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PREFACE

Biodiversitas, the Journal of Biological Diversity has successfully entering the tenth year since it firstly published in 2000. The journal has tremendously fulfilled all requirements for the third re-accreditation by Directorate General of Higher Education of National Education Department, Government of Indonesia to be the third consecutive period of becoming a "nationally accredited journal" at end of last year. The success should stimulate the improvement of the performance in term of management and quality of the contents as well as able to reach what we have planned to be an internationally recognized journal. This edition is the first publication fully in English instead of the bilingual version. Consequently, the management will only accept any articles or papers written in English for the next publication.

In line with the changes that already happened since the early of this year when the journal formally got the third nationally re-accredited journal, the *Biodiversitas* editorial board was also changed, in which many internationally recognized experts in the area of biodiversity were asked for their help to participate in forming the editorial board as *Managing Editor*. Thanks a lot for their willingness to join in the editorial board. These members of the board will eventually responsible for the quality of the journal since they could directly receive, select, comment, appoint peer reviewer or reject articles from the writers before the articles directed to the *Editor-in-Chief*. The accepted articles will be processed by *Secretary and Technical Editor* for printing purposes. The accepted articles will also be published electronically in editor's website to reach more readers internationally. List of *Editorial Boards* with their email- and geographical-addresses are always provided in each edition, therefore, writers could communicate with preferably paperless by email to help reducing global warming by reducing CO_2 emission due to transportation as well as reducing forest degradation needed for paper production.

The way of selecting the articles is also strictly done starting this edition in term of the content. This journal will only publish articles or papers of biodiversity in the level of gene, species or ecosystem of all organisms as well as cultural diversity that in turn could impact on biodiversity. Therefore, many subjects like (i) Molecular Biology and Genetics, (ii) Taxonomy, Biosystematics, and Phylogenetics, (iii) Ecology and Conservation Biology, as well as (iv) Etnobiology would fit with the contents of the journal. Articles received before this year, will be reconsidered and reselected based on the mention criteria. In order to improve the quality of our journal, I should take this difficult decision, and I feel sorry about this matter.

Wassalam,

Prof. Drs. Sutarno, M.Sc., Ph.D

EDITOR-IN-CHIEF

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Biodiversity Study of Black Coral (Order: Antipatharia) Collected from Manado, Indonesia based on rDNA Internal Transcribed Spacer (ITS) Sequences Analysis

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ABSTRACT

Biodiversity of black coral (order Antipatharia) collected from the Bunaken Marine Park (Manado Sea, Indonesia) has been studied based on the sequence of the Internal Transcribed Spacers (ITS) region of rDNA gene. The results of the study showed that the 18 species of Antipatharia were considered to be separated in two family groups, family Myriopathidae and Antipathidae-Aphanipathida. A significant species-specific signal has been detected among the families of Antipathidae and Aphanipathida. However, more studies-on different species were required to be clearly interpreted. The new species *Pseudocirrhipathes mapia*, the new genus *Reticulopathes*, and possibly a new taxon of the family Myriopathidae has been recognized based on ITS sequence data.

Key words: biodiversity study, Manado, rDNA ITS, Antipatharia, black coral.

INTRODUCTION

Antipatharia Milne-Edwards and Haime, 1857 (Cnidaria, Anthozoa) is the order of Black Coral. Five families of Antipatharia have been established, Antipathidae, Cladopathidae, Leiopathidae, Myriopathidae, and Schizopathidae. About 200 species of black coral have been recorded. Black coral could be found in all oceans at depths ranging from those of shallow waters to two thousands meters (Pax et al., 1987)

Antipatharians have been traditionally classified according to morphological and anatomical characters. The primary characters that are usually used in this term of systematic are the general morphology of the corallum, the pattern of ramification, the spines arising on the skeleton surface, the morphology of the polyps and the number of their mesenteries. Milne-Edwards and Haime (1857) had been proposed one of the first systems of classification that is entirely based on the skeletal features. The polypar characteristics are considered important also in most recent taxonomic works where the number of mesenteries, the size and position of tentacles around the mouth and the arrangement of zooids around the axis and the kind of ramification of the corallum are characters used in the genus separation (Cooper, 1909; Opresko, 1972).

In line with developing technology, more specific tools in biology such as electron microscope enhance placing of black coral in more specific taxon. The study of spines surface using scanning electron microscopy has recently been employed with success in taxonomic works, putting in evidence micro-differences in the deposition of skeletal material on the spines, which are often useful for species distinction.

At a specific level, although there is an increasing study of systematic of Antipatharia based on morphological and anatomical characters, the main problem in taxon definition by means of this approaches is the extreme plasticity of the colonies. The number of species obtained tends to overestimate because it may contains some ecotypes. For this reason, sequences data of DNA are an obvious source of additional evidences regarding black corals systematic relationships. Genetic consideration has been widely used to clarify one of the largest problems arising from identifying coral at the species level.

Genes of internal transcribed spacers (ITS) have been used for molecular markers at the species level of coral in order to study intra- and inter-specific diversity (Diekmann et al., 2001; van Oppen et al., 2002). Sequences of internal transcribed spacers of rDNA was also usefully used in resolving

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phylogenetic relationship of closely related taxa in some coral (Odorico and Miller, 1997; Takabayashi et al., 1998; van Oppen et al., 2000; Lam and Morton, 2003; Chen et al., 2004). The internal transcribed spacer (ITS) region, consisting of the ITS1, 5.8S, and ITS2 sequenced from protists (Hunter et al., 1997), animals (Gonzalez et al., 1990), plants (Baldwin et al., 1995), fungi (Lee and Taylor 1992), and macrophyte algae (Coleman et al., 1994 and Goff et al., 1994), typically provide phylogenetic resolution at or below the species level in each of these groups.

In this paper, in order to investigate biodiversity of black coral lived in the sea of Manado, genetic relationships of 18 species of black coral belonged to nine genera and three families have been performed. The application of a molecular approach to the systematic of this group represents an interesting possibility to test, by an alternative method, the traditional morphological taxonomy.

MATERIALS AND METHODS

Sample collection

Eighteen species of black coral belonged to three families and 9 genera of the order have been used in this study. All the specimens were collected by SCUBA diving on the shallow-water reefs of the Bunaken Marine Park (North Sulawesi, Indonesia) within a depth range of 10-50 m.

DNA Analysis

Antipatharian ITS rDNA sequences were obtained after isolation, amplification, and sequencing process. All procedures of this molecular analysis were conducted using facilities of the Molecular Genetic Laboratory of *Istituto di Biologia e Genetica, Dipartimento di Scienze del Mare, Facolta di Scienze, Universita Politecnica delle Marche*, Italy.

Isolation

Using protocol of Qiamp tissue kit (QIAGEN) the genomic DNA was isolated using primer RA2 5'-GTCCCTGCCCTTTGTACACA-3' and primer ITS 2.2 5'-CCTGGTTAGTTTCTTTTCCTCCGC-3' (Wörheide, 1998) for the internal transcribed spacer regions (ITS1 and ITS2).

Amplification

Thirty cycles of PCR were conducted using a *Perkin Elmer GeneAmp PCR System 2400* under following profiles: 94°C for 30 s; 52°C for 30 s; and 72°C for 60 s using HotStarTaq Master Mix Kit (Qiagen). The PCR product was then purified using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's recommended protocol.

Sequencing

Cycle sequencing reactions were done using DNA Sequencing Kit (Applied Biosystem) according to the protocol provided by the manufacturer with the same primers used in PCR. To obtain the sequences in both directions the forward primer 5'-CAACGGGCGGGCTGGTGCGCA-3' and the reverse primer 5'-TGCGCACCAGCCCGCCCGTTG-3' designed on the 5.8S rDNA have been used. The sequencing reaction product was purified using Qiagen DyeEx Spin Kit and then was sequenced on an automated DNA sequencer (ABI PRISM 310, Applied Biosystems). The sequences are about 850 *nt*. long except for *Cirrhipathes spiralis* (partial sequences of 642 *nt*). The nucleotide sequences have been deposited in GenBank (accession numbers AM404315-AM404329).

Phylogenetic analysis for biodiversity observation

The trees were produced by maximum parsimony (MP), maximum likelihood (ML), and neighbour-joining (NJ) methods, using the PAUP 4.8 beta version (Swofford, 1998) program. For the heuristic ML analysis, the optimum substitution model was determined using the Model test 3.06 program (Posada and Crandall, 1998). Once the appropriate model was determined (TrNef + G) (Tamure and Nei, 1993), ML analysis was performed with all the parameter values of the model (Gamma distribution shape parameter = 0.2411; substitution model: A-C 1.000; A-G 2.3660; A-T 1.000; C-G 1.000; C-T 3.4329; G-T 1.000; equal base frequencies). *Porites lutea* was used as outgroup.

RESULTS AND DISCUSSION

Results

Sequencing of the ITS region (spanning a partial sequence of the 5' end of the 18S gene, complete ITS1-5.8S-ITS2 and partial sequence of the 3' end of the 28S gene) results sequences ranged between 804 (*Pseudocirrhipathes mapia*) and 912 (*Cirrhipathes sp,*) nucleotides for the species of the family Antipathidae, and between 812 (*Myriopathes myriophylla*) and 836 (*Antipatella subpinnata*) nucleotides for the species of the family Myriopathidae, while the sequence of *Rhipidipathes reticulata* (Aphanipathidae) was 832 nucleotides long.

Figure 1 show that the 18 species of Antipatharia considered are separated in two groups, one containing the genera belonging to the family Myriopathidae and the other belonging to the families Antipathidae-Aphanipathidae. The distance matrix shows that in the first group genetic distances among the Indonesian Myriopathidae species *Cupressopathes abies*, *Myriopathes myriophylla* and *Myriopathes* sp.1, *Myriopathes* sp.2 and the Mediterranean species *Antipatella subpinnata* were very small (0.25% to 2.6%). The second group is divided into three well-differentiated clades: the first group, in basal position, is composed of the new species *Pseudocirrhipathes mapia*, the second is

made up of the genera *Cirrhipathes*, *Rhipidipathes*, and *Reticulopathes*, the last clade is made up of the genera *Stichopathes* and *Antipathes* and the species *Cirrhipathes* cf. *anguina* 2. The analysis showed that the species belonging to *Cirrhipathes* genus are not monophyletic. In fact although *Cirrhipathes spiralis* and *Cirrhipathes* sp. show a gentic distance of 0.99%, the two specimens of *Cirrhipathes anguina* display, respect to those mentioned above, a genetic distance not smaller than 9% and between them of 11.20%. Similarly, *Stichopathes* sp.2 is not clustered with *Stichopathes sp1* although shows a genetic distance of 3.53%. In the *Cirrhipathes-Rhipidipathes-Reticulopathes* group, the new genus *Reticulopathes* differs from the other species belonging to this group by genetic distances ranging from 11.06% (*Cirrhipathes* cf. *anguina* 1) to 14.80% (*Cirrhipathes* sp.).

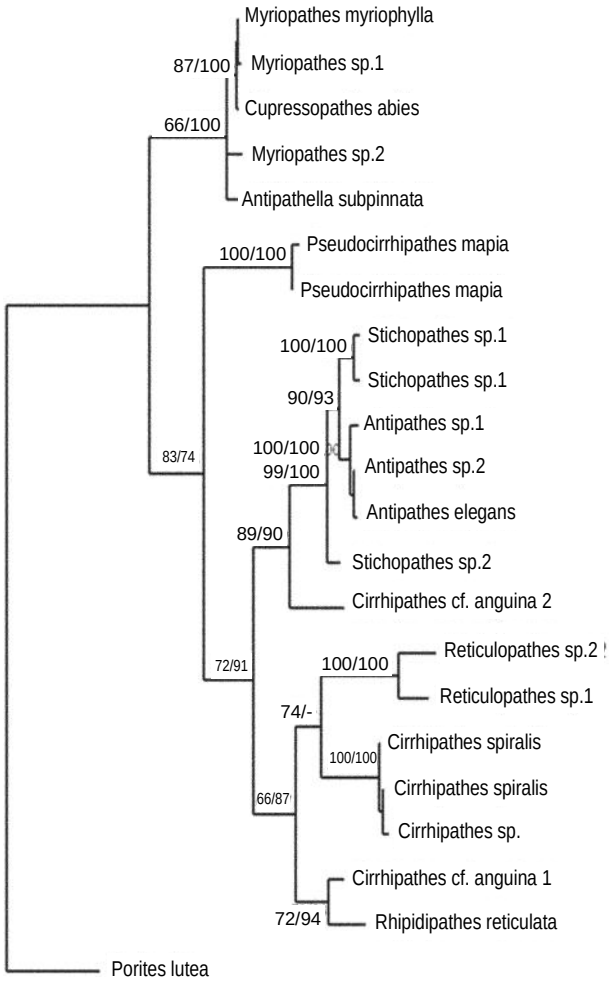

Figure 1. Phylogenetic tree obtained by Maximum Likelihood and Maximum Parsimony of the antipatharian species based on internal transcribed spacers rDNA. On the left ML value, on the right MP value.

The phylogenetic tree obtained by Neighbor-Joining method (Figure 2) presents the same tipology of the described tree with the exception of the species *Reticulopathes* which are separated from the *Stichopathes-Antipathes* group and the *Cirrhipathes-Rhipidipathes* group which is supported by a low bootstrap value (54%).

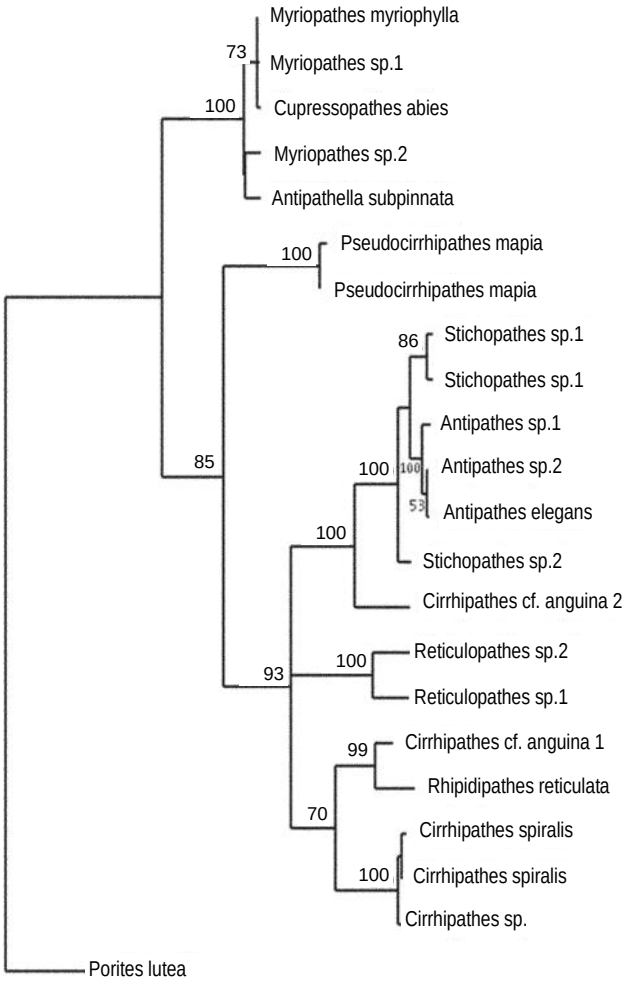

Figure 2. Phylogenetic tree obtained by Neighbor-Joining method.

Discussion

The results demonstrated that ITS genes are able to give information regarding biodiversity of black coral lived in around Manado. These results are interesting because they represent the opportunity to set, with the help of an alternative method, the value of the traditional taxonomy based on morphological characters. The data obtained from this analysis support the difference among the families Antipathidae, Aphanipathidae and Myriopathidae with the first two more closely related. The family of Myriopathidae and Antipathidae are genetically divergent from one another and obviously are not considered as sister taxa. The degree of genetic variation differs inside the families Antipathidae and Myriopathidae. In the Myriopathidae, the analysis by means of DNA sequences is not able to separate the

species of *Myriopathes* and *Cupressopathes* and a clear difference is detected only with the Mediterranean *Antipathella subpinnata*. Particularly the similarity among the species of *Cupressopathes* and *Myriopathes* could suggest the possibility of a reticulate evolution due to hybridization among these species (Diekmann et al., 2001; van Oppen et al., 2002). Hybridization among these species affects the similarity as shown both in genetic and morphological characters. The degree of hybridization of these genera might be very high since they share many ecological and reproductive traits. Morphologically similar species frequently live in close proximity to each another and have overlapping reproductive periods, thus opportunities for interspecific encounters among gametes. This hypothesis could clarify the high range of morphological variability of the colonies (size, height, thickness, branching) of the specimens "*Cupressopathes*-like".

Although the genetic distance among the species of Myriopathidae is not able to differentiate among them, it could be identified in the tree a group of new species or at least a new genus of *Myriopathes*-like antipatharians. When it is compared to the morphological data, the description confirms the existence of either new species or new genus of Myriopathidae.

ITS sequences data subdivided the Antipathidae cluster into different clades. The first clade comprises *Antipathes* sp.1, *Antipathes* sp.2, and *Stichopathes*. The second clade comprises the genus *Cirrhipathes*, while the last clade includes *Reticulopathes*. This present study has also shown that *Cirrhipathes* cf. *anguina* and *Cirrhipathes spiralis* are not clustered in a monophyletic group which indicates that placement of these two species need to be re-examined.

The separation of *Rhipidipathes reticulata* from the *Cirrhipathes* and *Antipathes* groups supports, although with low bootstrap values, the recent establishment (Opresko, 2004), based on polyp morphology and on the size and shape of spines, of the family Aphanipathidae. The agreement between the genetic variability and spine morphology, suggests that this morphological character is largely independent from the environmental cues and therefore particularly suitable for a diagnostic verification. To some extents, the ITS region of rDNA provides a great systematic resolution for Antipatharians and it's useful to distinguish among high taxa of the order. Further analysis of the data set supports the establishment of new genera and species. At the tip of the tree, *Pseudocirrhipathes mapia*, as a new undescribed species, is significantly different from the other groups.

CONCLUSION

The study showed that gene of ITS are able to distinguished a variety among three families of black coral (Antipatharia) collected from the Bunaken Marine Park (Manado Sea, Indonesia). A Significant species-specific signal has been detected among the families Antipathidae and Aphanipathidae, even studies on different species of the last were needs to be clearly interpreted. The new species *Pseudocirrhipathes mapia*, the new genus *Reticulopathes*, and possibly a new taxon of the family Myriopathidae have been recognized based on ITS sequence data.

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Selection of Indonesia Cassava (*Manihot esculenta* Crantz) Genotype as Source of β-Carotene

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ABSTRACT

Fourteen genotypes of Indonesia cassava (*Manihot esculenta* Crantz) of two generations were evaluated for β-carotene content. The β-carotene content of tubers and leaves were determined by spectrophotometry method. Other parameters such as water and ash contents were also evaluated. Results showed that β-carotene content of tubers of fourth generation (planted in 2006-2007) was higher than that of first generation (planted in 2002-2003), with the exception of Apuy, Iding and Sarewen genotypes. β-carotene content of tubers was lower than that in their leaves of fourth generation plants and that there was no correlation between both organs in terms of β-carotene content of tubers and their leaves except for Tim-Tim 40 genotype. β-carotene content of tubers in several genotypes i.e. Kalbar III (1.13 ppm), Local Muneng (1.03 ppm), Tim-Tim 29 (1.61 ppm) was higher than 1 ppm, and the highest value was found in Tim-Tim 40 (16.83 ppm) which was significantly different (5%) with other genotypes. Meanwhile the lowest content was found in Sarewen genotype as it could not be detected. Water and ash contents of the tubers were between 54 and 69% and between 0.20 and 0.79% respectively. As β-carotene is the precursor of vitamin A, consuming high β-carotene tubers are sufficient for daily requirements of vitamin A, although further study is needed.

Key words: cassava, *Manihot esculenta*, genotype, selection, β-carotene, tubers, leaves.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz.) is a very important crop in the tropics and is a staple food for over 800 millions people (Nassar et al., 2007). In the past, cassava is the main food, especially in the lack of food period (*paceklik*) in Indonesia. Nowadays, cassava is used as an alternative food in food diversity program to reduce on rice dependence. Cassava is one of the options are profitable because of the price is relatively cheaper (Setiawan, 1992).

Cassava is drought tolerant plant and they could be grown easily in land with low soil fertility. Therefore, they could be found in marginal areas (Sudarmonowati et al., 2002). Cassava originating from the South America, precisely in Brazil and they were spreading to almost all the world, including: Africa, Madagascar, India and China. Cassava is growing rapidly in agricultural countries and introduced to Indonesia in 1852 (Bappenas, 2007).

Carotene found in all green parts of the plant and most of them found in the yellow parts (Mutschler, 1991). Green or yellow vegetables and fruits usually have high content of carotene. There is a direct relationship between degrees of greenness vegetables with their carotene content. The greenest leaf contains higher content of carotene (Budiyanto, 2002). β-carotene is the most important provitamin, the two molecules of that provitamin can be formed vitamin A. Only up to 50% β-carotene were used to produce vitamin A. Carotenoid is a precursor of vitamin A, which is needed by the human body for growth, to establish immunity against the disease and to clarify the vision. β-carotene dosage for adults each day according to the WHO standard is 2.4 mg to 3.5 mg (Agbaje et al., 2007). In addition, maternal mortality will be dramatically reduced when pregnant women receive vitamin A or β-carotene supplements (Anderson et al., 2003). There are known 600 types of carotenoids, of which approximately 50 play important roles in the human diet (Lusty et al., 2006).

The objective of this research is to select Indonesian cassava genotype which has high β-carotene content mainly on tubers as a source of low-cost β-carotene for the community.

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MATERIALS AND METHODS

Source of materials and research location

Materials used in this research were the root and leaves of cassava plants obtained from the Plant and Animal Germplasm Garden, Research and Development Center for Biotechnology-LIPI Cibinong-Bogor, West Java, Indonesia. Analysis of β-carotene was conducted in Laboratory of Natural Material Chemistry, Research Center for Biotechnology-LIPI, Cibinong. This study was conducted from August to October 2007.

Sample preparation

Fresh cassava tubers were peeled and washed until clean. The clean tubers were sliced by using blender machine and washed with aquadest twice, and then filtered. After the sample separated into starch and pulp, the starch were then dried in the oven at a temperature of 50°C for 2 days. Leaf sample taken from fresh leaves were cut and dried in the oven at a temperature of 50° C for 1 day. The samples were then grinded to obtain leaves powder.

Determination of water content

Determination of water content was done according to Sudarmadji et al. (1984). Empty bowls and their lids were firstly dried in the oven with a temperature of 105° C for 30 minutes and cooled in desiccator, and then ± 5 gram of sample quickly weighed. Open cup, its contents and lid were dried in an oven at a temperature of 105 °C for 3-4 hours carefully to avoid contact between the cups with oven wall. The cups were then moved into desiccator, close the cover with the cup lid, and let it cool down for further weighing up again after cooling down. The samples were then redried to obtain a constant weight.

Determination of ash content

Determination of ash content was done according to Sudarmadji et al. (1984). Approximately 5 grams of sample were put into a porcelain cup that has been known its weight, then heat up in a furnace at a temperature of 550°C until grayish color (charcoal-out), cooled and then weight up to obtain a constant weight. The calculation was done based on the dry powder weight.

Determination of β-carotene content in spectrophotometer

Spectrophotometry is a method of analysis based on measurements of the interaction between electro-magnetic radiation and molecule of a chemical substance. The term of spectrophotometry means measurement of light energy absorption by a chemical system as a function of long wave radiation. If the light monochromatic or heterochromatic fell in a homogenous medium, some of them will be reflected, partly absorbed in the medium and the rest will be forwarded (Underwood, 1992).

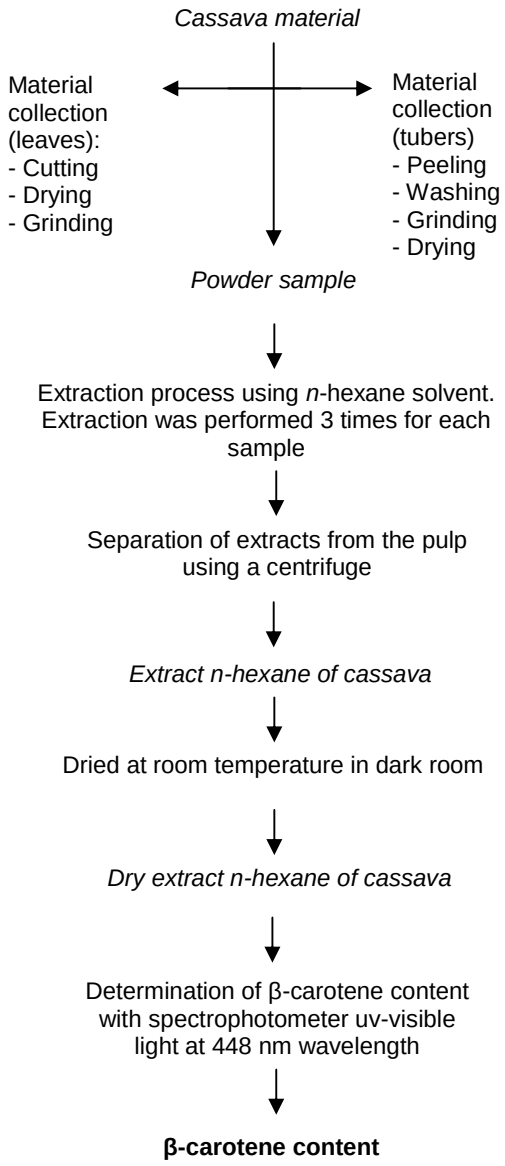

Figure 1. Flow chart of determination of β-carotene content of cassava tubers and leaves.

To analyze the content of β-carotene in cassava tubers and leaves, samples were extracted using the solvent of *n*-hexane. The extract was then dried up. The extracts were dissolved with technical methanol, and then measured with spectrophotometer (*Beckman DU650*) 448 nm wavelength. Extraction was performed 3 times. Solvent used for extraction was 5 ml of *n*-hexane (first extraction), while for the next extraction was 1 ml of *n*-hexane. Summary of the research procedures is presented in Figure 1.

Research design and statistical analysis

Research was arranged in the Complete Random Design with two replications. Data were analyzed using analysis of variance (ANOVA) and followed with Duncan's Multiple Range Test (DMRT) using SPSS 11.0.0. (2001).

RESULTS AND DISCUSSION

Tuber morphology

Morphological observations of 14 cassava genotypes after harvesting the tubers are presented in Table 1 and Figure 2. Some cassava genotypes have a brown and light brown skin color, while the rind color consists of cream and dark cream color. Among the 14 cassava genotypes tested, most of them have white color and only 3 genotypes that having yellow flesh tubers.

Table 1. Results of tuber morphological observation of 14 Indonesia cassava genotypes

Genotype	Skin Color	Rind Color	Flesh Color
Adira I	Light brown	Cream	Yellow
Adira IV	Brown	Cream	White
Apuy	Brown	Dark Cream	White
Gebang	Light brown	Cream	White
Iding	Brown	Cream	White
Kalbar III	Light brown	Dark Cream	White
Lelen	Brown	Cream	White
Local Muneng	Light brown	Dark Cream	White
Menti	Brown	Dark Cream	White
Parelek	Brown	Cream	White
Rawi	Brown	Dark Cream	White
Sarewen	Light brown	Cream	White
Tim-Tim 29	Light brown	Dark Cream	Yellow
Tim-Tim 40	Light brown	Cream	Yellow

β-carotene content

Tuber

The results of β-carotene determination indicated that the carotene content of some tuber were higher than 1 ppm, i.e. genotype Kalbar III (1.13 ppm), Local Muneng (1.03 ppm), Tim-Tim 29 (1.65 ppm) and Tim-Tim 40 (6.83 ppm) consecutively. The highest content of β-carotene found in this research was Tim-Tim 40, and it was indicated by its color in which more yellowy compared with Local Muneng. Extract produced from the Tim-Tim 40 has a color close to standard. Based on the results of organoleptic study, tubers of genotype Tim-Tim 40 have nice taste after being cooked (Priadi et al*.,* 2004). Red and yellow color of steamed flesh tuber indicated that there is high content of β-carotene (Nassar et al., 2007).

Tubers that have β-carotene content less than 1 ppm were Adira IV (0.41 ppm), Adira I (0.35 ppm), Gebang (0.32 ppm), Parelek (0.33 ppm), Menti (0.23 ppm), Apuy (0.13 ppm), Rawi (0.12 ppm), Lelen (0.09 ppm), Iding (0.03 ppm) genotypes, while the lowest β-carotene content was of Sarewen (0.00 ppm) genotype. The Sarewen that having lowest content of β-carotene was indicated by white color of its extract. Due to the very low content of β-carotene in Sarewen genotype, the method used was unable to determine it.

In general in each genotype, β-carotene content of leaf was much higher than that of the tuber. For example, the genotype Sarewen tubers has very small content of β-carotene, even zero, however, their leaves have higher content of β-carotene. The results of the determination of water, ash and β-carotene content of tubers is presented in Table 2.

Table 2. Results of the determination of water, ash and β-carotene content of tubers of 14 Indonesia cassava genotype

Genotype	Water content (%)	Ash content (%)	β-carotene (ppm)
Adira I	61.3350^{e}	0.5900^{b}	0.3515^{b}
Adira IV	61.2350^{e}	0.5900^{b}	0.4125^{b}
Apuy	63.0150^{d}	0.3950^{c}	0.1255^{b}
Gebang	56.2600^{g}	0.1950^{d}	0.3170^{b}
Iding	62.4000^{de}	0.5900^{b}	0.0310^{b}
Kalbar III	58.1600^{f}	0.7850^{a}	1.1305^{b}
Lelen	54.1750^{h}	0.6000^{b}	0.0920^{b}
Lokal Muneng	57.5250^{f}	0.7900^{a}	1.0260^{b}
Menti	67.2300^{b}	0.4000^{c}	0.2260^{b}
Parelek	68.8750^{a}	0.5900^{b}	0.3305^{b}
Rawi	54.6100^{h}	0.3950^{c}	0.1160^{b}
Sarewen	62.6200^{d}	0.5950^{b}	0.0000^{b}
Tim-Tim 29	68.0100^{ab}	0.2000^{d}	1.6050^{b}
Tim-Tim 40	66.0050^{c}	0.3950^{c}	16.8255^{a}

Note: Means followed by different letters in a column are significantly different (*P<0.05*) according to DMRT.

In tuber, the water content ranges from 54 to 69%. The highest water content was in the Parelek genotype, while the lowest one was in Lelen genotype. Ash content ranges from 0.20 to 0.79%. The highest ash content was in Kalbar III genotype, while the lowest one was in Tim-Tim 29 genotype. According to Adupa (1994), characteristic of edible cassava tubers contain 62-65% water and 0.3-1.3% ash content respectively. Therefore the genotype of Tim-Tim 40 which have highest β-carotene content is the most feasible to be consumed as a source of vitamin A.

Leaves

Results of β-carotene determination in the leaves sample were higher than those of tubers. The lowest content of β-carotene was 298.95 ppm (Local Muneng), and the highest one was 517.72 ppm (Tim-Tim 40). The complete results of water, ash and β-carotene content of leaves were presented in Table 2. In general, the content of β-carotene in the leaf was higher than i tuber. For the measurement of absorbance, leaf samples were diluted 10 times due to too large reading of absorbance.

Tables 2 and 3 show that there is no relationship between the content of β-carotene in the tubers an in the leaves, with exception in genotype of Tim-Tim 40 which has the highest β-carotene content both in the tuber and leaf. Nassar et al. (2007) reported that there was relationship between the content of β-carotene in tubers and leaves of local cassava of Brazil genotype.

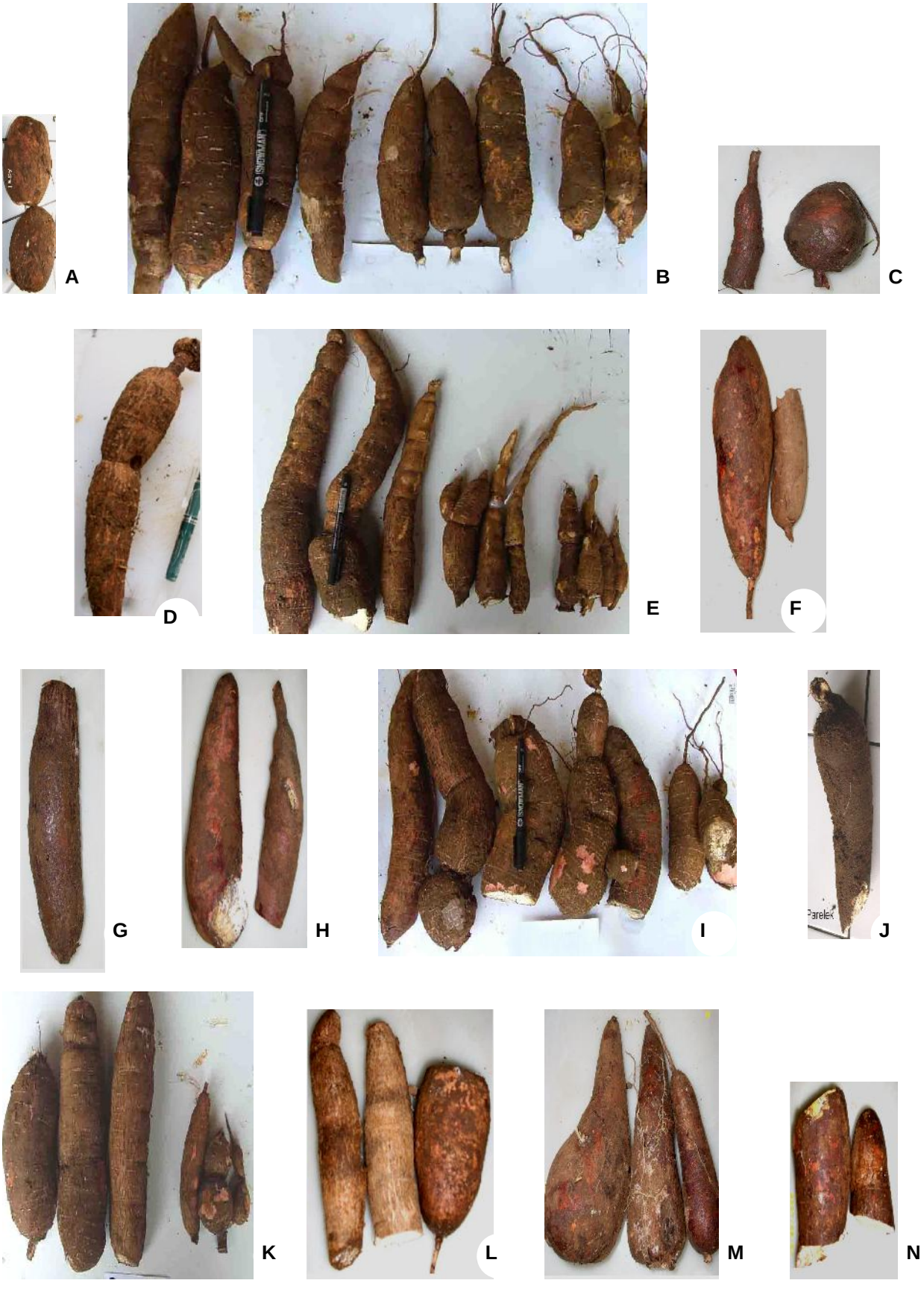

Figure 2. Morphology of 14 tubers of Indonesia cassava genotype. A. Adira I, B. Adira IV, C. Apuy, D. Gebang, E. Iding, F. Kalbar III, G. Lelen, H. Local Muneng, I. Menti, J. Parelek, K. Rawi, L. Sarewen, M. Tim-Tim 29, N. Tim-Tim 40. Bar = ± 10 cm.

Due to the low interest of consuming cassava, especially in Jakarta or other big cities in Indonesia, this study is expected to increase awareness of community about the importance of cassava as a source of low cost and easily obtained β-carotene. Beside of consuming the tubers, the leaves of cassava are also very potential for vitamin A intake in human consumption

Table 3. Results of the determination of water, ash and β-carotene content of leaves of 14 Indonesia cassava genotype.

Genotype	Water content (%)	Ash content (%)	β-carotene (ppm)
Adira I	68.6500^{bc}	1.6800^{fg}	323.4460^{gh}
Adira IV	69.0000^{bc}	1.7500^{ef}	474.3310^{b}
Apuy	67.4300^{cd}	2.3850^{b}	469.2595^{bc}
Gebang	68.7650^{bc}	1.4850^{gh}	388.1410^{e}
Iding	68.2950^{bcd}	2.4800^{b}	449.1345^{cd}
Kalbar III	66.8650^{de}	2.0000^{de}	354.6840^{f}
Lelen	64.9700^{f}	2.3950^{b}	305.1705^{hi}
Lokal Muneng	69.1050^{ab}	2.9000^{a}	298.9470^{i}
Menti	69.6650^{ab}	1.9900^{de}	467.7035^{bc}
Parelek	65.7750^{ef}	1.5950^{fgh}	350.7780^{f}
Rawi	67.0000^{de}	1.4000^{h}	446.3705^{d}
Sarewen	69.8300^{ab}	2.0900^{cd}	305.4100^{hi}
Tim-Tim 29	69.1600^{ab}	2.3000^{bc}	334.2685^{fg}
Tim-Tim 40	70.6250^{a}	1.0900^{i}	517.7195^{a}

Note: Means followed by different letters in a column are significantly different (*P<0.05*) according to DMRT.

Comparison between β-carotene on a different generation

Results of study indicated that the β-carotene content in the fourth generation which is growing in the 2006-2007 was higher than the first generation planted in 2002-2003, with exception in the genotype Apuy, Iding and Sarewen (Figure 2). β-carotene content difference between those generations is likely caused by the level of maturity in the tubers at harvesting time, because the fourth generation cassava was harvested in a mature age (12 months), while the first generation was harvested in a shorter period (8 months) due to the consideration of dry season. The carotenoid composition of foods are affected by factors like cultivar or variety, part of the plant consumed, stage of maturity, climate or geographic site of production, harvesting and post harvest handling, processing and storage (Rodriguez-Amaya, 2001).

Further research for examining protein and minerals content of tubers is required to increase value-added of Indonesia cassava genotypes.

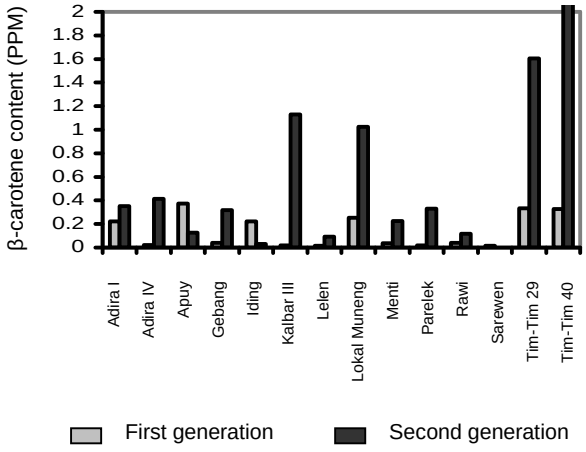

Figure 3. Comparison of β-carotene content in cassava tubers between first and fourth generation.

CONCLUSIONS

Range of β-carotene content in cassava tubers tested was 0,00-16,8 ppm. Cassava genotypes which have β-carotene content in tubers more than 1 ppm i.e. Kalbar III (1.13 ppm), Local Muneng (1.03 ppm), Tim-Tim 29 (1.60 ppm), and the highest one is in the genotype of Tim-Tim 40 (16.83 ppm), while the lowest (0.00 ppm) one was the genotype of Sarewen. β-carotene content in the leaf was higher than in their tubers. Tim-Tim 40 genotype is a potential genotype to be developed further as a source of β-carotene. Although some Indonesian cassava genotypes containing high β-carotene, further research is still needed for selecting genotypes that having higher β-carotene content that can be used to provide low cost β-carotene for the community.

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Primer Screening for *Dyera costulata* (Miq) Hook.f Random Amplified Polymorphic DNA Analyses

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ABSTRACT

Dyera costulata (Miq.) Hook.f (Apocynaceae) is a large tree of the lowland tropical rain forest of Southeast Asia, Thailand, Malay Peninsula and Indonesia, especially in Sumatra and Kalimantan (Borneo) islands. Its economic value was in its copious latex, used as gum in the manufacture of chewing gum. Today the timber of this species is much sought after for the manufacture of pencils and picture frames. Information on genetic diversity of the species is very limited. Hence studies were initiated to screen primers for RAPD analyses of *Dyera costulata* for use in genetic variation studies. Seventy one Operon primers (10 mer) were used to generate a total of 864 consistent and ambiguous amplification products ranging from 200 bp to 2.0 kb. Rare and genotype specific bands were identified which could be effectively used to distinguish the genotypes. 34 highly polymorphic primers (100%) are recorded from 71 primers used. Three primers (OPA-04, OPU-06, and OPU-07) produced highest variable RAPD profiles. The dendrogram separated the 8 genotypes into 2 groups. Genetic dissimilarity ranged from 0.07 to 0.71 %.

Key words: *Dyera costulata*, jelutong, primer screening, RAPD.

INTRODUCTION

Dyera costulata (Miq.) Hook.f (Apocynaceae), known as *jelutong* or "*jelutung*" in Indonesia, are spread in Sumatra and Kalimantan, and islands between them. Its timber is light and soft, easy to work and stable. The timber is used to manufacture of board, pencils, toys, packing board, photo frame, and furniture accessories. Although timber quality of *D. costulata* is moderate, it used for manufacturing of veneer and plywood. Its latex is used for manufacturing of chewing gum. Export of jelutong from Indonesia averaged 3600 t annually over the periods of 1988-1993. Kalimantan has always been the main area of the supply (Boer, 2001). Malaysia is also one of the exporters of the timber (Norwati, 2002).

The maintenance of genetic variation is considered essential for the long-term survival of a species since genetic diversity provides a species' evolutionary potential. Furthermore, a reduction in diversity through the loss of alleles reduces a population's ability to respond to biotic challenges (e.g. pathogens) and to changes in the abiotic environment (Pither et al., 2003). These make assessment of genetic diversity of a species is important. The species has not been studied genetically, even the total number of the chromosome has not been determined. The present study is conducted to obtain polymorphic primer suitable for distinguishing individual or variety of *D. costulata* for use in genetic diversity study and genetic identification (*fingerprinting)* based on Random Amplified Polymorphic DNA (RAPD). Study of genetic diversity of a species is becoming important for selection and domestication programs of *D. costulata* as well as for its conservation strategy. In this present research, individual/variety of *D. costulata* which represent genetic diversity from West Kalimantan, South Kalimantan, Jambi (DHL, Berbak) and from Palembang are used. Eight accessions of *D. costulata* collected from those areas are used for developing a protocol of DNA extraction and isolation, optimation of DNA amplification with Polymerase Chain Reaction (PCR), and primer using RAPD marker. RAPD analyses is used because of its simple and cost effective, and the analyses has been used with other species of tropical timber trees (Rath et al*.,* 1998; Shashidhara et al*.,* 2003; Pither et al*.,* 2003; Telles et al*.,* 2003; Mori et al*.,* 2004; Runo et al*.,* 2004; Singh et al*.,* 2005; Sarkhosh et al*.,* 2006) and used for other identification purposes (Poerba, 2003; Fernandez et al*.,* 2006; Parjanto et al., 2006,).

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MATERIALS AND METHODS

Materials

DNA materials consist of eight accessions of *D. costulata* collected from West and East Kalimantan, Jambi (DHL, Berbak) and Bogor Collection Garden of Dept of Forestry (from Palembang). The eight accessions were: 1 = DHL (Jambi), 2 = red jelutong (Jambi), 3 = green jelutong (Jambi), 4 = black jelutong (Jambi), 5 = West Kalimantan, 6 = South Kalimantan, 7 = Jambi, 8 = Palembang. DNA materials were leaf samples dried with silica gel collected from those areas using method for DNA sampling (Widjaja and Poerba, 2004).

Methods

DNA extraction

Total genomic DNA was isolated from dried silica leaves according to Delaporta et al. (1983) with the addition of RNase treatment (100 mg/mL). Isolated DNA was visualized for its quantity and quality by running them in 1% Agarose gel electrophoresis. Amplification of RAPD was performed in Takara Thermocycler according to Williams et al*.* (1990) with total volume of PCR reaction of 11 µl consisting of 0.2 nM dNTPs; 1X reaction buffer; 2mM $MgCl_2$; 1-5 ng of DNA sample ; 0.5 pmole of single primer; and 1 unit of *Taq* DNA polymerase (Promega). Ten arbitrary RAPD primers from Operon Kit A, B, C, N, and U (Tabel 2) were screened for their polymorphic level. PCR reaction was conducted twice to ensure the reproducibility of RAPD. Only bands that appeared on both PCR reactions were scored. PCR products were visualized in 2% agarose gel electrophoresis for 50 min at 50 Volt. This was followed by EtBr staining (0.15 µl/mL) before photographed in UV transilluminator. 100 bp plus ladder (Promega) was used as DNA's size standard.

Total plant genomic DNA was isolated from 0.5 g dried silica leaves with the addition of RNase treatment of 1 µL RNase A (100 mg/mL). Leaves were collected in the field into plastic bags containing 5-10 g silica gel as desiccant, and then stored in a freezer at -20°C upon arrival to the laboratory. With silica gel, the plant material could be kept at the ambient temperature for up to 14 days without degradation of DNA quality. About 0.5 g of silica-dried leaf tissue was grinded to a fine powder using a mortar, pestle, and liquid nitrogen. The powder were then transferred to a 2.0 ml micro tube and were added 700 µL of hot (65°C) extraction buffer which contained 2% CTAB, 2% PVP, 20mM EDTA, 100mM Tris-HCl pH 7.5, and 1.4M HCl and 0.2% mercaptoethanol. The microtube was then vortexed until mixed well and incubated at 65°C for 60 min with occasional inversion. Centrifuge in a Beckman centrifuge at 12000 rpm for 10 min at 28^0C. The top aqueous layer was then transferred to a fresh 2-mL tube. Equal volume (~700 µL) of chloroform: isoamyl alcohol (41: 1) was then added, mixed by gentle inversion and centrifuged at 12000 rpm for 10 min at 28^0C to precipitate the DNA.

The top aqueous layer was then transferred to a fresh 2-mL tube. Equal volume (~700 µL) of chloroform: isoamyl alcohol (24: 1) and 5 M sodium acetate was then added, mixed and centrifuged at 12000 rpm for 10 min at 28^0C to precipitate DNA. 700µL of the top aqueous layer was transferred, and the DNA was precipitated by adding a ¾ volume of cold isopropanol and mix by gentle inversion, and incubated at -20^0C for 30 minute. Mix well by using gentle inversion and centrifuge for 3 min at 12000 rpm at 28^0C. The top aqueous layer was discarded, and removed the DNA pellet with a hook. The pellet was then washed with absolute alcohol and air-dried for a night or vacuumed for 30 min. At this step, DNA can be stored at -20^0C or add 100µL TE buffer to dissolve the pellet. Isolated DNA was visualized for its quantity and quality by running them in 1% Agarose gel electrophoresis, by comparing with Lambda DNA/ *Eco*R1+Hind III marker.

DNA amplification

Optimum PCR conditions for RAPD was standardized with various quantities of template DNA (5, 10, 15, 20, and 25 ng), primer concentration (5, 10, 25 pmole), and MgCl2 concentration (0, 1, 2 and 3 mM). DNA amplification was conducted based on the methods of Williams et al*.* (1990) using a selected arbitrary primer which consist of 10 oligo nucleotide (10-oligomer), OPN-14 (Operon Technology), a polymorphic primer previously tested for *Alstonia scholaris (*Apocynaceae) (Poerba, 2005). PCR reactions (15 µL) contained 5-25 ng of genomic DNA; 1X reaction buffer, 0.2 nM of each of dATP, dCTP, dGTP, and dTTP (Promega); 2.5 mM of $MgCl_2$; 5-25 pmole of 10-mer primer (Operon); and 1unit of *Taq* DNA polymerase (Promega). A thermocycler (Takara) was programmed for one preamplification step of 2 min at 94°C, followed by 45 cycles, with a denaturation step of 1 min at 94°C, annealing step of 1 min at 36^0C, and extension step of 2 min at 72°C. After 45 cycles, it was then followed by 5 min extension at 72°C. After amplification, 5 µL of the samples was loaded and fragments separated by Mupid Mini Cell electrophoresis in 2.0% agarose gels using a 1 × TEA running buffer for 50 min at 50 Volt. After electrophoresis, the gel was staining in ethidium bromide solution with final concentration of 1µg/ml for 10 min. The result of RAPD was documented using Gel document system (Biorad, USA).

Primer screening

Based on the results of PCR optimation, a total of eighty primers (Kits A, C, N and U from Operon Technologies, Alameda, California) were screened for usefulness in an initial survey. Amplification of DNA was performed in Takara Thermocycler according to Williams et al*.* (1990) with total volume of PCR reaction of 15 µl consisting of 0.2 nM dNTPs; 1X

reaction buffer; 2mM $MgCl_2$; 10 ng of DNA sample; 5 pmole of single primer; and 1 unit of *Taq* DNA polymerase (Promega). Eighty arbitrary RAPD primers from Operon Kit A, C, N, and U (Tabel 2) were screened for their polymorphic level. PCR for amplifying the DNA preparations was carried out in 15-µL vol of reaction mixture. Reaction tube contained 10 ng of DNA, 1 unit of *Taq* DNA polymerase enzyme, 0.2 nM of each dNTP, 1.5µl reaction buffer, 2mM $MgCl_2$, and 5 pmol decanucleotide primers. Amplifications were carried out by using DNA thermocycler (Takara) with the following parameters: preamplification step of 2 min at 94°C, followed by 45 cycles, with a denaturation step of 1 min at 94°C, annealing step of 1 min, and extension step of 2 min at 72°C. After 45 cycles, followed by 5 min extension at 72°C. PCR products were subjected to agarose gel (2.0 w/v) electrophoresis for 50 min at 50 volt, in 1x TAE buffer, along with 100-bp DNA ladders (Promega) as molecular weight markers. DNA was stained with ethidium bromide and photographed under UV light. PCR reaction was conducted twice to ensure the reproducibility of RAPD. Only bands that appeared on both PCR reactions were scored. PCR products were visualized in 2% agarose gel electrophoresis for 50 min at 50 Volt. This was followed by EtBr staining (0.15 µl/mL) before photographed in UV transilluminator. 100 bp plus ladder (Promega) was used as DNA's molecular weight standard.

Data scoring

Each band in the RAPD fingerprint pattern will be considered as a separate putative locus. Only the loci with clearly amplified bands will be manually selected and scored for presence (1) and absence (0) of a band. The binary matrices of RAPD phenotypes will then be assembled for different analyses. A similarity matrix was constructed and subjected to cluster analysis following the UPGMA method by computer program NTSYS-pc version 1.8 (Rohlf, 1993). Measurement of genetic similarity for pair-wise accessions was based on the simple matching coefficients (Dunn and Everitt, 1982; Rohlf, 1993), while measurement of genetic dissimilarity for pair-wise accessions was subtraction of genetic similarity values by 1 (Dunn and Everitt, 1982).

RESULTS AND DISCUSSION

DNA isolation

The DNA extraction of 8 accession of *Dyera costulata* was conducted using CTAB method of Delaporta et al. (1983). Mechanical grinding step was necessary to disrupt the cell wall for the release of DNA. The extraction process involves breaking or digesting away cell walls to release the cellular constituents. This is followed by disruption of the cell membranes to release DNA into the extraction buffer. This is normally achieved by using detergents such as sodium dodecyl sulphate (SDS) or cetyltrimethylammonium bromide (CTAB). In this research, we used CTAB. The released DNA should be protected from endogenous nuclease. For this purpose, hexadecyltrimethylammonium bromide (EDTA) is often included in the extraction buffer to chelate magnesium ions, necessary cofactor for nucleases. The initial DNA extracts often contain large amounts of RNA, proteins, polysaccharides, tannins, and pigments, which may interfere with the extracted DNA and were difficult to separate. Most proteins were removed by means of denaturation and precipitation from the extract with chloroform and/or phenol. RNAs, on the other hand, were normally removed by means of treatment of the extract with heat-treated RNase A. Polysaccharide like contaminants were particularly problematic, and more difficult to remove. Polysaccharides can cause anomalous reassociation kinetics. They can also co precipitate with DNA after alcohol addition during DNA isolation to form highly viscous solutions. The addition of NaCl at concentrations higher than 0.5 M, together with CTAB, is known to remove polysaccharides. Antioxidants were commonly used to address problems related to phenolics. Examples include the use of ß-mercaptoethanol, ascorbic acid, bovine serum albumin (BSA) sodium azide, and polyvinylpyrrolidone (PVP). In this research PVP was used for removing phenolic compounds. The concentration of ß-mercaptoethanol commonly used in DNA extraction protocols is 0.2%. However, our experimentation showed that as little as 0.05% ß-mercaptoethanol was sufficient to decrease polyphenol oxidation in jelutong young leaves.

Results of the experiment showed that method of Delaporta et al. (1983) produced DNA of the eight accession of *D. costulata* with good quality and quantity; so that DNA amplification can be proceed.

PCR optimation

The RAPD technique, which uses single primers of arbitrary nucleotide sequence, allows random amplification of DNA sequences throughout the entire genome. Because RAPD polymorphisms result from either a nucleotide base change that alters the primer binding site, or from an insertion or deletion within the amplified region (Williams et al., 1993), polymorphisms usually result in the presence or absence of an amplification product from a single locus (Tingey et al., 1994). The products of these amplifications can be polymorphic and were useful as genetic markers. RAPD profiling is being increasingly used in genetic studies because of the easiness of methodology and the numerous polymorphic distinguishable. RAPD has proven to be quite efficient in detecting genetic variations (Williams et al. 1990). The technique is also very simple, fast, cost-effective,

highly discriminative, requiring very small amount of genomic DNA, without the need for blotting and radioactive detection, enabling assay to be performed at any stage of plant development (Fernandez et al*.,* 2006). RAPD fingerprinting techniques have been used for the identification of horticultural crop varieties, description of cultivar genotypes and for protecting breeder's rights. One of disadvantage of RAPD marker was its low reproducibility (Jones et al*.,* 1997). However, these can be minimized by optimizing PCR conditions. Different quality of amplification products can be observed based on number of bands and resolution of DNA fragments (Siregar et al*.,* 1998).

Results of the experiment showed that the best DNA amplification of *D. costulata* was performed with total volume of PCR reaction of 15 µl consisting of 0.2 nM dNTPs; 1X reaction buffer; 2mM $MgCl_2$; 10 ng of DNA sample; 5 pmole of single primer; and 1 unit of Taq DNA polymerase (Promega). Amplifications were carried out by using DNA thermocycler (Takara) with the following parameters: preamplification step of 2 min at 94°C, followed by 45 cycles, with a denaturation step of 1 min at 94°C, annealing step of 1 min at 36^0C, and extension step of 2 min at 72°C. After 45 cycles, followed by 5 min extension at 72°C, and cooling at 4^0C for 30 min. These PCR conditions produced more DNA amplicon and produced better DNA bands compared with other PCR conditions. Reproducibility of these selected primers was tested by repeating the PCR amplification for at least two times under the same amplification conditions. The results also showed that the optimized condition of PCR used in this experiment for *D. costulata* produced consistent DNA amplicon bands. Consistency of DNA bands produced indicated that RAPD markers can be used for distinguishing variation of *D. costulata* tested. Similar results was also observed in various plant for identifying individual plant, clone or cultivar (Shashidhara et al*.,* 2003; Mori et al*.,* 2004; Runo et al*.,* 2004; Fernandez et al*.,* 2006; Sarkhosh et al*.,* 2006).

Primer screening and RAPD profile analyses

For genetic identification purposes, primer used is important to be able to differentiate varieties or cultivars of the species. Primer screening, therefore, is an important step in genetic analyses. Optimum primer concentration for DNA amplification of different plant species depended on type of primer and plant species. Number of DNA amplification bands depended on how primer attached to its homolog at DNA template (Tingey et al., 1994).

Primers used for screening were arbitrary random primer consisted of 10-nucleotide and had a G+C content of 60-80%, and no palindromes. Results of DNA amplification showed that the eight accession of jelutong produced clear, scorable PCR products, so that they can be analyzed, except for primer OPC-7, OPC-9, OPC-10, OPC-13, OPC-17, OPU-04, OPU-09 and OPU-16. For the following experiment, only 71 primers were used i.e. 20 primers of Kit A, 14 primers of Kit C, 20 primers of Kit N and 17 primers of Kit U (Operon Technology) (Table 1). DNA amplification products of eight accession of jelutong showed different DNA profiles (Figure 1). The 71 RAPD primers resulted in 864 scorable band classes, ranging from 200 bp to 2 kb in size. The number of bands for each primer varied from 4 (OPC-16) to 19 (OPU-06) with an average of 12.1 bands per RAPD primer (Table 1).

The amplification products indicated that 9.26% were monomorphic, common to all the genotypes, and 90.74% were polymorphic bands. Among the selected primers OPU-06 produced maximum number of polymorphic 19 bands followed by OPU-01. Out of 71 primers used 34 (42.25%) were highly polymorphic (100% polymorphic). These results showed that the eight accessions of jelutong can be distinguished using these highly polymorphic primers. Similar results were observed in different plant species (Pither et al*.,* 2003; Poerba, 2003; Shashidhara et al*.,* 2003; Telles et al*.,* 2003; Mori et al*.,* 2004; Runo et al*.,* 2004; Singh et al*.,* 2005; Sarkhosh et al*.,* 2006; Fernandez et al*.,* 2006; Parjanto et al., 2006).

The profiles of the RAPD indicate that each primer could generate a major band (strong band) which could be used as RAPD markers for detecting the differences among the eight accessions. The differences in polymorphism may be due to the differences in amount of genetic variation that exist among the different accessions. The primers, and conditions for DNA amplification, chosen in this study produced reasonably consistent results.

Cluster analyses

The eight jelutong accessions grouped into two clusters in Figure 2. 'Red jelutong' resolved separately from the remaining jelutong accessions. Cluster II grouped seven accessions, which was separated into two subgroups of five accessions and two accessions. The first subgroup consisted of five accessions from Jambi (DHL), West Kalimantan, South Kalimantan, Jambi and Palembang. The second subgroup consisted of green jelutong and black jelutong, both from Jambi. Clustering analysis indicates similarities between accession 7 (Jambi) and 8 (Palembang).

The genetic dissimilarity value ranges from 0.09 to 0.71 was observed. The highest dissimilarity 0.71 was detected between genotypes No 2 and No 6, and between No 2 and No 7, and the least 0.09 between genotypes N0 5 and No 6 (Table 2). The high genetic dissimilarity values indicated that these jelutong accessions were highly separated to each other and resulted in their clustering in the dendrogram (Figure 2).

Table 1. List of primers, their sequences, total number of amplified fragments and number of polymorphic bands generated by PCR using 71 RAPD primers.

No	Primer code	Primer nucleotide sequence (5'- 3')	Total bands	Mono-morphic bands	Polymorphic bands
1	OPA-01	CAGGCCCTTC	11	0	11 (100%)
2	OPA-02	TGCCGAGCTG	13	0	13 (100%)
3	OPA-03	AGTCAGCCAC	12	0	12 (100%)
4	OPA-04	AATCGGGCTG	14	2	12 (85.71%)
5	OPA-05	AGGGGTCTTG	9	0	9 (100%)
6	OPA-06	GGTCCCTGAC	10	0	10 (100%)
7	OPA-07	GAAACGGGTG	14	0	14 (100%)
8	OPA-08	GTGACGTAGG	13	0	13 (100%)
9	OPA-09	GGGTAACGCC	15	0	15 (100%)
10	OPA-10	GTGATCGCAG	15	0	15 (100%)
11	OPA-11	CAATCGCCGT	14	0	14 (100%)
12	OPA-12	TCGGCGATAG	12	0	12 (100%)
13	OPA-13	CAGCACCCAC	12	3	9 (75%)
14	OPA-14	TCTGTGCTGG	9	0	9 (100%)
15	OPA-15	TTCCGAACCC	8	1	7 (87.5%)
16	OPA-16	AGCCAGCGAA	12	0	12 (100%)
17	OPA-17	GACCGCTTGT	10	1	9 (90%)
18	OPA-18	AGGTGACCGT	16	1	15 (93.75%)
19	OPA-19	CAAACGTCGG	12	0	12 (100%)
20	OPA-20	GTTGCGATCC	17	0	17 (100%)
21	OPC-01	TTCGAGCCAG	7	1	6 (87.5%)
22	OPC-02	GTGAGGCGTC	9	3	6 (66.67)
23	OPC-03	CCGCATCTAC	7	0	7 (100%)
24	OPC-04	CCGCATCTAC	5	0	5 (100%)
25	OPC-05	GATGACCGCC	17	1	16 (94.12%)
26	OPC-06	GAACGGACTC	18	1	17 (94.44%)
27	OPC-08	TGGACCGTCC	14	3	11 (78.57%)
28	OPC-11	AAAGCTGCGG	14	4	10 (71.43%)
29	OPC-12	TGTCATCCCC	9	0	9 (100%)
30	OPC-14	TGCGTGCTTG	9	0	9 (100%)
31	OPC-16	CACACTCCAG	4	0	4 (100%)
32	OPC-18	TGAGTGGGTG	6	1	5 (83/33%)
33	OPC-19	GTTGCCAGCC	12	3	9 (75%)
34	OPC-20	ACTTCGCCAC	7	0	7 (100%)
35	OPN-01	CTCACGTTGG	13	1	12 (92.31%)
36	OPN-02	ACCAGGGGCA	15	1	14 (73.33%)
37	OPN-03	GGTACTCCCC	14	0	14 (100%)
38	OPN-04	GACCGACCCA	19	4	15 (78.95%)
39	OPN-05	ACTGAACGCC	19	3	16 (84.21%)
40	OPN-06	GAGACGCACA	14	3	11 (78.57%)
41	OPN-07	CAGCCCAGAG	18	1	17 (94.44%)
42	OPN-08	ACCTCAGCTC	16	2	14 (87.5%)
43	OPN-09	TGCCGGCTTG	13	1	12 (92.31%)
44	OPN-10	ACAACTGGGG	14	7	11 (73.33%)
45	OPN-11	TCGCCGCAAA	16	0	16 (100%)
46	OPN-12	CACAGACACC	16	3	13 (81.25%)
47	OPN-13	AGCGTCACTC	14	3	11 (92.86%)
48	OPN-14	TCGTGCGGGT	12	0	12 (100%)
49	OPN-15	CAGCGACTGT	12	0	12 (100%)
50	OPN-16	AAGCGACCTG	7	2	5 (71.43%)
51	OPN-17	CATTGGGGAG	12	1	11 91.67%)
52	OPN-18	GGTGAGGTCA	9	4	5 (55.56%)
53	OPN-19	GTCCGTACTG	12	2	10 (83.33%)
54	OPN-20	GGTGCTCCGT	13	0	13 (100%)
55	OPU-01	ACGGACGTCA	16	0	16 (100%)
56	OPU-02	CTGAGGTCT C	10	0	10 (100%)
57	OPU-03	CTATGCCGAC	12	1	11 (91.67)
58	OPU-05	TTG GCGGCCT	9	4	5 (55.56%)
59	OPU-06	ACCTTTGCGG	19	0	19 (100%)
60	OPU-07	CCTGCTCATC	14	0	14 (100%)
61	OPU-08	GGCGAAGGTT	13	0	13 (100%)
62	OPU-10	ACCTCGGCAC	8	0	8 (100%)
63	OPU-11	AGACCCAGAG	11	0	11 (100%)
64	OPU-12	TCACCAGCCA	4	0	4 (100%)
65	OPU-13	GGCTGGTTCC	17	3	14 (82.53%)
66	OPU-14	TGGGTCCCTC	15	0	15 (100%)
67	OPU-15	ACGGGCCAGT	9	1	8 (88.89%)
68	OPU-17	ACCTGGGGAG	13	1	13 (92.86%)
69	OPU-18	GAGGTCCACA	9	1	12 (92.31%)
70	OPU-19	GTCAGTGCGG	11	2	9 (81.82%)
71	OPU-20	ACAGCCCCCA	10	4	6 (60%)
		Total	864	80	784 (90.74%)

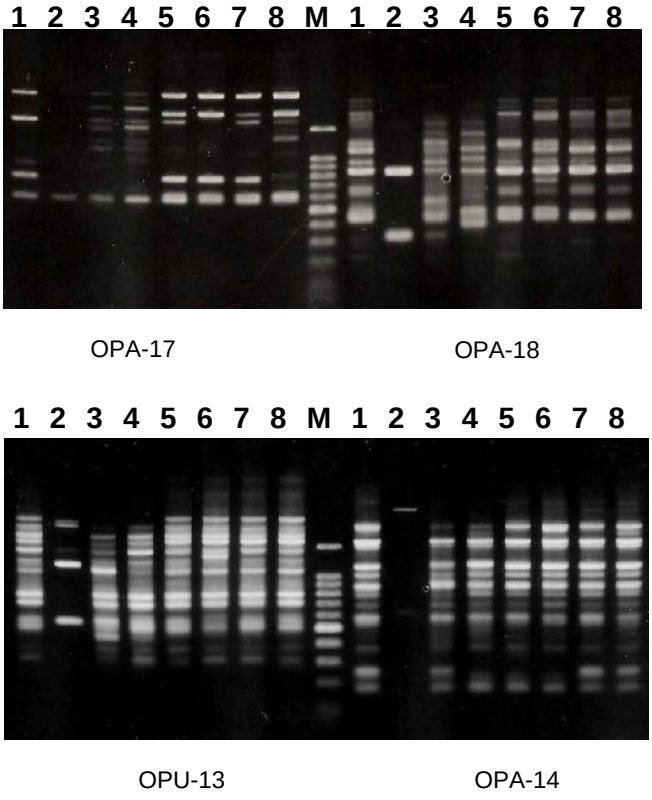

Figure 1. RAPD profile of eight jelutong accessions using four different primers. Note: 1-4 = Jambi, 1 = DHL, 2 = red jelutong, 3 = green jelutong, 4 = black jelutong, 5 = West Kalimantan, 6 = South Kalimantan, 7 = Jambi, 8 = Palembang

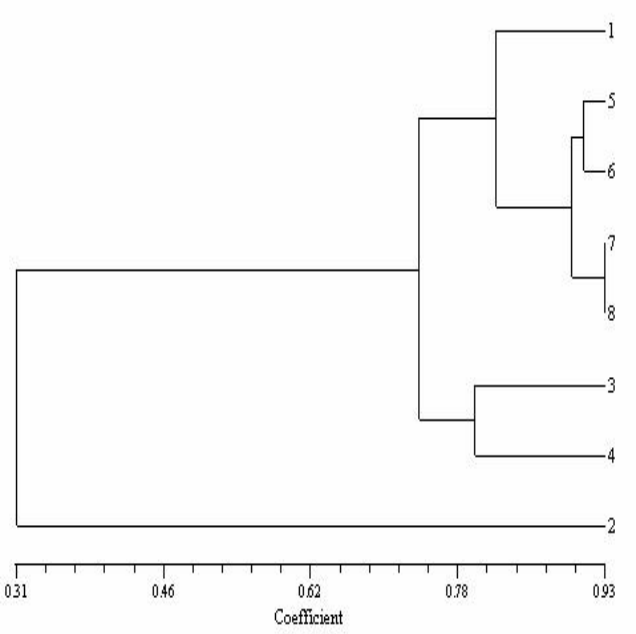

Figure 2. Dendrogram of eight accessions of jelutong. Note: 1-4 = Jambi, 1 = DHL, 2 = red jelutong, 3 = green jelutong, 4 = black jelutong, 5 = West Kalimantan, 6 = South Kalimantan, 7 = Jambi, 8 = Palembang.

Table 2. Genetic dissimilarity of eight jelutong accessions as Obtained from RAPD markers.

	1	2	3	4	5	6	7	8
1	0							
2	0.67	0.00						
3	0.25	0.67	0.00					
4	0.22	0.68	0.21	0.00				
5	0.16	0.70	0.24	0.27	0.00			
6	0.19	0.71	0.28	0.30	0.09	0.00		
7	0.19	0.71	0.26	0.30	0.10	0.10	0.00	
8	019	0.70	0.25	0.28	0.09	0.11	0.07	0.00

Note: 1-4 = Jambi; 1= DHL, 2 = red jelutong, 3 = green jelutong, 4 = black jelutong, 5 = West Kalimantan, 6 = South Kalimantan, 7 = Jambi, 8 = Palembang.

CONCLUSION

Optimized PCR condition for RAPD analyses of *D. costulata* was obtained to produce clear and consistent DNA amplification bands. The amplification of DNA was performed in Takara Thermocycler according to Williams et al*.* (1990) with total volume of PCR reaction of 15 µl consisting of 0.2 nM dNTPs; 1X reaction buffer; 2mM $MgCl_2$; 10 ng of DNA template ; 5 pmole of single primer; and 1 unit of *Taq* DNA polymerase (Promega). The DNA amplifications were carried out with the following parameters: preamplification step of 2 min at 94°C, followed by 45 cycles, with a denaturation step of 1 min at 94°C, annealing step of 1 min at 36^0C, and extension step of 2 min at 72°C. After 45 cycles, followed by 5 min extension at 72°C, and cooling at 4^0C for 30 min.

The 71 RAPD primers resulted in 864 scorable band classes, ranging from 200 bp to 2 kb in size. The number of bands for each primer varied from 4 (OPC-16) to 19 (OPU-06) with an average of 12.1 bands per RAPD primer. Out of the amplification products recorded, 90.74% were polymorphic bands with OPU-06 produced maximum number of polymorphic 19 bands Operon primers of OPA-04, OPU-06, and OPU-07 were highly polymorphic primers for distinguishing jelutong accessions tested.

The eight jelutong accessions grouped into two clusters. 'Red jelutong' resolved separately from the remaining jelutong accessions. Cluster II grouped seven accessions, which was separated into two subgroups of five accessions and two accessions. The first subgroup consisted of five accessions from Jambi (DHL), West Kalimantan, South Kalimantan, Jambi and Palembang. The second subgroup consisted of green jelutong and black jelutong, both from Jambi. Clustering analysis indicates similarities between accession 7 (Jambi) and 8 (Palembang).

Result of the experiment showed the accessions of jelutong showed a broad genetic variation and can be detected using highly polymorphic RAPD markers, such as OPA-04, OPU-06, and OPU-07. From the above study, we concluded that RAPD is reliable, rapid and inexpensive screening method to discriminate the jelutong genotype. RAPD analysis also revealed genetic diversity among these investigated species, which may be beneficial to crop improvement and the detection of genetic variation of the species. Furthermore, this technique is less restricting than other techniques like RFLPs (no hybridization and no use of radioisotopes), and therefore is more convenient for use in research. Knowledge on genetic diversity will help in the efficient management of jelutong germplasm by breeders and conservationist.

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Stomata Diversification and Phylogenenetic Analysis of 13 Species of Family Euphorbiaceae *sensu lato*

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ABSTRACT

Investigation on diversity of stomata from 13 species of family Euphorbiaceae has been carried out. Characters like type of stomata, position of stomata, presence/absence of ledge, and density both abaxial and adaxial leaf surface were examined. Stomata characters in the family were found quite diverse in this study. Monophyletic nature of Euphorbiaceae has been proved in this study on the basis of phylogenetic analysis using parsimoni method. Our data further suggested that the family can be classified into two major groups. However, surprisingly, genus *Phyllanthus* is non-monophyletic.

Key words: diversity, Euphorbiaceae, phylogenetic analysis, stomata.

INTRODUCTION

Euphorbiaceae *sensu lato* is one of the most cosmopolitan plant groups within angiosperm, consisting of about 300 genera and 7500 species (Jones and Luchsinger, 1987). Many members of the family play important role in supporting tropical rain forest, horticultural value for ornamental, traditional medicine, and food resources. Although classification of the family Euphorbiaceae has been clearly defined, phylogenetic relationships among genera within the family remains unresolved due to remarkable diversification of morphological characters.

Phylogenetic analysis to classify a group of plants based upon micromorphological characters on leaf surfaces has been carried out by many researchers. Several characters, such as presence/absence of stomatal ledge, differentiation of subsidiary cells, shape of epidermal cells, morphology of trichomes can provide valuable information in taxonomic and/or phylogenetic studies (Wu and Cutler, 1985; Yukawa et al., 1992; Sudarsono et al., 2005). In many studies, these characters appear to be relatively more consistence than those of macromorphological one (e.g. Yukawa et al., 1992). Moreover, stomatal type has not only good diagnostic value for such studies, but also it can be used as indicators for naturally taxonomical similarity (Fahn, 1991; Sharma, 1993).

The diversity of stomatal shape on 13 species of Euphorbiaceae were investigated in this study. Phylogenetic analysis was also further carried out in this study to infer evolutionary relationships among samples used in the family .

MATERIALS AND METHODS

Species examined in this study were listed in Table 1. All leaves were collected from wild plants that are grown around Bandung, West Java. Two methods, fresh dissection and parafin coated dissection were used according to Sass (1958) to investigate stomata. The former method was used to observe stomatal type, location, and density, whereas the latter method was used to observe the ledge of stomata. Mature leaves were cut into small pieces approximately 1x1 cm^2. These pieces were fixed in FAA (Formalin+Acetic Acid+Alcohol) 50% for minimum 24 hours. They were then dehydrated through alcohol-TBA (Tersier Butyl Alcohol) series, followed by critical point drying. They were coated with parafin and dissected using Microtom model Yamato KHKI PR-50 with 10 µm of thick. The specimens were subjected to fast green staining and examined with a light microscope with 40x10 magnification.

Density of stomata was obtained from stomatal index through calculating number of stomata that was divided by number of stomata plus number of epidermal cells, then was multiplied by 100 (Willmer, 1983). Five characters, i.e. type of stomata, position of stomata, the ledge, density of stomata both abaxial and adaxial surfaces were subjected to phylogenetic analysis using parsimony method conducted with PAUP version 4.0b10 (Swofford, 2002).

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All character states (listed in Table 1) were equally weighted and unordered (Fitch, 1971). All the data sets were analyzed by the heuristic search method with tree bisection-reconnection (TBR) branch swapping and the MULPARS option on, saving all most parsimonious trees. Evaluation of internal support of clades was conducted by the bootstrap analysis (Felsenstein, 1985) with 1,000 replicates, simple stepwise addition, TBR branch swapping, and the MULTREES option off. The number of steps, consistency indices (CI), and retention indices (RI) were calculated on one of the MPTs using the TREE SCORES.

A member of Cucurbitaceae (*Sechium edule*) was used as outgroup, because the family has been recognized as sister groups to family Euphorbiaceae *s.l.* (APG, 2003).

Table 1. Euphorbian species examined in this study and those data matrix for phylogenetic analysis.

No	Species	Character states				
		1	2	3	4	5
1	*Sechium edule* (Jacq.) Sw.**	0	0	0	0	0
2	*Phyllanthus niruri* L. (Yellow)*	1	0	1	1	1
3	*Phyllanthus niruri* L. (Green)*	1	0	1	1	1
4	*Phyllanthus acidus* (L.) Skeels	2	0	1	1	1
5	*Phyllanthus urinaria* L.	2	1	1	0	2
6	*Euphorbia pulcherrima* L.	0	1	1	1	2
7	*Codiaeum variegatum* (L.) A. Juss	2	1	0	1	2
8	*Sauropus androgynus* (L.) Merr.	2	1	1	1	2
9	*Manihot esculenta* Crantz	2	1	1	1	2
10	*Antidesma bunius* (L.) Spreng.	2	1	0	0	2
11	*Ricinus communis* L.	2	0	0	0	0
12	*Jatropha curcas* L.	2	0	0	0	0
13	*Claoxylon polot* (Burm.) Merr.	2	0	0	1	0
14	*Hevea brasiliensis* (Willd.) Muell.	2	1	1	0	2

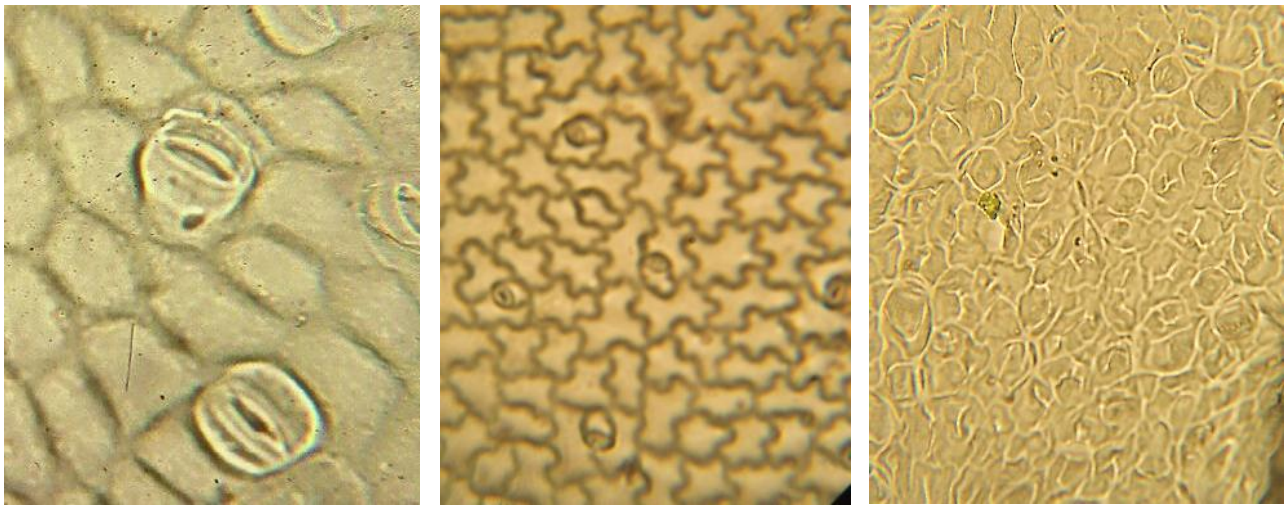
Note: * Variety of *P. niruri* that is distinguished by color of stem, yellow or green (Hadad et al., 1993). 1) Type of stomata = anomositic: 0, anisositic: 1, parasitic: 2; 2) Location of stomata = amphistomatic: 0, hypostomatic: 1; 3) Ledge = presence: 0, absence: 1; 4) Density on abaxial = ≤ 25/mm^2: 0, ≥25/mm^2: 1; 5) Density on adaxial = ≤ 10/mm^2: 0, ≥10/mm^2: 1, absence: 2. Scoring for density followed Yukawa et al. (1992). Species outgroup.

RESULTS AND DISCUSSION

In observing the type of stomata from the studied species we found three types of stomata: parasitic, anomositic, and anisositic (Figure 1). Among them, 10 species are parasitic, one species is anomositic (*Euphorbia pulcherrima)*, and two species are anisositic (*Phyllanthus niruri* Green and *Phyllanthus niruri* Yellow). Several specimens examined have amphistomatic stomata (located at both adaxial and abaxial leaf surfaces), except *P. acidus, C. variegatum, S. androgynus, A. bunius, H. brasiliensis*, *M. esculenta*, and *E. pulcherrima*, which have hypostomatic one (located at abaxial leaf surfaces). On the basis of position of stomata relative to its epidermal cell, phaneroporic stomata is found in all species studied. Information about the density of stomata is listed in Table 2. There are eight species characterized by absence of the ledge of all specimens observed, *P. niruri* Green*, P. niruri* Yellow*, P. urinaria, P. acidus, S. androgynus, H. brasiliensis*, *M. esculenta*, and *E. pulcherrima*. In contrast, existence of the ledge are identified in the remained species.

Table 2. Density of euphorbian stomata.

Species	Density (number of stomata/mm^2)	
	Abaxial	Adaxial
P. niruri Green	48,08	25,74
P. niruri Yellow	37,08	12,65
P. urinaria	29,53	15,68
P. acidus	12,53	-
E. pulcherrima	36,59	-
C. variegatum	29,55	-
S. androgynus	27,35	-
M. esculenta	53,70	-
A. bunius	18,17	-
R. communis	20,99	6,08
J. curcas	20,26	5,64
C. polot	33,53	1,30
H. brasiliensis	18,43	-



A **B** **C**

Figure 1. Three types of stomata in some species of Euphorbiaceae *s.l.* A. Parasitic, B. Anisositic, C. Anomositic. Bar = 100 mm.

Phylogenetic tree obtained (Figure 2) suggests that family Euphorbiaceae is tentatively monophyletic group. We state tentative because there were only 13 species included in the phylogenetic analysis. The family is further divided into two groups. Group 1 is composed of *P*. *urinaria*, *P*. *niruri* Green, and *P*. *niruri* Yellow. With the exception of *R. communis, J. curcas,* and *C. polot*, the rest of species are housed in Group 2. As mentioned earlier, from all specimens studied there are three types of stomata, notably, parasitic, anomositic, and anisositic. This is congruent with Cronquist (1981) that these three types of stomata commonly exist in the family Euphorbiaceae. Location of stomata in Euphorbiaceae could be amphistomatic or hypostomatic. According to Susetyoadi et al. (2004) these condition are often found in mesophyte plant. In many cases, stomata is more abundant on abaxial than adaxial, even totally absence. This is one mechanism in the mesophyte plant to prevent much lost of water. The mesophyte plant is also characterized by the position of stomata that is parallel with epidermal cells (phaneropore). Unlike the mesophyte plant, hydrophyte plant is identified with prominent stomata, whereas xerophyte plant with hidden stomata; these are clearly correlated with habitat, whether plenty or poor of water respectively.

The family Euphorbiaceae is also characterized with widely variety of stomatal density (Table 2). Environmental condition and genetic factor strongly influence morphogenesis of stomata (Willmer, 1983). Environmental factors that influence the density include availability of water, intensity of light, temperature, and concentration of CO_2. For instance, plant with high density of stomata usually growth in condition with fully intensity of sunlight, and vice versa. The plant growth with low humidity usually has much higher density than those with high humidity. Indeed, these all factors obviously give effect for respiration and transpiration in the plant.

The ledge of stomata is presence in several genera of family Euphorbiaceae. This structure is one of the main components for opening and closing of stomata, and giving a general outer shape of stomata (Yukawa et al., 1992). The stomatal ledge will therefore determine the shape opening of stomata. In some groups of plant, the ledge is considered to be useful phylogenetic markers. In general, stomata characters of Euphorbiaceae have several advantages for use as phylogenetic markers. No shortage data, stability in shape and size, and limited collapse and shrinkage on the stomata were found during preparation.

Composition of two groups in the family as depicted in Figure 2 is generally concordant with molecular data of the family (Hidayat et al., 2008). Group 1 is inhabited by species of genus *Phyllanthus*, except *P*. *acidus* that is distant position in Group 2. Plant herb, growth erect 30-50 cm, single leave, single flower emerging at each nodus are several combination synapomorphic characters that support Group 1 (Cronquist, 1981; Ogata and Iwasaki, 1995). In contrast, Group 2 consists of genera which is characterized by plant with small to big tree, reaching more than five meters in tall, compound leaf and flower, and hypostomatic stomata. Another morphological character that support Group 2 is that they have ovary with three locus and 1-2 ovule each (Backer and Bakhuizen v.d. Brink, 1963), except *A*. *bunius* and *H*. *brasiliensis*.

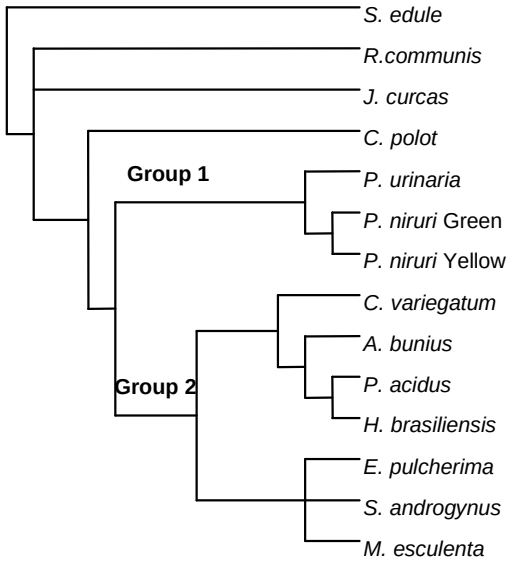


Figure 2. Strict consensus tree of 108 equally most parsimonious trees. Bootstrap value is not shown due to low percentages (Bootstrap Percentage/BP<50).

As we stated before, phylogenetic position of three species, namely, *R*. *communis, J*. *curcas*, and *C*. *polot* still remain unresolved. They are separated from Group 1 and 2. This indicates that character states we used do not sufficient in placing them to either Group. Surprisingly, this study revealed that *Phyllanthus* is non-monophyletic group. This result is consistent with our previous phylogenetic analysis using DNA sequences of internal transcribed spacers region (Hidayat et al., 2008). Furthermore, this information can be used as reference in attempts to perform taxonomic treatment for the genus.

This study is subjected to preliminary due to limited sampling and insufficient micromorphological information for the family. Further phylogenetic analyses with more micromorphological characters, such as trichome, and greater taxon sampling are desirable of the establishment more robust phylogenetic hypotheses for the family.

CONCLUSION

The results of this study showed that stomatal shapes in the family Euphorboaceae *s.l.* is quite

diverse. This property is important and useful in the phylogenetic reconstruction in order to understand diversity in the family. Using characters derived from stomata, monophyletic nature of the family has tentatively been addressed: our data generally revealed two major groups in the family. However, phylogenetic status of some species studied remains unclear. Stomatal data further suggets that genus *Phyllanthus* is non-monophyletic group.

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Biodiversity of Endophytic Fungi Associated with *Uncaria gambier* Roxb. (Rubiaceae) from West Sumatra

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ABSTRACT

Endophytic fungi are specific microbes that live at a specific ecosystem in nature. Various endophytic fungi have been known posses an ability to produce a broad range of biologically active substances. Totally 53 endophytic filamentous fungi were isolated from leaves, stems, fruits and roots of the two plant varieties of *Uncaria gambier* Roxb. (Rubiaceae), i.e. gambir udang and gambir nasi. Morphological observation of 53 isolated endophytic filamentous fungi was further divide into two classes, Coelomycetes and Hypomycetes. Fifteen fungi isolates are unidentified due to lack of specific morphological characters. Morphologically identified fungi at genera level are: *Aspergillus*, *Cladosporium*, *Diaporthe*, *Fusarium*, *Penicillium*, *Pestalotiopsis*, *Phoma* and *Phomopsis*. Chemotaxonomic analysis base on their TLC chromatogram patterns of the ethyl acetate extract are in agreement to morphological grouping.

Key words: endophytic fungi, *Uncaria gambier* Roxb., morphological character, chemotaxonomy.

INTRODUCTION

Microorganism live in association with plant (endophytic microbes) is commonly observed in nature (Bacon and White, 2000). They are commonly found in Coniferae, Gramineae, and Ericaceae (Okane et al., 2001a), bakau *Bruguiera gymnorrhiza* (Okane et al*.,* 2001b, 2001c), *Theobroma cacao* (Rubini et al*.,* 2005), and *Camellia sinensis* (Agusta et al., 2006). Endophytic microbes, especially endophytic fungi have been recognized as a potential source of divers array for bioactive secondary metabolites (Tan and Zou, 2001), and hence Owen and Hundley (2004) proposed they are "the chemical synthesizer inside plant". Interestingly, endpophytic fungi especially *Taxomyces andreanae* which lives in *Taxus brevifolia* is able to mimic their host metabolite, taxol (Stierle et al., 1993). More examples, an unidentified endophytic fungus isolated from the plant *Nothapodytes foetida* is also produce camptotecin under laboratory conditions (Puri et al., 2005). On the other hand, the endophytic fungus *Diaporthe* sp. isolated from *Camellia sinensis* grow in Ciawi, Bogor, West Java is selectively transform natural catechin into leucoantosianidin in a semisynthetic medium (Agusta et al., 2005). In addition, the fungus *Diaporthe* sp. is also produce two rare bisanthraquinones namely (+)-2,2'-epicytoskyrin and (+)-1,1'-bislunatin that show a high cytotoxicity effect against KB cells (Agusta et al., 2006).

Based on the above information, it is economically quite promising to explore the physiological ability of endophytic fungi to produce secondary metabolite with various interests. One important endophytic is those fungi which live in (*Uncaria gambier* (Hunter) Roxb.) which never been explored so far. This paper will report diversity of endophytic fungi which live in two varieties of *U. gambier* namely var. udang and var. nasi.

MATERIALS AND METHODS

Plant materials

The young stems of *U. gambier* var. nasi and *U. gambier* var. udang were collected from Lembah Harau, Limapuluh Kota District, West Sumatra Province, in August 2007 and taxonomically identified at the Herbarium Bogoriense, the Research Centre for Biology, Indonesian Institute of Sciences.

Isolation of the endophytic fungi

The young stems were cut into pieces (ca. 1 cm in length) and washes with tap water for 10 min. The pieces were treated with 70% aq. EtOH for 1 min,

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5.3% aq. sodium hypochlorite for 5 min, and 75 % aq. EtOH for 0.5 min, and then cut into two pieces. The cut stems were placed on *corn-meal malt agar* (CMMA) medium containing 2% gambir leaf extract and 0.05 mg/mL chloramphenicol in a Petri dish and incubated at 27^{o}C. After 3 d, individual colonies were transferred onto PDA in a Petri dish and then incubated again at 27^{o} C for several days with periodic check for the purity to obtain the 53 endopyhtic filamentous fungi. The pure culture of endopyhtic filamentous fungi were then preserved in the medium containing 10 % glycerol and 5 % trehalose and placed at -80°C (Nakagiri, 2005).

Identification of endopyhtic filamentous fungi

Taxonomic identification of the isolated endophytic filamentous fungi were done through observation of macroscopic and microscopic characters according to Barnett (1955), Domsch et al. (1980), Ellis (1971), Kobayashi (1970), Samson et al. (1995), Sutton (1980) Webster (1980), and Barnett and Hunter (1998). All of observe fungi were grown on Potato Dextrose Agar Media at 27ºC. The following features of the appearance of cultures are recorded: texture, color, surface, elevation and margin. Microscopic observation was done by microscope Olympus type BX-51 (Olympus, Japan).

Cultivation of fungi for secondary metabolite production

The endophytic filamentous fungi were inoculated into 10 mL *potato dextrose broth* (PDB) and *glucose yeast peptone* (GYP) (20 g glucose, 1 g *yeast extract*, 5 g peptone, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 1.0 g $CaCO_3$, 1 L H_2O, pH 6.44) in 100 mL Erlenmeyer flask. After cultivation for 3 weeks at room temperature (26-28^{o}C), the medium including the fungus bodies were extracted with ethyl acetate and analyzed by thin layer chromatography (TLC) with dichloromethane-methanol (20:1) as developing solvent. Profiles of TLC patterns are then observed under UV 254 nm and UV 365 nm, and by using spry reagents 1% Ce_2SO_4 in 10% H_2O_4.

RESULTS AND DISSCUSION

Totally 53 fungi isolates were obtained from *U. gambier* var. udang and var. nasi. From *U. gambier* var. udang were obtained 26 isolates, i.e. 7 isolates from (GUDP-1, GUDP3 ~ GUDP-8) from leaves, 12 isolates (GUBP-1, GUBP-3, GUBP-5 ~ GUBP-15) from stems, 6 isolates (GUFP-1 ~ GUFP-6) from fruits and 1 isolate (GUAP-1) from roots (Table 1). Whereas *U. gambier* var. nasi obtained 27 isolates, i.e. 8 isolates (GNDP-1 ~ GNDP-8) from leaves, 10 isolates (GNBP-1 ~ GNBP-10) from stems, 5 isolates (GNFP-1 ~ GNFP-5) from fruits and 4 isolates (GNAP-1 ~ GNAP-4) from roots (Table 2). Based on morphological characters (Table 1 and Table 2), show that there are 12 species of 8 genera. One isolate identified until family level, one isolate identified till class level and 15 isolates are sterile-hypha and thus could not be identified by morphological characters observation.

Morphological observation show that 2 fungi isolates obtained from roots (GNAP 2 and GNAP 5) belong to member of genus *Aspergillus*. One isolate obtained from leaf of *U. gambier* var. udang (GUBP 15) is also belonging to *Aspergillus*. Detail of morphological observations (microscopic and macroscopic) shows that there are four species of *Aspergillus* (Fig. 1A-D), which clearly shows that they are belong to different species. TLC chromatogram patterns of ethyl acetate extract of fungi cultures grown in PDB and GYP are also shows different profile reinforces those preposition.

In general, *Aspergillus* is able to grow on many kinds of habitat (cosmopolite), and the genera compose of 180 species of anamorph and 70 species of teleomorph (Samson et al., 1995). *Aspergillus* is commonly observed in soil and rhizosphere of horticulture (Moreau & Moss, 1979), and recently reported some species of *Aspergillus* are also evolve to be endophyte. In addition to well known endophytic fungi *Penicillium* and *Aspergillus* are residence inside plant tissues of *Melia azedarach* (Geris-dos-Santos et al., 2003). *Aspergillus fumigatus* CY018 is endophyte of *Cynodon dactylon* (Liu et al., 2004), and *Aspergillus niger* EN-13 is endophyte in alga *Colpomenia sinuosa* (Zhang et al., 2007).

On isolate fungus (GNDP-2) obtained from leaves and one isolate (GUFP-3) obtained from fruits of *U. gambier* var. nasi shows identical morphological characters each other. Both fungi are having micro- and macro-conidia as reproductive bodies. Half-moon shaped macroconidia (*pedicellate*) is a special asexual morphological characters of *Fusarium* (Summerel et al*.,* 2003). Both fungi isolates are also have similar number of macroconidia (Fig. 1E) and identical TLC patterns of ethyl acetate extract derived from fungus culture grown in PDB and GYP reinforce that both isolates are identical each other. So that, both fungi isolates must belong to the genus of *Fusarium* (Fig. 1E).

Most member of *Fusarium* are plant pathogenic, and recently some species is evolutionary adapted to plant tissue perform endophytic growth. *Fusarium oxysporum*, a non-pathogenic fungus associate with *Cucumis sativus,* can maintain the host survival under *Pythium ultimum* infection (Rubini et al*.,* 2005). *Fusarium sambucinum* associated with plant *Aphelandra tetragona* has unique ability to detoxify benzoxazolinone (BOA), a chemical constituent of their host (Zikmundova et al., 2002). Bacon and White (2000) reported intensively association between *Fusarium* and wild plant.

Coelomycetes are commonly isolated fungi in this research; out of 53 isolates obtained 11 isolates are belonged to these taxa. Coelomycetes is well known have strong association with higher plants as pathogenic or mutual symbiotic. Close relation

between plant and fungus enable to transfer material genetic among them (Tanaka et al., 1999). Many of Coelomycetes produce asexual spore (teleomorphic phase) as well as asexual spore (anamorphic phase) in their host, but fail to do so in a synthetic medium (Paulus et al., 2003). Member of Coelomycetes which produce asexual and sexual spores in culture could be identified into genera or special level. We

Table 1. Endophytic fungi isolated from the plant of *U. gambier* var. udang.

Plant organ	Isolates code	Taxon	Morphology description (*)
Root	GUAP-1	Dematiaceae	Colonies: effuse, greyish black, sterile. Mycelium: immersed and partly superficial, branched, septate, and dematiaceous.
Stem	GUBP-1	Dematiaceae	Colonies: effuse, dark grey to black, sterile. Mycelium: immersed, branched, septate, dematiaceous.
	GUBP-3	*Pestalotiopsis* sp.	Mycelia: immersed, branched, septate, hyaline to pale brown. Acervuli: dark, discoid or cushion-shaped, subepidermal. Conidia: dark, ellipsoid to fusifoid, 3-4 euseptate, with hyaline pointed end cells and 2-3 apical appendages.
	GUBP-5	nd	Colonies: hairy, creamish, sterile. Mycelium: immersed, branched, septate, hyaline.
	GUBP-6	Coelomycetes	Colonies: thick-cottony, creamish to pale green. Mycelium: immersed, branched, septate, hyaline. Sclerotia flattened, black, tuberculate.
	GUBP-7	nd	Colonies: thick-cottony, white creamish, sterile. Mycelium: immersed, branched, septate, hyaline.
	GUBP-9	Coelomycetes	Colonies: cottony, greyish-brown. Mycelium: immersed, branched, septate, hyaline. Sclerotia: numerous, flattened, black, tuberculate.
	GUBP-10	Coelomycetes	Colonies: thick-cottony, creamish to pale green. Mycelium: immersed, branched, septate, hyaline. Sclerotia: flattened, black, tuberculate.
	GUBP-11	Coelomycetes	Colonies: cottony, greyish-brown. Mycelium: immersed, branched, septate, hyaline. Sclerotia: numerous, flattened, black, tuberculate.
	GUBP-12	Dematiaceae	Colonies: effuse, dark brown to black, sterile. Mycelium: immersed, branched, septate, dematiaceous
	GUBP-13	*Phoma* sp.	Colonies: cream grey, dense aerial mycelium. Pycnidia abundant, formed in concentric dark grey zone. Conidia hyaline, ellipsoid to cylindrical.
	GUBP-14	Coelomycetes	Colonies: cottony, white with distinct dark grey zonation, produce orange exudates, sterile. Mycelium: immersed, branched, septate, hyaline.
	GUBP-15	*Aspergillus* sp.	Colonies: hairy-powdery, yellow. Conidial head dark yellow to orange, globose or radiate, uniseriate. Conidiophore smooth walled, colourless. Conidia smooth, globose or subglobose.
Leaf	GUDP-1	*Phomopsis* sp.	Mycelium: immersed, branched, septate, hyaline. Conidiomata: immersed, dark brown to black. α-conidia hyaline, fusiform, aseptate. β-conidia filiform, hyaline, aseptate.
	GUDP-3	Coelomycetes	Colonies: thick-cottony, white-creamish. Mycelium: immersed, branched, septate, hyaline. Sclerotia: flattened, black, tuberculate.
	GUDP-4	*Phoma* sp.	Colonies: cream grey with aerial mycelium, produce orange exudates. Pycnidia black. Conidia: hyaline, ellipsoid to cylindrical.
	GUDP-5	Dematiaceae	Colonies: effuse, dark grey to black, sterile. Mycelium: immersed, branched, septate, dematiaceous. Sclerotia: black, superficial.
	GUDP-6	nd	Colonies: hairy, white-creamish, sterile. Mycelium: immersed, branched, septate, hyaline.
	GUDP-7	*Pestalotiopsis* sp.	Mycelia: immersed, branched, septate, hyaline to pale brown. Acervuli: dark, discoid or cushion-shaped, subepidermal. Conidia: dark, ellipsoid to fusifoid, 3-4 euseptate, with hyaline pointed end cells and 2-3 apical appendages.
	GUDP-8	Coelomycetes	Colonies: cottony, white-greyish. Mycelium: immersed, branched, septate, hyaline. Sclerotia: numerous, flattened, black, tuberculate.
Fruit	GUFP-1	nd	Colonies: slow growing, cottony, white, sterile. Mycelium: immersed, branched, septate, hyaline.
	GUFP-2	Coelomycetes	Colonies: cottony, grey-dark brown. Mycelium: immersed, branched, septate, hyaline. Sclerotia: black, tuberculate.
	GUFP-3	*Fusarium* sp.	Colonies: cottony, white, formed pink concentric zone. Macroconidia: hyaline, several celled, curved or bent at pointed ends, with pedicellate. Microconidia: hyaline, 1-celled, ovoid or oblong.
	GUFP-4	nd	Colonies: hairy, white, sterile. Mycelium: immersed, branched, septate, hyaline.
	GUFP-5	nd	Colonies: hairy, pale brown, sterile. Mycelium: immersed, branched, septate, hyaline.
	GUFP-6	*Cladosporium* sp.	Colonies: effuse, velvety, dark olivaceous. Conidiophores dark, branched variously near apex, clustered or single. Conidia dark 1 or 2-celled, ovoid to cylindrical, in acropetalous chains.

Table 2 . Endophytic fungi isolated from the plant of *U. gambier* var. nasi.

Plant organ	Isolates code	Taxon	Morphology description (*)
Root	GNAP-2	*Aspergillus* sp.	Colonies: hairy-powdery, reverse colony brown-red. Conidial: head blue-green, radiate, uniseriate. Conidiophore: slightly granular, colourless. Conidia smooth, globose or subglobose
	GNAP-3	*Penicillium* sp.	Colonies: powdery, blue-grey. Conidiophore: simple branch, singly or less often in synnemata. Conidia: hyaline or light grey in mass, 1-celled, globose or ovoid.
	GNAP-5	*Aspergillus niger*	Colonies: hairy-powdery, black. Conidial head black, radiate. Conidiophore: smooth-walled or slightly ornamented, brown near apex. Conidia: ornamented, brown, globose to subglobose.
	GNAP-6	*Penicillium* sp.	Colonies: powdery, dark grey. Conidiophore: simple branch, singly or less often in synnemata. Conidia: hyaline or light grey in mass, 1-celled, smooth, globose or ovoid.
Stem	GNBP-1	*Pestalotiopsis* sp.	Mycelia: immersed, branched, septate, hyaline to pale brown. Acervuli dark, discoid or cushion-shaped, subepidermal. Conidia: dark, ellipsoid to fusifoid, 3-4 euseptate, with hyaline pointed end cells and 2-3 apical appendages.
	GNBP-2	nd	Colonies: thick cottony, pale brown, forming fruiting-body like, sterile. Mycelium: branched, septate, no clamp connection, hyaline.
	GNBP-3	*Pestalotiopsis* sp.	Mycelia: immersed, branched, septate, hyaline to pale brown. Acervuli: dark, discoid or cushion-shaped, subepidermal. Conidia: dark, ellipsoid to fusifoid, 3-4 euseptate, with hyaline pointed end cells and 2-3 apical appendages.
	GNBP-4	*Phoma* sp.	Colonies: cream grey with aerial mycelium, formed dark and light grey zonation, produce orange exudates. Pycnidia black. Conidia: hyaline, ellipsoid to cylindrical.
	GNBP-5	Coelomycetes	Colonies: cottony, white creamish, produce yellow exudates. Mycelium: immersed, branched, septate, hyaline. Sclerotia: black, tuberculate.
	GNBP-6	Coelomycetes	Colonies: thick-cottony, grey brownish. Mycelium: immersed, branched, septate, hyaline. Sclerotia: flattened, black, tuberculate.
	GNBP-7	nd	Colonies: cottony, white-brown-green, produce brown exudates, sterile. Mycelium: immersed, branched, septate, hyaline.
	GNBP-8	Coelomycetes	Colonies: thick-cottony, white brownish. Mycelium: immersed, branched, septate, hyaline. Sclerotia: flattened, black, tuberculate.
	GNBP-9	*Pestalotiopsis* sp.	Mycelia: immersed, branched, septate, hyaline to pale brown. Acervuli: dark, discoid or cushion-shaped, subepidermal. Conidia: dark, ellipsoid to fusifoid, 3-4 euseptate, with hyaline pointed end cells and 2-3 apical appendages.
	GNBP-10	*Diaporthe* sp.	Colonies: thick cottony white turn to dark yellow. Mycelium: immersed, branched, septate, hyaline. Sclerotia black, immersed. Ascoma a perithecium black, immersed in a stroma.
Leaf	GNDP-1	nd	Colonies: cottony, creamish-brown, produce brown exudates, sterile. Mycelium: immersed, branched, septate, hyaline.
	GNDP-2	*Fusarium* sp.	Colonies: cottony, white, formed pink concentric zone. Macroconidia: hyaline, several celled, curved or bent at pointed ends, with pedicellate. Microconidia hyaline, 1-celled, ovoid or oblong.
	GNDP-3	nd	Colonies: cottony, white-creamish, reverse colony brown, sterile. Mycelium: immersed, branched, septate, hyaline.
	GNDP-4	*Phoma* sp.	Colonies: cream grey with aerial mycelium, formed dakr and light grey zonation, produce orange exudates. Pycnidia black. Conidia: hyaline, ellipsoid to cylindrical.
	GNDP-5	*Aspergillus* sp.	Colonies: hairy-powdery, dark violet. Conidial: head globose or radiate, uniseriate. Conidiophore slightly granular, colourless. Conidia: ornamented, globose or subglobose
	GNDP-6	*Phoma* sp.	Colonies: cream grey with aerial mycelium, formed dakr and light grey zonation, produce orange exudates. Pycnidia black. Conidia: hyaline, ellipsoid to cylindrical.
	GNDP-7	*Phoma* sp.	Colonies: cream grey with aerial mycelium, formed dark and light grey zonation, produce orange exudates. Pycnidia black. Conidia: hyaline, ellipsoid to cylindrical.
	GNDP-8	*Pestalotiopsis* sp.	Mycelia: immersed, branched, septate, hyaline to pale brown. Acervuli dark, discoid or cushion-shaped, subepidermal. Conidia: dark, ellipsoid to fusifoid, 3-4 euseptate, with hyaline pointed end cells and 2-3 apical appendages.
Fruit	GNFP-1	nd	Colonies: cottony, white-creamish, sterile. Mycelium: immersed, branched, septate, hyaline
	GNFP-2	nd	Colonies: thick-cottony, white, sterile. Mycelium: immersed, branched, septate, hyaline
	GNFP-3	nd	Colonies: cottony, creamish-brown, produce brown exudates, sterile. Mycelium: immersed, branched, septate, hyaline.
	GNFP-4	nd	Colonies: cottony, white-creamish, reverse colony dark in center colony, sterile. Mycelium: immersed, branched, septate, hyaline.
	GNFP-5	nd	Colonies: cottony, white-grey-creamish, distinct zonation, sterile. Mycelium: immersed, branched, septate, hyaline

Note**:** nd = cannot identified due to lack of both asexual (anamorph phase) or sexual organelles (teleomorph phase) formation on PDA. (*) = The above identifications were conducted by incubation of endophytic fungi on PDA, at 26-28° C for 14-20 days.

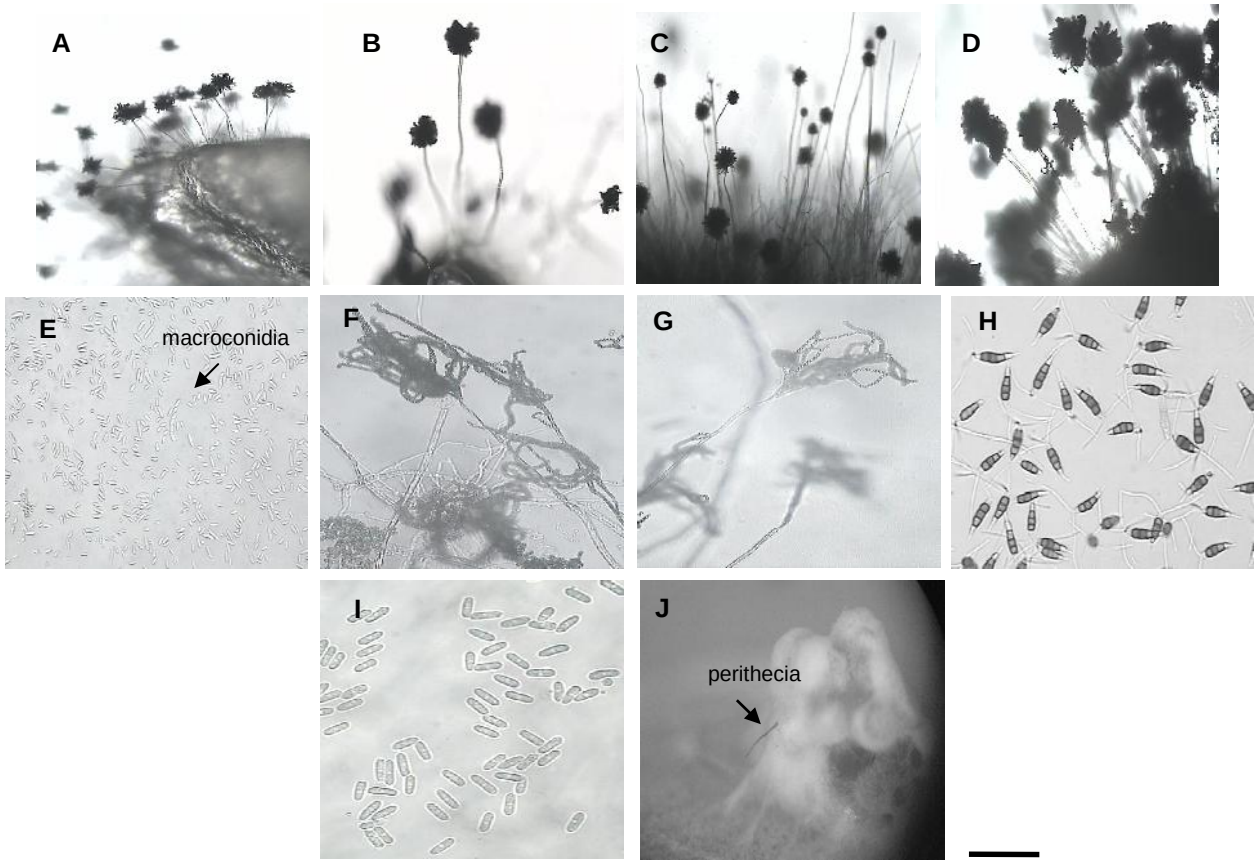

Figure 1. Microscopic structures of reproductive stages of endophytic fungi isolated from *U. gambier*. **A**. *Aspergillus* sp. GNAP-2; **B**. *Aspergillus* sp. GNDP-5; **C**. *Aspergillus* sp. GUBP-15; **D**. *Aspergillus niger*; **E**. *Fusarium* sp. GNDP-2); **F**. *Penicillium* sp. GNAP-3; **G**. *Penicillium* sp. GNAP-6; **H**. *Pestalotiopsis* sp.; **I**. *Phoma* sp.; **J**. *Diaporthe* sp. GNBP-10. Bar: A, B, C, D = 40 um, E, H, I = 10 um, F, G = 20 um, J = 50 um.

observed 13 isolates member of Coelomycetes produce asexual spores and identified into genera level, and they could be divided into 3 genera include *Pestalotiopsis* (GUBP-3, GUDP-7, GNBP-1, GNBP-3, GNBP-9, GNDP-8), *Phoma* (GUDP-4, GUBP-13, GNBP-4, GNDP-4, GNDP-6, GNDP-7) and *Phomopsis* (GUDP-1).

Pestalotiopsis is endophytic fungus which commonly isolated from higher plant than other member. Two isolates (GUDP-7, GUBP-3) isolated from var. udang, and 4 isolates (GNDP-8, GNBP-1, GNBP-3, GNBP9) from var. nasi are having identical morphological characters identified as *Pestalotiopsis*. Those isolates form spore as shown in Fig. 1H. Although they are morphologically identical each other, however TLC patterns of the ethyl acetate extract of culture medium are different. They form 4 different patterns employing that they are belong to 4 different species. The isolates of GNDP-8, GNBP-3 and GNBP-9 show their own specific TLC pattern, but GUDP-7, GUBP-3 and GNBP1 have identical profile and identified as *Pestalotiopsis* sp. GUDP.

From *U. gambier* var. udang obtained two isolates (GUDP-4, GUBP-13) which shows morphological characters similar to *Phoma*. The two fungus shows identical morphological characters similar to fungi namely GNDP-4, GNDP-6, GNDP-7, and GNBP-4 obtained from *U. gambier* var. nasi, thus the six fungi are identified as *Phoma* sp GUDP, and the six isolates shows similar TLC pattern (data not shown) which implies that those isolates produce similar secondary metabolites when grown in PDB and GYP. Anamorphic phase of *Phoma* is indicated by formation of conidia or phyliaspore inside pycnidia (Sutton, 1980). Grown on PDA, *Phoma* sp. GUDP produce a number phyliaspora (Fig. 1I).

One of the 14 isolated Coelomycetes (GNBP-10) produce black stick colored reproductive sexual (Fig. 1J), this is one character of *Diaporthe. Diaporthe* is endophytic fungi with wide range of host plant. Genera *Diaporthe* is a sexual stage (teleomorph), and mostly found in asexual stage (anamorphic stage) that belongs to genera of *Phomopsis* (Kobayashi, 1970). Morphologically *Diaporthe* is very difficult to be identified correctly since this genera is seldom to form perithecia grown in a synthetic medium. In a synthetic medium they mostly form pycnidia (Pioli et al., 2003).

Only two isolates (GNAP-3, GNAP-6) are isolated from root of var. nasi which identified as *Penicillium.* Species identification of *Penicillium* based on morphological observation is difficult (Samson et al., 1995). The two *Penicillium* isolated from var. nasi are also difficult to identify into species level. The two isolates have identical conidia (Fig. 1F and 1G), but

macroscopically the color of those isolates are different. The appearance of GNAP-3 colony is whitish blue, and GNAP-6 is deep blue. The TLC chromatogram patterns of fungi isolates (data not shown) are not identical implying that two isolates are different species. Generally *Penicillium* is saprophyte and some are parasitic to plant. *Penicillium* are endophytic in *P. janthinellum* and *Melia azedarach* (Marinho et al*.,* 2005), and *Taxus brevifolia* (Stierle et al., 1997).

We observed 15 sterile hypha, and they shows quite differs macroscopic view (Table 1 and Table 2). All of those isolates do not form sexual or asexual organelle grown in PDA till 60 days incubation. Identification for those isolates will be conducted in the future with molecular analyses of the ribosomal DNA.

CONCLUSIONS

We isolated 53 isolates of fungi from *U. gambier* var. nasi and var. udang. Based on morphological observation, those 53 isolates are belonged Coelomycetes and Hyphomycetes, 15 isolates are unidentified due to they form only sterile hypha. Identification at genera level, endophytic fungi obtained is *Aspergillus, Cladosporium, Diaporthe, Fusarium, Penicillium, Pestalotiopsis, Phoma,* and *Phomopsis*. Chemotaxonomy analyses based on TLC patterns of secondary metabolites extracted by ethyl acetate of cultures grown on PDB and GYP reinforce morphological identification, and grouping of endophytic fungi.

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Functional Importance of Seed Weight on the Seedling Establishment of *Syzygium bankense* (Hassk.) Merr. & L.M. Perry and *Quercus gemelliflora* Blume

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ABSTRACT

Seedling is the first and prominent phase of plant development. Seedling performance can be determined by seed and its embedded characteristics, among them is seed weight. In this study we would like to see how seed weight within species affect seedling establishment. The hypotheses tested were that heavier seed is regarded advantageous compared to lighter seed by functionally produce more competitive seedlings for better growth. Heavier seeds indeed resulted in higher shoot length than lighter seeds. The same pattern was also pronounced for the correlation between seed weight and shoot length of *Syzygium bankense* and *Quercus gemelliflora*. There was also a significant positive correlation between seed size and number of leaf within species.

Key words: seed weight, *Syzygium bankense, Quercus gemelliflora*, seedling establishment, germination.

INTRODUCTION

The world is presently dependent on a few plant species. Only 30 plant species provide 95% of the world's food (FAO, 1996). Over exploitation of particular species lead them into extinction. According to the International Union for Conservation of Nature (IUCN), more than 13% of plant species is threatened (Walter and Gillet, 1997). In addition to food source, plant is mainly used as timber. There are more than 29 000 vascular plant species in Indonesia, of which less than 40 species are utilized for timber. One of the crucial questions in tropical-forest management today is the future of lesser-known, also known as underutilized plant species. Hence, it is important to identify the lesser known species potential for timber resources. Furthermore, another important facet is to understand the ecology of species, including the germination ecology with respect to seed characteristics and seedling of the underutilized plant species.

Seedling is the first and prominent phase of plant development. It may determine the success rate of a species to accomplish further ontogeny form. Seedling establishment is, thus, important and critical to multi factor affecting species survival. Seedling performance can be determined by seed and its embedded characteristics, among them is seed size or seed weight. Seed weight encompasses amount of reserves that is contained by embryo to start its initial life phase. Seed weight ranges wide among species and within species. Difference of seed weight among species can be subjected to dispersal mode, and allocation pattern of mother tree. Seed weight and life history correlated with the formation of a soil seed bank determines further plant communities and vegetation structure (Honda, 2008). In addition, there are three mechanisms have been proposed to explain functional seed-seedling relationships (Leishman et al., 2000).

Two species were chosen, i.e. *Syzygium bankense* (Hassk.) Merr. & L.M. Perry and *Quercus gemelliflora* Blume to be tested in the study as both are recalcitrant seeds but perform different seedling bank characters. Seedling bank of *Q. gemelliflora* is abundant but not for *S. bankense*, this perhaps entitled to the difference of seed weight between species with regards to reserves in the cotyledon. However, we will focus on a within species analysis. A within-species analysis may therefore more clearly show the functional significance of seed size. Thus, in this study we would like to see how seed size within species affect seedling establishment. The hypotheses tested were that heavier seed is regarded

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advantageous compared to lighter seed by functionally produce more competitive seedlings for better growth. Heavier seeds referred to seeds with larger endosperms or storage tissues. The storage tissue of the seed can be considered to have two functions: sources of energy and of nutrients. The latter function could be expected to be especially important in nutrient-poor soils (Lee and Fenner, 1987).

MATERIALS AND METHODS

Seeds collection

Mother trees of *S. bankense* (V.B.116) and *Q. gemelliflora* (VIII.B.18) belong to the Bogor Botanic Gardens collection. The seeds of *S. bankense* were harvested from a single tree by picking. The seeds of *Q. gemelliflora* were collected from garden floor, produced by a single mother tree as it was not feasible to do climbing. *S. bankense* berries were cleaned from the flesh, then air dried for several hours and weigh individually before it was sown. *Q. gemelliflora* acorns were cleaned; nut and cap were separated, and nuts were then individually weighed before it was sown. The seeds collection and sowing were not carried out at the same time as fruiting and collection time differed.

Germination experiment and measurement

The sowing was carried out in a shaded glass house in Bogor Botanic Garden. The experiment was set up as random single factor design which involved eight replications, which represent four lighter seed and four heavier seed sowing blocks. Each replication consisted of thirty five seeds, giving a total measurement unit of 280 seeds/species. The observation was carried out after 105 days for *S. bankense*, and 61 days for *Q. gemelliflora*, as first leaf shed occurred on the respective days.

The sowing substrate used was nutrient poor sand. Seeds were sown on substrate surface without further being covered by the substrate. The seedlings were watered periodically with fixed sprinkler. There was neither nutrient addition nor dormancy breaking treatment to eliminate external facilitation effect on seedling establishment that can overshadow seed size role on seedlings.

The observation was periodically carried out to record: (i) germination percentage (%); (ii) shoot length (cm); and (iii) number of leaves. The last two were considered as seedling performance or establishment indicators. During the observation, leaf was accounted when the width was larger than 2 mm for *S. bankense*, and was larger than 1 cm for *Q. gemelliflora*. For branched *S. bankense*, the shoot length was counted as the sum length of branches and main stem between branch fork and cotyledon. The length of *Q. gemelliflora* forked seedlings was counted as the sum of seedling branches.

Data analysis

Seed weight determines germination percentage and seedling establishment, represented by number of leaf and shoot length, were analyzed. As the data remained not normally distributed and variance unequal upon various transformation applied, Chi square test was employed to point out differences among groups. Seeds did not germinate were left out from correlation analysis applied for seed weight and seedling establishment indicators. Furthermore, Spearman rank correlation coefficient was used to determine the presence and strength of relationship between seed weight and seedling performance. Statistical analysis was performed under SPSS 15.0.0 for Windows.

RESULTS AND DISCUSSION

Seed weight

The span of average seed weight categorized in smaller and larger seeds category of *S. bankense* was only two fold, being 0.0358 g and 0.0796 g; whereas, it was more than three fold, i.e. 2.0151 g and 6.9908 g, for *Q. gemelliflora*.

Germination

The germination of *S. bankense* and *Q. gemelliflora* follow epigeal and hypogeal pattern, respectively. The germination of *S. bankense* was faster than *Q. gemelliflora*. This can be attributed to the thickness of seed testa. The germination was uniform for *S. bankense*, meaning that seedling emerged almost at the same time. Larger seeds of *Q. gemelliflora* germinated following uniformity and were faster than smaller ones. The mean germination rates of *S. bankense* and *Q. gemelliflora* were 98.57% and 54.64%. Smaller seeds of *Q. gemelliflora* did not germinate until the end of observation (Figure 1).

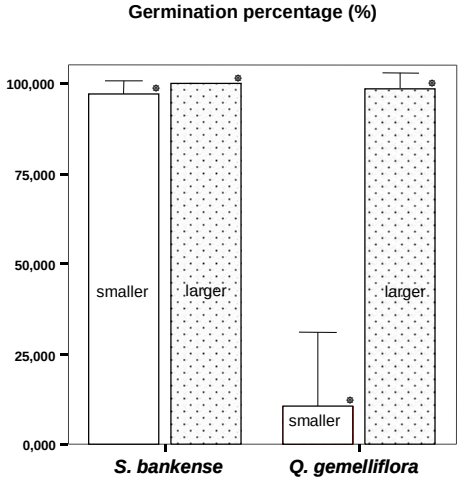

Figure 1. Germination percentage of smaller and larger seeds of *S. bankense* and *Q. gemelliflora*. Error bars show 95% CI of mean, bars show means.

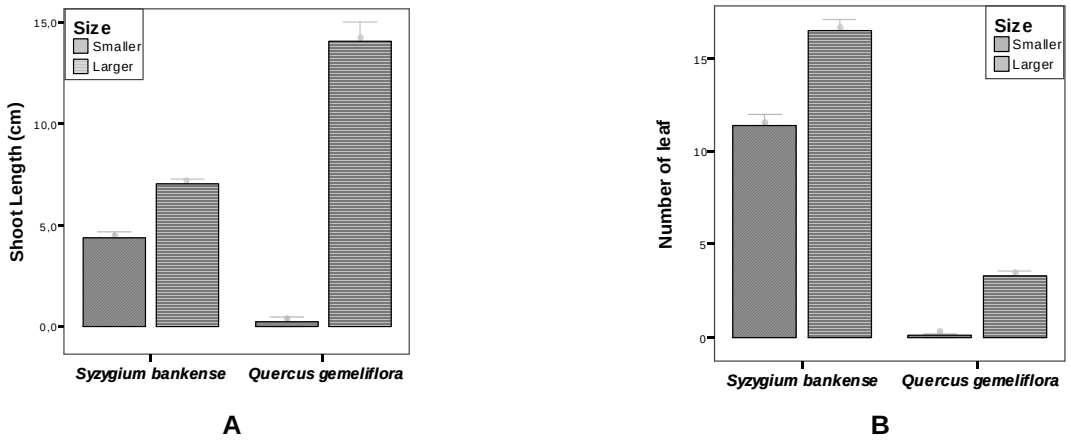

Figure 2.A. Shoot length emerged from smaller and larger seeds of *S. bankense (df*= 73, Chi sq= 188.913**) and *Q. gemelliflora (df=68, Chi sq*=171.286***)*. **B**. Number of leaf flushed from smaller and larger seeds of *S. bankense (df*= 21, Chi sq= 297.913**) and *Q. gemelliflora (df=7, Chi sq=88.597***)*. Error bars show 95% CI of mean, bars show means.

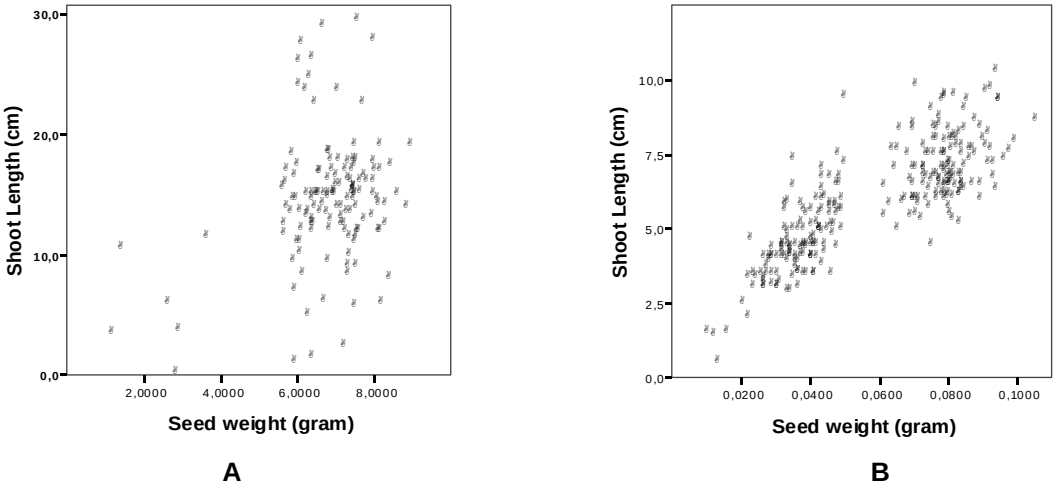

Figure 3.A. Seed weight of *Q. gemelliflora* correlates with seedlings' shoot length (Spearman's r= 0.292**, N=154); **B**. Seed weight of *S. bankense* correlates with seedlings' shoot length (Spearman's r=0.798**, N=276).

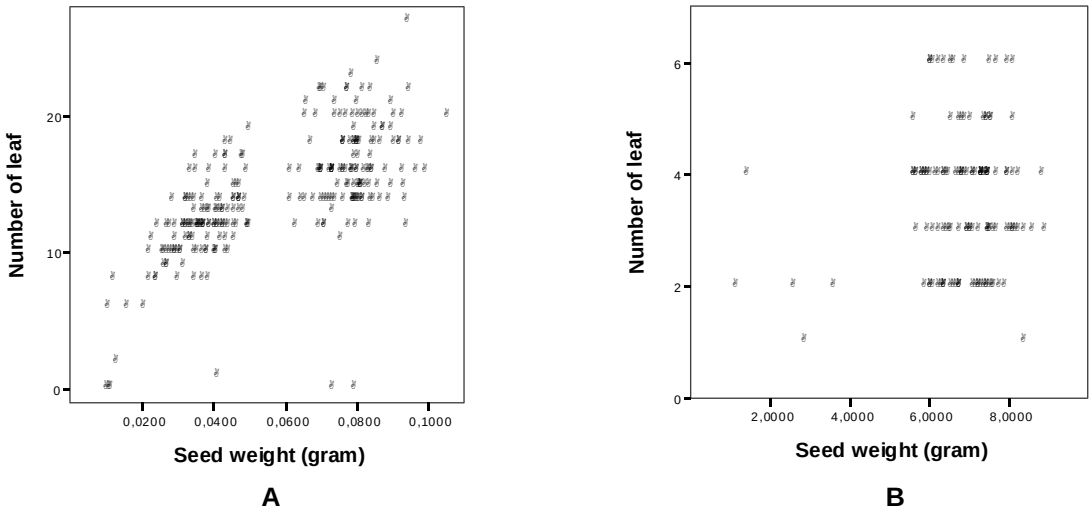

Figure 4.A. Seed weight of *S. bankense* positively correlates with number of leaf of emerged seedlings (Spearman's r=0.719**, N=276); **B**. Seed weight of *Q. gemelliflora* positively correlates with number of leaf of emerged seedlings (Spearman's r=0.219**, N=154).

Radicles, in fact, emerged from some seeds but hypocotyls elongation deteriorated at some point, indicating germination failure.

Seedling establishment

Heavier seeds indeed resulted in higher shoot length than lighter seeds. The shoot length of *S. bankense*, in general, was shorter than seedling of *Q. gemelliflora* (Figure 2A). The average leaf area of *Q. gemelliflora* was about a hundred and fifty fold larger than leaf area of *S. bankense*. Heavier seeds were provoked in more seedling leaves for both species. In general, *S. bankense* seedlings attributed with more leaves than *Q. gemelliflora* seedling (Figure 2B).

It can be referred that there was a positive correlation between seed weight and shoot length of *S. bankense* and *Q. gemelliflora*. The same pattern was also pronounced for the correlation between seed weight and shoot length of *S. bankense* and *Q. gemelliflora* (Figure 3A and 3B). Heavy seeds of *Q. gemelliflora* frequently produced more sprout from the cotyledon compared to *S. bankense*, whereas, smaller seeds of *Q. gemelliflora* produced single sprouted seedling. However, we did not measure the correlation between seed weight of *Q. bankense* and number of seedling sprouts produced. Smaller seeds of *Q. gemelliflora* produced less leaf per seedling, and slow developed leaf compared to seedlings leaves flushed from larger seeds. The survival (emergence) of smaller seed seedlings of *Q. gemelliflora* is lower than heavier ones.

There was also a significant positive correlation between seed size and number of leaf for within species relationship (Figure 4A and 4B). We did not measure other leaf characters than number. Nevertheless, it was apparent that except difference in number of leaf, there was no other leaf character difference, such as colour, width, or thickness.

Seed weight-germination

The ability of germination of *S. bankense* that did not differ regarding to weight were contrasted to of *Q. Bankense*. This was not fully in accordance with the expected pattern that germination or seedling emergence was regarded as seed size dependent. A flaw in the experiment design can be possibly subjected to the germination is that we did not look precisely at the emergence (from below substrate surface). Besides that, *Q. gemelliflora* seed collection from garden floor might result in accidental picking of small juvenile seeds. Insect attack can also be attributed to the discrepancy of seed size effect on seedling establishment on *Q. gemelliflora* as reported also in *Quercus suber* insect damage study by Branco et al. (2002), or other biotic factors on oaks (Andersson, 1992; Herrera, 1995).

As the seeds were not buried, germination is solely regarded as the protrusion of radicle from the seed coat, not the emergence from below substrate surface. In *S. bankense* seed size did not affect time of radicle emerging from testa (personal observation). It could be indeed affecting germination when it is referred as seedling emergence, given seeds have to be buried at a given depth. Other study by Jankowska-Blaszczuk and Daws (2007) found also that with increasing seed weight, germination become less dependent on light.

Seed weight-seedling establishment

A positive correlation between seed weight and number of leaf and shoot, representing seedling growth, also supported the theory that seed size determines competitive ability within species (Turnbull et al., 1999; Leishman and Westoby, 1994), but still not profound for between species. This can address the fact that not all larger sized seedlings were more advantageous to smaller ones. The finding was in accordance with a study by Gross (1984) which tested correlation between seed size and growth form of six perennial plants, and that within species difference in seed size has a significant effect for seedling growth on non competitive cover.

In nutrient poor substrate as in pure sand, seed weight is expected to give more advantage for seedlings. Nutrients, particularly Nitrogen compound, are known to have important role in germination (Broncano et al., 1998), and hence, in nutrient poor condition seed weight is expected to be more determining in seedling establishment. Besides leaf number and shoot growth, relative growth rate or aboveground biomass is a good indicator for seedling performance as it will give insight into the importance of morphological and physiological plasticity for seedling performance (Broncano et al., 1998).

Cotyledon type-seedling performance

We expected that phanerocotylar (photosynthetic) cotyledon produced by epigeal germination of *S. bankense* provided seedlings better capacity to grow than cryptocotylar cotyledon by *Q. gemelliflora* hypogeal germination. Apparently some of *S. bankense* are photosynthetic cotyledons; several days after germination they turned greenish. Without observation on growth increment we can not determine whether there was additional effect of cotyledon on seedling growth of *S. bankense* and *Q. gemelliflora*.

Larger seeds of *Q. gemelliflora* tend to have hypogeal cotyledons as epigeal type can not be supported, given the cotyledons of *Q. gemelliflora* were bulky and heavy. Correlations among seed mass, seedling type, and other seedling traits suggest the evolution of a coordinated set of traits for seedling establishment, defined by a gradient from smaller-seeded species with epigeal foliar cotyledons, orthotropic stems, and dichotomous root systems at one extreme, to larger-seeded species with hypogeal reserve cotyledons, plagiotropic stems, and taproots at the other extreme (Baraloto and Forget, 2007).

CONCLUSION

It was profound that heavier seed provides germination and establishment advantage of the two underutilized species tested namely *S. bankense* and *Q. gemelliflora*. Hence, it is necessary to take seed size into consideration when planning a nursery for underutilized species development program.

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A Comparison on Biodiversity between Private Conservation and Wildlife Reserve Forests in Riau by using Macro-moths as an Indicator

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ABSTRACT

A study on biodiversity of the two forest management types, private conservation forest PT. A and wildlife reserve forest of Suaka Margasatwa Giam Siak Kecil, Riau by using a rapid assessment approach with macro-moths as an indicator was conducted from 23 October to 6 November 2007. Four sample sites were established in Giam Siak Kecil Wildlife Reserve, whereas three sample sites were performed in private conservation forest PT. A. The results show that the diversity indexes based on Fisher's α of the private forest PT. A was higher than those of wildlife reserve forest Giam Siak Kecil, they were 67.98 and 47.86, respectively. The species composition of the two forests is different, pyralid moths dominate at Giam Siak Kecil. On the contrary, Geometrid moths dominate at private conservation forest PT. A. The results indicated that diversity index and species composition in Giam Siak Kecil is influenced by habitat changes and decrease on floral diversity due to illegal logging. Moreover, a low faunal similarity which is indicated by Jaccard's index that is only 0.218 showed that the samples represent significant different communities.

Key words: biodiversity, conservation, macro-moths, Fisher's α index.

INTRODUCTION

The forest of Sumatra is increasingly being modified by men in a variety of ways. This not only happen to the protected forests but also to the wildlife or nature reserve forests and national parks such as in Giam Siak Kecil Wildlife Reserve (or Game Reserve), Tesso-Nelo National Park and other areas in Sumatra. Illegal logging, clearing for timber industry or crop plantations and agricultural encroachment threatened the biodiversity of the very survival of extensive lowland of peat swamp forest in this island. Therefore, it is not surprising if the World Bank is predicted that the lowland forests in Sumatra will no longer exist unless some efforts taken to reduce the rate of deforestation (Sukara, 2005). The impact of the deforestation themselves in Riau has been well documented together with the social and political issues behind these pressures. The most significant direct impact of the deforestation and the environmental destruction in Riau is floods that occur in several villages and cities in Riau during 2007-2008 that has never occurred before. The other impact is biodiversity lost due to forest conversion for oil plantations and pulp industries that are conducted by local governments and private companies in order to generate their own incomes. Biodiversity lost may not directly and immediately influent to our society but it will change the whole ecosystem. Thereby, monitoring of biodiversity is a very important approach for early detection of ecosystem change. Realizing the ecosystem changes in our surrounding will provide us a better future plan.

Measurements of biodiversity in a certain region can be conducted in many different ways, depend on what the level of measurements will be achieved. There are three levels of biodiversity, genes, species and ecosystems. Measurements of genetic diversity refer to the variations of genes within a species. This covers distinct population of the same species or genetic variations within population. Measurements of genetic diversity were applied mainly to domesticated species and population held in zoo or botanic garden for conservation purposes. This method requires an advanced technology such as PCR and DNA sequencing. In species diversity, measurement is based on the species diversity in simple. This species diversity refers to variety of species within region that can be measured based on morpho-species richness. The last category, ecosystem diversity, is harder to measure than species or genetic diversity because the "boundaries" of communities-association of species-and ecosystem are elusive. Therefore, it is not surprising that species diversity is more common

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in use to evaluate or measure the biodiversity of forest even to monitoring the impact of human activities to forest ecosystem in certain region such as forest fire, land clearing, illegal logging, land conversion etc.

Floristic and structural changes are often obvious to the casual observer, as are the effect of these on vertebrate groups. Very much less is known of the less apparent but possibly more serious costs to major part of biodiversity such as micro-organisms and invertebrate, particularly insects, and to the role they play in the continuing health of the forest and the survival of their component. Thus, insect is more suitable than vertebrata as indicator of the state of forest ecosystem and for monitoring the impact of changes. The importance of insect and their value as indicators is discussed by Holloway (1980), Brown (1991), and Holloway and Stork (1991).

A number of insect groups are currently championed for use as indicator in tropical rain forest ecosystem, but no single group appears paramount when assessed in relation to criteria for good indicator group, such as: (a) ease and objective in sampling; (ii) taxonomic tractability; (iii) ecological generality combined with fine-grained habitat fidelity (including low blurring of pattern through mobility); and (iv) rapid response to disturbance. Night-flying moths satisfy most of these criteria. They can be collected in a large number by using a light tarp and also can be found in numbers in most vegetation type. In addition, Lepidopterist can do rapid sorting to species level based on good taxonomic characters in wing marking and genital structure permit accurate cross-reference between samples. This is important factor to conduct a rapid assessment on biodiversity of forest. Therefore, macro-moth is chosen as indicator to evaluate and compare biodiversity between two areas of forest conservation in Riau Province, Giam Siak Kecil Wildlife reserve and private conservation forest PT. A. By knowing the status of its biodiversity, we expect that management plan on the both conservation forests can be improved.

Sites of study

The research was conducted from 23 October to 6 November 2007 on wildlife reserve forest Giam Siak Kecil and private conservation forest PT. A Bukit Batu belong to a timber forest industry, in Riau Province (see Figure 1).

Natural forest of Giam Siak Kecil Wildlife Reserve

This natural forest is located on the eastern side of Sumatra, approximately 45 km south west of Bengkalis strait. The site is located along and southwest of a 50 km section of Sungai Siak Kecil. The northern boundary is approximately 40 km from Dumai. The area consists of swamp forest, peat swamp forest and floodplain lakes. North and northeast lays a large peat dome with reportedly some of the deepest peat in Sumatra. Along the river numerous shallow floodplain lakes occur that are connected to the Siak Kecil by narrow streams. Much of the forest is secondary, having been selectively logged prior to gazettal. This natural forest covers about 100,000 ha; almost 30% of the total area is peat swamp forest.

Private conservation forest at Bukit Batu

The area consists of swamp forest, peat swamp forest and floodplain lakes and covers about 70.000 ha. The vegetation type of this forest is more or less similar to natural forest at Giam Siak Kecil, as a secondary forest, surrounded by timber industrial forests belongs to a private company.

MATERIALS AND METHODS

Collecting adult moths

Sampling was conducted using light traps equipped with a 160 watt mercury vapor light and a 2 X 2.5 m white screen. The light trap was set up at the open area within the forest. Moths attracted to the light trap and lied at the white screen were collected into an ethyl acetate-killing bottle. Large moths (wing span > 5 Cm) were collected by using an insect net, followed by injection of absolute ammonia at the thorax. All specimens collected at the night then were pined using insect pins No 3 and 4 the next morning while the specimens are still in fresh condition.

Preservation

Preservation of the specimens is conducted at the Laboratory of Entomology, Division of Zoology, Research Center for Biology, Cibinong Bogor, West Java, Indonesia. All moth specimens were labeled based on the field collection data. Their wings were spread and then dried up using oven at 45-50°C for 3-5 days, depends on the condition of specimens. Shorting and identification to specific level were performed as described by Kristensen (1999), Inoue et al. (1982); Common (1990), Nassig et al. (1996), Holloway (1997), Robinson et al. (1994). Kuroko and Lewvanich (1993), Kobes (2000), and Holloway et al. (2001). All the materials are deposited at Museum Zoologicum Bogoriense, the Indonesian Institute of Sciences.

Data analysis

The diversity measure for species-richness to be used throughout this discussion is the α–statistic of Fisher et al. (1943). Fisher's alpha diversity index, defined implicitly by the formula: $S=a*\ln(1+n/a)$ where S is number of taxa, n is number of individuals and a is the Fisher's alpha. Justification for this on grounds of the frequent approximation of light-trap moth samples to a log-series distribution of abundance among the species is given by Taylor et al. (1976) and, within a South East Asian context, by Barlow and Woiwod (1989). Wolda (1983) demonstrated that

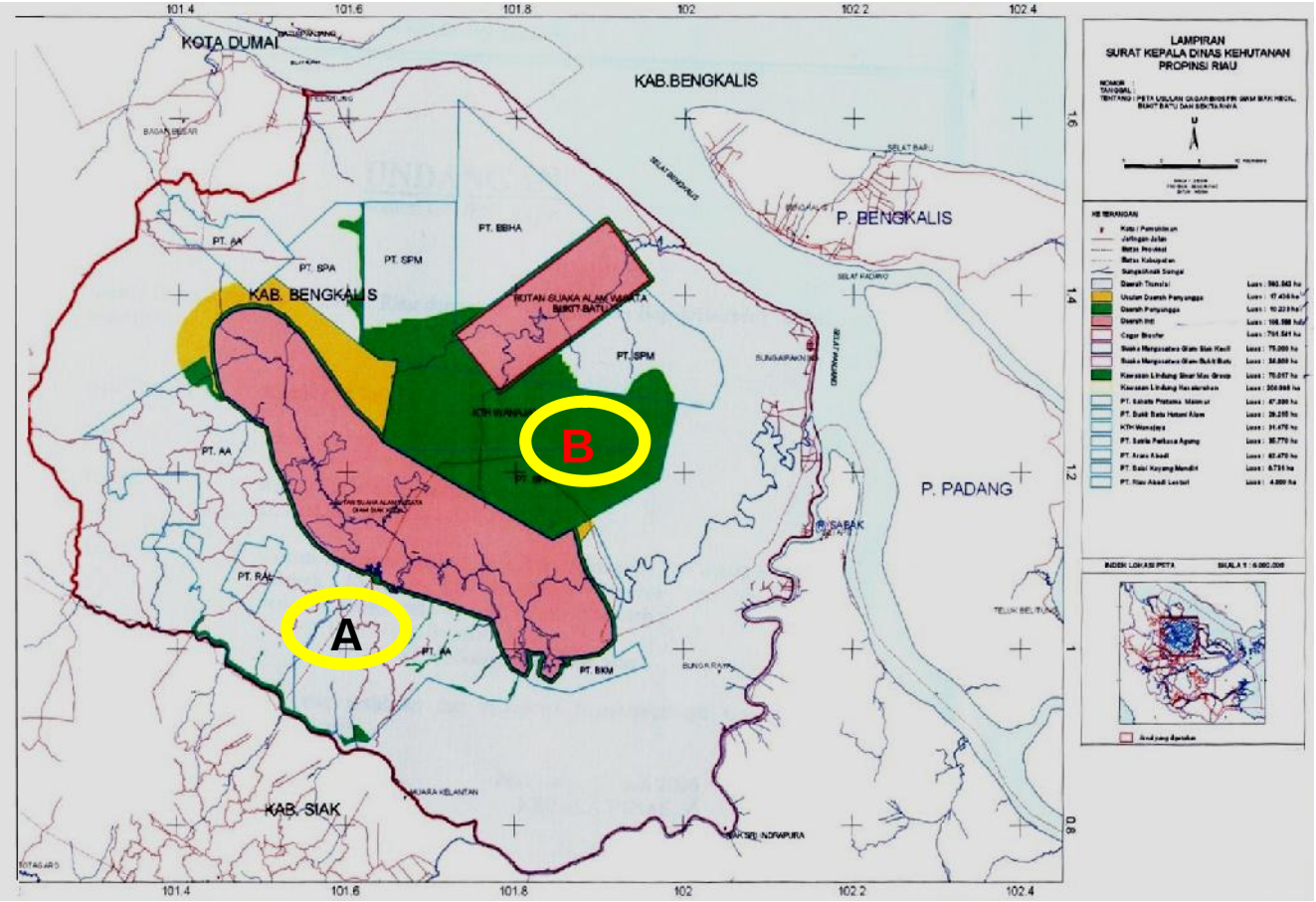

Figure 1. Site A is Nature Forest Giam Siak Kecil and site B is private conservation forest PT. A. in Riau.

this statistic was the most sample-size independent of a number of frequently used diversity measure. For comparison among two sites, Jaccard index of similarity is used. This is a robust measure of beta diversity and widely used in biodiversity research.

In addition, we have chosen extrapolation method, which gave an estimate of the total number species from empirical samples. Let *N* be the total number of individuals in the sample, *s* the total number of species, and *Ni* the number of individuals of species number *i*. The expected number of species $E(S_n)$ in a sample of size *n* and the variance $V(S_n)$ are then given:

$$E(S_n) = \sum_{i=1}^{s} \left[1 - \frac{\binom{N-N_i}{n}}{\binom{N}{n}} \right]$$

$$V(S_n) = \sum_{i=1}^{s} \left[\frac{\binom{N-N_i}{n}}{\binom{N}{n}} \left(1 - \frac{\binom{N-N_i}{n}}{\binom{N}{n}} \right) \right] + 2 \sum_{j=2}^{s} \sum_{i=1}^{j-1} \left[\frac{\binom{N-N_i-N_j}{n}}{\binom{N}{n}} - \frac{\binom{N-N_i}{n}\binom{N-N_j}{n}}{\binom{N}{n}\binom{N}{n}} \right]$$

All this methods were implemented in "Methodological Ecology" software (Krebs, 1998).

The value of light collecting

A correct statistical analysis first requires a certain number of replicate samples, which, secondly, have to be drawn randomly. Both conditions are not met in the present study. They are hardly achievable in these kinds of studies at all, because the use of light or light trap is a selective method and the number of replicate usually remains low. Nonetheless, the use of light in collecting Lepidoptera yields the highest proportion of the total species spectrum at a given locality. It is nearly impossible to get similar part of taxocenosis by applying other methods

RESULT AND DISCUSSION

The record of species collected from this study is available on request and all the specimens are deposited at Museum Zoologicum Bogoriense. These results in Table 1 show that the short collecting time spent across all sites in these forests makes the results only a fragment of the actual existing Lepidoptera fauna which is only 81 of 92 species (88%) at Giam Siak Kecil and 112 of 125 species

(89.6%) at private conservation forest PT. A. In addition, the number of families recorded from these two forests is quiet low, 18 families, or about 1/3 of the families of moths that exist in Indo-Malayan region (Holloway et al., 2001). Moreover, total species number of moths at the indicated areas is very low, only 162 species from 18 families (Table. 1).

Table 1. Species richness of moth collected at Giam Siak Kecil Wildlife Reserve (GSK) and private conservation forest PT. A, Bukit Batu (S= species number, ST= total species).

Locality	Taxa	S	%	ST	Species with 1 individual		Species with >2 individuals		Esti-mated species
GSK	Sphingidae	5	6.17		2		3		
	Geometridae	17	21.00		8		9		
	Noctuidae	14	16.47		9		5		
	Pyralidae	25	30.86		15		10		
	Arctiidae	4	4.93		0		4		
	Limacodidae	3	3.70	81	0	42	3	38	92
	Lasiocampidae	4	4.93		2		2		
	Notodontidae	1	1.23		1		0		
	Nolidae	4	4.93		2		2		
	Cossidae	2	2.47		2		0		
	Thyrididae	1	1.23		0		1		
	Agamidae	1	1.23		1		0		
PT. A	Sphingidae	7	6.25		3		4		
	Geometridae	32	28.57		18		14		
	Noctuidae	10	8.91		5		5		
	Pyralidae	22	19.64		7		15		
	Arctiidae	9	8.03		2		7		
	Limacodidae	7	6.25		3		4		
	Lasiocampidae	5	4.46		3		2		
	Notodontidae	1	0.89		1		0		
	Bombycidae	1	0.89	11	1	56	0	56	125
	Yponomeutidae	1	0.89	2	1		0		
	Nolidae	4	3.57		3		1		
	Cossidae	2	1.78		1		1		
	Thyrididae	6	5.35		0		2		
	Saturniidae	2	1.78		2		0		
	Lymantriidae	5	4.46		5		0		
	Drepanidae	1	0.89		0		1		
	Psychidae	1	0.89		1		0		

The species number at conservation forest PT. A is higher than those at Giam Siak Kecil Wildlife Reserve, they were 112 species (12 Families) and 81 species (17 families), respectively. Diversity indexes based on Fisher's α consistently revealed a greater number than that of Giam Siak Kecil; they were 67.98 and 47.86, respectively. Jaccard coefficient between two forests is very low (0.218) (Table 2).

The result shows that Families Geometridae, Pyralidae and Noctuidae is the most dominant among other families at the two sites. The most significant different between two forests is their species composition. Pyralidae were found to be dominant at Giam Siak Kecil, approximately 30.86% of the total moths; whereas family Geometridae is up to 28.57% at conservation forest PT. A.

Table 2. Index Diversity and Index similarity of moth collected at Giam Siak Kecil (GSK) and PT. A, Bukit Batu

Locality	N	S (F)	Alpha index	Jaccard's index
GSK	212	81	47.86	0.128
PT. A	285	112	67.98	

The results of the study show that the diversity of moth fauna is very low at both two forests. This is a general trend on moth diversity at the low land forests (< 100 m above sea level), especially at the peat swamp forest, as has been reported by Sutrisno (2005). He reported that the number of species in Sebangau National Park collected during 8 nights is only 100 species of 12 families with Fisher's alpha index is 50.91. More over, his study at Nusa Barong Nature Reserve, a low land forest, (<15 m), within 5 nights shows the Fishers's α index is only 34.58. The results of this study also have a similar trend with those found in Sulawesi where the low land forest (<100 m) has lower diversity than a primary high land forest at or even than secondary forest (Holloway, 1987).

In general, vegetation, latitude and altitude are the most significant factors that determine the moth diversity (Beck and Kitching, 2007). The larvae of moths indeed often show great specificity to host plants (Robinson, 1975; Holloway, 1976; Hebert, 1980; Inoue et al., 1982; Common, 1990; Robinson et al., 1994). Thus, the low diversity of the moth of the two forests is caused by availability of their host plants at these areas. Indeed, only a certain plant such as Euphorbiaceae, Dipterocarpaceae, Rubiaceae, Myristicaceae, Lauraceae, and Sapotaceae which is able to adapt to the acid environment with unfertilized soil.

Another factor that influent to the low of floral diversity in these areas is illegal loggings. Illegal loggings have caused the decrease of floral diversity and caused the change of vegetation in this area. Finally, it will influence to the moth diversity as has been reported by Holloway (1998). He compared the moth diversity at illegal logging forests and primary forests in Danum Valley, Sabah. He found a significant result, there have been decreased up to 2/3 species at illegal logging forests. Moreover, a study on butterflies in Buru Island and pyralid moths on Mount Kinabalu, Sabah also gives a similar phenomenon (Hill et al., 1995; Fiedler and Schulze, 2004).

The result of this study also shows that the moth diversity at Giam Siak Kecil is lower than private conservation forest PT. A. Giam Siak Kecil Wildlife Reserve has been illegally logged and land cleared in many areas especially on the areas that close to the local settlements. There are many evidences prove that Giam Siak Kecil has been logged for a long time such as presence of canals and changes of characteristic vegetation. There are many artificial

canals that have been used to transport logs from the deep forest to the Giam Siak Kecil river previously. This activity has caused many changes to the ecosystem such as subside of water levels and changes of vegetation. Subside of water level has caused many large trees fall down due to its root has no ability to support them anymore since the upper peat soil go down follow the decrease of water levels.

Illegal logging has caused the decrease on species tree but increase on its density since young trees and liana trees growth everywhere after illegal loggings. For example the density at Giam Siak Kecil is higher than private conservation, they are 587.7 and 566.3 and the Mischung coefficient of Giam Siak Kecil is also higher than private conservation, which are 3.9 and 2.3 (Partomihardjo, pers. comm.). Mischung coefficient, a proportion number of individual/area and the species number, indicates the complexity of a sampling site. High values indicate that the species number at the sampling site is low. Based on this parameter, private conservation forest PT. A Bukit Batu has higher floral diversity than that of Giam Siak Kecil.

The vegetation of conservation forest PT. A. is relatively more conserve due to its geographical position and its access limitation. This forest is far from local settlements and located at deep inside of the private pulp forests. Therefore, this forest is less disturbed from illegal logging. As a healthy forest at the peat swamp forest, its vegetation is dominated by very large tree Dipterocarpaceae, (*Shorea teysmanniana* and *Shorea uliginosa*). These species have been reported by Istomo (2002) and Partomihardjo (2007) as the main supporting component of the vegetation at the peat swamp forests in Sumatra. Indeed, this private forest conservation is well-protected and less disturbed. The results of the study also showed that many species of moths that usually found at the primary forest were also found at this forest such as *Attacus atlas* and *Loepa megacore* (Saturniidae).

The other difference between the two forests can be seen on its species composition. In Giam Siak Kecil, family Pyralidae is dominant while in private conservation forest family Geometridae is dominant. Pyralidae is mostly medium size moths which its larva has various behaviors such stem borers, leaf roller and leaf eaters. These larvae frequently found in open habitat areas (grasses or Poaceae). On the contrary, most Geometrids are phytophagus that inhabit the green canopy of the threes. This result is also show a similar pattern on the study of moth diversity at Meru Betiri National Park, Nusa Barong Nature Reserve, and Sebangau National Park (Sutrisno, 2005; 2007). Indeed, moth composition can tell us the natural condition of vegetation of a certain area and can be used to evaluate the changes of forest vegetations (Beck et al., 2002).

Based on the results, it is clear that Giam Siak Kecil Wildlife Reserve as well as other wildlife and nature reserves in Sumatra urgently need attention from both central and local governments to improve its management by involving local peoples to protect and stop loss of biodiversity and other disasters such as floods, forest fires and drought. But it does not means, that private companies in Riau have no responsibility. They should actively participate in stopping global warming through providing more areas to become conservation forests in order to maintain an equilibrium ecosystem.

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Phagocytosis Effectivity Test of Phenylbutenoid Compounds Isolated from Bangle (*Zingiber cassumunar* Roxb.) Rhizome

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ABSTRACT

This study was conducted to determine the immunostimulant activity of several phenylbutenoid derivatives isolated from bangle (*Zingiber cassumunar* Roxb) rhizome: **[1]** [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol], **[2]** [(*E*)-4-(2',4',5'-tri-methoxyphenyl)but-3-en-1-ol] and **[3]** [(E)-4-(3',4',1-trimethoxyphenyl)but-3-en-1-ol]. Immunostimulant activity was done by stimulating macrophage cells of mouse peritoneum. The result showed that [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol] compound had highest immunostimulant activity (99.0%) compared to compound 2 (93.7%) and 3 (80.0%).

Key words: *Zingiber cassumunar* Roxb, immunostimulant, phenylbutenoid derivative, [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol], [(*E*)-4-(2',4',5'-trimethoxyphenyl)but-3-en-1-ol]and [(E)-4-(3',4',1-trimethoxy phenyl)but-3-en-1-ol].

INTRODUCTION

Phagocytosis process is one of non-specific defense mechanism of the body against some of foreign agent/body including pathogen microorganism. The primary cell that take important role in phagocytosis (phagocyte cell) is mononuclear (monocyte and macrophage) and polymorphonuclear or granulocyte (neutrophil). Effective phagocytosis process in early microorganism invasion can prevent illness. Microorganism destruction in the process of body defense mechanism is divided into several steps which are chemotaxis or phagocyte cells movement to the infection site, and then phagocyte cells bind it through non-specific receptor. If the microorganism already in the phagocyte cells, lysosomes fused with phagosome to form phagolysosome and microorganism is destroyed by microbicidal mechanism (Baratawijaya, 1991). Several plant species have been known as immunostimulant such as *Echinaceae angustifolia* and *Z. officinale.* The common natural immunostimulants are interleukin, interferon (INF), monoclonal antibody, crestin and lentinan, while the synthetic immunostimulant are levamisol, isoprinosin dipeptide muramil.

Several studies of phytochemical compounds and biological activity of *Zingiber cassumunar* Roxb had been done previously. The main component of *Z. cassumunar* rhizome essential oil were triquinacene 1,4-bis (methoxy), (Z)-ocimene and terpinen-4-ol (Bhuiyan et al., 2008). The result of the isolation work showed that its rhizome contained several phenylbutenoid compounds, curcuminoid, and sesquiterpene (zerumbon). Curcuminoid (cassumunin A and cassumunin B) isolated from *Z. cassumunar* may possess a potent protective action on living cells suffering from oxidative stress (Nagano et al., 1997). Some of the phenylbutenoid had the biological activity as cyclooxygenase inhibitor, cytotoxic, antiinflammation (Ozaki et al*.*, 1991; Han et al., 2003, 2005; Fachriya et al., 2007), phenylbutenoid dimmer proved to have cytotoxic activity (Han et al*.*, 2004), and acted as antioxidant, antiinflammation and anticancer (cytotoxic) (Murakami et al*.*, 2002), and curcuminoid is potential as antioxidant (Nagano et al*.*, 1997). Other study concluded that *Z. cassumunar* rhizome performs biological activity as bowel/ intestine relaxant (Kanjanapothi et al*.*, 1987), lipase enzyme activator (Darusman et al*.*, 2001) and hepatoprotector (Arafah, 2005), but its role for phagocytosis activity has not been studied yet.

The aim of this study was to determine the potential of several phenylbutenoid compounds isolated from bangle (*Z. cassumunar* Roxb) rhizome: **[1]** [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol], **[2]** [(*E*)-4-(2',4',5'-tri-methoxyphenyl)but-3-en-1-ol] and **[3]** [(E)-4-(3',4',1-trimethoxyphenyl)but-3-en-1-ol] as immunostimulant.

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MATERIALS AND METHODS

Materials

Rhizome of bangle (*Z. cassumunar*) was obtained from Bogor Botanical Garden and identified for its scientific name in Herbarium Bogoriense, Research Center for Biology, Cibinong-Bogor, West Java, Indonesia. The rhizome was thin sliced and dried in the oven with temperature of 40-50^{o}C. Dried rhizome was powdered with grinder. Macrophage cells were obtained from peritoneum of *Swiss Webster* male mouse (*Mus musculus*) 2 month old, 20-30 g body weight. *Staphylococcus epidermidis* isolates as foreign agent that will be phagocyted by macrophage cell from mouse peritoneum *in-vitro.* Bacteria growth medium: *Mueller-Hinton Agar* (MHA) (Sigma) and Nutrient Broth (NB) (Sigma). Reagent and solvent: Phosphate Buffer Saline (PBS) pH ± 7.4, dimethyl sulfoxide (DMSO), Na_2EDTA 0.2 M, Giemsa, tripan blue, methanol, ethanol, ethyl acetate, *n*-hexane, and aquadest. Levamisol HCl as control positive (200 µL)

Equipments

Equipments used in this study were incubator, autoclave for sterilization, laminar air flow, centrifuge, touch mixer, hemocytometer Nechbauer, spectrophotometer microscope, animal cages and animal balance, pH meter, analytical balance, rotary vacuum evaporator, percolator, Eppendorf pipette , petridish, ose, vial, object glass, sterilized glassware.

Isolation and purification of bioactive compounds

Dried rhizome powder of *Z. cassumunar* (1 kg) macerated in methanol 80% for 24 hours, the filtrate was separated and placed in the erlenmeyer and composited. The composed filtrate was evaporated with rotary evaporator with the temperature of 60^{o}C. The evaporation continued with water bath (petridish had been weighed previously). Maceration and percolation was done until the filtrate was clear. This crude extract was dissolved in methanol 50%, and placed in the separating funnel. Fractionation was done based on solvent polarity of *n*-hexane, ethyl acetate and residue (MeOH/water) consecutively. Every fraction was shacked several times until the fraction was clear. Every fraction was concentrated with rotary evaporator with the temperature of 35^{o}C. The evaporation was continued with until the fraction was concentrated. Three fractions were obtained, n-hexane, ethyl acetate and MeOH/water. Every fraction was tested for phagocytosis activity and capacity of mouse peritoneum macrophage cells. High effectivity was purified by chromatography technique.

Chromatography column of ethyl acetate fraction was used as stationary phase SiO_2 and mobile phase in gradient system with the eluent of *n*-hexane/ethyl acetate in the composition of 9: 1, 8: 2 up to 4: 6 respectively, each 2,5 L. Based on the result of Thin Layer Chromatography (TLC), this extract composed of 4 fractions. Every fraction was composited and evaporated. The dried fraction was tested for its phagocytosis activity and capacity of mouse peritoneum macrophage cells. Purification of bioactive compounds of the fraction by HPLC was done based on its immunostimulant activity; this was done by stationary phase SiO_2 and mobile phase *n*-hexane/ethyl acetate (4: 6). Lead us to four isolated compounds and three of them were pure compounds (**[1]**, **[2]** and **[3]**) which can be elucidated and to be determined as phenylbutanoids derivatives.

(*E*)-4(3',4'-dimethoxyphenyl)but-3-en-1-ol (**[1]**) isolated as a pale yellow oil, UV $\lambda_{max:}$ 259.70, 214.20 nm, IR ν_{max}: 3491-3334 (broad, OH), 1514 (aromatic), 2997, 2932, 2836 cm^{-1}(weak, short chain aliphatic). MS m/z 208 $[M]^+$, 190, 177, 146 ,77. ^{1}H-NMR; δ 2.46 (q, 2H), 3.74 (t, 2H), 3.86 (s, 3H), 3.88 (s, 3H), 6.06 (m, 1H), 6.42 (d, 1H), 6.79 (d, 1H), 6.88 (dd, 1H), and 6.91 (d, 1H). ^{13}C-NMR; δ 36.49 (2), 55.95 (3), 56.06 (3), 62.24 (2), 108.87 (1), 111.27 (1), 119.27 (1), 124.50 (1), 130.54 (0), 132.58 (1), 148.69 (0), and 149.14 (0).

(*E*)-4(2',4',5'-trimethoxyphenyl)but-3-en-1-ol (**[2]**) isolated as a pale yellow oil, UV $\lambda_{max:}$ 314.20, 258.70, 210.80 nm, IR ν_{max}: 3539-3320 (broad, OH), 1510 (aromatic), 2996, 2928, 2849 cm^{-1} (weak, short chain aliphatic). MS m/z 238 $[M]^+$, 207, 192, 161, 151, 77. ^{1}H-NMR: δ 2.51 (q, 2H), 3.76 (t, 2H), 3.82 (s, 3H), 3.87 (s, 3H), 3.89 (s, 3H), 6.06 (m, 1H), 6.49 (s, 1H) , 6.75 (d, 1H) and 6.97 (s, 1H). ^{13}C-NMR: δ 36.99 (2), 56.81 (3), 56.66 (3), 57.27 (3), 62.37 (2), 97.82 (1), 109.78 (1), 118.21 (1), 124.75 (1), 127.19 (1), 143.45 (0), 149.34 (0) and 151.11 (0).

(*E*)-4(3',4'-dimethoxy phenyl)but-3-en-1-methoxy-1-ol (**[3]**) isolated as a pale yellow oil, UV $\lambda_{max:}$ 262.90, 212.20 nm, IR ν_{max}: 3387 (broad, OH), 1514 (aromatic), 2996, 2928, 2835 cm^{-1}(weak, short chain aliphatic). MS m/z 238 $[M]^+$, 207, 190, 177, 161, 46, 77. ^{1}H-NMR; δ 3.64 (m, 2H), 3.87 (m, 1H), 3.89 (s, 3H), 3.91 (s, 3H), 5.90 (q, 1H), 6.59 (d, 1H), 6.83 (d, 1H), 6.94 (dd, 1H), and 6.96 (s, 1H). ^{13}C-NMR; δ 56.02 (3), 56.13 (3), 56,72 (3), 65.76 (2), 83.87 (2), 108.87 (1), 111.21 (1), 120.15 (1), 123.85 (1), 129.37 (1), 134.36 (0), 149.25 (0) and 149.37 (0).

Immunostimulant activity test

Immunostimulant activity was determined by phagocytosis activity and capacity of mouse peritoneum macrophage cells. Phagocytosis activity value is the percentage of active macrophage cells in 100 macrophage cells.

$$\text{Phagocytosis activity (\%)} = \frac{\text{Number of active macrophage cells}}{\text{Total of macrophage cells}} \times 100\%$$

Phagocytosis capacity = the number of bacteria phagocyted by 50 macrophage cells.

Before phagocytosis test was carried out, viability test was conducted previously to determine the macrophage cells viability and the total number of cells. Viability was expressed by the percentage of viable macrophage cells to total number of macrophage cells. Viability value should not be less than 95%.

Phagocytosis test

200 μL of bacteria suspension, 200 μL macrophage cells and 200 μL sample were placed in the test tube, and then incubated for 30 minutes, 37^0C. After incubation period, the sample was added 50 μL Na_2EDTA 0.2 M to stop phagocytosis process. The control positive was 200 μL levamisol HCl, and negative control was 200 μL phosphate buffer saline (PBS). After incubation period, the thin slides was made by dropping 100 μL suspension to object glass and spread with the spreader, dried, and then fixed with MeOH for 6 minutes. Thin slides were stained with Giemsa 2% and left in the stain for 45 minutes. Put in the acetic acid 1% 4 times, washed under tap water and allowed to air-dried. The thin slides were observed under the microscope with the magnification of 10x100 to determine macrophage cells activity and capacity (Wagner and Jurcic, 1991).

RESULTS AND DISCUSSION

The most active fraction of *Z. cassumunar* rhizome was screened for its immunostimulant properties lead to 4 compounds, but one of them was not pure (*impurity*), so that the structure elucidation was done toward 3 compounds. The result of structure elucidation showed that the compounds identified as: **[1]** [(*E*)-4-(3',4'-dimethoxy phenyl)but-3-en-1-ol], **[2]** [(*E*)-4-(2',4',5'-tri-methoxy phenyl)-but-3-en-1-ol] dan **[3]** [(E)-4-(3',4',1-trimethoxy phenyl)but-3-en-1-ol] and the first compound was the major compound (Figure 1). All of the three compounds were oily liquid.

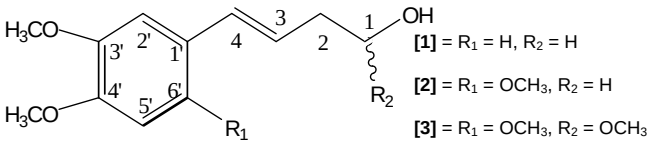

Figure 1. Structure of phenylbutenoid derivatives of bangle rhizome.

The result of phagocytosis test by using 500 μg of the sample showed that the phagocytosis capacity and activity of compound **[1]** were1069.3 and 99.0%, while the compound **[2]** were (612.33 and 90.7%) and compound **[3]** (691.22 and 88.0%) (Figure 2 and 3).

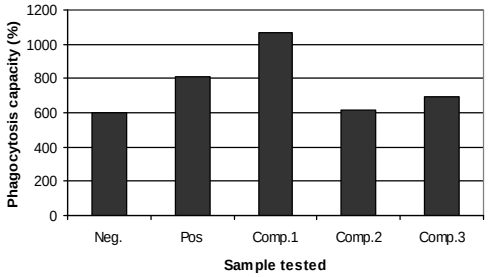

Figure 2. Phagocytosis capacity of phenylbutenoid compound from *Z.cassumunar* rhizome.

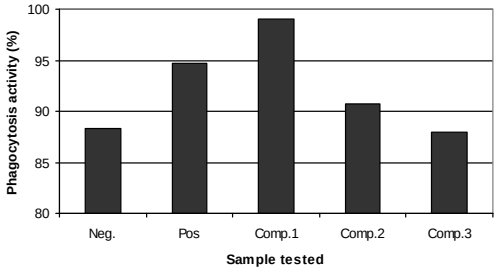

Figure 3. Phagocytosis activity of phenylbutenoid compound from *Z.cassumunar* rhizome.

Compound **[1]** did not have cyclooxygenase (COX-2) enzyme inhibitor (Han et al., 2005). Cyclooxygenase is the enzyme that has important role in arachidonic acid metabolism to prostaglandin E_2, chemical mediator that inhibit interleukin-2 and interferon γ production (NK cell activation attack tumor cell) by lymphocyte T. Prostaglandin E_2 also produced by tumor cell to suppress immune system related to tumor and cause immunodeficiency. The compound which had cyclooxygenase inhibitor properties can be used as cytotoxic, while macrophage activation for attacking tumor cell stimulated by lymphokin (chemostatic (CFM), migration inhibitor factor (MIF) and macrophage activator factor (MAF)) released by T cell stimulated by tumor antigen. The mechanism of macrophage stimulation and cyclooxygenase inhibition had different path although both of them had cytotoxic effect. Compound **[1]**. might not have cyclooxygenase inhibition properties but had the ability to stimulate macrophage cell. The study of immunostimulant activity showed that there is no correlation/relation between the structure and its activity of natural compound. Several chemical compound that had immunostimulant properties can be classified into two groups which are the compound with low molecular weight (alkilamid, phenolic compound, alkaloid, quinon, saponin, sesquiterpene, diterpene, triterpene) and compound with high molecular weight (Wagner, 1999).

Phenylbutenoid compound isolated from *Z. cassumunar* was a derivative of phenolic compound which had aromatic ring with one or more hydroxyl substitution. Phenolic compound as antioxidant

specified by its ability in capturing free radical and ROS (*reactive oxygen species*) (Huang et al., 1992). Free radical and active oxygen is one of foreign body that suppress body immune; and antioxidant properties related to the immunostimulant properties. Double bond outside the aromatic ring of phenolic compound might determine its reactivity. Besides, the more aromatic nucleus being substituted result in lowering π bond thereby lowering its reactivity and also activity and capacity of phagocytosis. Double bond in the side chain block by methoxy group subsistent in compound **[2]** and **[3]** result in decreasing reactivity so that the immunostimulant activity of both compound were lower compare to compound **[1]**.

CONCLUSION

All of the three compounds isolated from *Z. cassumunar* Roxb rhizome which were, **[1]** (*E*)-4-(3',4'-dimethoxyphe-nyl)but-3-en-1-ol), **[2]** ((*E*)-4-(2',4',5'-trimethoxyphenyl)but-3-en-1-ol dan **[3]** (*E*)-4-(3',4',1-trimethoxyphenyl)but-3-en-1-ol) showed the activity as immunostimulant through stimulation mouse macrophage cells. Compound **[1]** (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol, had higher activity compare to two other compounds (compound **[2]** and **[3]**). The *in vivo* immunostimulant activity test of active compound from *Z. cassumunar* Roxb rhizome and its relationship of structure and activity need further research.

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The Effect of Crude Extract of *Pandanus conoideus* Lamb. var. Yellow Fruit on Apoptotic Expression of the Breast Cancer Cell Line (T47D)

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ABSTRACT

A mechanism controlling a growing cancer cells is by a programmed cell death (apoptosis). The wildtype-*p53* enable to stop cleaves that follow DNA repair or cell death (apoptosis). The mutation of wt-*p53* caused loosing its ability to inhibit cancer cells proliferation. Healing methods like surgery, radiation, immunotherapy and chemotherapy still have some weaknesses, and clinical medicine to cancer is also still has any dissatisfactory. Much of chemotherapy was not given optimal result yet, because no specific action to cancer cells only, but also to the normal cells. These problems encourage important effort to find specific and sensitive anticancer. Empirical evidence indicates that the crude extract of *Pandanus conoideus* Lamb var. yellow fruit has potential effect as an anticancer. Method of Freshney was used in growing T47D cell line, counting cells was done by direct counting, and apoptotic evaluation was done by TUNEL enzymatic labeling assay. The results of the research demonstrated that the LC50 of yellow fruit extract are 0.25 µL/mL. The percentage of apoptotic of 0.125 µL/mL, 0.0625 µL/mL, and 0.03125 µL/mL are 34.38±2.26, 30.03±3.87 and 21.07±1.14 respectively.

Key words: T47D, Apoptotic, *p53*, *Pandanus conoideus* Lamb. var. yellow fruit.

INTRODUCTION

There are more than 30 kinds of *Pandanus* sp. (Widiyanto, 2006). Fourteen taxon from Pandanaceae were classified into red fruit, and wellknown for its beneficial use, while the rest of them belong to the group of yellow fruit, namely awone mengkaki by local people of Serui. *Pandanus conoideus* Lamb. at present is known by local people in that area as the red fruit, and this plant endemically growing in Papua. Four varieties of *P. conoideus* are cultivated by peoples in that area because its economical value as medicinal plant. Those are *P. conoideus* var. long red fruit, short red fruit, brown fruit, and yellow fruit. The importance of those fruits as traditional medicine provide prominent source as of new anticancer agent originated from Indonesia. The red fruit plant at present undergo over-exploitation, and its population reduced drastically due to utilisation of its fruits by traditional medicine producers both from Indonesia and other country. Empirical experience shows that *P. conoideus* var. yellow fruit (later abbreviated as yellow fruit) could act as anticancer, however, the mechanism underlying the inhibition of cancer cell by this fruit is still not fully understood yet.

The yellow fruit has been analysed by I Made Budi the person who found the red fruit, it contain tocopherol and β-carotene higher than that of red fruit (personal communication, 2007). Natural subtances found in this fruit such as carotene (9,500 ppm), β-carotene (240 ppm), tocopherol (10,400 ppm), and also oleic acid, linoleic acid and decanoic acid of omega 3 and omega 9 are known as powerfull antioxidant, aiding in preventing many deseases including cancer.

Breast cancer is the most common cause of death from cancer among women in the world. Mostly the victim of breast cancer (60-70%) was because of too late in testing it, so that it causes their death (Klauber-DeMore et al*.,* 2001). It globally takes the second place of women death after cervic cancer. In Indonesia, victim of breast cancer gradually increases by year, whereas United State reports that 27 in 100.000 (18%) of death are caused by breast cancer (Tjindarbumi and Mangunkusumo, 2002; Meiyanto et al*.,* 2006).

The main problem of chemoterapi is in it's low selection in anti-cancer medicine (Valeriote et al*.,* 2002; Kinghom et al*.,* 2003; Jenie and Meiyanto 2007). The use of radiation for theraphy like chemotheraphy and hormonal therapy, could result in

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any other effects to human body such as hairfall, skin getting darker (Jiang et al*.,* 2004). This problem has lead to promote the use of traditional medicine, which is generally believed to have less side effect (Sugiyanto et al*.,* 2003). One of the strategy to find the compound that works as target of its action to several gene which regulate the growth or proliferation of the cells (Gibbs, 2000).

The T47D breast cancer cell line (from ATCC, American Tissue and Culture Collection) is cell taken from epitelium of mamae ductus cell suffering malignation. This cell has a gene mutation of *p53* in the positive of amino acid 194th, with the fenilalanin amino acid (M;194F) (Nigro et al*.,* 1989). The mutation at the *p53* gene often found following a non regulated genetic as long as carsinogenesis in mostly tumor kind, included the breast cancer and cancer-derived cell lines (Smardova et al*.,* 2005). In this cells *p53* mutation occurred at the 194 residue (in the zinc binding domain L2), so that *p53* loose its function. If the *p53* is not link with DNA, so that the potential of regulating cell cycle and apoptosis could be reduce or completely lost (Schafer et al*.,* 2000).

The main principle of the effecti**v**ity and potential selection of anti cancer could be dealed with mutated *p53*, so that the apoptosis of cancer cells could proceeded. In general, the objective of the reseach were to promote the use of the variety of plantation in Indonesia, by using the yellow fruit especially as an agent which is cytotoxic, and provide scientific reasons of the use of the yellow fruit as a cancer medicine in society. The research on the cytotoxicity effect of yellow fruit extract toward cell line of cancer T47D should be investigated by observing the effect of cytotoxicity in inhibition mechanism of the cell growth (cycle of the cell) and apoptosis especially in relation with the expression of the *p53* gene.

MATERIALS AND METHODS

The T47D growth was monitored according to the method described elsewhere (Freshney, 2000). The cells linkage was counted using 20 µL of cell suspesion, added with 180 µL of tryphan blue, and the cells was then counted with haemocytometer at the fase contrast microscope. The total of cells found then multiplied with liquidity factor and number 10^4/mL (Freshney, 1987). The extract of the yellow fruit was taken from fresh yellow fruit. Solution of the test made by dissolving DMSO (dimethyl sulfoxide) 0.25% filtered with microfilter with diameter of 0.22 µm until the suspencion is in homogenious condition and then put in a sterile cup as main solution. Cytotoxicity test was done by pouring in 100 µL complete medium consist of cell suspension with closeness around $2x10^5$ cells/mL into each well 96 hole micro culture.

The effect of inhibited kinetic proliferation after treatment with yellow fruit toward cell T47D, inhibition test of proliferation kinetics was done for **72** hours, by counting the growth of cells (12, 24, 48, 72).

Apoptosis observation was done with TUNEL enzymatic labelling assay. Cell suspension was dropped to the slides and was incubated in poly-L lysine, fixed with 4% formaldehide in the PBS prior to permeabilization by Triton X-100. This step was followed by washing and rewashing using PBS. DNA was labelled with fluorescence-12-dUTP after TdT enzyme treatment. Slides were then covered with plastic coverslip and incubated at 37^{o}C for 1 hour, by avoiding direct exposure to sunlight/roomlight. To stop the reaction, coverslip was removed and SSC (Sodiu**m** Saline Citrate) was added for 2x5 minutes. Slide were washed with PBS before addition of propidium iodine, then rewashed with PBS. Sample were analized under fluorescence microscope. The apoptotic cell will appear in green, while non-apoptotic in red. The treatment to control the positive TUNEL was done by adding DNA-ase I enzyme after permeabilization with TritonX-100 and beeing washed with PBS.

RESULTS AND DISCUSSION

The results given in Table 1. shows that the extract of yellow fruit inhibits the growth/proliferation of T47D cell. This experiment was performed to know the cytotoxicity potential of the yellow fruit extract toward T47D cell with parameter of LC_{50} percentage of T47D cell.

Table 1. The percentage of T47D cell death after treatment with the crude extract of *P. conoideus* var. yellow fruit

Concentration (µL/L)	The percentage of death			Mean ±SD
	I	II	III	
Control	0	0	0	0±0
4	100	100	100	100±0
2	92.68	95	92.68	93.45±1.34
1	72.09	68.18	70.45	70.24±1.96
0.5	59.57	58.33	59.57	59.16±0.72
0.25	50	50	50	50±0
0.125	40.38	39.62	48.83	42.94±5.11
0.0625	32.14	31.58	31.58	31.77±0.32
0.03125	26.66	26.23	26.23	26.37±0.25
0.0015625	20.63	19.05	19.05	19.58±0.91
0.0078125	13.64	13.64	13.43	13.57±0.12
0.00390625	5.7	7.04	5.7	6.15±0.77

LC_{50} of yellow fruit toward T47D cell after 24 hours incubation is 0.25 µL/mL. According to Ueda et al*.* (2002) the extract of plantation with LC_{50} < 100 g/mL is potential to be developed as anti cancer. In this experiment, DMSO was used as solvent despite of water since the extracted compound was difficult to dissolve in water. Nogaki et al. (1998) reported that DMSO does not disturbing the growth of HL-60 cell and HSC-40, so that it can be used as solvent. We assumed that 0.7% DMSO used in this experiment will not significantly influence percentage of life and morphology of T47D cell.

Based on Figure 1. Significant effect of the yellow fruit extract on T47D death cell was observed.

Correlation between extract concentration and the level of of expression of *p53* mutant is shown with r value of 0.967. This figure shows that the extract concentration under LC_{50} inhibited the level of expression of *p53* significantly. This possibly caused by the excistance of the bioactive compound (tocopherol, β-carotene and carotene) in the extract which inhibit proliferation of T47D cells. Research by Carlisle et al*.* (2000) found that tocopherol could induce apoptosis and promote the expression of *p53* in the lung cancer (HLF cell). Moreover, the combination of tocopherol and vitamine A shown that it could inhibit the growth of metastasis in breast cancer cell in transgenic experiment (Albright et al*.,* 2004). The activition of the *p53*-wildtype might happen due to the reactivation of *p53* mutant. Beside this, there is also possibility that the reactivation of biological function of *p53*-wtp which was done by some compound in the yellow fruit.

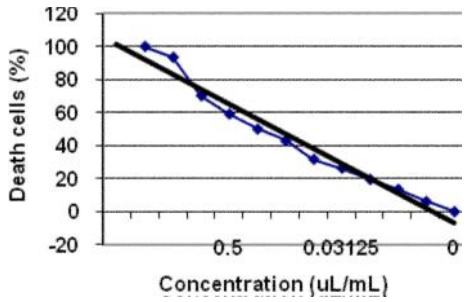

Figure 1. The effect of the crude extract of *P. conoideus* var. yellow fruit on death of T47D cell.

In general, the treatment with yellow fruit extract could significantly inhibit the growth of T47D cell as indicated by doubling time test (Table 2). Addition extract of yellow fruit with dosis of 0.125 uL/mL made a reaction the doubling time from 20.362 hours become 37.989 hours (1.86 time), while at the concentration of 0.0625 µL/mL and 0.03125 µL/mL its reaction time longer and the value of doubling time become 27.376 hours (1.34 times) and 23.220 hours (1.14 times).

Table 2. The regression similarity between number of cells in certain incubation time and the doubling time.

Material	Concen-tration (µL/mL)	The line equation of the incubation time versus the amount of cells	Slope value	R^2	Doubling time value
Crude extract of P. conoideus var. yellow fruit	Control	Y=0.1988x+0.562	0.1988	0.923	20.322
	0.03125	Y=0.159X + 0.91	0.159	0.929	23.220
	0.0625	Y=0.125X +1.18	0.125	0.944	27.376
	0.125	Y=0.088X +1.259	0.088	0.899	37.989

The discovery of the compound potential as cytostatic agent for *p53* is very important in the effort of healing cancer. This is due to the cell cancer in human being is usually caused by abnormal function of the *p53*, so that the growth of cancer could be inhibitted by applying such compound.

Based on the promoting effect of cytostatic due to the addition of yellow fruit extract, there is a possibility of activating *p53*-wildtype so that it will be the process of apoptosis. Nuclear fragmentation as marker of apoptosis in a microscopic scale show increased in the treatment of the extract in the concentration close to the LC_{50}.

Apoptotic test to know the effect of the yellow fruit extract in inducing apoptosis in T47D cells wad carried out by TUNEL enzymatic assay staining, and observed under the fluorescens microscope. The positive apoptosis cells will be in bright green color, whereas the viable cells will be in orange color (Rode et al., 2004). Both viable cells and apoptotic cells are presented in Figure 2. TUNEL is a quick method to identify and know the cells quantity which getting apoptosis in cell culture treatment (Wyllie, 2000; Wieder, 2005; Darzynkiewicz et al*.,* 2008).

Decreasing amount of viable cells after treated by yellow fruit extract indicated the blocking of cell proliferation that occurs related to the decreasing of protein synthesis as needed in the process of proliferation. The TUNEL enzymatic assay staining was employed to evaluate morphological change of T47D. The cells that undergo apoptosis appear as green colour, while the cells that experiencing the first apoptosis, their plasma membrane is still intact and will be in orange colour, but begin to form the chromatin condensation that resulting in green spots. It thus can be diferentiated between viable and necrotic cells. The percentage of apoptosis of T47D cells after 24 hours incubation with yellow fruit extract is presented in Table 3.

Table 3. The average percentage of apoptosis of T47D cells after 24 hours incubation with crude extract of *P. conoideus* var. yellow fruit.

Concentration (µL/mL)	Apoptosis (%±SD)
0.125	34.38±2.26
0.0625	30.03±3.87
0.03125	21.07±1.14

The death cells include (i) apoptosis,the progammed cells death that physiologically to balance and it is marked by DNA fragmentation, chromatin condensation, decreasing the cells size, cytoplasmic prominent and forming apoptosis body (ii) necrosis, death cells pathologically bringing of inflamation (King, 2000).

The *p53* protein code by *p53* gene is located in short arm of 17st human chromosom. Two types of *p53* protein is recognized i.e *p53* protein wild type and mutant type. The amount of *p53* protein wild type in nucleus is in small amount, labile and has short half life time so it could not be detected by immunohistochemical staining technique. This protein

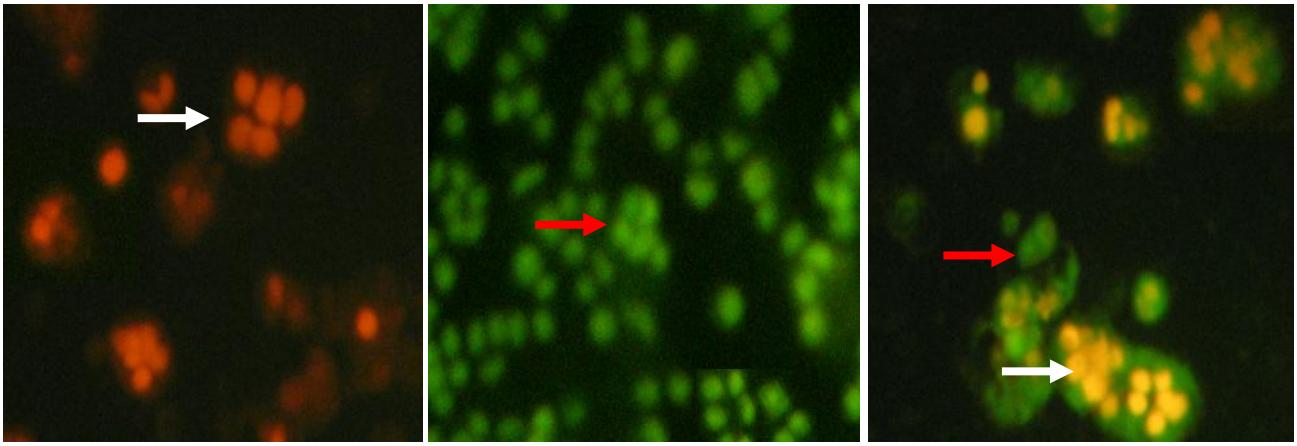

Figure 2. T47D cells after the treatment of crude extract of *P. conoideus* var. yellow fruit stained by TUNEL *enzymatic assay*. A. Negative control, B. Positive control of T47D cells undergo apoptotic (red arrow), C. Viable T47D cell (white arrow).

contribute to block cells proliferation, transcription, DNA repair and apoptosis, whereas *p53* protein mutant type contribute in blocking the *p53* protein wild type until cells proliferation loss its resistance (Brock, 1993).

Controling cell cycle is done in order to perform a normal cycle. Cdk (*cyclin dependent kinase*) like Cdk 4 Cdk 6, Cdk 2 along with *cyclin* (*cyclin* D, *cyclin* E, *cyclin* A and *cyclin* B) are main substances involved in cells cycle, which bringing on the movement from G1 to S or from G2 to M (Guardavoccaro et al*.,* 2000). MPF (*Maturation Promoting Factor*) along with Cdk and cycling become progressively trigger the cell cycle. The *p53* protein function as blocking of cell cycle if DNA damage taken place, and if a serious damage occurs could resulted in an apoptosis (Brown and Wouters, 1999).

CONCLUSION

The crude extract of *Pandanus conoideus* Lamb. var. yellow fruit is potential as anti cancer. The results of the research evidence that the LC_{50} of yellow fruit extract are 0.25 µL/mL. The percentage of apoptotic in the dosage of 0.125 µL/mL, 0.0625 µL/mL and 0.03125 µL/mL are 34.38±2.26, 30.03±3.87 and 21.07±1.14 respectively.

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The Effect of Seed Maturity, Temperature and Storage Period on Vigor of *Picrasma javanica* Bl. Seedling

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ABSTRACT

A study on the effect of seed maturity, temperature and period of storage on vigor of *Picrasma javanica* Bl. seedling was conducted at the Macropropagation Laboratory, Research Centre of Biology, LIPI, Cibinong, from March to September 2008. The research was arranged using Randomized Block Design with 3 factors and 3 replications, in which each replication had 20 samples. The first factor was stage of seed maturation with 2 levels i.e. pre-mature and mature stage; the second factor was storage temperature with 3 levels i.e. ambient temperature (28±1ºC), 20ºC and 5ºC; and the third factor was storage period with 4 levels i.e. 0, 1, 2, 3 months. The result showed that the germination time of *Picrasma javanica* seed was varies between 11-23 days. The mature seeds had better growth as compared to pre-mature seeds. Storage on the ambient temperature (28±1ºC) caused decrease of seed vigor, so is not suggested to seeds store in the ambient temperature. The storage under lower temperature (5ºC and 20ºC) was able to maintain seed vigor until 3 months storage.

Key words: seeds maturity, temperature, storage period, vigor, *Picrasma javanica* Bl. seed.

INTRODUCTION

The synonym of *Picrasma javanica* Bl. is *Picrasma nepalensis* A.W. Bennet, *Picrasma philippinensis* Elmer. The plant belongs to Simaroubaceae family. The local name of this plant is '*ki pahit', 'kaju paek'* which can be grown in Java island at 150-1400 m altitude (Heyne, 1997), or even at 1500 m (Hidayat, 2003). The bark of this plant can be used as a febrifuge to substitute for quinine (Heyne, 1997; Hidayat, 2003). The bark has been isolated and tested of its anti malaria activity *in vitro* using hexane extraction method (Saiin et al., 2003). Extract of leaves, seeds, stem and root of *P. javanica* Bl. consist of anti bacteria compound (Arbain and Sargent, 1987). Laloo et al. (2006) reported the use of *P. javanica* as anti-malaria medicine. Moreover Praptiwi et al. (2007) said that chemically active compound of bark extract of 'ki pahit' consist of alkaloid, flavonoid, saponin, tannin and steroid.

Heyne (1997) and Laloo et al. (2006) stated that the distribution of '*kaju paek'* in nature is rare recently. Uji (1995) and Bahktiar (2005) reported that *P. javanica* in Sumatra is accounted as endangered species. It is caused by the harvesting of material medicinal plant was done by logging, at the same time the cultivation not yet intensive done. The utilization of '*ki pahit'* bark as a material of medicine requires some adult trees or minimum has bark.

To fulfill the necessity, some effort needs to be carried out for large scale cultivation of this plant. In the effort of these cultivation, optimal number, best quality and good continuity of seed stokes are needed. Sutarno and Utami (2007) said that seed viability of *P. javanica* is low i.e. 10-15%, and in 30-32ºC temperate. Seed viability with 4.8-20.8ºC pre treatment during 51 days resulted higher viabilities and uniform seedlings i.e. 90-100%.

In the effort to support the supply of seeds stocks with best quality, continuous and optimal number of seeds stocks, study about the effect of seeds maturity, temperature and storage period on vigor of *P. javanica* seedling is needed. The objective of the study is to know vigor of *P. javanica* seed during storage period. From the result of the study, it is expected to obtain data about technique of seed selection and seed storage of *P. javanica*, so that the seeds with high quality will always available.

MATERIALS AND METHODS

This study was carried out at the Macropropagation Laboratory, Research Centre of Biology, LIPI, Cibinong-Bogor, West Java, Indonesia from March to September 2008. *P. javanica* obtained from plant collection of Bogor Botanical Gardens was used as raw material in this study.

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Seed was harvested and separated into two different stages of maturity based on morphology characteristic of fruit peels color; pre-mature represented by white orange, and mature by green tosca. Seeds storage were done by wrapping with aluminum foil, and further germinated on plastic boxes upon each treatment. Prior germination, part of seeds were taken as sample to examine water content by cutting about 5 g the seeds, oven at 105ºC until constant was obtained, the water content based on fresh weight (ISTA, 1985). Besides, ion leakage of the seeds also tested by soaking of 5 g seeds in 50 mL deionized water during 24 hours at 15ºC, its water solution measured with conductivity meter in μScm^{-1} per g of seeds (Hanson, 1983).

The study was arranged by using Randomized Block Design in Factorial with 3 factors and 3 replications, in which each replication had 20 samples (for germination percentage), whereas for seeds vigor was taken 3 samples of seedling aged 2 months. The first factor was stage of seed maturity with 2 levels i.e. pre-mature and mature stage; the second factor was storage temperature with 3 levels i.e. ambient temperature (28±1ºC), 20ºC and 5ºC; and the third factor was storage period with 4 levels i.e. 0, 1, 2, 3 months.

The observation was started from germination seeds until 2 months ages. The parameters were observed i.e. germination percentage (to know the seeds viability), seedling height, number of leaves, leaves length, leaves width, stem diameter, number of roots and seedling fresh weight (for vigor parameters). Seedling in germination boxes was watered daily, to maintain the relative humidity.

RESULT AND DISCUSSIONS

The result showed that time of germination of *Picrasma javanica* seed was varies between 11-23 days. After 2 months cultivation to germination of seed can be observed. At ebony seeds, the variation of time germination between 10-30 days (Sumiasri and Setyowati, 2006). The result of this study also showed that time of storage *P. javanica* seeds at ambient temperature during 2-3 months could not maintain seeds viability (germination percentage = 0%), in the pre-mature or mature seeds. Even on pre-mature seeds storage during 1 month at the ambient temperature seeds viability decrease until 0% (Table 6). It can be said that seeds storage of *P. javanica* at the ambient temperature cause death of seeds.

Seeds maturity stage

Seeds maturity is significantly correlated to seeds viability of *P. javanica*. It can be shown from the result of statistical analysis with significant different in all parameter observed at 5% Duncans test (DMRT). The mature seeds gave a better growth response as compare to pre-mature seeds shown by germination percentage (59.86; 44.44 %), seedling height (10.63; 8.20 cm), number of leaves (2.18; 1.87 leave), leaves length (3.44; 2.98 cm), leaves width (1.72; 1.47 cm), stem diameter (0.09; 0.08 cm) and number of root (6.45; 5.32), respectively. Although seedling fresh weight statistically showed no significant difference, but the fresh weight of mature seeds were higher than that of pre-mature seeds (Table 1).

Similar condition was also reported by Hartutiningsih and Utami (1998) in which seeds maturity stage of '*palem kipas*' was highly significant on its germination. The best germination showed by mature seed. Study on the '*palem putri*' study also revealed that seeds mature affected on germination rate and viability of seeds (Utami and Hartutiningsih, 2000). Moreover, the study of *Brucea javanica* seeds showed that the best germination percentage was in mature seed (Setyowati and Utami, 2008). According to Sutopo (1985) said that seed which harvested before physiologically mature stage has no high viability, at this stage, seeds were estimated lack of nutrient and impaired of embryonic development. The reason is after fruiting, weight and measurement of the seeds is increasing, until physiologically mature. The increasing of nutrient namely carbohydrate, lipid and protein, depends on species of seeds (Byrd, 1983). The reserve nutrient is substances which is used for hydrolysis during germination and transferred to embryo axis for seedling growth. Thus resulting on seed germination or seedling derived from mature seed to grow better than that from pre-mater seeds.

Seed water content

According to seed water content condition, early water content of pre-mature seeds is higher than mature seeds (Table 2), and there were no significant difference during storage. The pre-mature seeds showed decreasing water content during storage in 3 months. The water content about 39.47-33.14% for pre-mature seeds and 26.55-30.28% for mature seeds. The degradation of seeds viability estimated is caused by water content which is higher in pre-mature seeds. Sutopo (1985) said that seeds viability with high water content will undergo quick degradation. High water content will increase enzymes activities to accelerate respiration process, so that nutrition degradation was higher. The heat energy and humid condition stimulate microorganism which could damage seeds.

In this study, it could not decrease of water content, because in the preliminary study the water content could decrease viability of *P. javanica*, and estimated that the seeds belong to semi recalcitrant (N.W. Utami, 2009, private communication). On the contrary with '*adas*' seeds which belong to orthodox species, which could maintain of viability upon storage with low water content and low temperature (Utami and Sutarno, 2008).

Storage temperature

The storage temperature significantly affected to viability of *P. javanica* seeds. The seeds storage under ambient temperature (28±1ºC), which is 20ºC and 5ºC temperature, indeed able to maintain seeds viability and vigor, based on mean of germination and growth seedling parameters (Table 3). The storage temperature of 20ºC and 5ºC were no significantly different, except on leaves length and seedling fresh weight. The storage at 5ºC better than 20ºC (Table 3). According to Sutopo (1985), the high temperature on seeds storage causes seeds damage, due increased evaporation of liquid substances inside seeds, which lead to lose imbibition capacity and germination viability. The embryo protoplasm could be died because a part or all of seeds dryness. Sutopo (1985) also said that the optimum temperature for long term seed storage is between (-18) ºC to 0ºC.

Storage period

Generally, seeds without storage (0 month) posses better vigor compared with seeds which is stored (1, 2, and 3 months), these were demonstrated by all of observed parameters, except on germination percentage and seedling height, a control which is lower than 1 month seeds storage, but no significant difference based on Duncans Test 5% (Table 4). This phenomenon only showed variation of germination since no significant different was observed based on statistical analysis. Although 1 month storage showed highest mean (seedling high 10.75 cm; germination percentage 58.06 %) than 2, 3 months storage and control, but no significant different with control.

The result indicated from Table 4 known that 3 months period was range time where mature seeds of 'ki pahit' still endure seeds viability with high water content of fresh seeds, with storage temperature under 20ºC.

Table 1. The single effect of seeds maturity stage to viability and vigor of *Picrasma javanica* seedlings.

Seed maturated stage	Germination percentage (%)	Seedling height (cm)	Number of leaves (leaf)	Leaves length (cm)	Leaves width (cm)	Stem diameter (cm)	Number of root	Seedling fresh weight (g)
Pra-mature	44.44 b	8.20 b	1.87 b	2.98 b	1.47 b	0.08 b	5.32 b	0.28a
Mature	59.86 a	10.63 a	2.18 a	3.44 a	1.72 a	0.09 a	6.45 a	0.30a

Note: Number followed by the same letters on the same column was not significantly different based on DMRT 5%

Table 2. Effect of seeds maturity, temperature and storage period on water content of *Picrasma javanica* seeds

Seed maturated stage	Water content (%)									
	Control (pre-treatment)	1st month			2nd month			3rd month		
		Ambient temperature (28±1ºC)	20ºC	5ºC	Ambient temperature (28±1ºC)	20ºC	5ºC	Ambient temperature (28±1ºC)	20ºC	5ºC
Pre-mature	37.37	38.92	39.47	37.41	38.16	35.69	34.29	36.27	35.81	33.14
Mature	29.57	29.28	29.98	30.28	29.06	26.99	26.55	28.71	27.11	26.67

Table 3. Effect of storage temperature to viability and vigor of *Picrasma javanica* seedling

Storage temperature (ºC)	Germination percentage (%)	Seedling height (cm)	Number of leaves (leaf)	Leaves length (cm)	Leaves width (cm)	Stem diameter (cm)	Number of root	Seedling fresh weight (g)
Ambient temperature (28±1)	24.58 b	4.14 b	0.90 b	1.54 c	0.74 b	0.04 b	2.83 b	0.13 c
20	70.21 a	12.10 a	2.67 a	3.88 b	1.95 a	0.10 a	7.10 a	0.34 b
5	61.74 a	12.04 a	2.51 a	4.24 a	2.10 a	0.10 a	7.77 a	0.39 a

Note: Number followed by the same letters on the same column was not significantly different based on DMRT 5%

Table 4. Effect of storage period to viability and vigor of *Picrasma javanica* seedlings

Storage period	Germination percentage (%)	Seedling height (cm)	Number of leaves (leaf)	Leaves length (cm)	Leaves width (cm)	Stem diameter (cm)	Number of root	Seedling fresh weight (g)
0	50.83 ab	9.59 ab	2.61 a	4.32 a	1.94 a	0.10 a	7.67 a	0.40 a
1	58.06 a	10.75 a	2.18 b	3.02 b	1.64 b	0.08 b	5.69 b	0.26 b
2	56.39 a	8.53 b	1.67 c	2.71 b	1.37 c	0.07 c	6.35 b	0.22 b
3	42.35 b	8.65 b	1.61 c	2.75 b	1.39 c	0.07 d	3.67 c	0.26 b

Note: Number followed by the same letters on the same column was not significantly different based on DMRT 5%

Table 5. Effect of seeds maturity, temperature and storage period to ion leakage of *Picrasma javanica* seeds.

Seed maturated stage	Ion leakage (μScm^{-1})									
	Control (pre-treatment)	1st month			2nd month				3rd month	
		Ambient temperature (28±1ºC)	20ºC	5ºC		Ambient temperature (28±1ºC)	20ºC	5ºC		Ambient temperature (28±1ºC)
Pre-mature	4440.00	7580.63	5803.98	4388.11	21802.58	8856.62	3637.05	476285.20	14295.43	4917.18
Mature	4780.61	6658.77	3669.43	4559.53	24311.33	4303.43	4141.13	374338.90	7918.09	4705.04

Table 6. Effect of treatment combination on seed maturity, temperature and period storage to viability and vigor of *Picrasma javanica* seedling.

Seed maturated stage	Storage temperature (ºC)	Storage periode (month)	Germination percentage (%)	Seedling height (cm)	Number of leaves (leaf)	Leaves length (cm)	Leaves width (cm)	Stem diameter (cm)	Number of root	Seedling fresh weight (g)
Pre-mature	ambient 28±1	0	53.33	8.91	2.22	4.11	1.88	0.10	6.89	0.38
		1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20	0	53.33	8.91	2.22	4.11	1.88	0.10	6.89	0.38
		1	76.67	13.17	3.00	3.31	1.90	0.10	6.44	0.32
		2	86.67	13.11	2.56	4.20	2.04	0.11	9.33	0.34
		3	60.00	10.83	2.78	3.67	1.96	0.10	4.56	0.40
	5	0	53.33	8.91	2.22	4.11	1.88	0.10	6.89	0.38
		1	63.33	10.33	2.61	3.39	1.82	0.10	6.22	0.34
		2	83.33	12.28	2.55	4.50	2.04	0.10	10.89	0.41
		3	53.33	11.78	2.22	4.33	2.10	0.10	5.66	0.33
Mature	ambient 28±1	0	48.33	10.28	3.00	4.53	2.00	0.10	8.44	0.42
		1	85.00	13.95	2.00	3.67	2.013	0.10	7.33	0.26
		2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20	0	48.33	10.28	3.00	4.53	2.00	0.10	8.44	0.42
		1	75.00	12.22	2.89	3.52	1.71	0.10	6.56	0.30
		2	85.00	13.39	2.56	3.36	1.86	0.11	8.78	0.22
		3	76.67	14.89	2.33	4.39	2.23	0.10	5.78	0.36
	5	0	48.33	10.28	3.00	4.53	2.00	0.10	8.44	0.42
		1	88.33	14.83	2.55	4.22	2.36	0.10	7.56	0.36
		2	83.33	12.39	2.33	4.22	2.29	0.12	9.113	0.36
		3	81.67	16.61	2.67	4.78	2.34	0.10	7.33	0.51

This could also be observed on ion leakage test, in which mature seeds storage at 5ºC during 3 months shows no increasing of ion leakage concentration i.e. 4559.53; 4141.13 and 4705.04 μScm^{-1}, for storage 1, 2 and 3 months, respectively, there were no significantly different with control (4780.61 μScm^{-1}). However, seeds storage at 20ºC showed a few ion leakages i.e. 7918.09 during 3 month storage. The higher enhancement of ion leakage was shown by storage at ambient temperature i.e. 374338.90 μScm^{-1}. Consistently, storage at ambient temperature causes a highly increase of ion leakage concentration. The ion leakage was a reflection on cell membrane degradation. Higher ion leakage concentration, more clear to know of seeds damage indication (Copeland, 1976). It causes decreasing of seeds viability.

Effect of treatment combination

The pre-mature seeds which is stored at the ambient temperature (28±1ºC) were unable to maintain its viability, so the storage of seeds at 1 month could not germinated (0%) (Table 6). The mature seeds could be germinated at 1 month storage; however at 2 months storage the seeds could not be germinated. This phenomena was caused by ion leakage enhancement which become higher and higher during storage at the ambient temperature, i.e. the early of ion leakage of pre-mature seeds (4440.00 μScm^{-1}); increase to 7580.63; 21802.58; 476285.20 μScm^{-1}, for storage on 1, 2 and 3 months, respectively (Table 5). Decreasing of seeds viability during storage perhaps caused by plasma membrane damage so that ion leakage occurred. Transportation of substance, carbohydrate hydrolysis and transpiration enzyme activities were disturbed and causes on germination failed (Hanson, 1983). Increasing on ion leakage was indicated by increasing of ions and soluble substance in soaking solution. It shows positive correlation between increasing of ion leakage and seeds deterioration (Hartutiningsih and Utami, 1996/1997).

The seeds storage until 3 months at 5 and 20ºC, still could maintain seeds viabilities and vigor, for

mature or pre-mature seeds (Table 6). However, seeds viability and seedlings growth of mature seeds are better than pre-mature seeds; therefore mature seeds were preferable recommended for seeds storage (Sutopo, 1985). That food supplement transferred for growth of seedling after germination, so generally viability and vigor of mature seeds are better than pre-mature seeds (Byrd, 1983).

The germination percentage on control of pre-mature seeds was 53.33% higher than mature seeds (48.33%) (Table 6), because ion leakage stage of mature seeds ((4780.61 μScm^{-1}) are higher than pre-mature seeds (4440.00 μScm^{-1}) (Tabel 5). The phenomena perhaps caused by physical condition of seeds selected as sample in this study, where the seeds sample was selected randomly. The germination percentage increased during storage at 20ºC and 5ºC, it was supported by data of concentration ion leakage where it were not highly significant different with control. During storage at 20ºC and 5ºC, the seeds still undergo maturation process, so that food supplement in the embryo was optimum enough for germination process, this causes the increasing on seeds germination.

CONCLUSSIONS

The conclusions of this study were (i) the first germination of *Picrasma javanica* seed was varies between 11-23 days., (ii) The mature seeds had better growth as compared to pre-mature seeds, (iii) Storage on the ambient temperature (28±1ºC) caused decrease of seed vigor, (iv) The storage under lower temperature (5ºC and 20ºC) was able to maintain seed vigor until 3 months storage.

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