UNIVERSITI PUTRA MALAYSIA

MOLECULAR GENETIC CHARACTERIZATION OF DIFFERENT ACCESSIONS OF CENTELLA ASIATICA

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By

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

MOLECULAR GENETIC CHARACTERIZATION OF DIFFERENT ACCESSIONS OF *CENTELLA ASIATICA*

By

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January 2003

Chairperson : **Assoc. Prof. Siti Khalijah Daud, Ph.D.**

Faculty : **Science and Environmental Studies**

Centella asiatica or locally known as "pegaga" belongs to the *Apiceae* family. This medicinal plant is one of the most important medicinal herbs, and widely used in health foods, pharmaceutical and cosmetic industries. Twelve accessions of *C. asiatica* planted in MARDI, originated from different locations in Peninsular Malaysia, were used for this study. Phenotypic differences among these accessions are not very distinct, thus this study is undertaken to determine whether there are any genetic differences.

DNA markers, unaffected by environmental or physiological factors, have potential utility in the characterization of plant species. High discriminating power of this class of markers demonstrated uniformity and stability within genetically complex cultivars. Good quality DNA was extracted from leaf samples using conventional hexadecyltrimethylammonium bromide (CTAB) method. Two PCR-based DNA markers

system, namely Amplified Fragment Length Polymorphisms (AFLPs) and Long Primer Randomly Amplified Polymorphic DNA (LP-RAPDs), were employed. This study has successfully analyzed the genetic relationships among the accessions of *C. asiatica*. Two phylogenetic trees had been constructed from the unweighted pair group method with arithmatical average (UPGMA) pairwise analyses. Genetic distances was calculated based on the Dice similarity index. From both analyses, the CA01 and CA02 as well as CA05 and CA06 were closer (D=0.119) within the same cluster indicating that they are closely related. Based on the genetic distances, CA10 represented as highest distant group in the LP-RAPDs analyses whereas CA03 represented as highest distant group in the AFLPs analyses. Furthermore, diagnostic band with highest molecular weight (3000 bp) was found in CA10 by using long primer PEH A3. The amplification of CA03 genotypes with AFLP primer pair ACG/CTA has shown a unique DNA profile. In addition, CA03 is easily distinguish from other accessions morphologically due to its wavy shape of the plant leaf.

Different levels of genetic diversity among the accessions suggested that all the accessions are genetically non identical. CA05 showed the lowest percentage of polymorphism within the accession, approximately 6.18 % (LP-RAPDs) and 8.15% (AFLPs). Both techniques employed 6 primers and adequate DNA markers were obtained. However, the AFLP technology produced relatively greater amount of DNA markers, 3616 polymorphic bands, compared to LP-RAPD which had only 773 polymorphic bands. Both marker systems have successfully described genetic diversity of *C. asiatica* although both the AFLPs and LP-RAPDs are dominance inherited markers.

Abstrak tesis yang dikemukakan kepada Senat Universit Putra Malaysia sebagai Memenuhi keperluan untuk ijazah Master Sains.

PENENTUAN CIRI-CIRI GENETIK MOLEKUL ANTARA ASESI *CENTELLA ASIATICA* YANG BERLAINAN

Oleh

WONG SOOK MUN

Januari 2003

Pengerusi : Prof. Madya Siti Khalijah Daud, Ph.D.

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Centella asiatica atau dikenali sebagai pegaga oleh penduduk tempatan tergolong dalam Famili *Apiceae*. Ia merupakan herba yang penting dalam industri perubatan dan kosmetik. Dua belas asesi pegaga yang ditanam di MARDI diperolehi dari lokasi yang berlainan di Semenanjung Malaysia. Perbezaan fenotip antara asesi ini tidak berapa ketara. Oleh itu, kajian ini dijalankan untuk menentukan perbezaan dari segi genetiknya.

Penentu DNA yang tidak dipengaruhi oleh faktor-faktor sekitaran atau fisiologi mempunyai potensi untuk digunakan dalam mencirikan spesies tumbuhan. Kuasa diskriminasi yang tinggi bagi kelas penanda DNA ini dapat menunjukkan keseragaman dan kestabilan dalam tanaman yang mempunyai genetic yang kompleks.

DNA yang berkualiti diekstrak daripada semua sampel daun dengan kaedah CTAB yang konvensional. Dua kaedah berasaskan PCR iaitu AFLP dan LP-RAPD telah digunakan. Kajian ini berjaya menganalisis perhubungan genetik antara asesi-asesi pegaga. Dua pokok filogenetik telah dibina berdasarkan analisis UPGMA yang berasaskan Indeks Keserupaan Dice. Keputusan menunjukkan antara asesi CA01 dan CA02 serta antara asesi CA05 dan CA06 adalah berkait rapat antara satu sama lain dengan jarak genetik bernilai 0.119. Berdasarkan jarak genetik, CA10 merupakan asesi yang mempunyai hubungan genetik yang paling jauh daripada asesi yang lain berdasarkan LP-RAPDs, manakala CA03 merupakan kumpulan yang paling jauh jarak genetiknya dengan asesi yang lain berdasarkan penanda AFLPs. Selain itu, kewujudan jalur penentu yang paling berat jisim molekulnya (3000 bp) bagi CA10 dengan primer panjang PEH A3 dan juga keunikan corak DNA bagi CA03 dengan pasangan primer ACG/CTA telah menyokong keputusan tersebut. Selain itu, perbezaan CA03 amat ketara berbanding dengan asesi lain dari segi morfologi iaitu mempunyai daun berbentuk ombak.

Perbezaan aras diversiti genetik yang berlainan telah ditunjukkan di antara asesi dan ini menunjukkan tidak wujud kesamaan genetik di antara dua belas asesi pegaga tersebut. Asesi CA05 telah menunjukkan peratus polimorfisme terendah dalam asesi, iaitu 6.18 % (LP-RAPD) dan 8.15 % (AFLP). Kedua-dua teknik menggunakan 6 primers dan penanda DNA yang mencukupi telah diperolehi. Walau bagaimanapun, teknologi AFLP telah menghasilkan jumlah DNA yang agak banyak, berjumlah 3616 jalur polimorfisme iaitu jauh lebih banyak berbanding dengan 773 jalur polimorfisme yang dihasilkan oleh LP-RAPD.

Kedua-dua sistem penanda ini berjaya menggambarkan diversiti genetik pegaga walaupun kedua-dua AFLP dan LP-RAPD adalah penanda yang bersifat dominans.

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I certify that an Examination Committee met on 8th January 2003 to conduct the final examination of Wong Sook Mun on her Master of Science thesis entitled "Molecular Genetic Characterization of Different Accessions of *Centella asiatica*" in accordance with Universiti Pertanian Malaysia (Higher Degreee) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are follows:

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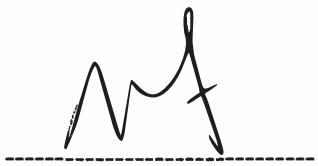
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



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ABBREVIATIONS AND SYMBOLS

%	Percent
°C	Degree centrigrade
λ	Lamda
γ	Gamma
bp	Base pair
kb	Kilo base
U	Units
ml	Milliliter
mm	Micrometer
µl	Microliter
µM	Micromolar
µg	Microgram (s)
1 X	One time
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTPs	Deoxynucleotide triphosphates
mer	Oligomer
EDTA	Ethylenediaminetetracetic acid
A_{280}	absorbance at wavelength 280 nm
A_{260}	absorbance at wavelength 260 nm
nM	nanometer
mM	milimolar
DNA	Deoxyribonucleic acid
$MgCl_2$	Magnesium chloride
OD	Optical density
RAPD	Randomly Amplified Polymorphic DNA
LP	Long primer
AFLP	Amplified Fragment Length Polymorphisms
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecylsulphate
UV	Ultra violet
TBE	Tris-borate EDTA
RNA	Ribonucleic acid
w/v	Weight per volume
V	Voltage
UPGMA	Unweighted pair-group method with arithmetical averages

CHAPTER 1

INTRODUCTION

A medicinal plant is defined as any plant, one or more of its structures, containing substances that can be used for therapeutic semi-synthesis. Morphologically, medicinal plant species can be classified into trees, shrubs, herbs and ferns. There are approximately 500,000 plant species occupied the terrestrial habitat. About 7% to 14% (35,000 to 70,0000) of these species are used as medicinal plants worldwide. A large portion of these medicinal plants is found in the tropical rainforest biome (Allegra, 1984).

In Malaysia, rainforest biome covers around 58.1% (19.12 million hectares) of the country's total land area. This area supports over 20,000 plant species, of which more than 2000 species were reported of having medicinal values. About 200 medicinal plants species used by different ethnic groups. The most popular local medicinal plant species, such as *Centella asiatica* (Pegaga), *Eurycoma longifolia* (Tongkat Ali), *Kaemferia galanga* (Cekur), *Zingiber officinale* (Halia), *Cymbopogan citratus* (Serai), *Curcums domestica* (Kunyit), *Andrographs paniculate* (Hempedu bumi), are widely consumed traditionally (Aziz, 1973).

Amongst these medicinal plants, *Centella asiatica* (Linn.) Urban belonging to *Apiceae* (also known as umbelliferae) family, was chosen in this study.

The study is part of the Malaysia-MIT Biotechnology Partnership Program (MMBPP). The twelve accessions of *C. asiatica* were coded as CA01, CA02, CA03, CA04, CA05, CA06, CA07, CA08, CA09, CA10, CA11, and CA12.

All the accessions were sampled from different places in Peninsular Malaysia. These accessions were collected and planted in nursery by a senior scientist, Madam Indu Bala Jaganath from the Strategic Environment and Natural Resource Center, MARDI.

Centella asiatica is indigenous to the Southern United States, but is widely distributed in Asia and South Africa (Grieve, 1974). This plant has increased its popularity as a vegetable crop due to its medicinal and nutritional values. It is commonly used as a source of raw material in health food, pharmaceutical and cosmetic industries. The World Health Organization (WHO) had recommended *C. asiatica* as one of the most important medicinal plant species to be conserved and cultivated (Belcaro *et al*., 1989).

For improving any reasonable gene conservation program, the precise understanding of the organization of the existing genetic diversity and its factors are emphasized. Thus, biotechnological approaches have a significant impact and play a major role in plant genetic resources conservation.

Traditional breeding procedures are mainly based on the evaluation of morphological characteristics on individual plants, in which environmental factors can influence the results. To overcome this drawback, molecular markers that unaffected by external environmental conditions can be used

effectively in evaluating genetic variation within plant species (Powell *et al*., 1996).

According to Waugh and Powell (1996), genetic variation or polymorphisms revealed by molecular markers could help to select priority areas for conservation and provide vital information for the development of genetic sampling and improvement. Various types of molecular markers, such as allozymes, RFLPs (Restriction Fragment Length Polymorphisms), RAPDs (Randomly Amplified Polymorphic DNA), AFLPs (Amplified Fragment Length Polymorphisms), are widely used to distinguish between species and between varieties within a species. Recently, these technologies have received great attention in agriculture and horticulture (Cooke, 1995; Smith, 1995; Movell *et al*., 1995; Cooke and Reeves, 1998).

A broad application of these markers has contributed to plant genetic resources management and also plant breeding programs (Bretting and WildrleChner, 1995). From various studies, DNA fingerprinting have proven to be more powerful tools than isozyme markers for assessing genetic diversity and polymorphisms among individuals in a particular species or even different populations of closely related species (Weising *et al*., 1995).

The LP-RAPDs assay is a modified RAPD protocol using longer primer, 18 to 24 oligomers, to replace the normal RAPD primer, which having only 8 to 10 bases in length (Gillings and Holley, 1997). This technique is based on the use