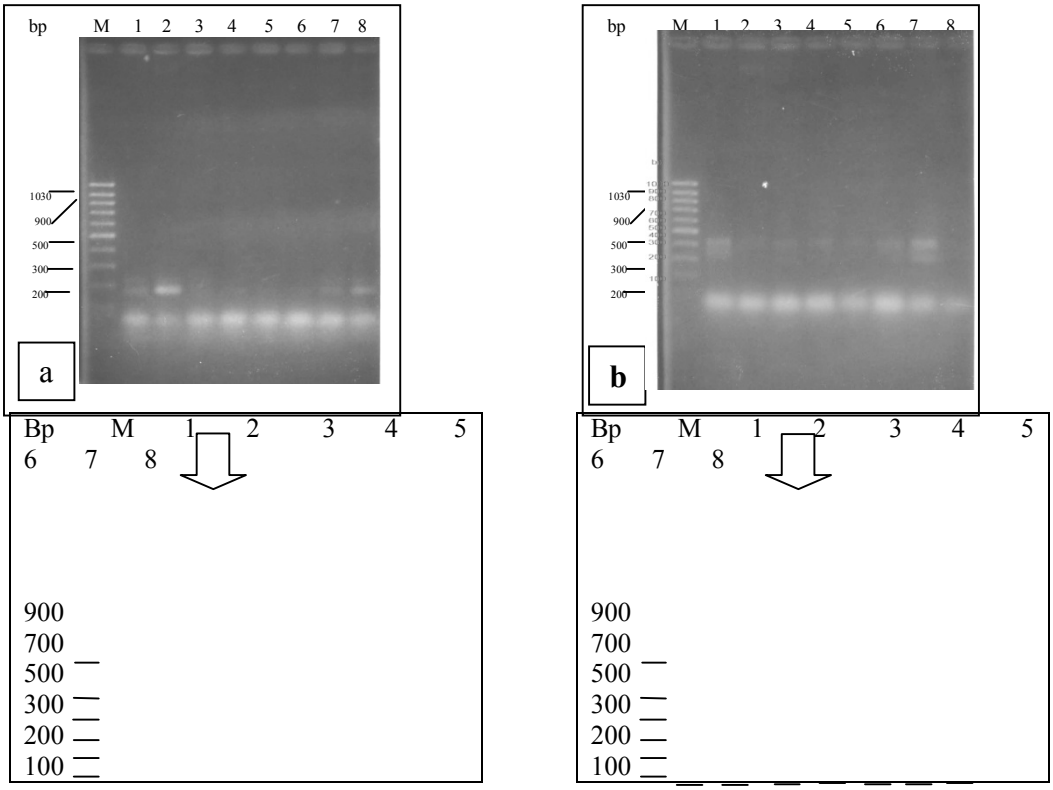

Figure 2. Electrophoresis and Electrophoregram of 5 Shallot Varieties using Yield1 Primer (a) and Yield2 Primer (b)

M = 100 bp DNA ladder

1.Super Phillip	3. Bali Kuning	5. Sumenep	7. Bali Hijau
2. Bali Hijau	4. Ampenan	6. Ampenan	8. Super Phillip

Based on the results of measurements with a spectrophotometer, it showed that DNA purity values were ranging from 0.568 µg / µl up to 1.971 µg / µl, with an average of 1.023 µg / µl. The average ratio (A_{260}/A_{280}), which was measured with a spectrophotometer, was 1.690 with values in the range of 1.559 to 1.822. The measurement was done on the DNA samples after RNase addition. The results of measuring the value of purity of DNA are presented in Table 1.

Based on the electrophoregram, it indicated that the DNA banding pattern formed would tend to be uniform but was still able to distinguish between varieties based on two pairs of primers used, and were indicated by the length of the DNA bases shown. Yield1 primer produced the band sizes between 100 to 300 bp (base pair), with the total band number of 12 from 5 varieties evaluated, as shown in Figure 2a. Yield2 primer produced 11 bands with the size of 300 to 450 bp, as shown in Figure 2b.

Electrophoresis product using yield1 primer as shown in figure 2a indicated that Variety Bali Kuning, Ampenan, and Sumenep were homozygous, while Super phillip and Bali Hijau were heterozygous for the yield character. Figure 2b showed that Bali Hijau in lane 2 was homozygous but heterozygous in lane 7 when yield2 primer was used. That was also true for Ampenan in lane 4 and 6. It indicated that the electrophoresis product was unstable by using the yield2 primer. This research showed that only Sumenep variety which was homozygous, but Ampenan variety could also be considered as candidate to be used as homozygous parent.

CONCLUSION

Based on the result of electrophoresis using SSR molecular marker in this research, it was concluded that three shallot varieties of five varieties tested were heterozygous while the other two varieties were homozygous. These two homozygous shallot varieties were Sumenep and Ampenan, which could then be used as the parent stocks to produce a genetically good true seeds either for inbred or hybrid variety.

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