UNIVERSITI PUTRA MALAYSIA

ISOLATION, CHARACTERIZATION AND APPLICATION OF DNA MICROSATELLITE MARKERS IN MUNGBEAN (VIGNA RADIATA L. WILCZEK) AND OTHER SELECTED LEGUMES

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By

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Chairman: **Prof. Dr. Tan Soon Guan**

Faculty: **Science and Environmental Studies**

Mungbean (subgenus *Ceratotropis*) is an important food source in many parts of the world, particularly in Asia and Southeast Asia. It is an important source of plant protein and calcium, and is a good substitute for meat. Although it is an important crop, little is known about its genetic background. DNA markers, in particular microsatellites, are able to provide insights regarding the genetic structure and background of populations and thus would be of great benefit in mungbean improvement programs.

Three techniques were used to isolate microsatellite loci in mungbean, namely direct amplification of length polymorphism (DALP), 5' anchored PCR and random hybridizing microsatellites (RAHM). A total of 107 repeat sequences were identified of which 80% were microsatellite loci and 20% were cryptic simple regions. The majority of microsatellites were found using the 5' anchored PCR procedure which

proved to be the most efficient technique in the present study, while DALP did not produce any microsatellite.

Fourty-four microsatellite primer pairs were designed based on the mungbean DNA sequences obtained. Out of these, eight were unable to amplify the mungbean genome or gave irreproducible banding patterns and were subsequently discarded. The remaining 36 primer pairs gave excellent results upon polymerase chain reaction (PCR) amplification. In addition to mungbean-specific primers, six orthologous primer pairs from the common bean (*Phaseolus vulgaris*) were successfully used to amplify the mungbean genome. Thus, a total of 42 reproducible microsatellite markers were developed for use in mungbean.

Twenty-four primer pairs were used to evaluate the genetic variability in 11 populations representing three species of wild *Vigna* in Peninsular Malaysia. The three species of wild *Vigna* were *V. trinervia* (Beranang 1, Beranang 2, Banting, Triang, Bentong 1, Tangkak 1, Merapoh and Kg. Paya Mas), *V. reflexo-pilosa* (Bentong 2 and Tangkak 2) and *V. mungo* (Bukit Serdang). Primer pairs LR7315B and VJ3144B amplified two loci consistently, thus both these loci were scored independently, making the total number of loci scored for all the populations 26. The number of alleles per locus ranged from 1 to 12 with 4.6 as the average number. The total observed and expected heterozygosity across all 11 populations were 0.2858 and 0.4472, respectively. Characterization of the populations showed relatively high levels of genetic variation compared to previous studies using allozymes markers. Genetic distances were highest between populations of *V. trinervia* and *V. mungo*. Cluster analysis correctly differentiated the 11 populations according to their species.

Fourty-two primer pairs were used to evaluate levels of genetic variability in 49 mungbean accessions. The number of alleles per locus ranged from 1 to 6 with an average of 2.2 alleles. The total observed and expected heterozygosity across all 49 mungbean accessions were 0.1676 and 0.2423, respectively. It was found that the genetic variability in the cultivated mungbean accessions was more than twice lower than in wild *Vigna* populations. This indicates the availability of genomic materials for introgression into cultivated mungbean. In the cluster analysis of mungbean accessions, no definite association between geographical origin and genetic distance was found.

The fourty-two primer pairs developed for mungbean were also used to test for cross-amplifications in 15 other legume species. All the primers pairs were able to amplify the genomes of more than three legume species each. This indicates high levels of conservation among the DNA sequences of legumes. The successful cross-amplifications of mungbean primers in other legume species will save considerable amounts of time and valuable resources since the development of specific microsatellite markers for each legume species of interest is no longer a necessity.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai keperluan ijazah Doktor Falsafah

PEMENCILAN, PENCIRIAN DAN PENGGUNAAN PENANDA MIKROSATELIT DNA DALAM KACANG HIJAU (VIGNA RADIATA L. WILCEK) DAN LEGUM-LEGUM TERPILIH

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Kacang hijau (subgenus *Ceratotropis*) merupakan sumber makanan yang penting di kebanyakkan tempat di dunia, terutamanya di Asia dan Asia Tenggara. Ia merupakan sumber penting bagi protein tumbuhan dan kalsium, dan juga merupakan alternatif bagi daging. Walaupun ia merupakan suatu tanaman kontang yang penting, tidak banyak maklumat yang ada mengenai latarbelakang genetiknya. Penanda DNA, khususnya mikrosatelit, dapat memberi maklumat mengenai stuktur genetik dan latarbelakang populasi dan justeru, membantu dalam program peningkatan kacang hijau.

Tiga teknik telah digunakan untuk memencil lokus mikrosatelit dari kacang hijau iaitu, *direct amplification of length polymorphism (DALP), 5' anchored PCR* dan *random hybridizing microsatellites (RAHM)*. Sejumlah 107 jujukan berulang telah dikenalpasti di mana 80% terdiri dari lokus mikrosatelit dan 20% adalah kawasan-

kawasan *cryptic simple*. Kebanyakkan mikrosatelit dijumpai melalui teknik *5' anchored PCR* di mana ia merupakan teknik yang paling berkesan dalam kajian ini, manakala DALP tidak menghasilkan sebarang mikrosatelit.

Empatpuluh empat pasangan primer mikrosatelit telah direka berdasarkan jujukan DNA kacang hijau yang diperolehi. Daripada jumlah ini, lapan primer tidak dapat mengamplifikasikan genom kacang hijau atau memberikan corak jalur yang tidak menyakinkan dan ia tidak digunakan. Baki 36 pasangan primer menghasilkan keputusan yang baik selepas amplifikasi tindakbalas rantai polimerase (PCR). Di samping primer spesifik kacang hijau, enam pasangan primer ortologus dari kacang biasa (*Phaseolus vulgaris*) telah berjaya digunakan untuk mengamplifikasi genom kacang hijau. Oleh itu, sejumlah 42 penanda mikrosatellit telah dihasilkan untuk kegunaan dalam kacang hijau.

Duapuluh empat pasangan primer telah digunakan untuk menilai variasi genetik dalam 11 populasi yang terdiri daripada tiga spesies liar *Vigna* di Semenanjung Malaysia. Ketiga-tiga spesies liar *Vigna* adalah *V. trinervia* (Beranang 1, Beranang 2, Banting, Triang, Bentong 1, Tangkak 1, Merapoh dan Kg. Paya Mas), *V. reflexo-pilosa* (Bentong 2 dan Tangkak 2) dan *V. mungo* (Bukit Serdang). Pasangan primer LR7315B dan VJ3144B mengamplifikasi dua lokus secara konsisten dan ia telah dicatat secara berasingan, menjadikan jumlah keseluruhan lokus yang dicatit dalam semua populasi sebanyak 26. Bilangan alel setiap lokus berjulat antara 1 hingga 12 dengan purata bilangan alel setiap lokus sebanyak 4.6. Jumlah heterozigositi cerapan dan jangkaan bagi kesemua 11 populasi adalah 0.2858 dan 0.4472, masing-masing. Pencirian populasi menunjukkan kadar variasi genetik yang tinggi berbanding kajian

sebelumnya yang menggunakan penanda alozim. Jarak genetik adalah paling tinggi di antara populasi *V. trinervia* dan *V. mungo.* Analisa kelompok telah dapat membezakan 11 populasi tersebut menurut jenis spesiesnya.

Empatpuluh dua pasangan primer telah digunakan untuk menilai kadar variasi genetik dalam 49 jenis kacang hijau. Bilangan alel setiap lokus berjulat antara 1 hingga 6 dengan purata bilangan alel setiap lokus sebanyak 2.2. Jumlah heterozigositi cerapan dan jangkaan bagi kesemua 49 jenis kacang hijau adalah 0.1676 dan 0.2423, masing-masing. Hasil keputusan menunjukkan variasi genetik dalam kacang hijau yang dikultur adalah dua kali ganda lebih rendah daripada populasi liar *Vigna.* Ini menunjukkan terdapatnya bahan-bahan genomik sedia ada untuk dipindahkan ke kacang hijau yang dikultur. Dalam analisis kelompok aksesi kacang hijau, didapati tiada hubungan antara asalusul geografi dan jarak genetik.

Empatpuluh dua pasangan primer yang dihasilkan bagi kacang hijau telah digunakan untuk menguji amplifikasi-merentasi 15 jenis spesies legum. Kesemua pasangan primer dapat mengamplifikasi genom lebih daripada satu jenis spesies legum. Ini menunjukkan kadar pemuliharaan yang tinggi di antara jujukan DNA legum. Kejayaan amplifikasi-merentasi spesies legum dapat menjimatkan masa serta sumber penting memandangkan proses penghasilan penanda mikrosatelit khusus bagi spesies yang dikaji tidak lagi diperlukan.

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I certify that an Examination Committee met on the 28th January 2003 to conduct the final examination of Vijay Kumar on his Doctor of Philosophy thesis entitled "Isolation, Characterization and Application of DNA Microsatellite Markers in Mungbean (*Vigna radiata* L. Wilczek) and Other Selected Legumes" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as 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.

Vijay Kumar
Date: 6/03/2003

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ABBREVIATIONS

α	alpha
AFLP	amplified fragment length polymorphism
ATP	adenosine triphosphate
β	beta
bp	base pairs
Ci	Curie
dH_2O	distilled water
ddH_2O	double distilled water
deH_2O	deionized water
dNTP	deoxyribonucleotide
kb	kilobase
λ	lambda
LB	Luria-Bertani
mCi	millicurie
mM	millimolar
ng	nanogram
nmol	nanomole
PCR	polymerase chain reaction
pm	picomole
RAHM	random hybridising microsatellites
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rpm	revolution per minute
µg	microgram
µl	microliter
µM	micromolar
UPGMA	unweighted pair-group method with arithmetic mean
V	volts
w/v	weight/volume
xg	centrifugal force

CHAPTER 1

INTRODUCTION

Mungbean is an important food crop in the developing world, particularly in Asia where 98% of the world's mungbean is grown. It is an excellent and cheap source of plant protein and calcium, and is well-suited for cultivation throughout the tropics and subtropics. It is used for food, forage and green manure.

Although it is an important crop, little is known about the genetic background of mungbean. This lack of information has lead to the loss of genetic variability or diversity in the plant. Such a loss cannot be regenerated since variation within a species is the result of very long evolutionary processes. Therefore, mungbean accessions that show high genetic variability are considered important genetic resources as they have greater potential for improvement and serve as invaluable resource for different selection criteria, especially when planning breeding or crossbreeding programs.

However, before implementing any breeding and/or crossbreeding programs, it is important that the genetic variability within and between accessions or varieties is determined. This would facilitate efficient sampling and utilisation of resources. The plant breeder can then use this genetic information to make informed decisions regarding the choice of genotypes to cross for the development of the cultivars or to facilitate the identification of diverse parents to cross in order to maximise heterosis or hybrid-vigour.

The estimation of genetic variation is often limited by the availability of polymorphic genetic markers. Traditionally, variation has been studied using morphological characters but with limitations. The use of isozymes to study variation brought renewed interest in the field and is still used by many researchers, as it is an efficient and cost-effective marker, however it was the use of DNA markers that led to a phenomenal increase of studies in population genetics.

Several types of DNA markers have been developed in recent years to assist in genetic analysis. These include Restriction Fragment Length Polymorphisms (RFLP), Amplified Fragment Length Polymorphisms (AFLP), Random Amplified Polymorphic DNA (RAPD), Direct Amplification of Length Polymorphisms (DALP), Random Amplified Microsatellites (RAM), and Variable Number of Tandem Repeats (VNTR) such as minisatellites and microsatellites. In spite of the many types of markers available, the most efficient and effective marker system is microsatellites which is also known as simple sequence repeats (SSR). Unfortunately, microsatellite markers have not been developed in mungbean because of the difficulty of their isolation. As a result, other types of markers such as isozymes, RFLP, RAPD, RAM and proteinase inhibitors have been used for phylogenetic and taxonomic purposes in mungbean with varying degrees of success.

1.1 Objectives

The mungbean is a convenient plant for genetic studies due to several reasons. It has a short life cycle, is naturally self-pollinated and easily cross-pollinated, a diverse germplasm collection is available and it may possibly serve as a model system for other commercially important legumes.

The primary objectives of this study are to:

1. identify and isolate microsatellite markers in mungbean,
2. characterize the microsatellite markers,
3. apply these microsatellite markers to study the genetic structure of wild and cultivated *Vigna* species.

The specific objectives of this study are to:

1. develop efficient methodologies for the isolation of microsatellite markers in mungbean,
2. estimate levels of heterozygosity and genetic distance using microsatellite markers in wild *Vigna* species collected from Peninsular Malaysia and cultivated accessions of mungbean.
3. estimate phylogenetic relationships among cultivars of mungbean,
4. test for cross-amplification of microsatellite primers in closely related species,
5. evaluate the inheritance of microsatellites from parents to offsprings.