ความหลากหลายทางพันธุกรรมของ Asian *Vigna* วิเคราะห์โดยใช้เครื่องหมายโมเลกุล AFLP และ SSR และการผสมข้ามชนิดระหว่าง *Vigna radiata* และ *Vigna* spp.

นางสาววรภา สีหลักษณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีการผลิตพืช
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2548

ISBN 974-533-540-1

GENETIC DIVERSITY OF Asian *Vigna* ANALYZED BY AFLP AND SSR MOLECULAR MARKERS AND INTERSPECIFIC HYBRIDIZATION BETWEEN *Vigna radiata* AND *Vigna* spp.

Worapa Seehalak

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Crop Production Technology

Suranaree University of Technology

Academic Year 2005

ISBN 974-533-540-1

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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วรภา สีหลักษณ์ : ความหลากหลายทางพันธุกรรมของ Asian *Vigna* วิเคราะห์โดยใช้เครื่องหมายโมเลกุล AFLP และ SSR และการผสมข้ามชนิดระหว่าง *Vigna radiata* และ *Vigna* spp. (GENETIC DIVERSITY OF ASIAN *Vigna* ANALYZED BY AFLP AND SSR MOLECULAR MARKERS AND INTERSPECIFIC HYBRIDIZATION BETWEEN *Vigna radiata* AND *Vigna* spp.) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.ปิยะดา ทิพยผ่อง, 160 หน้า. ISBN 974-553-540-1

จากการสำรวจในอดีตพบว่า ประเทศไทยเป็นแหล่งหนึ่งของ Asian *Vigna* การศึกษานี้มีวัตถุประสงค์เพื่อ (1) ประเมินความหลากหลายทางพันธุกรรมของ Asian *Vigna* จากประเทศไทยและประเทศใกล้เคียงโดยใช้เทคนิค AFLP (2) สำรวจและเก็บรวบรวม Asian *Vigna* ในจังหวัดเชียงใหม่และแม่ฮ่องสอน (3) วิเคราะห์ตัวอย่างที่เก็บมาโดยใช้เทคนิค SSR เพื่อตรวจสอบระดับ heterozygosity ในประชากรและหาความสัมพันธ์ระหว่างพืชแต่ละชนิด และ (4) ประเมินอัตราการผสมติดในการผสมข้ามชนิดระหว่าง *V. radiata* และ *Vigna* spp. การทดลองแบ่งเป็น 4 ส่วนตามวัตถุประสงค์ของการทดลอง การทดลองในส่วนแรกแสดงให้เห็นว่า ภาคเหนือของไทยและรัฐ Mandalay ของพม่า อาจจะเป็นแหล่งที่มีการนำเอา *V. umbellata* ป่ามาเพาะปลูก *V. hirtella* ที่วิเคราะห์แบ่งเป็น 2 กลุ่มที่มีความแตกต่างกันมาก แสดงว่าทั้ง 2 กลุ่มนี้อาจจะเป็นคนละชนิด ทั้งนี้จะต้องมีการศึกษาเพิ่มเติมถึงความแตกต่างระหว่าง 2 กลุ่มนี้ต่อไป ส่วน *V. mungo* ที่มีอยู่ในธรรมชาติในภาคเหนือของประเทศไทยนั้น เป็นพันธุ์ป่าของไทย ไม่ได้เกิดจากการปนเปื้อนมากับพันธุ์ปลูกที่ไทยนำเข้ามาจากประเทศอินเดีย จากการสำรวจในส่วนที่ 2 นั้น พบ Asian *Vigna* ทั้งหมด 4 ชนิด คือ *V. hirtella, V. minima, V. tenuicaulis* และ *V. umbellata* แต่ละชนิดพบในสภาพแวดล้อมที่ต่างกัน คือ *V. minima* ส่วนใหญ่พบบริเวณที่ร่มในป่าผลัดใบ *V. tenuicaulis* และ *V. umbellata* พบในที่ได้รับแสงมาก ส่วน *V. hirtella* พบในที่แสงรำไร อย่างไรก็ตาม ในบางประชากรพบพืชเหล่านี้หลายชนิดขึ้นอยู่ใกล้เคียงกัน การทดลองในส่วนที่ 3 ซึ่งตรวจสอบ heterozygosity ในประชากรนั้น พบว่าค่า heterozygous loci ต่อต้นมีระดับต่ำ โดยที่ *V. hirtella* มีค่านี้สูงสุด และตัวอย่างที่เก็บมา มีต้นหนึ่งที่มีอัลลีล ของ *V. hirtella* ร่วมกับ *V. minima* แสดงให้เห็นถึงการผสมข้ามโดยธรรมชาติระหว่างพืช 2 ชนิดนี้ จากการทดลองสามส่วนแรกนี้ แสดงให้เห็นว่าประเทศไทยมีศักยภาพในการเป็นแหล่งของความหลากหลายของ Asian *Vigna* ที่อยู่ใน subgenus *Angulares* รวมถึงแนวโน้มในการเกิดพันธุกรรมใหม่จากการผสมข้ามชนิดที่เกิดขึ้นตามธรรมชาติ ซึ่งอาจนำไปสู่การเกิดสปีชีส์ใหม่ ส่วนการทดลองสุดท้ายซึ่งเป็นการผสมข้ามชนิดนั้น การผสมสำเร็จเฉพาะคู่ผสมระหว่างถั่วเขียวพันธุ์ปลูก (*Vigna radiata*)

และ พันธุ์ป่า (*V. radiata* var. *sublobata*) ไม่ว่าจะใช้ถั่วเขียวพันธุ์ปลูกเป็นพ่อหรือแม่ก็ตาม ลูกผสมที่ได้มีลักษณะผสมระหว่างพ่อแม่และไม่เป็นหมัน ซึ่งได้รับการยืนยันจากการวิเคราะห์โดยใช้เทคนิค ISSR ว่า ลูกผสมนี้เกิดจากการผสมข้ามจริง นอกจากนี้ยังได้ลูกผสม 2 ต้นของ *V. mungo* x *V. stipulacea* จากการเพาะเลี้ยงคัพภะ แต่ลูกผสมตายหลังจากที่ย้ายจากอาหารเพาะเลี้ยงไปยังเวอร์มิคูไลท์ จึงไม่ได้ต้นลูกผสมที่สมบูรณ์

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา ยงยุทธ สัมฤทธิ์

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา

WORAPA SEEHALAK : GENETIC DIVERSITY OF ASIAN *Vigna* ANALYZED BY AFLP AND SSR MOLECULAR MARKERS AND INTERSPECIFIC HYBRIDIZATION BETWEEN *Vigna radiata* AND *Vigna* spp. THESIS ADVISOR : ASST. PROF. PIYADA THIPYAPONG, Ph. D. 160 PP. ISBN 974-553-540-1

AFLP/ASIAN *Vigna*/ DIVERSITY/ INTERSPECIFIC HYBRIDIZATION/ SSR/ THAILAND/ *Vigna radiata*

Based on the previous exploration, Thailand appeared to be a center of Asian *Vigna*. Therefore, the objectives of this study were to (1) determine genetic diversity of Asian *Vigna* species in Thailand and neighboring regions using AFLP techniques, (2) explore and collect Asian *Vigna* in Chiang Mai and Mae Hong Son provinces, (3) analyze individuals from collection sites by using SSR to gain an insight into the level of heterozygosity in populations and to determine the relationships among species, and (4) evaluate cross compatibility between *V. radiata* and *Vigna* spp. According to the objectives, this study was divided into four parts. The first part suggested that northern Thailand and neighboring Mandalay state of Myanmar were the probable centers of domestication for *V. umbellata*. While *V. hirtella* consisted of two well differentiated subgroups, suggesting that taxonomic revision may be necessary. Naturally growing *V. mungo* populations in northern Thailand appeared to be true wild species not contaminated from the Indian cultigens introduced to Thailand. From the exploration of Asian *Vigna* species in the second part, four *Vigna* species were found including *V. hirtella, V. minima, V. tenuicaulis*, and *V. umbellata*. These

species grew in different habitats. *V. minima* generally grew in shaded areas in the under-story of dry deciduous forests. *V. tenuicaulis* and *V. umbellata* were found in open area whereas *V. hirtella* grew in semi-shaded area. However, more than one species were found growing in close proximity at some sites. The third part of study showed low level of heterozygous loci per plant. The maximum number of heterozygous loci per plant was found in *V. hirtella*. One accession of *V. hirtella* showed high heterozygosity sharing alleles between *V. hirtella* and *V. minima*, suggesting that natural outcrossing might occur. The first three experiments show that Thailand has the potential to be a center of diversity for section *Angulares* of Asian *Vigna* as well as the tendency for new genetic recombination from natural interspecific hybridization to occur that could lead to speciation. Only the cross between *V. radiata* and its wild relative (*V. radiata* var. *sublobata*) was successful when using *V. radiata* either as paternal or maternal parent. The hybrids were fertile and showed intermediate morphology between their parents. These hybrids were confirmed by ISSR analysis that they were true hybrids. Two hybrid plants from *V. mungo* x *V. stipulacea* were obtained from embryo culture. However, they died soon after transferring from the media to vermiculite.

School of Crop Production

Academic Year 2005

Student's Signature Worapon Seehalak

Advisor's Signature Pinda Thipwong

ACKNOWLEDGEMENTS

This research could not be completed without the support of many people. With this I would like to express appreciation to the following persons:

Prof. Dr. Aree Waranyuwat, former advisor, for giving me the chance of receiving research fund through the Royal Golden Jubilee Ph. D. program, his kind support, guidance, and editing this thesis until nearly thesis defence.

Dr. Duncan Alexander Vaughan, co- advisor, Dr. Norihiko Tomooka, Dr. Akito Kaga, and other members in Crop Evolutionary Dynamics Team, National Institute of Agrobiological Sciences, Tsukuba, Japan, a very special appreciation is given to them for all materials used in this research, chemical reagents, knowledge, their advice, their kind assistance during my research works in Japan, and their kind support for everything.

Asst. Prof. Dr. Piyada Thipyapong, advisor, for her very kind guidance and editing this thesis.

Prof. Dr. Paisan Laosuwan and Prof. Dr. Peerasak Srinives, committee, for their very kind advice and assistance.

Assoc. Prof. Jutharat Attajarusit, chairperson, for her kind guidance.

Asst. Prof. Hatsachai Boonjung, committee, for his kind advice and the equipment for measuring GPS.

The Thailand Research Fund (TRF) for financial support of this study through the Royal Golden Jubilee Ph. D. program.

The personnels at the Plant Entomology and Pathology Laboratory, and SUT farm for very kind support.

Seniors, juniors, friends, and my relatives both in Thailand and Japan for their assistance and encouragement.

Chai Nat Field Crops Research Center especially Miss Sumana Ngampongsai for *Vigna* seeds, knowledge, and allowing a visit to the center.

Most of all, I would like to thank my mother for her encouragement and her kind financial support as well.

Worapa Seehalak

CONTENTS

Page

ABSTRACT (THAI).....I

ABSTRACT (ENGLISH).....III

ACKNOWLEDGEMENTS.....V

CONTENTS.....VII

LIST OF TABLES.....X

LIST OF FIGURES.....XII

CHAPTER

I: INTRODUCTION

1.1 Rationale of the study.....1

1.2 The objectives.....4

1.3 References.....4

II: REVIEW OF THE LITERATURE

2.1 The exploration of *Vigna* in Thailand.....9

2.2 Molecular markers for evaluating genetic diversity.....11

2.3 Species relationships in the Asian *Vigna*.....20

2.4 Interspecific hybridization.....23

2.5 References.....31

III: GENETIC DIVERSITY OF *Vigna* GERMPLASM FROM THAILAND AND NEIGHBORING REGIONS REVEALED BY AFLP ANALYSIS

3.1 Abstract.....49

CONTENTS (CONTINUED)

Page

3.2 Introduction.....50

3.3 Materials and Methods.....52

3.4 Results and Discussion.....59

3.5 Conclusion.....79

3.6 References.....80

IV: COLLECTION OF *Vigna* species SUBGENUS *Ceratotropis* IN CHIANG MAI AND MAE HONG SON PROVINCES

4.1 Abstract.....85

4.2 Introduction.....85

4.3 Materials and Methods.....86

4.4 Results and Discussion.....87

4.5 Conclusion.....91

4.6 References.....91

V: SSR ANALYSIS OF SYMPATRIC *Vigna* species OF NORTHERN THAILAND

5.1 Abstract.....99

5.2 Introduction.....100

5.3 Materials and Methods.....101

5.4 Results and Discussion.....107

5.5 Conclusion.....120

5.6 References.....121

CONTENTS (CONTINUED)

Page

VI: INTERSPECIFIC HYBRIDIZATION BETWEEN *Vigna radiata* AND *Vigna* spp.

6.1 Abstract.....124

6.2 Introduction.....125

6.3 Materials and Methods.....126

6.4 Results and Discussion.....130

6.5 Conclusion.....146

6.6 References.....147

VII: CONCLUSION AND RECOMMENDATION.....150

APPENDIX.....152

BIOGRAPHY.....160

LIST OF TABLES

Table **Page**

2.1 Production of viable F_1 hybrids between five Asian *Vigna* cultigens.....27

2.2 Results of interspecific crosses and germination test.....29

2.3 Results of embryo culture of interspecific hybrids among *Vigna* species.....29

2.4 Summary of results on interspecific hybridization.....30

2.5 Results of embryo culture of interspecific hybrids among *Vigna* species.....31

3.1 Adapter and primer sequence for AFLP analysis.....59

3.2 Summary of AFLP fragments and diversity statistics calculated for each species.....71

3.3 Gene diversity statistics for population groups of *V. umbellata* and *V. mungo*.....72

3.4 Genetic distance within and between species/subgroup based on Innan's nucleotide diversity.....73

4.1 Date, routes, species and numbers of collected seeds.....87

4.2 Average seed and hilum size of each accession.....92

4.3 Passport data of accessions collected.....96

5.1 Populations analyzed.....102

5.2 Primers used, number of alleles and heterozygous plant detected from each primer.....106

5.3 SSR comparison among species.....109

5.4 Phenetic distance between populations within *V. minima* constructed using Simple Matching Coefficient.....111

LIST OF TABLES (CONTINUED)

Table **Page**

5.5 Phenetic distance between population within *V. hirtella* and *V. tenuicaulis* constructed using Simple Matching Coefficient.....111

5.6 Phenetic distance between species constructed using Simple Matching Coefficient.....112

6.1 List of ISSR primers screened and their nucleotide sequences.....129

6.2 The results of crosses among different species of *Vigna*.....131

6.3 The morphological characteristics of hybrids and their parents.....135

1A. Materials used in the AFLP analysis.....153

2A. Ingredients in White's medium.....157

LIST OF FIGURES

Figure **Page**

3.1 Variation in seed shape and color of different components of the *V. umbellata* crop complex, *V. mungo* and *V. unguiculata*.....56

3.2 Distribution of Thai accessions analyzed.....57

3.3 Unrooted neighbor-joining tree of *Vigna* species analyzed in section *Angulares* (except *V. trinervia*).....75

3.3a Enlargement of *V. umbellata*.....76

3.4 Unrooted neighbor-joining tree of species in section *Ceratotropis*, *V. trinervia* and *V. unguiculata*.....77

3.4a Enlargement of *V. mungo*: *V. mungo* var. *mungo* and *V. mungo* var. *silvestris*....78

5.1 UPGMA dendrogram of all accessions based on Jaccard's similarity Coefficient.....113

5.2 Sketch map of site 17.....116

5.3 An unrooted neighbor-joining tree showing the genetic relationship between individuals of population 2.....118

5.4 Sketch map of site 2.....119

5.5 Relationship among *Vigna* species of northern Thailand.....121

6.1 An example of shrunken F_1 seeds of *V. radiata* var. *sublobata* x *V. mungo*.....134

6.2 Seedling of *V. radiata* var. *sublobata*, *V. radiata*, and *V. radiata* var. *sublobata* x *V. radiata*.....136

LIST OF FIGURES (CONTINUED)

Figure **Page**

6.3 Plant morphology of *V. radiata* var. *sublobata*, *V. radiata*, and *V. radiata* var. *sublobata* x *V. radiata*.....136

6.4 Stem pubescence and leaf vein color of *V. radiata* var. *sublobata*, *V. radiata*, and *V. radiata* var. *sublobata* x *V. radiata*.....137

6.5 Stipule of *V. radiata* var. *sublobata*, *V. radiata*, and *V. radiata* var. *sublobata* x *V. radiata*.....137

6.6 Inflorescence of *V. radiata* var. *sublobata*, *V. radiata*, and *V.radiata* var. *sublobata* x *V. radiata*.....138

6.7 Young flowers of *V. radiata* var. *sublobata*, *V. radiata*, and F_1 of *V. radiata* var. *sublobata* x *V. radiata*.....138

6.8 Flowers of *V. radiata* var. *sublobata*, *V. radiata*, and F_1 of *V. radiata* var. *sublobata* x *V. radiata*.....139

6.9 Pod length of *V. radiata* var. *sublobata*, F_1 of *V. radiata* var. *sublobata* x *V. radiata*, and *V. radiata*.....139

6.10 Seed size of *V. radiata* var. *sublobata*, F_1 of *V. radiata* var. *sublobata* x *V. radiata*, and *V. radiata*.....140

6.11 F_1 hybrid plant from *V. mungo* x *V. stipulacea*, 72 days after rescuing the embryo.....143

6.12 ISSR polymorphism among *V. radiata* var. *sublobata*, *V. radiata*, and *V. radiata* var. *sublobata* x *V. radiata* amplified with primer 841.....145

1A. The example of passport data sheet.....158

CHAPTER I

INTRODUCTION

1.1 Rationale of the study

The genus *Vigna* includes seven subgenera: *Vigna*, *Haydonia*, *Plectotropis*, *Macrorhyncha*, *Ceratotropis*, *Lasiospron*, and *Sigmoidotropis*. Each subgenus is distributed in different geographical areas. In Asia, *Plectotropis*, *Macrorhyncha*, and *Ceratotropis* are found but the latter is most common and thus often called the Asian *Vigna*. Species in this subgenus are mostly diploid whose chromosome numbers are 2n=2x=22 except *V. reflexo-pilosa* which is tetraploid and its chromosome number is 2n=4x=44. There are 21 species in this subgenus and eight of these are used for human or animal food (Tomooka et al., 2002).

Moth bean (*V. aconitifolia* [Jacq.] Maréchal) is used by people in South Asia for its green pods, cooked ripe seeds (Purseglove, 1974, quoted in Tomooka et al., 2002) and bean sprouts (Jain and Mehra, 1980, quoted in Tomooka et al., 2002). Azuki bean (*V. angularis* [Willd.] Ohwi & Ohashi), a traditional crop of East Asia and northeastern Vietnam, is used to make sweet bean jam and sweet bean soup (Tomooka et al., 2002). Black gram (*V. mung*o [L.] Hepper) is traditionally cultivated as fallow crop after rice cultivation in India (Sivaprakash et al., 2004). Mungbean (*V. radiata* [L.] Wilczek), an ancient and well-known crop in Asia (Tomooka et al., 1991), is used to make a soup in South Asia and various kinds of sweets, bean jam, sweeten

bean soup, vermicelli and bean sprouts in Southeast and East Asia (Tomooka et al., 2002). Creole bean (*V. reflexo-pilosa* Hayata var. *glabra* [Maréchal, Mascherpa & Stainier] N. Tomooka & Maxted) has been used as a forage crop in West Bengal and is cultivated in Mauritius (Baudoin and Maréchal, 1988, quoted in Tomooka et al., 2002). In Vietnam, it is used in the same way as mungbean (Kobayashi et al., 1994, quoted in Tomooka et al., 2002). Jungli bean (*V. trilobata* [L.] Verdc.) is cultivated as a cover crop and a cattle fodder in India. Tuapée (Thai) (*V. trinervia* [Heyne ex Wall] Tateishi & Maxted) is used as cover crop for young rubber plantations in Thailand (Tomooka et al., 2002). Immature pods of rice bean (*V. umbellata* [Thunb.] Ohwi & Ohashi) are eaten in northern Thailand. Seeds are also used to make sweets (Takeya and Tomooka, 1997, quoted in Tomooka et al., 2002) and are usually boiled and used as a vegetable (Purseglove, 1974, quoted in Tomooka et al., 2002).

Today in several parts of Asia, wild or weedy relatives of the Asian *Vigna* are also gathered for their pods and/ or seeds for human consumption. In India *V. radiata* var. *sublobata* is gathered and eaten. Some people in India gather seeds of *V. trilobata* for food (Jain and Mehra, 1980, quoted in Tomooka et al., 2002). In Japan wild azuki is not used, but weedy azuki bean has been eaten by people as a substitute for the cultivated form (Yamaguchi, 1992, quoted in Tomooka et al., 2002).

Genetic diversity has an important role in plant breeding program. The loss of genetic diversity, in part due to the conventional breeding programs associated with modern agricultural practices, has been dramatic in many cultivated species (Wilkes, 1983, quoted in Li et al., 2001). In consequence, the narrow genetic base of the elite germplasm has increased the potential vulnerability to pests and abiotic stress (Li et

al., 2001). Therefore, the wild relatives and even alien species are needed to broaden the genetic bases of breeding program through interspecific hybridization (Singh and Ocampo, 1997, quoted in Nguyen et al., 2004; Stoskopf, 1993). Therefore, plant breeders can succeed their breeding programs by relying on the diversity or variation in plant populations. A comprehensive understanding of the amount and pattern of genetic variation that exists within and between the available cultivated and wild accessions is important for enhancing genetic potential because the diverse germplasm may include traits needed for effective improvement of the crop (Robertson et al., 1997, quoted in Nguyen et al., 2004). Moreover, plant diversity study also reveals the relationship among plants in a population which guides the researcher to understand their evolution, indicates the centers of origin (Xu-xiao et al., 2003), provides prediction for heterosis and helps establish a core collection management to sample a maximum of genetic variation in a minimum number of accessions (Ntundu et al., 2004). Currently, the knowledge of wild-weedy-crop relationship is important for lending insight into the potential consequences of transgenic crop plant releases (Boudry et al., 1993, quoted in Xu et al., 2000).

Among the cultivated Asian *Vigna*, mungbean (*V. radiata*) is the most important. Although there have been many studies of the diversity of cultivated mungbean (Tomooka et al., 2004, quoted in Saravanakumar et al., 2004), little is known about the diversity of its wild progenitor, *V. radiata* var. *sublobata*. Similar to other cultigens and their wild relatives, the knowledge of them is relatively lacking. The reason may be that these groups of species are found in three different eco-geographic regions between which scientific communication tends to be hampered by ethno-linguistic and historic factors (Tomooka et al., 2002).

In the part of interspecific hybridization, this makes variation occur as described above and it is used for transformation of the desirable trait(s) from a species to another. Interspecific hybridization can also exhibit evolution; the species which closely related have high possibility of success in crossing.

1.2 The objectives

1. To determine intra-specific genetic diversity of different *Vigna* species in Thailand and determine their relationships to one another using AFLP analysis.
2. To explore and collect Asian *Vigna* species in Chiang Mai and Mae Hong Son province.
3. To analyze the collected individuals by using SSR techniques to gain an insight into the level of heterozygosity in populations and to determine the relationships among species based on the SSR profile.
4. To evaluate cross compatibility between *V. radiata* and *Vigna* spp. and confirm the hybrids using morphological comparison between hybrids and respective parents and using ISSR marker to verify the hybrids.

1.3 References

Baudoin, J. P. and Maréchal, R. (1988). Taxonomy and evolution of the genus *Vigna*. In S. Shanmugasundaram and B. T. McLean (eds.). **Mungbean: Proc. of the Second Int. Symp.** (pp 2-12). AVRDC, Shanhua, Tainan: Taiwan. Quoted in N. Tomooka, D. A. Vaughan, H. Moss and N. Maxted. (2002). **The Asian *Vigna*: Genus *Vigna* Subgenus *Ceratotropi*s Genetic Resources.** The Netherlands: Kluwer Academic. 270 p.

Boudry, P., Morchen, M., Saumitou-Laprade, P., Vernet, P. and Van Dijk, H. (1993). The origin and evolution of weed beets: consequences for the breeding and release of herbicide-resistant transgenic sugar beets. **Theor. Appl. Genet.** 87: 471-478. Quoted in R.-Q. Xu, N. Tomooka and D. A. Vaughan. (2000). AFLP markers for characterizing the azuki bean complex. **Crop Sci.** 40: 808- 815.

Jain, H. K. and Mehra, K. L. (1980). Evolution, adaptation, relationships and uses of the species of *Vigna* cultivated in India. In R. J. Summerfield and A. H. Bunting. (eds.). **Advances in legume science (Volume 1 of the Proc. of the International Legume Conf.).** (pp 459-468). Kew: U.K. Quoted in N. Tomooka, D. A. Vaughan, H. Moss and N. Maxted. (2002). **The Asian *Vigna*: Genus *Vigna* Subgenus *Ceratotropi*s Genetic Resources.** The Netherlands: Kluwer Academic. 270 p.

Kobayashi, T. N., Shimada, N., Thang, N. Q. and Tung, L. T. (1994). Exploration and collection of grain legume germplasm in Vietnam. **Annual Report on Exploration and Introduction of Plant Genetic Resources (NIAR, Tsukuba, Japan)** 10: 141-169. Quoted in N. Tomooka, D. A. Vaughan, H. Moss and N. Maxted. (2002). **The Asian *Vigna*: Genus *Vigna* Subgenus *Ceratotropi*s Genetic Resources.** The Netherlands: Kluwer Academic. 270 p.

Li, C. D., Fatokun, C. A., Ubi, B., Singh, B. B. and Scole, G. (2001). Determining genetic similarities among cowpea breeding lines and cultivars by microsatellites markers. **Crop Sci.** 41: 189-197.

Ntundu, W. H., Bach, I. C., Christiansen, J. L. and Andersen, S. B. (2004). Analysis of genetic diversity in bambara groundnut [*Vigna subterranean* (L.) Verd.]

landraces using amplified fragment length polymorphism (AFLP) markers. **Afri. J. Biotech.** 3(4): 220-225.

Purseglove, J. W. (1974). **Tropical Crops**. London, UK: Longman. 719 p. Quoted in N. Tomooka, D. A. Vaughan, H. Moss and N. Maxted. (2002). **The Asian *Vigna*: Genus *Vigna* Subgenus *Ceratotropi*s Genetic Resources.** The Netherlands: Kluwer Academic. 270 p.

Robertson, L. D., Ocampo, B. and Singh, K. B. (1997). Morphological variation in wild annual *Cicer* species in comparison to the cultigen. **Euphytica** 95: 309-319. Quoted in T. T. Nguyen, W. J. Taylor, R. J. Redden and R. Ford. (2004). Genetic diversity estimated in *Cicer* using AFLP analysis. **Plant Breeding** 123: 173-179.

Singh, K. B. and Ocampo, B. (1997). Exploration of wild *Cicer* species for yield improvement in chickpea. **Theor. Appl. Genet.** 95: 418-423. Quoted in T. T. Nguyen, W. J. Taylor, R. J. Redden and R. Ford. (2004). Genetic diversity estimated in *Cicer* using AFLP analysis. **Plant Breeding** 123: 173-179.

Sivaprakash, K. R., Prashanth, S. R., Mohanty, B. P. and Parida, A. (2004). Genetic diversity of black gram (*Vigna mungo*) landraces as evaluated by amplified fragment length polymorphism markers. **Curr. Sci.** 86 (10): 1411-1416.

Stoskopf, N. C. (1993). **Plant Breeding: Theory and Practice.** Colorado, USA: Westview Press. 531 p.

Takeya, M. and Tomooka, N. (1997). The illustrated legume genetic resources database on the world wide web. **Misc. Publ. Inst. Agrobiol. Resour.** 11: 1-93. Quoted in N. Tomooka, D. A. Vaughan, H. Moss and N. Maxted. (2002). **The**

Asian *Vigna*: Genus *Vigna* Subgenus *Ceratotropi*s Genetic Resources. The Netherlands: Kluwer Academic. 270 p.

Tomooka, N., Lairungreang, C., Nakeeraks, P., Egawa, Y. and Thavarasook, C. (eds.). (1991). **Mungbean and the Genetic Resources: the Subgenus *Ceratotropis* in Ecological Studies on Tropical Food Legumes in Relation to Adaptation to Cropping Systems in Thailand.** Tropical Agriculture Research Center, Japan and Chai Nat Field Crops Research Center, Thailand.

Tomooka, N., Vaughan, D. A. and Kaga, A. (2004). Mungbean. In R. J. Singh and P. P. Jauhan (eds.). **Genetic Resources, Chromosome Engineering and Crop Improvement: Grain Legumes.** Boca Raton, FL: CRC Press. 390p. Quoted in P. Saravanakumar, A. Kaga, N. Tomooka and D. A. Vaughan. (2004). AFLP and RAPD analyses of intra- and interspecific variation in some *Vigna* subgenus *Ceratotropis* (Leguminosae) species. **Aust. J. Bot.** 52: 417-424.

Tomooka, N., Vaughan, D. A., Moss H. and Maxted N. (2002). **The Asian *Vigna*: Genus *Vigna* Subgenus *Ceratotropi*s Genetic Resources.** The Netherlands: Kluwer Academic. 270 p.

Wilkes, G. (1983). Current status of crop plant germplasm. **CRC Crit. Rev. Plant Sci.** 1: 133-181. Quoted in C.-D. Li, C. A. Fatokun, B. Ubi, B. B. Singh and G. J. Scoles. (2001). Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. **Crop Sci.** 41: 189-197.

Xu-xiao, Z., Vaughan, D., Tomooka, N., Kaga, A., Xin-wang, W., Jian-ping, G. and Shu-min, W. (2003). Preliminary study on geographical distribution and evolutionary relationships between cultivated and wild azuki bean (*Vigna*

angularis var. *angularis* and var. *nipponensis*) by AFLP analysis. **Plant Genet. Res.: Charact. and Util.** 1: 175-183.

Yamaguchi, H. (1992). Wild and weed azuki beans in Japan. **Econ. Bot.** 46: 384-394. Quoted in N. Tomooka, D. A. Vaughan, H. Moss and N. Maxted. (2002). **The Asian *Vigna*: Genus *Vigna* subgenus *Ceratotropis* Genetic Resources.** The Netherlands: Kluwer Academic. 270 p.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 The exploration of *Vigna* in Thailand

Ceratotropis species (Asian *Vigna*), which was expected that many of its wild species could be found in Thailand, was subdivided into three groups based on seedling characteristics including the Mungbean group (section *Ceratotropis*), the Azuki bean group (section *Angulares*), and the intermediate group (section *Aconitifoliae*) (Tateishi, 1996, quoted in Tomooka et al., 2002). The species of "Mungbean group" distributed mainly in the Indian Subcontinent and the species of "Azuki group" distributed mainly in the East Asia. Thus in Thailand, which is located between the Indian Subcontinent and the East Asia, wild species belonging to both "Mungbean group" and "Azuki group" were expected to be found (Tomooka, 1991). *Ceratotropis* is a well defined, easily identified group with all the typical *Vigna* characters well expressed: peltate stipule, asymmetric flower, keel and style curved to the left side with their tip facing a sharp pointed unilateral pocket of the keel, style protruding beyond the internal stigma by a long straight beak. The flowers of *Ceratotropis* always show different shades of yellow, but are never purple, violet or white as is often found in the other groups of *Vigna* (Maréchal et al., 1978, quoted in Tomooka et al., 1991). Thus, collectors use the character of this yellow flower as the first criterion to determine whether that plant belongs to *Ceratotropis*, then the morphology of the plant is observed in more details.

The explorations of wild *Vigna* species in Thailand were mostly for collecting germplasm and searching for desirable traits that could be used in breeding programs. The previous exploration in Thailand showed many *Vigna* species growing in each parts of the country except in southern part where there has been no report. The first exploration report of *Vigna* in Thailand was in 1989 when Lairungreung et al. (1991) explored the northern and central Thailand including Phitsanulok, Uttaradit, Phrae, Nan, Phayao, Chiang Rai, Chiang Mai, Lampang, Tak, Kamphaeng Petch, Nakhon Sawan, and Chai Nat provinces. From this exploration, 16 samples of 4 *Vigna* species were collected which were *V. grandiflora*, *V. umbellata* var. *gracilis*, *V. minima* subsp. *gracilis* and the other unknown species. However, this species was close to *V. reflexo-pilosa*. In 1993, Pichitporn et al. (1993), exploring the northern provinces of Thailand adjacent to Myanmar, China, and Laos, found *V. umbellata* var. *grandiflora* and *V. minima* on the route from Mae Sariang to Mae Hong Son. At Chai Nat, they found both *V. umbellata* var. *gracilis* and *V. grandiflora*. In 1997, Tomooka et al. (1997) explored the central and northern Thailand including Chai Nat, Phichit, Phitsanulok, Sukhothai, Tak, Lampang, Chiang Mai, Mae Hong Son, and Chiang Rai. They collected seed samples and root nodules of *V. umbellata*, *V. grandiflora*, *V. hirtella*. *V. minima*, *V. trinervia*, *V. mungo* var. *silvestris*, and *V. unguiculata*. Then in 2000, Ngampongsai et al. (2000) collected *V. umbellata* var. *gracilis* from Tak and Kanchanaburi and collected *V. unguiculata* at Chiang Rai. The exploration of Tomooka et al. (2000) at Kanchanaburi, Ratchaburi, Phetchaburi, Prachuab Khiri Khan, Saraburi, Nakhon Ratchasima, Chantaburi, Rayong, Phitsanulok, Nan, Phetchabun, Udon Thani, Nakhon Pathom, and Surin, found many *Vigna* species including *V. umbellata*, *V. hirtella*, *V. exilis*, *V. trinervia*, *V. minima*, *V. grandiflora*,

and *V. unguiculata*. Although there were many *Vigna* species collected in Thailand, there have been few reports about the relationships among *Vigna* species collected in Thailand.

2.2 Molecular markers for evaluating genetic diversity

The study of morphological variability, which is still the only approach used in many species, is the classical method to assess genetic diversity. An assessment of genetic diversity based only on morphological and agronomic traits might be biased because distinct morpholotypes can be influenced by environmental factors and even by a few mutations. However, with molecular marker techniques, powerful tools have been developed so that genetic resources can be accurately assessed and characterized (Capo-chichi et al., 2003). The most common techniques used for measuring genetic variability are isozymes, restriction fragment length polymorphism (RFLP), and numerous genetic marker assays based on polymerase chain reaction (PCR) such as random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), and amplified fragment length polymorphism (AFLP) (Karp et al., 1996, quoted in Yee et al., 1999).

Isozyme, structurally different molecular forms of an enzyme system with the same catalytic function, can mark allelic variation at single structural gene locus because amino acid alterations in isozymes are caused by changes in the nucleotide sequence of the coding gene. However, differentiation analysis among 21 wild *Vigna* accessions collected in Thailand and from germplasm using three isozymes showed clear differences among species but not among varieties (Ngampongsai et al., 2000). Genetic diversity study in bambara groundnut (*V. subterranea* (L.) Verdc.) showed

that domesticated accessions had lower genetic diversity than wild accessions (Pasquet et al., 1999). Moreover, isozyme can be used to find phylogenetic relationship such as the case of tetraploid species which isozyme analysis revealed that *V. reflexo-pilosa* and *V. glabrescens* should be derived from interspecific hybridization between *V. trinervia* and *V. minima* (Egawa et al., 1996, quoted in Tomooka et al., 2000).

RAPD is generated by PCR with single short oligonucleotide of arbitrary sequences. It provides genetic information at the DNA level with relative ease (Williams et al., 1990, quoted in Kaga et al., 1996). RAPD has been used to detect genetic variation, evaluate intraspecific variation (Dos Santos et al., 1994; Thormann et al., 1994), and reveal interspecific relationships.

Investigation of genetic variation among 5 species in the subgenus *Ceratotropis*, genus *Vigna* using RAPD analysis showed that wild forms were always grouped with their most closely related cultivated forms. The largest intraspecific variation was found in *V. radiata* (mungbean) in which wild form (*V. radiata* var. *sublobata*) was distinctly different from each other and from cultivated form (Kaga et al., 1996). Xu et al. (2000) used RAPD analysis to characterize genetic variation in three forms of *V. angularis*: cultivated, wild, and weedy forms. Genetic variation was highest in the wild form, followed by the weedy form and the lowest in the cultivated form. However, the results from RAPD analysis may bias the data obtained because there is the possibility that similar size fragments from RAPD analysis probably have no sequence homology (William et al., 1993, quoted in Kaga et al., 1996). Hence, some of the similar size fragments would be proven to be whether homologous to each other by Southern hybridization (Kaga et al., 1996).

AFLP, a novel PCR-based molecular marker assay (Vos et al., 1995), is simple, requires only small amounts of DNA, can be performed without the use of radioactive (Karp et al., 1996), and does not require prior sequence information (Sivaprakash et al., 2004). Moreover, AFLP is fast, less labor intensive, reproducible, and providing much information because it has the capacity to detect a high number of loci and high level of polymorphism. Compare to other techniques, AFLP combines the features of easy handling of RAPD (Williams et al., 1990, quoted in Sivaprakash et al., 2004) and the robustness of RFLP (Botstein et al., 1980, quoted in Sivaprakash et al., 2004). This is in agreement with the experiment of Yee et al (1999) comparing RAPD and AFLP. Their results showed that AFLP was more efficient than RAPD for detecting polymorphism in azuki accessions because the number accession pairs that could not be differentiated by AFLPs was less than RAPD. The explanation is that the two marker techniques may target at different portions of the genome (Karp et al., 1996, quoted in Yee et al., 1999). Therefore, this technique provides a new powerful tool for detection and evaluation of genetic variation in germplasm collections and in the screening of biodiversity (Matthes et al., 1998), and it has proven to be extremely proficient in revealing diversity at below the species levels (Karp and Edwards, 1995, quoted in Capo-chichi et al., 2003). The result from evaluating banding similarity provides information for parental selection in a breeding program and if there is high level of polymorphism, the ability to resolve genetic relationships among genotypes is high.

AFLP technology has been applied in many diversity studies including, for example, the *Cicer* species which AFLP analysis showed cultivated species (chickpea) close to a wild species. That wild species was hypothesized to be the

progenitor of the cultivated species (Nguyen et al., 2004). Cluster analysis of bambara groundnut [*Vigna subterranea* (L.) Verd] based on AFLP data revealed that bambara groundnut from Tanzania constitute two major groups according to their geographic origins and also phenotypic characters (Ntundu et al., 2004). AFLP analysis within cowpea [*Vigna unguiculata* (L.) Walp.] showed extensive gene flow between wild and domesticated cowpea that formed a large crop-weed complex distributed over the entire African continent. This is the reason why the localization of domestication of cowpea is still unclear (Coulibaly et al., 2002). In bluebunch wheatgrass, AFLP has been used for analyzing genetic diversity between two cultivars and a multiple-origin polycross. There were no AFLP markers that could completely distinguish two cultivars, therefore discrete morphological differences between these cultivars most likely result from natural or artificial selection (Larson et al., 2000). The genetic diversity of *Mucuna* studied by AFLP analysis showed separated clusters confirming the phenological difference (Capo-chichi et al., 2003). The study of genetic variation in *Moringa oleifera* of Kenya using AFLP revealed two sources of germplasm introduced to Kenya from India and Malawi. These provenances are important in the conservation and exploitation of *M. oleifera* genetic resources (Muluvi et al., 1999). Moreover, AFLP has been used for studying clonal structure in dwarf bamboo (*Sasa senanensis*), which the result exhibited the largest single clone occurring over a distance of about 300 m. (Suyana et al., 2000). Comparison between the AFLP phylogenetic analysis and the mtDNA tree in the Taiwanese bamboo viper revealed that AFLP tree did not support the mtDNA tree, suggesting that no contemporary barriers to gene flow exist between individuals from the two mtDNA lineages (Creer et al., 2004). AFLP markers used to examine spatial

distribution of native black poplar (*Populus nigra* subsp. *betulifolia*) in Britain revealed a general correlation between geographic proximity and genetic similarity (Winfield et al., 1998).

In the case of genus *Vigna* subgenus *Ceratotropis* species, *V. trinervia* has been confused with *V. radiata* var. *sublobata* and *V. bourneae*. However, by AFLP analysis *V. radiata* var. *sublobata* and *V. trinervia* were distinguished and *V. bourneae* was confirmed to be a variety of *V. trinervia* (Saravanakumar et al., 2004). Moreover, there were the AFLP analyses used for studying *V. minima* complex and related species in East Asia (Yoon et al., 2000), azuki bean complex in Japan (Vaughan et al., 2000), geographical distribution of azuki bean (Xu-xiao et al., 2003), and genetic diversity among black gram landraces (Sivaprakash et al., 2004).

Microsatellites, known as short tandem repeats (STRs) or simple sequence repeats (SSRs), consist of one to five base-pair repeated units that are dispersed throughout eukaryotic genomes, have high mutation rates ($2.5 \times 10^{-2} - 10^{-5}$ mutations per generation; Weber and Wong, 1993, quoted in Clauss et al., 2002). Variations in the number of repeats can be detected by PCR, with the development of primers (20-30 base pairs) specifically built for amplification and complementary to single sequences flanking the microsatellite (Geribello Priolli et al., 2002). SSRs combine desirable marker properties including unambiguous designation of alleles, even dispersal, selective neutrality, high reproducibility, rapid and simple genotyping assays (Li et al., 2001), multi-allelic and codominant nature, and high polymorphism between individuals within populations or closely related genotypes, (Powell et al., 1996, quoted in Métais et al., 2002).

The applications of SSRs span over different areas ranging from phylogenetic to forensic DNA studies, conservation management of biological resources (Jarne and Lagoda, 1996, quoted in Krishna et al., 2004), gene flow studies (Wang et al., 2004), evolutionary and population structure studies of related species (Métais et al., 2002), cultivar identification, and pedigree analysis (Geribello Priolli et al., 2002), migration and colonization rates, and mating systems (Luikart and England, 1999, quoted in Clauss et al., 2002). Moreover, microsatellites have been recognized as useful molecular markers in marker-assisted selection (MAS), analysis of genetic divergence in plants (Krishna et al., 2004), and genome mapping in many organisms (Schuler et al., 1996, quoted in Krishna et al., 2004) because they are single locus markers which provide an unambiguous means of defining linkage group homology across mapping populations (Cregan et al., 1999).

In rice breeding, SSRs were used for predicting heterosis. The results showed alleles contribute to the heterosis and alleles reduce heterosis. These are useful for hybrid rice breeding program with SSR marker-assisted selection (Liu and Wu, 1998). SSRs in rice were also used to cluster bacterial blight resistant/susceptible lines into different groups based on the resistant genes present in them (Davierwala et al., 2001). Population genetic structure analysis of a wild rice in China using microsatellites revealed that differentiation among populations was correlated with geographical distance and in agreement with the previous allozyme and RAPD studies (Zhou et al., 2003).

Genetic diversity assessment can be used to evaluate genetic redundancy, for example, among sorghum accessions maintained by the U.S. National Plant Germplasm (NPGS). The variance analysis from SSRs data could indicate that it

should be possible to reduce the number of sorghum accessions held by NPGS without seriously jeopardizing the overall amount of genetic variation contained in these holdings (Dean et al., 1999).

In soybean, SSRs have been used to assess diversity between elite genotypes and plant introductions (PIs) that may assist with the transfer of favorable alleles from PIs into elite soybean cultivars (Narvel et al., 2000). The relationship among North American soybean ancestors and plant introductions (PIs) was also assessed to select the PIs genotypes districted from the majority of the ancestors. These PIs are useful for widening genetic base of soybean (Brown-Guedira et al., 2000). In Brazil, SSR markers have characterized the large number of soybean cultivars eligible to receive protection under the Brazilian Cultivar Protection Act because characterizing by morphological and biochemical traits were limited. A dendrogram based on the SSR loci profiles showed good agreement with the cultivar pedigree information (Geribello Priolli et al., 2002).

SSR analysis in cowpea breeding lines also generated the dendrogram in agreement with their pedigree (Li et al., 2001). The uses of SSR markers to assess genetic relationships have been done among individuals within one population of wild azuki bean. The results showed 3 of 20 individuals were heterozygous genotypes indicating outcrossing, although azuki bean is a predominant self-pollinating species (Wang et al., 2004).

SSRs were successful in cross-species amplification in plants, especially to closely related genera. According to the SSR analysis of soybean, the primers used to amplify SSRs in soybean could amplify SSRs also in other species within *Glycine* and other genera which were *Kennedia*, *Vigna*, *Vicia*, *Trifolium*, *Lupinus*, and *Albizia*.

However, cross-species amplification outside the genus was much lower than the inside (Peakall et al., 1998). In the study using primer sequence flanking to $(GATA)_n$ in rice, these primers could also amplify DNA of maize, wheat, barley, and oat indicating that these $(GATA)_n$-containing loci are conserved across different cereal genera (Davierwala et al., 2001).

Compare with other molecular markers, the data generated by microsatellites are similar to that of allozymes, except that the number of alleles and heterozygosity revealed is almost always much higher (Ciofi et al., 1998). SSRs showed greater diversity than evaluated by RAPDs because of the presence of multiple alleles (Brown-Guedira et al., 2000) and they provide power of discrimination equal to or greater than RFLP in a more cost-effective manner (Smith et al., 1997, quoted in Li et al., 2001). Comparison of genetic diversity among soybean genotypes evaluated by DNA amplified by 20 SSR primer pairs and four SNP (single nucleotide polymorphism) primers exhibited distinguished clustering. SSR analysis revealed three main groups according to geographic origin. On the other hand, two main groups were classified using SNP analysis. This might imply that SSR was more informative than SNP, which may be due to the fact that SNP was surveyed on the smaller genomic region than SSR (Tanya et al., 2001).

Another molecular marker that has become a choice for genetic diversity studies of many crops is intersimple sequence repeat (ISSR) (Promper et al., 2003). This marker was modified from SSR-based marker system. ISSRs are amplified by PCR in the presence of primer homologous to SSR sequences which PCR products could be separated on either agarose or polyacrylamide gels (Kumar et al., 2001) combined with either silver staining or isotopic detection (Liu and Wendel, 2001). ISSR

technique is nearly identical to RAPD technique except that primer sequences are designed from microsatellite regions. Higher stringency of amplification in the form of longer primers (16-25 bp) and elevated annealing temperatures (45-60°C) makes more reproducible than RAPD markers. The important advantage is that it combines the high degree of DNA polymorphism detected by conventional multilocus probes in DNA fingerprinting experiments with the technical simplicity and speed of the PCR method, facilitating large-scale experiments (Kumar et al., 2001).

The technique combines the advantage of AFLP and microsatellite (SSR) analysis to the taxonomic universality of RAPD. Unlike SSR analysis, it does not require prior sequence information for primer design, and it can overcome some of the technical limitations of RFLP and RAPD (Assefa et al., 2003).

ISSR has been successfully used in DNA fingerprinting, genetic diversity studies (Promper et al., 2003) such as apple, pear, rice, trifoliate orange, common bean, and lupin (Assefa et al., 2003), genotyping, genome mapping, and phylogenetic studies in crops such as strawberry, soybean, and potato (Sica et al., 2005) and it is also considered especially suitable for forensic investigations where reproducibility of the results is required to settle the disputes (Kumar et al., 2001). Moreover, ISSR has been employed for genetic linkage maps; for gene tagging, for detecting somaclonal variation, somatic hybrids and microspore-derived plants (Archak and Baldev, 2005).

In the study of clonal diversity in the rare *Calamagrostis porteri* ssp. *insperata* (Poaceae), ISSR markers detected more genotypes within populations than did allozyme and more diversity than RAPD markers (Esselman et al., 1999). Comparison of RAPD, STS, ISSR, and AFLP markers used for assessment of genetic diversity in hop (*Humulus lupulus* L.) showed that the patterns of ISSR reactions were

very similar in agarose to RAPD reactions and in acrylamide to AFLP reactions. Although the level of polymorphism revealed by ISSR was lower than that revealed by RAPD and AFLP, the cluster analyses were very similar (Patzak, 2001).

2.3 Species relationships in the Asian *Vigna*

Previous researchers using RAPD (Tomooka et al., 1995) and isozyme (Egawa et al., 1996; Ping, 1996) to analyze relationships among Asian *Vigna* have recognized two groups within this subgenus, the azuki bean group and mungbean group. Based on the RAPD analysis, *V. aconitifolia* and *V. trilobata*, which afterward they were put in section *Aconitifoliae*, have made cluster with species in section *Ceratotropis*. Recently since more materials have been analyzed and a high level of polymorphism has been detected by using molecular techniques such as proteinase inhibitors (Kanarev et al., 2002), intergenic spacer of cpDNA (Doi et al., 2002) and AFLP (Tomooka et al., 2002b), three sections have been found in subgenus *Ceratotropis*, section *Aconitifoliae*, *Angulares*, and *Ceratotropis*. The relationships among these three sections analyzed by AFLP showed that all species in section *Ceratotropis* and *Aconitifoliae* were well differentiated compared with most of the species in section *Angulares* except *V. trinervia*. Section *Ceratotropis* is positioned between *Angulares* and *Aconitifoliae*. Section *Ceratotropis* is more closely related to section *Aconitifoliae* than section *Angulares* based on inter-section genetic distance. Section *Angulares* exhibits a complex pattern of genetic diversity. The intra-section genetic distance is highest in section *Aconitifoliae* and lowest in *Angulares*. This suggests, if the rate of evolution is the same for each section, that section *Angulares* is the most recently evolved section and section *Aconitifoliae* is the ancestral section, as has been shown

in the study of rDNA ITS sequence using *V. unguiculata* as an outgroup. Moreover, genetic distance based on rDNA-ITS sequence showed that the genetic diversity within section *Angulares* is 44% of that in *Aconitifoliae*, and 69% of that in *Ceratotropis*. It is possible that species in section *Angulares* have evolved over a relatively short period compared with other sections, and that the evolutionary history of *Angulares* might be approximately half of that of *Aconitifoliae* (Doi et al., 2002).

The relationship among and within species will be reviewed only in the species that would be used in these experiments.

V. exilis is a recently discovered species which grows naturally on limestone outcrops in Southeast Asia. There is less research on this species, however, based on AFLP analysis results of Tomooka et al. (2002b), it is closely related to *V. umbellata* and *V. hirtella*.

V. hirtella accessions were divided into 2 sub-clusters. An accession in one sub-cluster has morphology fit to the taxonomic key based on morphology for *V. hirtella*. More precise analysis using higher number of AFLP bands may be necessary.

According to the new taxonomic treatment of Tateishi (1985) *V. nakashimae* was considered as a subspecies of *V. minima* subsp. *nakashimae* and *V. riukuensis* was considered as a variety of *V. minima* subsp. *minima* var. *minor*. Based on AFLP analysis using 12 primer combinations *V minima* clustered with *V. minima* subsp. *nakashimae* and *V. minima* subsp. *minima* var. *minor*. However, the high degree of divergence between *V. minima* and *V. minima* subsp. *nakashimae* and *V. minima* subsp. *minima* var. *minor* seems reasonable to consider *V. nakashimae* and *V. riukuensis* as separate species within the *V. minima* (Yoon et al., 2000).

V. tenuicaulis is close to *V. nepalensis* based on morphological analysis and AFLP analysis also showed it closing to *V. nepalensis* and *V. angularis* (Tomooka et al., 2002b)

V. hirtella, *V. minima* and *V. tenuicaulis* have a high level of within species diversity. The main area where these species are distributed is mainland Southeast Asia. In Thailand and Myanmar, these species sometimes grow sympatrically, however, they show high level of morphological variation in this area. Based on the hybridization experiments using some accessions of these three species, they showed cross compatibility. Therefore, gene flow between these taxa, as has been recorded for other species complexes, cannot be ruled out (Tomooka et al., 2002b).

V. trinervia, which was sometimes confused with *V. radiata* var. *sublobata* (Tateishi and Maxted, 2002, quoted in Saravanakumar et al., 2004), is sometimes used as a cover crop in rubber plantations in Thailand and Malaysia, and has been collected from various parts of Sri Lanka and Southeast Asia (Tomooka et al., 2004, quoted in Saravanakumar et al., 2004). It is also reported from Madagascar, the Komoros and Tanzania. Within its natural range, *V. trinervia* var. *trinervia* accessions collected from Sri Lanka are generally found at high altitude (usually over 1000 m.), whereas in Southeast Asia (Myanmar, Thailand, and peninsular Malaysia) it grows at low altitude (below 100 m.) (Saravanakumar et al., 2004). This species is intermediate between the three sections. It is clustered in section *Angulares* according to its flower and seedling morphology. However, *V. trinervia* has dull seed coat and mature pod with brown hairs that are common characteristics of species in section *Ceratotropis* and are not seen in other species of section *Angulares* (Tomooka et al., 2002a). *V. trinervia*, while included in section *Angulares*, made a distinct cluster from other section

Angulares species based on AFLP and RAPD analysis (Saravanakumar et al., 2004). The rate of evolution of *V. trinervia* based on rDNA-ITS sequence is not statistically different from sections *Aconitifoliae* and *Ceratotropis* but is statistically different from other species in section *Angulares* (Doi et al., 2002). The intermediate position of *V. trinervia* suggests this may be a useful species to facilitate gene transfer among sections of the subgenus *Ceratotropis* (Tomooka et al., 2002a).

2.4 Interspecific hybridization

Interspecific hybridization (crossing of different species) plays an important role for transferring the desirable traits from one species to another, increasing genetic variation (Wijaya et al., 2002), clarifying the taxonomic relationship by testing several interspecific combinations (Kribben, 2002), investigating natural selection and speciation process, being a prominent tool for theoretical and empirical studies in evolutionary biology (Klaus, 1997), and recently being used to construct genetic linkage map (Kaga et al., 2000).

However, crossing barriers occurred which were the result of incongruity. Sexual barriers preventing interspecific hybridization have been distinguished into pre- and post- fertilization barriers (Van Tuyl and De Jeu, 1997). Pre-fertilization barrier on the stigma surface which arrests pollen germination or pollen tube entry into the stigma is one of the frequent barriers particularly in pollination with distantly related species (Borone et al., 1992, quoted in Onus, 2000). The causative factors for the failure of pollen germination can be the lack of adhesion, hydration, and suitable conditions on the stigma (Onus, 2000). For example, the pollen of many species requires calcium and boron for germination and it has been shown that the stigma

provides these elements to pollen (Bednarska, 1991, quoted in Onus, 2000). Sometimes the pollen tubes are unable to utilize stylar nutrients probably because of a lack of nutrients in the transmitting tissue or a lack of suitable enzymes in the pollen tubes (Labarka and Loweus, 1973, quoted in Onus, 2000). Crossing among *Fagopyrum* species showed pollen tubes inhibited in the stigma with thickening wall and burst tips or the pollen tubes probably failed to grow through the full length of the style with swallen tips and strong callose deposition. Moreover, difference of style types also showed in different degree of congruity even in same combination of two species (Woo et al., 1995). To overcome pre-fertilization barriers, a range of techniques such as bud pollination, stump pollination, use of mentor pollen and grafting of the style have been applied successfully (Van Tuyl and De Jeu, 1997).

Post-fertilization barriers, which have been found for example in *Cicer* species (Mallikarjuna, 1999), *Vicia* species (Wijaya et al., 2002), and *Vigna* species (Ngampongsai et al., 1995), are exemplified by the fact that the pollen grains germinate on the stigma and fertilization takes place. The embryos abort soon after (Mallikarjuna, 1999) because of the abnormality in embryo development at early stage. The study of Woo et al. (1995) on *Fagopyrum* found that the abnormal embryo showed irregular shape of embryo, isolated suspensor, obscure and vacuolate cells without seeing any nuclei, and 5 abnormal stages of hybrid embryo development. Some of zygotes did not divide whereas surrounded with normal divided free endosperm nuclei and cells with induced membranes filled with embryo-sac cavity. Young hybrid embryo developed, some of them formed long and bar-shaped proembryos but the endosperm degenerated leaving the embryo sac unfilled. The shrunken embryos without any normal cell were observed in most of cross-

combinations. This abnormal stage happened also early at 3 DAP (days after pollination), and mainly at 5 DAP. The developed embryos were isolated from micropylar part since the collapse of suspensor. At this stage, embryos were at a near globular stage, but endosperm cells could not be seen. In such instances the method by which hybrids can be obtained is by embryo recue (ovary, ovary slice, ovule and embryo culture) (Van Tuyl and De Jeu, 1997; Mallikarjuna, 1999; Woo et al., 1995). However, the responses of embryo cultures varied in different crosses.

The falling of hybrid pods before maturing is rescuable by using embryo culture techniques (Ngampongsai et al., 1995) or application of the mixture of growth regulators [gibberellic acid (GA) + 1-naphthalene acetic acid (NAA) + kinetin (KN)] upon pollinated pistils to prevent initial pod abscission (Mallikarjuna, 1999), or treating the emasculated and not pollinated flowers with gibberellic acid (GA_3) (Wijaya et al., 2002)

Although the F_1 hybrid plants can be obtained, the F_2 seeds might not be produced because of the sterility of F_1 (Ngampongsai et al., 1995), which is caused by low pollen viability (Ngampongsai, 2000). Pollen viability and seed setting of F_1 plants can be increased by applying with NAA, GA_3, and colchicine (Saengchot et al., 2000)

In the instance of interspecific hybridization among *Vigna* species, crossing mungbean with black gram and rice bean was successful when mungbean was used as maternal parent while black gram and rice bean were used as pollen parent. F_1 seeds of *V. mungo* x *V. radiata* were light, hollow, and shrunken. (Verma and Yadava, 1986, quoted in Ngampongsai et al., 1995; Chen et al., 1983, quoted in Ngampongsai et al., 1995). Crossing of *V. angularis* with *V. radiata* and *V. umbellata* was succeeded when their embryos were cultured but the hybrids obtained from *V. radiata* x *V.*

angularis were sterile (Chen et al., 1983, quoted in Ngampongsai et al., 1995; Siriwardhane et al., 1991, quoted in Ngampongsai et al., 1995). This was similar to the results from crossing between *V. glabrescens* and *V. mungo, V. umbellata*, and *V. radiata* which the hybrid embryos must be rescued (Egawa et al., 1988, quoted in Ngampongsai et al., 1995; Chen et al., 1989, quoted in Ngampongsai et al., 1995). The results of interspecific hybridization among subgenus *Ceratotropis* species genus *Vigna* were summarized in Table 2.1.

Table 2.1 Production of viable F_1 hybrids between five Asian *Vigna* cultigens.[(1)]

Pollen parent	Seed parent				
	V. aconitifolia	*V. radiata*	*V. mungo*	*V. angularis*	*V. umbellata*
V. aconitifolia	-	-	-	-	-
V. radiata	inviable seedling	-	no viable embryos	inviable seedlings	no viable embryos
V. mungo	-	viable seedlings[+]	-	inviable seedlings[+]	no viable embryos
V. angularis	-	viable seedlings[+]	inviable seedlings[+]	-	viable seedlings[+]
V. umbellata	-	viable seedlings[+]	viable seedling[+]	no viable embryos	-

[+] by embryo culture

[(1)] Note modified from "Evolution of grain legumes. III. Pulses in the genus *Vigna*", by Smatt, 1985, Expl. Agric, 21, 87-100, quoted in Ngampongsai et al. (1995).

Cross-compatibility between *V. radiata* and *V. grandiflora* exhibited 21.2% pod setting when *V. radiata* was used as a female parent. However, the hybrid pods were wilting at 8 to 10 DAP. A total of 16 hybrid embryos were cultured and 16 young seedlings were obtained but there has been no report about the hybrid plants obtained from these embryo cultures (Egawa et al., 1991).

The study of Ngampongsai et al. (1995) in crossing among 5 *Ceratrotopis* species (Table 2.2) showed high percentage of pod setting when *V. radiata* was used as maternal parent. Embryo rescues were successful in the crosses that used *V. glabrescens* and *V. radiata* as maternal parents (Table 2.3). Then hybrid embryos were cultured in White's agar medium supplemented with 200mg/l yeast extract to generate stem and transferred into B5 medium supplemented with 2.0 mg/l IBA to generate roots.

Crossings among 9 *Ceratotropis* species were studied by Egawa et al. (1996). Their results were summarized as shown in Table 2.4. Some cross combinations showed hybrid pod setting but there were no viable seeds obtained because the pods dried out before reaching maturity. The hybrid plants from *V. glabrescen* x *V. trinervia* were obtained through embryo rescue as shown in Table 2.5.

Table 2.2 Results of interspecific crosses and germination test. [1]

Cross combination	No. of flowers pollinated	No. of pods set (%)	No. of seeds sown	No. of plants obtained
V. radiata x V. mungo	124	7 (5.6)	48	2
V. radiata x V. umbellata	30	9 (30.0)	25	1
V. radiata x V. aconitifolia	39	0 (0.0)	0	-
V. radiata x V. angularis	123	0 (0.0)	0	-
V. mungo x V. radiata	105	6 (5.7)	19	0
V. mungo x V. aconitifolia	32	0 (0.0)	0	-
V. mungo x V. angularis	68	4 (5.9)	21	0
V. umbellata x V. radiata	15	2 (13.3)	3	0
V. angularis x V. radiata	60	0 (0.0)	0	-
V. angularis x V. mungo	44	0 (0.0)	0	-
V. angularis x V. aconitifolia	1	0 (0.0)	0	-

[1] Note from "Embryo culture of interspecific hybrids among *Vigna* species", by Ngampongsai et al., 1995, Proc. sixth Mungbean Workshop (pp 91-102) Nakhon Ratchasima: Suranaree University of Technology.

Table 2.3 Results of embryo culture of interspecific hybrids among *Vigna* species. [1]

Cross combination	No. of embryos cultured	No. of embryos germinated (%)	No. of seedling obtained (%)	No. of plants obtained (%)
V. radiata x V. mungo	14	10 (71)	5 (36)	1 (7)
V. radiata x V. glabrescens	51	18(35)	10 (20)	2 (3)
V. glabrescens x V. radiata	27	27 (100)	11 (41)	3 (11)
V. glabrescens x V. angularis	3	3 (100)	3 (100)	0 (0)

[1] Note from "Embryo culture of interspecific hybrids among *Vigna* species", by Ngampongsai, et al., 1995, Proc. sixth Mungbean Workshop (pp 91-102) Nakhon Ratchasima: Suranaree University of Technology.

Table 2.4 Summary of results on interspecific hybridization. [(1)]

Cross combination	No. of flowers pollinated	No. of pods set (%)	No. of viable seeds obtained
V. angularis (w) *x V. minima*	11	1 (9.1)	2
V. angularis (w) *x V. trinervia*	5	0 (0.0)	0
V. glabrescens x V. reflexo-pilosa	10	6 (60.0)	18
V. glabrescens x V. trinervia	24	20 (83.3)	0
V. glabrescens x V. umbellata (w)	2	0 (0.0)	0
V. minima x V. trinervia	74	8 (10.8)	0
V. nakashimae x V. minima	9	5 (55.5)	5
V. nakashimae x V. trinervia	17	12 (70.6)	0
V. radiata x V. minima	10	4 (40.0)	0
V. radiata x V. trinervia	27	8 (29.6)	0
V. radiata x V. umbellata (w)	1	1 (100.0)	0
V. radiata (w) *x V. trinervia*	17	9 (52.9)	27
V. radiata (w) *x V. umbellata* (w)	7	4 (57.1)	0
V. reflexo-pilosa x V. glabrescens	13	3 (15.4)	4
V. reflexo-pilosa x V. minima	69	6 (8.7)	0
V. reflexo-pilosa x V. trinervia	133	43 (32.3)	0
V. reflexo-pilosa x V. umbellata (w)	33	5 (15.1)	0
V. trinervia x V. minima	293	11 (3.8)	21
V. trinervia x V. radiata	9	0 (0.0)	0
V. trinervia x V. radiata (w)	21	0 (0.0)	0
V. trinervia x V. umbellata (w)	48	2 (4.2)	0
V. umbellata (w) *x V. minima*	17	11 (64.7)	0
V. umbellata (w) *x V. trinervia*	11	1 (9.1)	0

[(1)] Note from "Cross-compatibility among the subgenus *Ceratotropis* species of the genus *Vigna*", by Egawa et al., 1996b, Phylogenetic differentiation of mungbean germplasm (subgenus *Ceratotropis* of the genus *Vigna*) and evaluation for breeding program, pp 9-18.

Table 2.5 Results of embryo culture of interspecific hybrids among *Vigna* species. (1)

Cross combination	No. of embryos cultured	No. of plants obtained (%)
V. reflexo-pilosa x V. trinervia	4	0 (0)
V. glabrescens x V. trinervia	23	10 (43)
(V. glabrescens x V. reflexo-pilosa) x V. trinervia	1	1 (100)

(1) Note from "Cross-compatibility among the subgenus *Ceratotropis* species of the genus *Vigna*", by Egawa et al., 1996b, Phylogenetic differentiation of mungbean germplasm (subgenus *Ceratotropis* of the genus *Vigna*) and evaluation for breeding program, pp 9-18.

2.5 References

Archak, S. and Baldev, A. B. (2005). Inter simple sequence repeats (ISSR) analysis [On-line]. Available: http://nbpgr.delhi.nic.in/nrc_dna_rev/manual/t1/t1_ practical3 .pdf.

Assefa, K., Merker, A. and Tefera, H. (2003). Inter simple sequence repeat (ISSR) analysis of genetic diversity in tef [*Eragrostis tef* (Zucc.) Trotter]. **Hereditas** 139: 174-183.

Bednarska, E. (1991). Calcium uptake from the stigma by the germinating pollen in *Primula officinalis* L. and *Ruscus aculeatus* L. **Sex. Plant. Repro.** 4: 36-38. Quoted in A. N. Onus. (2000). Structure of the stigma and style in *Capsicum eximium* and the effects of pollination. **Turk. J. Bot.** 24: 337-346.

Borone, A., del Giudice, A. and Ng, N. Q. (1992). Barriers to interspecific hybridization between *Vigna unguiculata* and *Vigna vexillata*. **Sex. Plant. Repro.** 5: 195-200. Quoted in A. N. Onus. (2000). Structure of the stigma and

style in *Capsicum eximium* and the effects of pollination. **Turk. J. Bot.** 24: 337-346.

Botstein, D., White, R. L., Skolnik, M. and Davis, R. W. (1980). **Am. J. Hum. Genet**. 32: 314-331. Quoted in K. R. Sivaprakash, S. R. Prashanth, B. P. Mohanty, and A. Parida. (2004). Genetic diversity of black gram (*Vigna mungo*) landraces as evaluated by amplified fragment length polymorphism markers. **Curr. Sci.** 86 (10): 1411-1416.

Brown-Guedira, G. L., Thomson, J. A., Nelson, R. L. and Warburton, M. L. (2000). Evaluation of genetic diversity of soybean introductions and North American ancestor using RAPD and SSR markers. **Crop Sci.** 40: 815-823.

Capo-chichi, L. J. A., Weaver, D. B. and Morton, C. M. (2003). The use of molecular markers to study genetic diversity in *Mucuna*. **Trop. Subtrop. Agroecosyt.** 1: 309-318.

Chen, N. C., Baker, L. R. and Honma, S. (1983). Interspecific crossibility among four species of *Vigna* food legumes. **Euphytica** 32: 925-937. Quoted in S. Ngampongsai, S. Pichitporn, W. Promkum, P. Srinives, and Y. Egawa. (1995). Embryo culture of interspecific hybrids among *Vigna* species. In **Proc. Sixth Mungbean Workshop** (pp 91-102). Nakhon Ratchasima: Suranaree University of Technology.

Chen, H. K., Mok, M. C., Shanmungasundaram, S. and Mok, D. W. S. (1989). Interspecific hybridization between *Vigna radiata* and *V. glabrescens*. **Theor. Appl. Genet.** 78: 641-647. Quoted in S. Ngampongsai, S. Pichitporn, W. Promkum, P. Srinives, and Y. Egawa. (1995). Embryo culture of interspecific

hybrids among *Vigna* species. In **Proc. Sixth Mungbean Workshop** (pp. 91-102). Nakhon Ratchasima: Suranaree University of Technology.

Ciofi, C., Funk, S. M., Coote, T., Cheesman, D. J., Hammond, R. L., Saccheri, I. J. and Bruford, M. W. (1998). Genotyping with microsattellite markers. In A. Karp, P. Isaac and D. S. Ingram. (eds.). **Molecular Tools for Screening Biodiversity, Plants and Animals** (pp 195-201). London: Chapman & Hall.

Coulibaly, S., Pasquet, R. S., Papa, R. and Gepts, P. (2002). AFLP analysis of the phenetic organization and genetic diversity of *Vigna unguiculata* L. Walp. reveals extensive gene flow between wild and domesticated types. **Theor. Appl. Genet.** 104: 358-366.

Creer, S., Thorpe, R. S., Malhotra, A., Chou, W.-H. and Stenson, A. G. (2004). The utility of AFLPs for supporting mitochondrial DNA phylogeographical analyses in the Taiwanese bamboo viper, *Trimeresurus stejnegeri*. **J. Evol. Biol.** 17: 100-107.

Cregan, P. B., Shoemaker, R. C. and Specht, J. E. (1999). An integrated genetic linkage map of the soybean genome. **Crop Sci**. 39: 1464-1490.

Davierwala, A. P., Ramakrishna, W., Chowdari, V., Ranjekar, P. K. and Gupta, V. S. (2001). Potential of $(GATA)_n$ microsatellites from rice for inter and intra-specific variability studies. **BMC Evol. Biol.** 1: 7

Dean, R. E., Dahlberg, J. A., Hopkins, M. S., Mitchell, S. E. and Kresovich, S. (1999). Genetic redundancy and diversity among ‘Orange’ accessions in the U.S. National Sorghum collection as assessed with simple sequence repeat (SSR) markers. **Crop Sci.** 39: 1215-1221.

Doi, K., Kaga, A., Tomooka, N. and Vaughan, D. A. (2002). Molecular phylogeny of genus *Vigna* subgenus *Ceratotropis* based on rDNA ITS and *atpB-rbcL* intergenic spacer of cpDNA sequences. **Genetica** 114: 129-145.

Dos Santos, J. B., Nienhuis, J., Skroch, P., Tivang, J. and Sloum, M. K. (1994). Comparison of RAPD and RFLP genetic markers in determining genetic similarity among *Brassica oleracea* L. genotypes. **Theor. Appl. Genet.** 87: 909-915.

Egawa, Y., Bujang, I. B., Chotechuen, S., Tomooka, N. and Tateishi, Y. (1996a). Phylogenetic differentiation of tetraploid *Vigna* species, *V. glabrescens* and *V. reflexo-pilosa*. **JIRCAS Journal** 3: 49-58. Quoted in N. Tomooka, Y. Egawa and A. Kaga. (2000). Biosystematics and genetic resources of the genus *Vigna* subgenus *Ceratotropis*. In **7th MAFF International Workshop on Genetic Resources Wild Legumes** (pp 37-62). Tsukuba, Japan: National Institute of Agrobiological Resources.

Egawa, Y., Chotechuen, S., Tomooka, N., Ngampongsai, S., Thavarasook, C. and Kitbamroong, C. (1996b). Cross-compatibility among the subgenus *Ceratotropis* species of the genus *Vigna*. In Y. Egawa and S. Chotechuen (eds.). **Phylogenetic Differentiation of Mungbean Germplasm (Subgenus *Ceratotropis* of the Genus *Vigna*) and Evaluation for Breeding Program** (pp 9-18). Japan International Research Center for Agricultural Sciences (JIRCAS), Japan and Chai Nat Field Crops Research Center, Thailand.

Egawa, Y., Chotechuen, S., Tomooka, N., Thavarasook, C. and Kitbamroong, C. (1996c). Phylogenetic relationships within the *Ceratotropis* species of the genus *Vigna* detected by isozyme analysis. In Y. Egawa and S. Chotechuen (eds.).

Phylogenetic Differentiation of Mungbean Germplasm (Subgenus *Ceratotropis* of the Genus *Vigna*) and Evaluation for Breeding Program (pp 9-18). Japan International Research Center for Agricultural Sciences (JIRCAS), Japan and Chai Nat Field Crops Research Center, Thailand.

Egawa, Y., Nakagara, M. and Fernandez, G. C. J. (1988). Cytogenetical analysis of tetraploid *Vigna glabrescens* by interspecific hybridization involving diploid Asian *Vigna* species. In **Proc. Second Mungbean International Symposium** (pp 200-204). Bangkok: AVRDC. Quoted in S. Ngampongsai, S. Pichitporn, W. Promkum, P. Srinives and Y. Egawa. (1995). Embryo culture of interspecific hybrids among *Vigna* species. In **Proc. Sixth Mungbean Workshop** (pp 91-102). Nakhon Ratchasima: Suranaree University of Technology.

Egawa, Y., Tomooka, N., Lairungreang, C., Nakeeraks, P. and Thavarasook, C. (1991). The possibility to use *V. mungo* (black gram) and *V. grandiflora* for mungbean breeding. In N. Tomooka, C. Lairungreang, P. Nakeeraks, Y. Egawa, and C. Thavarasook (eds.). **Mungbean and the Genetic Resources: The Subgenus *Ceratotropis* in Ecological Studies on Tropical Food Legumes in Relation to Adaptation to Cropping Systems in Thailand** (pp 39-43)**.** Tropical Agriculture Research Center, Japan and Chai Nat Field Crops Research Center, Thailand.

Esselman, E. J., Jianqiang, L., Crawford, D. J., Winduss, J. L. and Wolfe, A. D. (1999). Clonal diversity in the rare *Calamagrostis porter*i ssp. *insperata* (Poaceae): comparative results for allozymes and random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers. **Mol. Ecol.** 8: 443-451.

Geribello Priolli, R. H., Mendes-Junior, C. T., Arantes, N. E. and Contel, E. P. B. (2002). Characterization of Brazillian soybean cultivars using microsatellite markers. **Gen. Mol. Biol.** 25: 185-193.

Jarne, P. and Lagoda, P. J. L. (1996). Microsatellite, from molecules to populations and back. **Trends Ecol. Evol.** 11: 424-429. Quoted in G. K. Krishna, J. Zhang, M. Burow, R. N. Pittman, S. G. Delikostadinov, Y. Lu and N. Puppala. (2004). Genetic diversity analysis in valencia peanut (*Arachis hypogaea* L.) using microsatellite markers. **Cell. Mol. Biol. Lett.** 9: 685-697.

Kaga, A., Ishii, T. Tsukimoto, K., Tokoro, E. and Kamijima, O. (2000). Comparative molecular mapping in *Ceratotropis* species using an interspecific cross between azuki bean (*Vigna angularis*) and rice bean (*V. umbellata*). **Theor. Appl. Genet.** 100: 207-213.

Kaga, A., Tomooka, N., Egawa, Y., Hosaka, K. and Kamijima, O. (1996). Species relationships in the subgenus *Ceratotropis* (genus *Vigna*) as revealed by RAPD analysis. **Euphytica** 88: 17-24.

Kanarev, A. V., Tomooka, N. and Vaughan, D. A. (2002). Proteinase inhibitor polymorphism in the genus *Vigna* subgenus *Ceratotropis* and its biosystematic implications. **Euphytica** 123: 165-177.

Karp, A. and Edwards, K. J. (1995). Molecular techniques in the analysis of the extent and distribution of genetic diversity. In **Molecular Genetic Techniques for Plant Genetic Resources. Report of an IPGRI Workshop.** Italy: Rome. Quoted in L. J. A. Capo-chichi, D. B. Weaver and C. M. Morton. (2003). The use of molecular markers to study genetic diversity in *Mucuna*. **Trop. Subtrop. Agroecosyt.** 1: 309-318.

Karp, A., Seberg, O. and Buiatti, M. (1996). Molecular techniques in the assessment of botanical diversity. **Ann. Bot. (London)** 78: 143-149. Quoted in E. Yee, K. K. Kidwell, G. R. Sill and T. A. Lunpkin. (1999). Diversity among selected *Vigna angularis* (azuki) accessions on the basis of RAPD and AFLP markers. **Crop Sci.** 39: 268-275.

Klaus, S. (1997). Evolutionary genetics of *Daphnia* species complexes-hybridism in syntopy. [On-line]. Available: http:// aslo.org/doalog/199704-1.html.

Kribben, F. J. (2002). Interspecific hybridization with *Arabidopsis*. [On-line]. Available: http://www.arabidopsis.org/ais/1965/kribb-1965-aaglg.html.

Krishna, G. K., Zhang, J., Burow, M., Pittman, R. N., Delikostadinov, S. G., Lu, Y. and Puppala, N. (2004). Genetic diversity analysis in valencia peanut (*Arachis hypogaea* L.) using microsatellite markers. **Cell. Mol. Biol. Lett.** 9: 685-697.

Kumar, L. D., Kathirvel, M., Rao, G. V. and Nagaraju, J. (2001). DNA profiling of disputed chilli samples (*Capsicum annum*) using ISSR-PCR and FISSR-PCR marker assays. **Forensic Sci. Int.** 116: 63-68.

Labarka, C. and Loweus, F. (1973). The nutritional role of pistil exudate in pollen tube wall formation in *Lilium longifolium*. II. Production and utilization of exudate from stigma and stylar canal. **Plant Physiol.** 52: 87-92. Quoted in A. N. Onus. (2000). Structure of the stigma and style in *Capsicum eximium* and the effects of pollination. **Turk. J. Bot.** 24: 337-346.

Lairungreang, C., Tomooka, N., Nakeeraks, P., Egawa, Y. and Thavarasook, C. (1991). Wild *Ceratotropis* species collected in northern Thailand. In N. Tomooka, C. Lairungreang, P. Nakeeraks, Y. Egawa and C. Thavarasook (eds.). **Mungbean and the Genetic Resources: The Subgenus *Ceratotropis* in**

Ecological Studies on Tropical Food Legumes in Relation to Adaptation to Cropping Systems in Thailand (pp 45-49)**.** Tropical Agriculture Research Center, Japan and Chai Nat Field Crops Research Center, Thailand.

Larson, S. R., Jones, T. A., Hu, Z.-M., McCracken, C. L. and Palazzo, A. (2000). Genetic diversity of bluebunch wheatgrass cultivars and a multiple-origin polycross. **Crop Sci.** 40: 1142-1147.

Li, C.-D., Fatokun, C. A., Ubi, B., Singh, B. B. and Scoles, G. J. (2001). Determinating genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. **Crop Sci.** 41: 189-197.

Liu, B. and Wendel, J. F. (2001). Intersimple sequence repeat (ISSR) polymorphisms as a genetic marker system in cotton. **Mol. Ecol. Notes** 1: 205-208.

Liu, X. C. and Wu, J. L. (1998). SSR heterogenetic patterns of parents for marking and predicting heterosis in rice breeding. **Mol. Breed.** 4: 263-268.

Luikart, G. and England, P. (1999) Statistical analysis of microsatellite DNA data. **Trends Ecol. Evol.** 14: 253-256. Quoted in M. J. Clauss, H. Cobban and T. Mitchell-Olds. (2002). Cross-species microsatellite markers for elucidating population genetic structure in *Arabidopsis* and *Arabi*s (Brassicaeae). **Mol. Ecol.** 11: 591-601.

Mallikarjuna, N. (1999). Ovule and embryo culture to obtain hybrids from interspecific incompatible pollination in chickpea. **Euphytica** 110: 1-6.

Maréchal, R., Mascherpa, J. M. and Stainier, F. (1978). Etude taxonomique d´ un groupe complexe d´espéces des genres Phaseolus et Vigna(Papilionaceae) sur la base de données morphologiqueset olloniques, traitées par l´ analysis informatique. **Boissiera** 28: 1-237. Quoted in N. Tomooka, C. Lairungreang, P.

Nakeeraks, Y. Egawa and C. Thavarasook (eds.). (1991). **Mungbean and the Genetic Resources: The Subgenus *Ceratotropis* in Ecological Studies on Tropical Food Legumes in Relation to Adaptation to Cropping Systems in Thailand.** Tropical Agriculture Research Center, Japan and Chai Nat Field Crops Research Center, Thailand.

Matthes, M. C., Daly, A. and Edwards, K. J. (1998). Amplified fragment length polymorphism (AFLP). In A. Karp, P. Isaac and D. S. Ingram. (eds.). **Molecular Tools for Screening Biodiversity, Plants and Animals** (pp 195-201). London: Chapman & Hall.

Métais, I., Hamon, B., Jalouzot, R. and Peltier, D. (2002). Structure and level of genetic diversity in various bean types evidenced with microsatellite markers isolated from a genomic enriched library. **Theor. Appl. Genet.** 104: 1346-1352.

Muluvi, G. M., Sprent, J. I., Soranzo, N., Provan, J., Odee, D., Folkard, G., McNicol, J. W. and Powell, W. (1999). Amplified fragment length polymorphism (AFLP) analysis of genetic variation in *Moringa oleifera* Lam. **Mol. Ecol.** 8: 463-470.

Narvel, J. M., Fehr, W. R., Chu, W.-C., Grant, D. and Shoemaker, C. (2000). Simple sequence repeat diversity among soybean plant introductions and elite genotypes. **Crop Sci.** 40: 1452-1458.

Ngampongsai, S. (2000). Interspecific hybridization of mungbean |*Vigna radiata* (L.) Wilczek| and related *Vigna* species using embryo rescue techniques. In **Proc. National Mungbean Research Conf. VIII** (pp 1-6). Nakhon Pathom: Kasetsart University.

Ngampongsai, S., Pichitporn, S., Promkum, W., Srinives, P. and Egawa, Y. (1995). Embryo culture of interspecific hybrids among *Vigna* species. In **Proc. Sixth**

Mungbean Workshop (pp 91-102). Nakhon Ratchasima: Suranaree University of Technology. (in Thai).

Ngampongsai, S., Watanasit, A., Tanomsub, W. and Tanomsub, S. (2000). Exploration, collection and evaluation of wild *Vigna* species. In **Proc. National Mungbean Research Conf. VIII** (pp 21-30). Nakhon Pathom: Kasetsart University.

Nguyen, T. T., Taylor, P. W. J., Redden, R. J. and Ford, R. (2004). Genetic diversity estimates in *Cicer* using AFLP analysis. **Plant Breeding** 123: 173-179.

Ntundu, W. H., Bach, I. C., Christiansen, J. L. and Andersen, S. B. (2004). Analysis of genetic diversity in bambara groundnut [*Vigna subterranea* (L.)Verdc] landraces using amplified fragment length polymorphism (AFLP) markers. **African J. Biotechnol.** 3: 220-225.

Pasquet, R. S., Schwedes, S. and Gepts, P. (1999). Isozyme diversity in bambara groundnut. **Crop Sci.** 39: 1228-1236.

Patzak, J. (2001). Comparison of RAPD, STS, ISSR and AFLP molecular methods used for assessment of genetic diversity in hop (*Humulus lupulus* L.) **Euphytica** 121: 9-18.

Peakall, R., Gilmore, S., Keys, W., Morgante, M. and Rafalski, A. (1998). Cross-species amplification of soybean (*Glycine max*) simple sequence repeats (SSRs) within the genus and other Legume genera: Implications for the transferability of SSRs in plants. **Mol. Biol. Evol.** 15: 1275-1287.

Pichitporn, S., Thavarasook, C. and Tomooka, N. (1993). Exploration and collection of wild *Vigna* in northern Thailand. In **Proc. Fifth Mungbean Workshop** (pp 9-13). Nongkhai: Dept. Agric. Extension and Dept. Agriculture. (in Thai).

Ping, L. (1996). Varietal verification of *Ceratotropis* species using isozyme. [On-line]. Available: http: www.arc-avrdc.org/pdf_files/Luping

Powell, W., Machray, G. C. and Provan, J. (1996). Polymorphism revealed by simple sequence repeats. **Trends Plant Sci.** 1:215–222. Quoted in I. Métais, B. Hamon, R. Jalouzot and D. Peltier. (2002). Structure and level of genetic diversity in various bean types evidenced with microsatellite markers isolated from a genomic enriched library. **Theor. Appl. Genet.** 104: 1346-1352.

Promper, K. W., Crabtree, S. B., Brown, S. P., Jones, S. C. and Bonney, T. M. (2003). Assessment of genetic diversity of Pawpaw (*Asimina triloba*) cultivars with intersimple sequence repeat markers. **J. Amer. Soc. Hort. Sci.** 128: 521-525.

Saengchot, S., Srinives, P., Kaveeta, R. and Lamseejan, S. (2000). A study on increasing pod setting in interspecific hybrid between *Vigna* spp. In **Proc. National Mungbean Research Conf. VIII** (pp 200-206). Nakhon Pathom: Kasetsart University.

Saravanakumar, P., Kaga, A., Tomooka, N. and Vaughan, D. A. (2004). AFLP and RAPD analyses of intra- and interspecific variation in some *Vigna* subgenus *Ceratotropis* (Leguminosae) species. **Aust. J. Bot.** 52: 417-424.

Schuler, D., Boguski, M.S. and Stewart, E.A. (1996). A gene map of the human genome. **Science** 273: 540-546. Quoted in G. K. Krishna, J. Zhang, M. Burow, R. N. Pittman, S. G. Delikostadinov, Y. Lu and N. Puppala. (2004). Genetic diversity analysis in valencia peanut (*Arachis hypogaea* L.) using microsatellite markers. **Cell. Mol. Biol. Lett.** 9: 685-697.

Sica, M., Gamba, G., Montieri, S., Gaudio, L. and Aceto, S. (2005). ISSR markers show differentiation among Italian populations of *Asparagus acutifolius* L. **BMC Genetics** 6: 17-23.

Siriwardhane, D., Egawa, Y. and Tomooka, N. (1991). Cross-compatibility of cultivated azuki bean (*Vigna angularis*) and rice bean (*V. umbellata*) with their wild relatives. **Plant Breeding** 107: 320-325. Quoted in S. Ngampongsai, S. Pichitporn, W. Promkum, P. Srinives and Y. Egawa. (1995). Embryo culture of interspecific hybrids among *Vigna* species. In **Proc. Sixth Mungbean Workshop** (pp 91-102). Nakhon Ratchasima: Suranaree University of Technology.

Sivaprakash, K. R., Prashanth, S. R., Mohanty, B. P. and Parida, A. (2004). Genetic diversity of black gram (*Vigna mungo*) landraces as evaluated by amplified fragment length polymorphism markers. **Curr. Sci.** 86: 1411-1416.

Smatt, J. (1985). Evolution of grain legumes. III. Pulses in the genus *Vigna*. **Expt. Agric.** 21: 87-100. Quoted in S. Ngampongsai, S. Pichitporn, W. Promkum, P. Srinives and Y. Egawa. (1995). Embryo culture of interspecific hybrids among *Vigna* species. In **Proc. Sixth Mungbean Workshop** (pp 91-102). Nakhon Ratchasima: Suranaree University of Technology.

Smith, J. S. C., Chin, E. C. L., Shu, H., Smith, O. S., Wall, S. J., Senior, M. L., Mitchell, S. E., Kresovitch, S. and Ziegle, J. (1997). An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from RFLPs and pedigree. **Theor. Appl. Genet.** 95: 163–173. Quoted in C-D. Li, C. A. Fatokun, B. Ubi, B. B. Singh and G. J. Scoles. (2001). Determining

Genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. **Crop Sci.** 41: 189-197.

Suyana, Y., Obayashi, K. and Hayashi, I. (2000). Clonal structure in a dwarf bamboo (*Sasa senanensis*) population inferred from amplified fragment length polymorphism (AFLP) fingerprints. **Mol. Ecol.** 9: 901-906.

Tanya, P., Srinives, P., Toojinda, T., Vanavichit, A., Ha, B. K., Bae, J. S., Moon, J. K. and Lee, S. H. (2001). Evaluation of genetic diversity among soybean genotypes using SSR and SNP. **Korean J. Crop Sci.** 46: 334-340.

Tateishi, Y. (1985). A revision of the azuki bean group, the subgenus *Ceratotropis* of the genus *Vigna* (*Leguminosae*). Ph.D. Thesis, Tohoku University, Japan. Quoted in M. S. Yoon, K. Doi, A. Kaga, N. Tomooka and D. Vaughan. (2000). analysis of the genetic diversity in the *Vigna minima* complex and related species in East Asia. **J. Plant Res.** 113: 375-386.

Tateishi, Y. (1996). Systematics of the species of *Vigna* subgenus *Ceratotropis*. In P. Srinives, C. Kitbamroong, and S. Miyazaki. (eds.). **Mungbean Germplasm: Collection, Evaluation and Utilization for Breeding Program** (pp 9-24). JIRCAS, Japan. Quoted in N. Tomooka, D. A. Vaughan, H. Moss and N. Maxted. (2002). **The Asian *Vigna*: Genus *Vigna* subgenus *Ceratotropis* Genetic Resources.** The Netherlands: Kluwer Academic. 270 p.

Tateishi, Y. and Maxted, N. (2002). New species and combinations in *Vigna* subgenus *Ceratotropis* (Piper) Verdcourt (Leguminosae, Phaseoleae). **Kew Bulletin** 57: 625-633. Quoted in P. Saravanakumar, A. Kaga, N. Tomooka and D. A. Vaughan. (2004). AFLP and RAPD analyses of intra- and interspecific variation

in some *Vigna* subgenus *Ceratotropis* (Leguminosae) species. **Aust. J. Bot.** 52: 417-424.

Thormann, C. E., Ferreira, M. E., Camargo, L. E. A., Tivang, J. G. and Osborn, T. C. (1994). Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. **Theor. Appl. Genet.** 88: 973-980.

Tomooka, N. (1991). Taxonomic position of mungbean and its close relatives, the subgenus *Ceratotropis* (A literature review). In N. Tomooka, C. Lairungreang, P. Nakeeraks, Y. Egawa and C. Thavarasook (eds.). (1991). **Mungbean and theGenetic Resources: The Subgenus *Ceratotropis* in Ecological Studies on Tropical Food Legumes in Relation to Adaptation to Cropping Systems in Thailand** (pp 25-31)**.** Tropical Agriculture Research Center, Japan and Chai Nat Field Crops Research Center, Thailand.

Tomooka, N., Chotechuen, S., Boonkerd, N., Taengsan, B., Nupllean, S., Vaughan, D., Egawa, Y., Tokoyama, T. and Tateishi, Y. (1997). Collection of seed and nodule samples from wild subgenus *Ceratotropis* species (genus *Vigna*) in central and northern Thailand. In **Annual Report on Exploration and Introduction of Plant Genetic Resources** 13: 189-206. National Institute of Agrobiological Sciences. (in Japanese with English summary).

Tomooka, N., Srinives, P., Boonmalisan, D., Chotechuen, S., Taengsan, B. and Ornnaichart, P. (2000). Field survey of high temperature tolerant Asian *Vigna* species in Thailand. In **Annual Report on Exploration and Introduction of Plant Genetic Resources** 16: 171-186. National Institute of Agrobiological Sciences. (in Japanese with English summary).

Tomooka, N., Vaughan, D. A., Kaga, A. and Jayasuriya, A. H. M. (2003). Advances in understanding the genus *Vigna* subgenus *Ceratotropis* species in Sri Lanka. **Conservation and Use of Crop Wild Relatives** (pp 133-150). Quoted in P. Saravanakumar, A. Kaga, N. Tomooka and D. A. Vaughan. (2004). AFLP and RAPD analyses of intra- and interspecific variation in some *Vigna* subgenus *Ceratotropis* (Leguminosae) species. **Aust. J. Bot.** 52: 417-424.

Tomooka, N., Lairungreang, C., and Egawa, Y. (1995). Taxonomic position of wild *Vigna* species collected in Thailand based on RAPD analysis. In **Proc. of the workshop on mungbean germplasm** (pp 31-40). Bangkok.

Tomooka, N., Vaughan, D. A., Moss H. and Maxted N. (2002a). **The Asian *Vigna*: Genus *Vigna* subgenus *Ceratotropis* genetic resources.** The Netherlands: Kluwer Academic. 270 p.

Tomooka, N., Yoon, M. S., Doi, K., Kaga, A. and Vaughan, D. A. (2002b). AFLP analysis of a *Vigna* subgenus *Ceratotropis* core collection. **Gen. Res. Crop Evol.** 49: 521-530.

Van Tuyl, J. M. and De Jeu, M. J. (1997). Methods for overcoming interspecific crossing barriers. In V. K. Sawhney and K.R. Shivanna (eds.). **Biotechnology and Crop Production Improvement** (pp 273-293). UK: Cambridge University Press.

Vaughan, D., Tomooka, N., Xu, R. Q., Konarev, A., Doi, K., Kashiwaba, K. and Kaga, A. (2000). The *Vigna angularis* complex in Japan. In **7th MAFF International Workshop on Genetic Resources of Wild Legumes** (pp 160-176). Tsukuba, Japan: National Institute of Agrobiological Resources.

Verma, S. N. P. and Yadava, H. (1986). Improvement of greengram through interspecific hybridization. Quoted in S. Ngampongsai, S. Pichitporn, W. Promkum, P. Srinives and Y. Egawa. (1995). Embryo culture of interspecific hybrids among *Vigna* species. In **Proc. Sixth Mungbean Workshop** (pp 91-102). Nakhon Ratchasima: Suranaree University of Technology.

Vos, P., Hogers, R., Bleeker, M., Rijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. **Nucleic Acid Res.** 23: 4407-4414. Quoted in M. S. Yoon, Doi, K., A. Kaga, N. Tomooka and D. Vaughan. (2000). Analysis of the genetic diversity in the *Vigna minima* complex and related species in East Asia. **J. Plant Res.** 113: 375-386.

Wang, X. W., Kaga, A., Tomooka, N. and Vaughan, D. A. (2004). The development of SSR markers by new method in plants and their application to gene flow studies in azuki bean [*Vigna angularis* (Willd.) Ohwi &Ohashi]. **Theor. Appl. Genet.** 109: 352-360.

Weber, J. and Wong, C. (1993). Mutation of human short tandem repeats. **Hum. Mol. Genet.** 2: 1123-1128. Quoted in M. J. Clauss, H. Cobban and T. Mitchell-Olds. (2002). Cross-species microsatellite markers for elucidating population genetic structure in *Arabidopsis* and *Arabi*s (Brassicaeae). **Mol. Ecol.** 11: 591-601.

Wijaya, A., Link, W. and Moellers, C. (2002). Interspecific hybridization in *Vicia faba*. [On-line]. Available: http://www.gwdg.de/~pbzhome/Zuechtung/Andi/hybride.html.

William, J. G. K., Hanafey, M. K., Rafalski, J. A. and Tingey, S. V. (1993). Genetic analysis using random amplified polymorphic DNA markers. **Methods Enzymol.**

218: 704-740. Quoted in A. Kaga, N. Tomooka, Y. Egawa, K Hosaka and O. Kamijima. (1996). Species relationships in the subgenus *Ceratotropis* (genus *Vigna*) as revealed by RAPD analysis. **Euphytica** 88: 17-24.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. **Nucleic Acid Res.** 18: 6531-6535. Quoted in A. Kaga, N. Tomooka, Y. Egawa, K. Hosaka and O. Kamijima. (1996). Species relationships in the subgenus *Ceratotropis* (genus *Vigna*) as revealed by RAPD analysis. **Euphytica** 88: 17-24.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. **Nucleic Acid Res.** 18: 6531-6535. Quoted in K. R. Sivaprakash, S. R. Prashanth, B. P. Mohanty and A. Parida. (2004). Genetic diversity of black gram (*Vigna mungo*) landraces as evaluated by amplified fragment length polymorphism markers. **Curr. Sci.** 86: 1411-1416.

Winfield, M. O., Arnold, G. M., Cooper, F., Le Ray, M., White, J., Karp, A. and Edwards, K. J. (1998). A study of genetic diversity in *Populus nigra* subsp. *betulifolia* in the Upper Severn area of the UK using AFLP markers. **Mol. Ecol.** 7: 3-10.

Woo, S. H., Tai, Q. S. and Adachi, T. (1995). Possibility of interspecific hybridization by embryo rescue in genus *Fagopyrum*. **Curr. Adv. Buckwheat Res.** 6: 225-237.

Xu, R., Tomooka, N., Vaughan, D. A. and Doi, K. (2000). The *Vigna angularis* complex: Genetic variation and relationships revealed by RAPD analysis, and

their implications for *in situ* conversation and domestication. **Gen. Res. Crop Evol.** 47: 123-134.

Xu-xiao, Z., Vaughan, D., Tomooka, N., Kaga, A., Xin-wang, W., Jian-ping, G. and Shu-min, W. (2003). Preliminary study on geographic distribution and evolutionary relationships between cultivated and wild azuki bean (*Vigna angularis* var. *angularis* and var. *nipponensis*) by AFLP analysis. **Agric. Sci. in China** 2: 368-376.

Yee, E., Kidwell, K. K., Sill, G. R. and Lunpkin, T. A. (1999). Diversity among selected *Vigna angularis* (azuki) accessions on the basis of RAPD and AFLP markers. **Crop Sci.** 39: 268-275.

Yoon, M. S., Doi, K. Kaga, A., Tomooka, N. and Vaughan, D. A. (2000). Analysis of the genetic diversity in the *Vigna minima* complex and related species in East Asia. **J. Plant Res.** 113: 375-386.

Zhou, H.-F., Xie, Z.-W. and Ge, S. (2003). Microsatellite analysis of genetic diversity and population genetic structure of a wild rice (*Oryza rufipogon* Griff.) in China. **Theor. Appl. Genet.** 107: 332-339.

CHAPTER III

GENETIC DIVERSITY OF *Vigna* GERMPLASM FROM THAILAND AND NEIGHBORING REGIONS REVEALED BY AFLP ANALYSIS

3.1 Abstract

Thailand is a center of diversity for section *Angulares* of the Asian *Vigna* (genus *Vigna* subgenus *Ceratotropis*) in which four *Vigna* species have been cultivated. Using newly collected wild and cultivated germplasm of *Vigna* from Thailand and outgroup accessions, AFLP analysis was conducted to clarify genetic diversity and relationships. The results suggested that cultivated *V. umbellata* and *V. mungo* evolved from wild relatives in a single domestication event. *V. umbellata* was poorly differentiated from its wild and weedy relatives compared to *V. mungo*. The results suggested that northern Thailand and neighboring Mandalay state of Myanmar were the probable centers of domestication for *V. umbellata* since wild accessions from this area and cultivated rice bean from a wide area in Asia were not greatly diverged. *V. minima*, *V. tenuicaulis* and *V. exilis* accessions in Thailand are well differentiated with considerable intra-specific variation. *V. hirtella* consisted of two well differentiated subgroups, suggesting that the taxonomic revision may be necessary. Close genetic relationships between *V. radiata* and *V. grandiflora*, between *V. mungo* and *V. trinervia* are confirmed. Naturally growing *V. mungo* populations in northern

Thailand appeared to be true wild species as they were well differentiated from the Indian wild and Thai cultivated populations. The origin of naturally growing cowpea in Thailand needs to be further studied using more comprehensive set of materials. This study clarifies inter- and intra-specific genetic diversity and inter-specific relationships of Thai *Vigna* species.

3.2 Introduction

Four *Vigna* cultigens are cultivated in Thailand. Among them, mungbean [*Vigna radiata* (L.) Wilczek] is the most important crop (Srinives and Yang, 1988). Rice bean [*V. umbellata* (Thunb.) Ohwi & Ohashi] is a traditional food crop cultivated mainly in the mountainous area in northern Thailand. Black gram [*V. mungo* (L.) Hepper] has been recently introduced to the central part of Thailand as an export crop (Chainuwat et al., 1988). These three crops belonging to the genus *Vigna* subgenus *Ceratotropis* are called the Asian *Vigna* (Tomooka et al., 2002a). Cowpea [*V. unguiculata* (L.) Walp.] is a traditional food legume grown throughout Southeast Asia including Thailand, although its origin is considered to be in Africa (Ng and Maréchal, 1985). This crop belongs to the genus *Vigna* subgenus *Vigna*. Wild relatives of these cultigens distributed in Thailand are considered to be useful genetic sources for breeding programs.

In the past, the wild *Vigna* species in Thailand were poorly documented. This is mainly because of the poor representation of *Vigna* specimens conserved in major herbaria in Europe (Maréchal et al., 1978). Based on recent systematic collections of the wild *Vigna* in Thailand, many new germplasm accessions and herbarium

specimens have been collected and are conserved (Tomooka, 1991, Tomooka et al., 1997, Tomooka et al., 2000).

Based on new germplasm and herbarium specimens, eight wild species of *Vigna* subgenus *Ceratotropis* are recognized in Thailand (Tomooka et al., 2002a). These are *V. mungo* var. *silvestris* Lukoki, Maréchal & Otoul, *V. grandiflora* (Prain) Tateishi & Maxted, *V. umbellata*, *V. exilis* Tateishi & Maxted, *V. hirtella* Ridley, *V. minim*a (Roxb.) Ohwi & Ohashi, *V. trinervia* (Heyne ex Wall.) Tateishi & Maxted and *V. tenuicaulis* Tomooka & Maxted. Among them, *V. exilis* and *V. tenuicaulis* are recently described (Tateishi and Maxtd, 2002, Tomooka et al., 2002b). While mungbean is the most important cultigen in Thailand, the wild form of this species has not been collected in Thailand. In contrast, wild and weedy forms of rice bean (*V. umbellata*) have frequently been found mainly in northern Thailand. Wild forms of black gram (*V. mungo* var. *silvestris*) were recorded for the first time in 1996 (Tomooka et al., 1997). However, it is not clear whether this is a native wild species or an escape from cultivation. In addition, the origin of naturally growing populations of *V. unguiculata* is unknown (Tomooka et al., 1997), since the natural distribution of wild cowpea (*V. unguiculata* var. *spontanea*) has been considered to be limited to only Africa.

The Asian *Vigna* has been studied using a variety of biochemical and molecular techniques such as RAPD, AFLP, and SSR (Tomooka et al., 2002c; Konarev et al., 2002). These studies used a limited number of accessions of each species. The only studies of the Asian *Vigna* in East Asia tried to assess genetic diversity using a large numbers of accessions of different species (Yoon et al., 2000; Xu et al., 2000a and b; Zong et al., 2003). There has been no report of detailed studies of the Asian *Vigna* in

Thailand using molecular techniques. The objectives of this study were to determine intra-specific genetic diversity of different *Vigna* species in Thailand and determine their relationships to one another using AFLP analysis.

3.3 Materials and Methods

Plant materials

Materials analyzed are listed in Appendix (Table 1A). All materials are diploid species with 2n = 2x = 22 chromosomes. They were analyzed in two sets since the number of accessions included in this study could not be fitted on to one gel to enable reliable comparison. The first set included 96 accessions of closely related species in section *Angulares* except *V. trinervia*. Most of the accessions analyzed in this set were of the *V. umbellata* (rice bean; 57 accessions) crop complex that is widely distributed across Thailand. *V. umbellata* accessions were divided into 4 types based on status when collected (wild or cultivated) and seed size and color. These types were wild accessions with typical seed size and color (wild A), wild accessions with seeds of similar size to typical wild accessions but with atypical seed coat color (wild B), wild accessions with seeds of larger size than typical wild seeds and atypical seed color (wild C) and cultivated accessions (Figure 3.1a). Three wild accessions from Myanmar and one cultivated accession each from Nepal and Japan were included to compare the diversity of Thai *Vigna* with that of neighboring countries. The other species in this set were 9 accessions of *V. exilis*, 8 accessions of *V. hirtella*, 13 accessions of *V. minima*, and 9 accessions of *V. tenuicaulis*.

The second set included other *Vigna* species found in Thailand that were not so closely related; they were: three species in section *Ceratotropis* including 3

accessions of *V. grandiflora*, 13 accessions of *V. mungo*, and one accession of *V. radiata*; 5 accessions of *V. trinervia* in section *Angulares*; and 2 accessions of *V. unguiculata* in subgenus *Vigna*. For both *V. mungo* and *V. unguiculata* both cultivated and wild forms (Figure 3.1b and 3.1c) were included. To enable the diversity of Thailand *Vigna* to be compared with that of neighboring countries, accessions of *V. hirtella, V. minima, V. tenuicaulis* and *V. umbellata* from Myanmar and *V. trinervia* from Malaysia were included. Cultivated accessions of *V. mungo* from Australia and India were also included for evaluation the geographical differentiation among cultivars. For the same reason, one accession each from Nepal and Japan of cultivated *V. umbellata* were examined. The distribution status of accessions from Thailand of each species used in this study is shown in Figure 3.2a-g.

DNA extraction

Five seeds of each accession were planted in pots in the greenhouse. Total genomic DNA of each population was extracted from bulked sample of young primary leaves by a modification of a previously published procedure (Dellaporta et al., 1983). DNA concentrations were estimated and standardized against known concentrations of λ DNA on 1% agarose gels.

AFLP analysis

AFLP analysis followed the procedure described in Zong et al. (2003). Briefly, the restriction reaction was carried out with 0.5 μg genomic DNA digested with *Eco*RI and *Mse*I. *Eco*RI and *Mse*I adapters were ligated to the fragments. PCR

preamplification was carried out with E00 and M00 primers without addition of selective nucleotide using diluted restriction ligation mixture as template DNA.

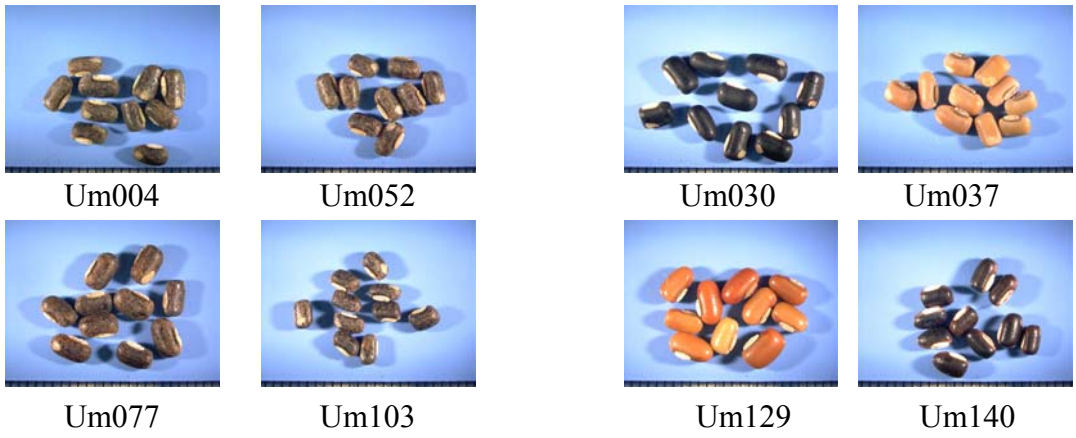
Selective amplification was carried out with *Eco*RI and *Mse*I primers with 3 selective nucleotides added using diluted preamplification products as template DNA. The selective AFLP primers used are shown in Table 3.1. For set 1, all accessions of species in section *Angulares* except *V. trinervia*, the 6 primer pairs used were E32/M32, E33/M33, E39/M39, E47/M47, E50/M50 and E63/M63. For set 2, which included all accessions of *V. trinervia*, *V. unguiculata* and species in section *Ceratotropis* of subgenus *Ceratotropis*, only the first three primer pairs were used (E32/M32, E33/M33, E39/M39). More primers were required for set 1 because the materials analyzed were closely related, so a larger number of primers were considered necessary to resolve differences among and within species. Amplified products were run in 6% acrylamide gel and the banding patterns were visualized according to the silver staining method described by Panaud et al. (1996).

Data analysis

AFLP bands were scored as present (1) and absent (0), and only bands showing unambiguous polymorphism were entered into a data matrix.

Two methods were used to estimate diversity. The first method estimates genetic distance at the species level based on inferred nucleotide diversity (π x 1000) designed especially for AFLP data (Innan et al., 1999). The average value of all the pairwise distances was regarded as a measure of nucleotide polymorphism (nucleotide diversity). A neighbor-joining analysis was conducted by PHYLIP ver. 3.57 on the basis of the pairwise distance (Felsenstein, 1992) and an unrooted tree was

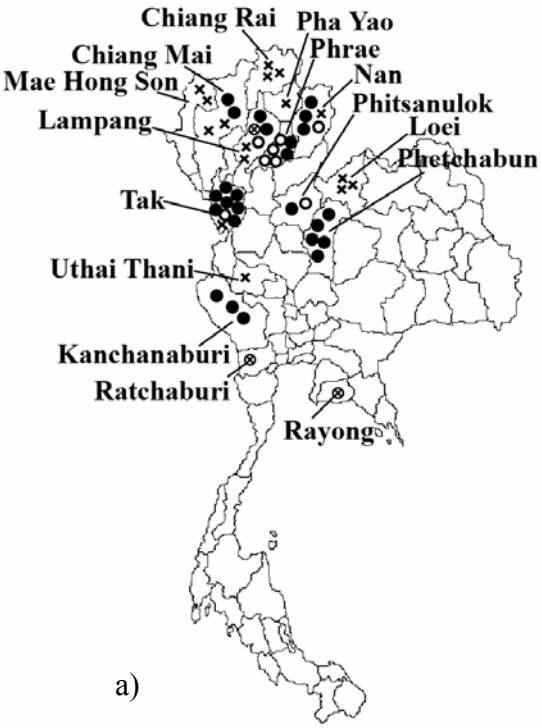
constructed using Treeview ver. 3.2. For wild and cultivated accessions of *V. umbellata* and *V. mungo*, POPGENE software ver 1.32 was used to calculate total genetic diversity (Ht), within-population gene diversity (Hs), relative magnitude of gene differentiation among populations (Gst), Nei's gene diversity (h) and Shannon's diversity index (I).



a) *Vigna umbellata* crop complex

b) *Vigna mungo* **c) *Vigna unguiculata***

Figure 3.1 Variation in seed shape and color of different components of the *V. umbellata* crop complex (a), *V. mungo* (b) and *V. unguiculata* (c).


Chiang Rai
Pha Yao
Chiang Mai
Phrae
Mae Hong Son
Nan
Phitsanulok
Lampang
Loei
Phetchabun
Tak
Uthai Thani
Kanchanaburi
Ratchaburi
Rayong

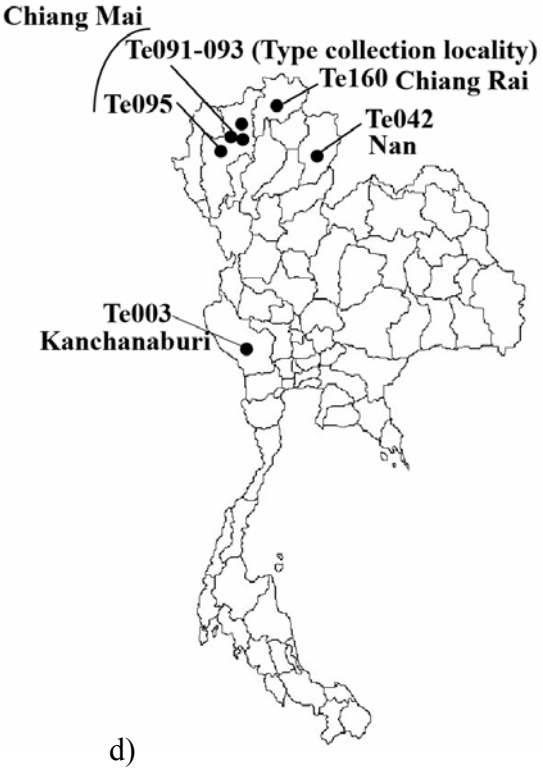
a)


Kanchanaburi
Ratchaburi
Petchaburi
Saraburi

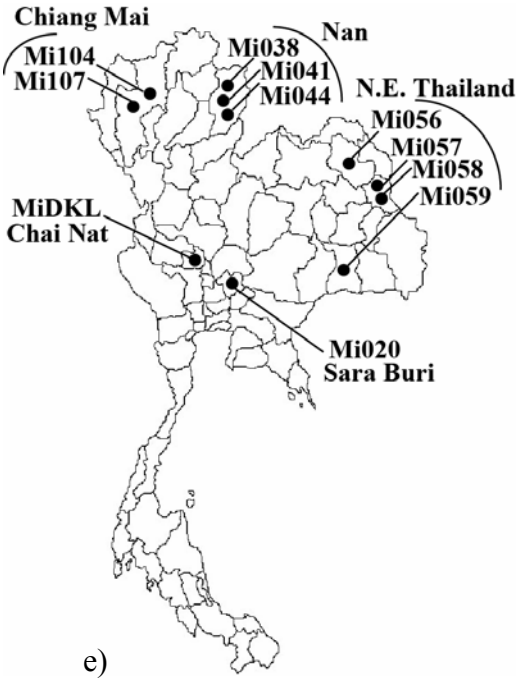
b)


Hi111
Hi112
Hi171
Hi105
Hi101
Hi021

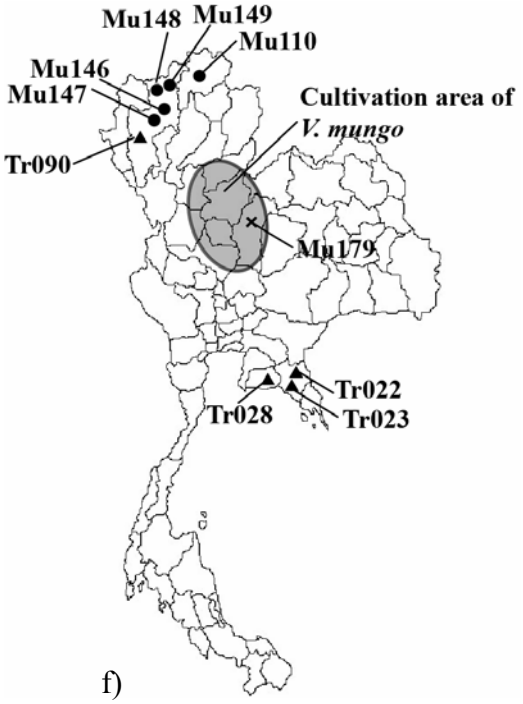
c)


Chiang Mai
Te091-093 (Type collection locality)
Te160 Chiang Rai
Te095
Te042
Nan
Te003
Kanchanaburi

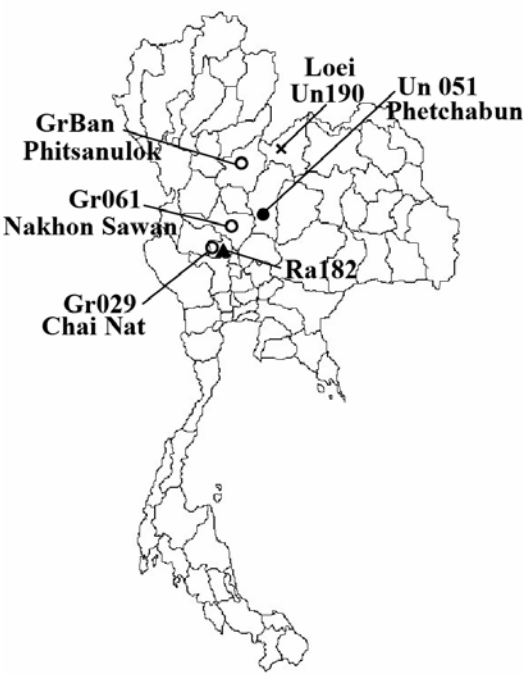
d)



e)



f)



g)

Figure 3.2 Distribution of Thai accessions analyzed:

a) *V. umbellata* (cultivated, ✕ ; wild (A) ● ; wild (B) ○ ; wild (C) ⊗);

b) *V. exilis*;

c) *V. hirtella* (a) ▲ , *V. hirtella* (b) ● ;

d) *V. tenuicaulis*;

e) *V. minima*;

f) *V. trinervia* (▲), *V. mungo* var. *mungo* (✕); *V. mungo* var. *silvestris* (●);

g) *V. grandiflora* (○), *V. radiata* (▲), *V. unguiculata*, cultivated (●); *V. unguiculata* wild (✕)

Table 3.1 Adapter and primer sequence for AFLP analysis.

Name	Enzyme	Type	Sequence (5'-3')
EA	*Eco* RI	Adapter	CTCGTAGACTGCGTACC CTGACGCATGGTTAA
MA	*Mse* I	Adapter	GACGATGAGTCCTGAG TACTCAGGACTCAT
E00	*Eco* RI	Primer + 0	GACTGCGTACCAATTC
E32	*Eco* RI	Primer + 3	GACTGCGTACCAATTCAAC
E33	*Eco* RI	Primer + 3	GACTGCGTACCAATTCAAG
E39	*Eco* RI	Primer + 3	GACTGCGTACCAATTCAGA
E47	*Eco* RI	Primer + 3	GACTGCGTACCAATTCCAA
E50	*Eco* RI	Primer + 3	GACTGCGTACCAATTCCAT
E63	*Eco* RI	Primer + 3	GACTGCGTACCAATTCGAA
M00	*Mse* I	Primer + 0	GATGAGTCCTGAGTAA
M32	*Mse* I	Primer + 3	GATGAGTCCTGAGTAAAAC
M33	*Mse* I	Primer + 3	GATGAGTCCTGAGTAAAAG
M39	*Mse* I	Primer + 3	GATGAGTCCTGAGTAAAGA
M47	*Mse* I	Primer + 3	GATGAGTCCTGAGTAACAA
M50	*Mse* I	Primer + 3	GATGAGTCCTGAGTAACAT
M63	*Mse* I	Primer + 3	GATGAGTCCTGAGTAAGAA

3.4 Results and Discussion

(a) Set 1

For the first set of accessions (see Appendix, Table 1A) AFLP analysis revealed a total of 1074 bands, of which 1021 (95.1%) were polymorphic (Table 3.2a). Neighbor-joining cluster analysis of this set of accessions revealed 6 groups of accessions consisting of 4 species and 2 subgroups within *V. hirtella* (Figure 3.3). All species showed geographical differentiation. The average number of amplified bands per accession, number of polymorphic bands and estimated nucleotide diversity (π x 1000) are summarized for these 6 groups (Table 3.2a). Average number of amplified bands ranged from 302.7 [*V. hirtella* (a)] to 337.8 (*V. umbellata*). Percentage of polymorphic bands ranged from 37.9% [*V. hirtella* (b)] to 76.0% (*V. minima*).

Nucleotide diversity for each species/subgroup ranged from 6.928 (*V. umbellata*) to 18.25 [*V. hirtella* (a)].

V. umbellata (rice bean)

V. umbellata is a minor cultigen but its prolific vegetative growth and high seed production suggest it may be a more useful cover crop, forage or food legume than realized hither-to-fore. This crop has been used as a staple food mainly in mountainous region of mainland Southeast Asia and southern China where it is cultivated under shifting cultivation agricultural systems. These are considered to be the center of origin and diversity.

A total of 57 accessions consisting of different components of this crop complex were analyzed (Figure 3.2a, Table 3.3a). Cultivated accessions consisted of 16 Thai landraces, 1 Nepalese and 1 Japanese landrace. Wild accessions consisted of 36 from Thailand and 3 from Myanmar. Although the number of accessions analyzed was more than other species and came from a wider geographic range, *V. umbellata* showed the lowest level of nucleotide diversity (6.928) (Table 3.2a).

Genetic variation parameters for the four population types of *V. umbellata*, typical wild [wild (A)], wild with small seeds and atypical seedcoat color [wild (B)], wild with larger seeds and atypical seedcoat color [wild (C)] and cultivated, are summarized in Table 3.3a. Average number of amplified bands was less in cultivated (330.6) and large, colored seeded wild (C) accessions (331.8) compared with typical wild (A) accessions (339.0) and highest in small, colored seeded wild (B) accessions (348.6). Percentage of polymorphic bands was smallest in large colored seeded wild (C) (18.7%) and highest in typical wild (A) accessions (48.1%). Within-population

gene diversity estimates (Hs) (Table 3.3) were low in cultivated (0.084) and large, colored seeded wild (C) (0.074), compared with high in typical wild (A) (0.132) and wild, small colored seeded (B) accessions (0.101). The tendency of within-population gene diversity was consistent with the results based on Nei's gene diversity (h) and Shannon's gene diversity index (I).

All cultivated accessions clustered together except two accessions found in a farmer's seed lot in Lampang province (Um086, 087) (Figure 3.1a, 3.2a, 3.3a). These two accessions positioned outside of the main Thai cultivated accessions. Um086 has tan color seed and Um087 has tan with black mottled seed. The genetic position based on AFLP analysis of these two accessions suggests they have originated from wild gene introgression into the cultigen. Therefore, these two cultivated accessions were possibly originated from the natural hybridization from wild to cultivated rice bean. Owing to preference or market demand, some Thai farmers report that they select only red or tan seeded *V. umbellata*. Farmers reported that during cultivation mottled color seeds were frequently found and discarded. Hence, these off-type may form escaped populations. These two accessions have an intermediate position, as do wild (C) accessions (Um012, 027, 082), between the cultivated and wild cluster. Three accessions [wild (C)] from roadside habitats (Um012, Um027, Um082) have considerably larger seeds and seed color not found in typical wild *V. umbellata* [wild (A)] (Figure 3.1, Figure 3.2). If these accessions have escaped from cultivation, they should be included in the cluster with cultivated accessions. However, AFLP results show this is not the case. Their position is intermediate between cultivated and wild populations (Figure 3.3a). Intermediate seed size and AFLP results as well as seed color similar to some cultivated accessions suggested these populations originated

from natural hybridization between wild and cultivated rice bean. Japanese and Nepalese cultivated accessions were included in the same cluster as Thai cultivated accessions but were significantly differentiated from each other and from other Thai cultivated accessions (Figure 3.3a). All the cultivated accessions of *V. umbellata* analyzed, including those from Japan and Nepal, form a single cluster within the wild accessions. This suggests a single domestication event for *V. umbellata*.

Cultivated accessions analyzed were very weakly differentiated from wild accessions (Figure 3.3a). The lack of differentiation between the wild and cultivated accessions suggested that northern Thailand was the area where domestication occurred. In both Thailand and Myanmar farmers reported collecting wild *V. umbellata* for consumption (Tomooka et al., 2003). However, frequent post-domestication gene flow between wild and cultivated forms may also explain the lack of differentiation as has been suggested for African cowpea, *V. unguiculata* (Coulibaly et al., 2002). To better understand the domestication of rice bean, analysis of materials from Southeast Asia and southern China materials is required. Within wild populations, three accessions from Kanchanaburi province (Um002, 004, 005) were highly differentiated from other Thai wild accessions. Although other wild accessions were closely related to each other, geographic differentiation was observed (Figure 3.3a). Generally, accessions from the same province clustered together, and most of the exceptional cases were observed for wild (B) accessions with seed color not found in typical wild (A) accessions from the same location (indicated with arrows in Fig 3.3a). Three wild (A) accessions from Myanmar (Um157, 158, 159) were not significantly differentiated from Thai cultivated and Thai wild populations. The lack of variation based on inferred nucleotide diversity in *V. umbellata* accessions

from northern Thailand, a region in the center of distribution of this crop complex, might imply that this is a recently evolved species. The productive system of *V. umbellata* and its ecological habitat may also help explain the low diversity. Isozyme and SSR (microsatellite) studies of plant species with different breeding systems have revealed that annual, inbreeding species have higher inter population variation than perennial, outcrossing species (Hamrick and Godt, 1990; Kuroda et al., 2002).

Field observations of *V. umbellata* in Thailand suggested that it has a perennial habit based on its very thick stem (Tomooka et al., 1997). It has a conspicuous inflorescence consisting of several large, bright yellow flowers raised above the leaf canopy. These characteristics suggest it is adapted to outbreeding. The prominent inflorescence with large, bright yellow of *V. umbellata* is in contrast to the other species of section *Angulares* analyzed here that have smaller flowers and less prominent inflorescence. Thus, outcrossing may be more common in *V. umbellata* than other *Vigna* species from Thailand analyzed. In addition, during germplasm collection many insects including bees were observed visiting flowers of *V. umbellata*. The frequent existence of populations having atypical seed color [wild (B) and wild (C)] may be the result of gene flow between cultivated and wild populations. However, inter- and intra-population genetic structures, rate of outcrossing and annual/perennial habit of *Vigna* species require clarification. It is necessary to analyze breeding system and population structure to clarify why the genetic diversity is much lower than other *Vigna* in Thailand.

V. exilis

This is the first detailed study of *V. exilis* that has a very restricted habitat on limestone outcrops scattered across parts of west and central Thailand (Tateishi and Maxted, 2002; Tomooka et al., 2002b). *V. exilis* was genetically well differentiated from other species and was most closely related to *V. umbellata* of species analyzed (Figure 3.3, genetic distance = 25.723, Table 3.4a). This partly supports the hypothesis of Tateishi (1985) who suggested *V. umbellata* being a recently evolved species from *V. exilis* based on morphological studies of the Asian *Vigna* species.

Among the nine accessions of *V. exilis* analyzed, geographic differentiation was detected. Eight accessions were collected from western and southwestern region of Bangkok and one from northwest of Bangkok (Ex060) (Figure 3.2b). The accession from northwest of Bangkok showed the highest level of differentiation. The disjunctive distribution of populations of this species might help explain the high level of inter-population diversity of this species (Table 3.2a).

V. hirtella

Neighbor-joining clustering of accessions identified based on morphological characteristics as *V. hirtella* formed two genetically distinct subgroups [*V. hirtella* (a): 3 accessions and *V. hirtella* (b): 5 accessions] (Figure 3.2d, Figure 3.3a). Genetic distance based on nucleotide diversity between the two subgroups was 27.869 (Table 3.4). *V. hirtella* (a) subgroup showed higher nucleotide diversity (18.25) than *V. hirtella* (b) subgroup (7.8) (Table 3.2a). In *V. hirtella* (b), the accessions from Myanmar were well differentiated from the accessions collected in northern Thailand. Although *V. hirtella* (a) consisted of three Thai accessions, each accession was well

differentiated. However, for *V. hirtella* (b) the level of genetic differentiation among accessions was relatively small (Figure 3.2d, Figure 3.3). Previous studies have suggested that materials identified as *V. hirtella* may consist of two taxa (Tomooka et al., 2002c). This study also suggests this, since the genetic distance between *V. umbellata* and *V. exilis* (25.723) was less than between the two subgroups (27.869) of *V. hirtella* (a and b) (Table 3.4a). *V. hirtella* was first described in peninsular Malaysia. An accession collected in peninsular Malaysia close to the collection site of the type specimen and therefore considered to be true *V. hirtella* was included in previous analysis (Tomooka et al., 2002c). Judging from DNA similarity with Malaysian accession, *V. hirtella* (a) in this study was probably true *V. hirtella*. The average altitude of the three *V. hirtella* (a) accessions is 628m, while that of the five *V. hirtella* (b) accessions is 1243m. It remains to be determined whether clear morphological differences exist between these two groups of *V. hirtella* accessions.

V. minima

Among species examined in this set of accessions, *V. minima* (13 accessions) showed the highest nucleotide diversity (15.458) and geographic differentiation was observed. Distinct sub-clusters were formed by four accessions from northeast Thailand (Mi056, 057, 058, 059), 2 accessions from Chiang Mai province (Mi104, 107) and 3 accessions from Nan province (Mi038, 041, 044) (Figure 3.2c, Figure 3.3). Accessions from Myanmar (Mi166, 168) and central provinces of Thailand (MiDKL, Mi020) were the most diverged of the *V. minima* accessions.

V. minima is the only species in section *Angulares* that is found as scattered plants on the forest floor. It is widely distributed in Southeast and East Asia (Tomooka et al.,

2002a). The high level of genetic differentiation reflected the wide distribution of the analyzed accessions (Figure 3.2b). The isolation of this species in forests on different mountain ranges across Thailand and in remaining sporadic patches of forest in northern Thailand probably accounts for the high level of population divergence.

V. tenuicaulis

V. tenuicaulis is a recently described species (Tomooka et al., 2002b). Nine *V. tenuicaulis* accessions showed the third highest nucleotide diversity (12.866). A high level of genetic differentiation was observed between Myanmar (Te161, 162) and Thai accessions (Figure 3.3). Among Thai accessions, the accession (Te003) that was not collected in northern Thailand was the most diverged. Accessions collected from locations in close proximity in northern Thailand each showed high inter-accession genetic diversity. Three accessions (Te091, Te092: Type plant, Te093) that had morphological differences in pod shape and bracteole color were collected at the same site (the collection site of Type specimens, Figure 3.2e). However, they showed a high level of genetic diversity.

(b) Set 2

A total of 526 bands were amplified and 513 (97.5%) showed polymorphism (Table 3.2b). Highest number of bands was amplified in *V. grandiflora* (184.3) and lowest for *V. trinervia* (147.8). Percentage of polymorphic bands ranged from 26.7% (*V. unguiculata*) to 50.9% (*V. mungo*). Nucleotide diversity (π x 1000) was lowest in *V. trinervia* (7.698) and highest in *V. grandiflora* (22.300) (Table 3.2b).

Section *Ceratotropis* consists of four species; three of them were analyzed here. *V. subramaniana* is only known from South Asia and was not included. The center of diversity for this section is South Asia (Tomooka et al., 2002a) and in Thailand only a few wild populations of species in this section have been found. While Thailand is an important producer of mungbean (*V. radiata*) its presumed wild ancestor was not in Thailand. *V. unguiculata* belonging to the subgenus *Vigna* was also included to investigate genetic relationship between cultivated and naturally growing population. Each species analyzed is highly differentiated compared with those analyzed in section *Angulares* species (Table 3.4).

V. grandiflora

V. grandiflora was most closely related to *V. radiata* of species analyzed (Table 3.4b, Figure 3.4). Results of analyzing Thai representatives of section *Ceratotropis* species and *V. trinervia* confirmed the previous studies that *V. grandiflora* was more closely related to *V. radiata* than *V. mungo* (Tomooka et al., 2002c). Although Niyomdham (1992) proposed a treatment of *V. grandiflora* as a variety of *V. radiata*, AFLP distance suggests this to be a distinct species. Although the three accessions analyzed were collected from a limited area in central Thailand (Fig 3.2f), they showed the highest nucleotide diversity (Table 3.2b).

V. mungo (black gram)

Accessions of *V. mungo* include cultivated accessions from Thailand (Figure 3.2g), India and Australia, and wild accessions from India and Thailand. However, nucleotide diversity was low (8.095) compared with *V. grandiflora* (22.3) (Table

3.2b). Cultivated accessions, wild accessions from India and wild accessions from Thailand formed three distinct sub-clusters (Figure 3.4a). Among three sub-clusters, wild populations from Thailand showed the highest gene diversity (Table 3.3b).

This research is the first study that analyzes both wild and cultivated *V. mungo* by molecular techniques in details. The results showed that cultivated *V. mungo* was probably monophyletic since cultivated accessions from Thailand, India and Australia were all clustered together and separated from accessions of its presumed wild ancestor *V. mungo* var. *silvestris* (Figure 3.4a). In addition, all cultivated accessions were differentiated from both Thai and Indian wild accessions. This genetic relationship contrasted with that of the *V. umbellata* crop complex discussed above. India has been proposed the most likely country where *V. mungo* was domesticated (Chandel et al., 1984). However, based on the genetic distance between analyzed wild and cultivated germplasm of *V. mungo*, this could not be confirmed. A larger number of wild *V. mungo* accessions covering its distribution range especially from Myanmar should be included to clarify the area of domestication of this crop complex.

The analyzed wild accessions showed that wild accessions from Thailand are well differentiated from wild accessions from India and cultivated accessions from India, Thailand and Australia. Recently, populations of wild *V. mungo* have been found in Thailand. In addition, *V. mungo* was introduced to Thailand within the last 50 years (Srinives, 2004, pers. comm.). Consequently, it is unclear whether naturally growing *V. mungo* populations in Thailand are true wild *V. mungo* or recently introduced wild contaminants of cultivated *V. mungo* that have become naturalized. Judging from the genetic distance between wild *V. mungo* populations in Thailand and cultivated and wild populations from India, and the geographic distance between the main area of

black gram cultivation in Thailand and where wild populations have been collected (Figure 3.2g), they appeared to be truly native to Thailand.

V. trinervia

V. trinervia was most closely related to *V. mungo* of species analyzed in this set (Table 3.4b, Figure 3.2g). *V. trinervia* of section *Angulares* was confirmed to be more closely related to *V. mungo* than the other section *Ceratotropis* species analyzed here (Saravanakumar et al., 2004). In contrast to *V. grandiflora*, accessions of *V. trinervia* showed a lower level of nucleotide diversity although accessions were from widely separated locations in northern Thailand (Tr090), southern Thailand (Tr022, 028, 023) and Malaysia (Tr163) (Table 3.4b, Figure 3.4).

V. unguiculata (cowpea)

One accession each of cultivated and wild growing *V. unguiculata* were analyzed. Relatively high nucleotide diversity (10.29) was recorded (Table 3.2b) and relatively high genetic differentiation was observed between these two accessions (Figure 3.4).

Cowpea was domesticated in Africa but is considered an ancient introduced crop in Asia (Ng and Maréchal, 1985). The wild progenitor of cowpea (*V. unguiculata* var. *spontanea*) is reported only from Africa. However in Thailand, there are naturally growing black seeded cowpea populations. Plants of these populations have easy shattering pods and seed size is much smaller than present cowpea landraces cultivated in Thailand (Figure 3.1c). In the present study, wild and cultivated accessions from Thailand were compared. A high degree of genetic differentiation between these two populations suggested that the wild population was not a recent

escape from landrace cowpea. A large number of wild populations and landraces should be analyzed to clarify the origin of wild small seeded cowpea in Thailand.

Table 3.2 Summary of AFLP fragments and diversity statistics calculated for each species.

Species	No. of accessions	Average no. of amplified bands/accession	No. of polymorphic bands/total no. of amplified bands/species (%)	π x 1000[a]
a) section *Angulares* subgenus *Ceratotropis* species (AFLP bands generated by 6 primer pairs)				
V. exilis	9	316.9	320/505 (63.4)	12.681
V. hirtella (a)	3	302.7	217/418 (51.9)	18.25
V. hirtella (b)	5	320.4	152/401 (37.9)	7.834
V. minima	13	306.5	453/596 (76.0)	15.458
V. tenuicaulis	9	307.2	305/475 (64.2)	12.866
V. umbellata	57	337.8	281/497 (56.5)	6.928
Total	96		1021/1074 (95.1)	
b) other *Vigna* species (AFLP bands generated by 3 primer pairs)				
V. trinervia	5	147.8	68/182 (37.4)	7.698
V. grandiflora	3	184.3	106/240 (44.2)	22.3
V. mungo	13	166.5	115/226 (50.9)	8.095
V. radiata	1	158.0	-	-
V. unguiculata	2	156.5	48/180 (26.7)	10.29
Total	24		513/526 (97.5)	

[a] π = Innan's nucleotide diversity (Innan et al., 1999)

Table 3.3 Gene diversity statistics for population groups of *V. umbellata* and *V. mungo*.

Population groups	No. of accessions	Average no. of amplified bands/ accession	Polymorphic bands/total no. of amplified bands (%)	Ht[a]	Hs[b]	Gst[c]	h[d]	I[e]
a) *V. umbellata*								
wild A[f]	29	339	239/497 (48.1)		0.132		0.135	0.21
wild B	7	348.6	130/497 (26.2)		0.101		0.101	0.15
wild C	3	331.8	93/497 (18.7)		0.074		0.074	0.11
cultivated	18	330.6	144/497 (29.0)		0.084		0.084	0.13
All accessions	57	337.8	281/497 (56.5)	0.131	0.104	0.205	0.134	0.21
b) *V. mungo*								
Thai wild	5	166.6	38/226 (16.8)		0.061		0.061	0.09
Indian wild	2	161	14/226 (6.2)		0.026		0.026	0.03
cultivated	6	168.3	24/226 (10.6)		0.045		0.045	0.06
All accessions	13	166.5	115/226 (50.9)	0.171	0.044	0.745	0.161	0.25

[a]Ht = total genetic diversity

[b] Hs = within-populations gene diversity

[c]Gst = relative magnitude of gene differentiation among populations

[d]h = Nei's (1973) gene diversity

[e]I = Shannon's gene diversity index (Shannon and Weaver, 1949)

[f] see text for description of wild A, B and C

Table 3.4 Genetic distance within and between species/subgroup based on Innan's nucleotide diversity (π x 1000).

a) section *Angulares* species

	V. minima	*V. hirtella (a)*	*V. hirtella (b)*	*V. tenuicaulis*	*V. exillis*	*V. umbellata*
V. minima	**15.458**[a]					
	3.371[b]					
	78[c]					
V. hirtella (a)	**35.321**	**18.250**				
	3.254	*3.424*				
	39	3				
V. hirtella (b)	**36.041**	**27.869**	**7.834**			
	1.724	*1.896*	*2.766*			
	65	15	10			
V. tenuicaulis	**35.723**	**32.141**	**27.284**	**12.866**		
	2.144	*2.288*	*1.884*	*2.834*		
	117	27	45	36		
V. exilis	**32.684**	**32.146**	**31.772**	**32.831**	**12.681**	
	2.087	*2.537*	*2.013*	*2.375*	*3.931*	
	117	27	45	81	36	
V. umbellata	**34.072**	**27.697**	**29.665**	**29.819**	**25.723**	**6.928**
	2.458	*2.233*	*1.286*	*1.77*	*1.569*	*1.635*
	741	171	285	513	513	1596

b) other *Vigna* species

	V. trinervia	*V. mungo*	*V. grandiflora*	*V. radiata*	*V. unguiculata*
V. trinervia	**7.698**				
	4.737				
	10				
V. mungo	**63.271**	**8.095**			
	2.883	*4.261*			
	65	78			
V. grandiflora	**82.659**	**87.327**	**22.300**		
	5.597	*8.831*	*15.854*		
	15	39	3		
V. radiata	**101.610**	**79.508**	**61.760**		
	11.854	*4.024*	*10.144*		
	5	13	3		
V. unguiculata	**105.006**	**116.468**	**82.659**	**84.675**	**10.290**
	6.656	*7.575*	*5.597*	-	-
	10	26	15	2	1

[a] Average genetic distance

[b] Standard Error

[c] Number of comparisons

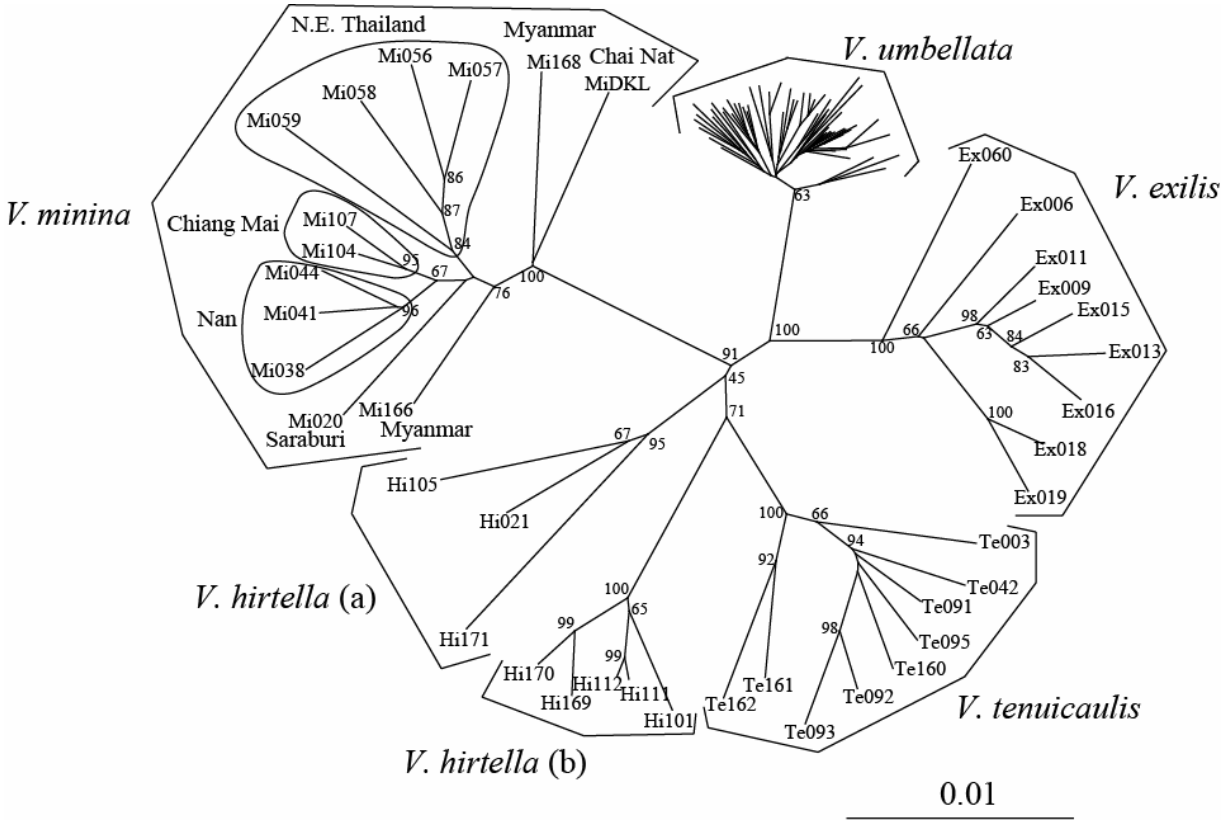


Figure 3.3 Unrooted neighbor-joining tree of *Vigna* species analyzed in section *Angulares* (except *V. trinervia*) based on a matrix of inferred nucleotide diversity using AFLP variation to prepare the matrix. Percent bootstrap values based on 1000 replications over 50% are shown.

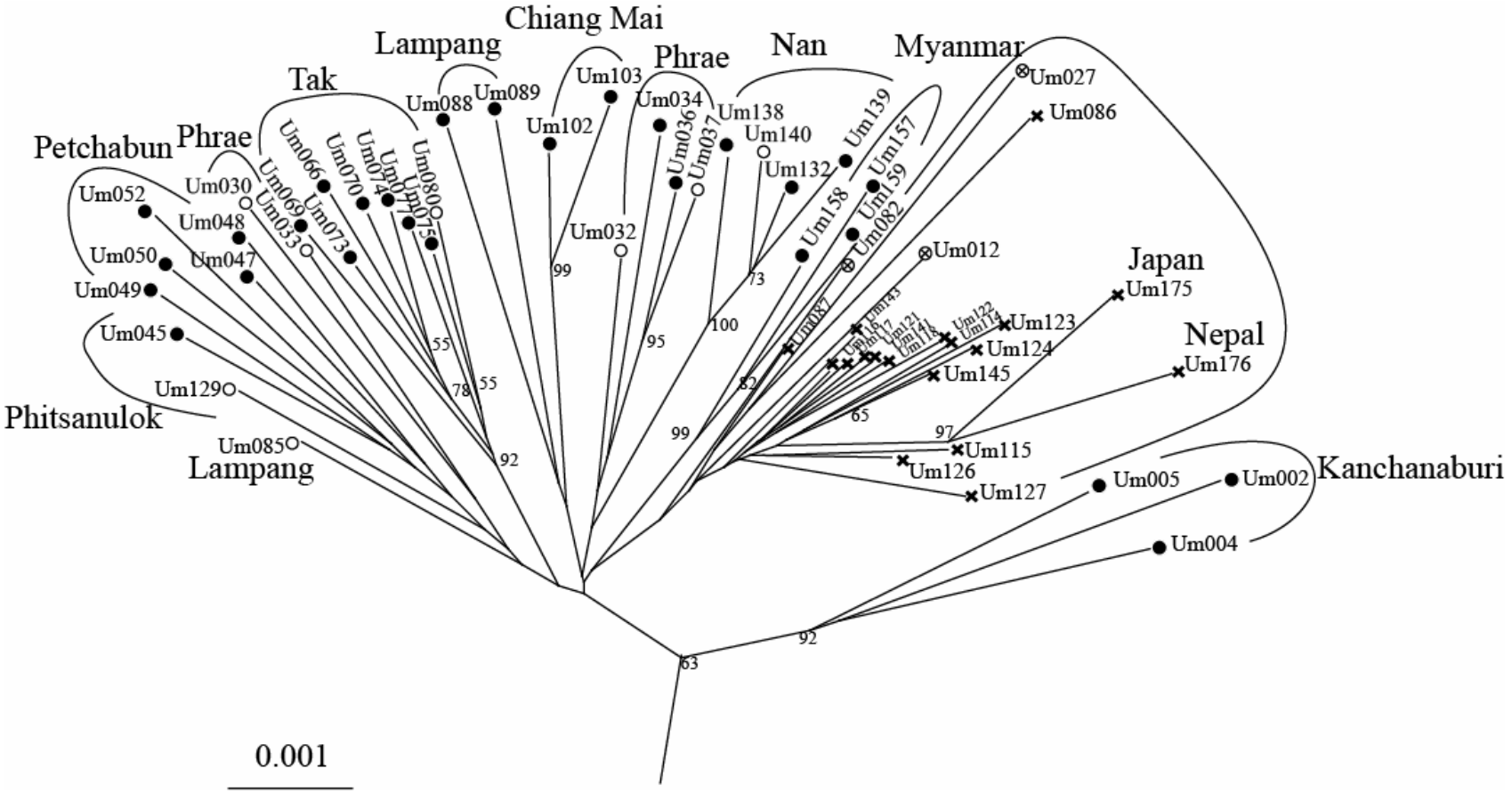


Figure 3.3a Enlargement of *V. umbellata* (cultivated, ✕; wild (A) ● ; wild (B) ○ ; wild (C) ⊗).

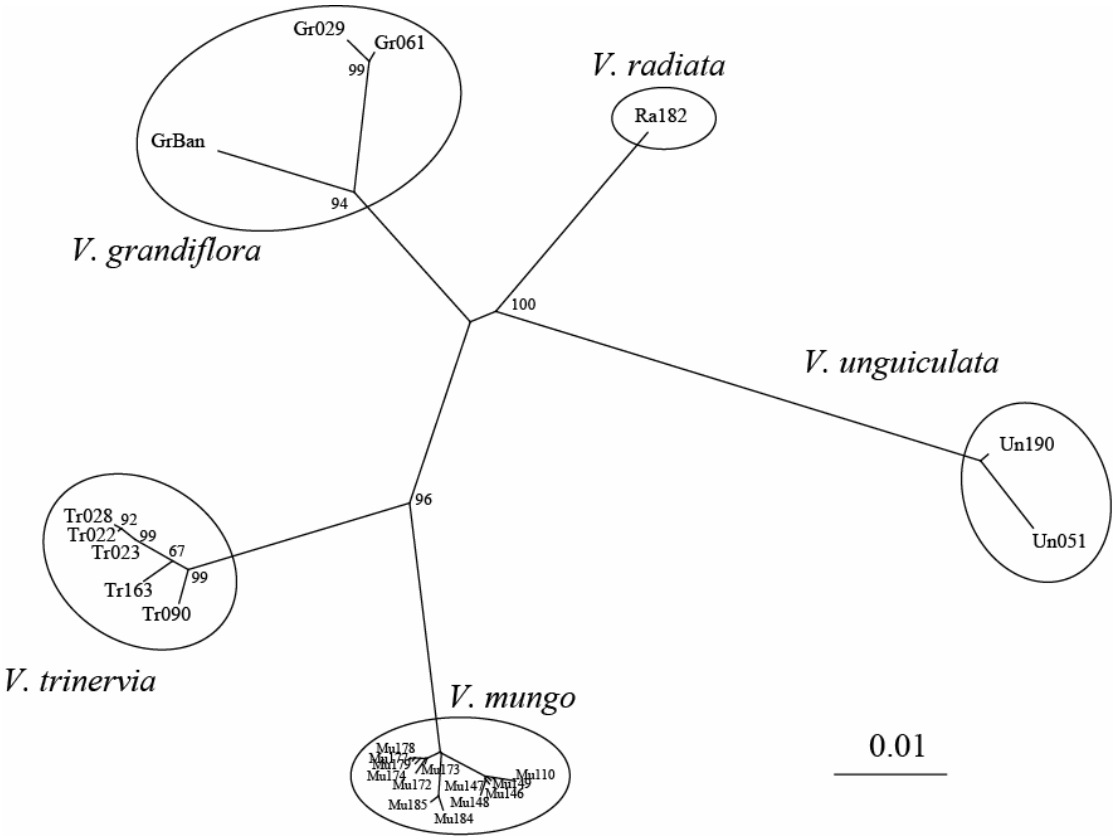


Figure 3.4 Unrooted neighbor-joining tree of species in section *Ceratotropis*, *V. trinervia* and *V. unguiculata* based on a matrix of inferred nucleotide diversity using AFLP variation to prepare the matrix. Percent bootstrap values based on 1000 replications over 50% are shown.

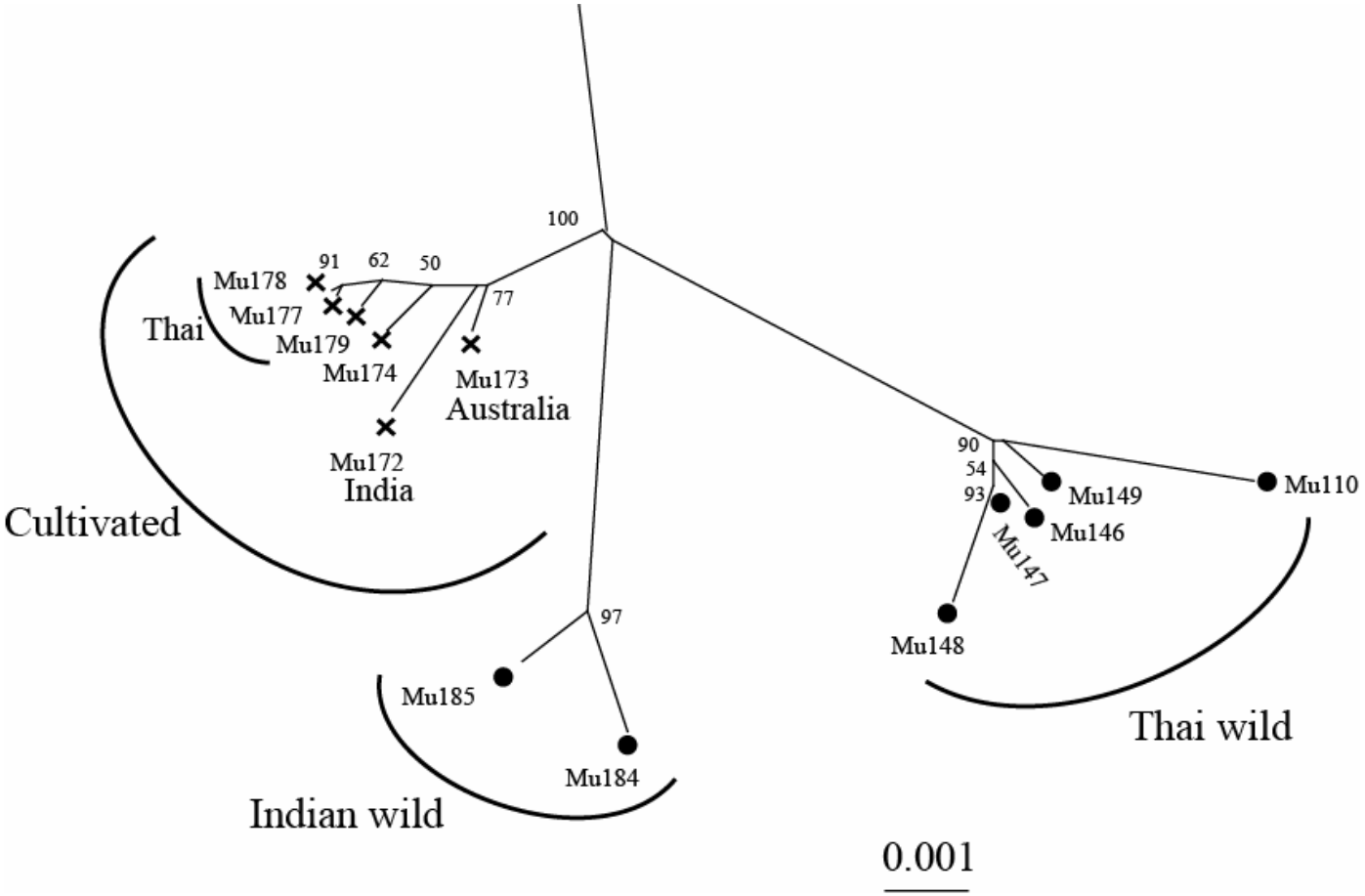


Figure 3.4a Enlargement of *V. mungo*. *V. mungo* var. *mungo* (✕) and *V. mungo* var. *silvestris* (●).

3.5 Conclusion

The genetic diversity analysis of section *Angulares* species of the subgenus *Ceratotropis* except *V. trinervia* based on AFLP markers revealed 6 groups consisting of 4 species which were *V. umbellata*, *V. exilis*, *V. hirtella*, *V. minima*, and *V. tenuicaulis*. The geographic differentiation was observed in every analyzed species. From this analysis, *V. umbellata* was hypothesized to be a recently evolved species from *V. exilis* because of its lowest nucleotide diversity among all species analyzed here and it is most closely related to *V. exilis*. Among accessions of this species, natural hybridization between wild and cultivated rice bean was found. *V. hirtella* was divided into two subgroups and the level of genetic distance between them was high. These two subgroups might be the distinct species. They need to be further determined morphological differences in details. *V. minima* showed the highest level of nucleotide diversity because of their wide distribution. *V. tenuicaulis* showed high inter-accession genetic diversity even the analyzed accessions were collected at the same site.

For the other species which were in section *Ceratotropis* and *V. trinervia*, the differentiation among them was higher than those section *Angulares* species analyzed here. The results confirmed the previous studies that *V. grandiflora* was a distinct species from *V. radiata*. *V. grandiflora* showed high nucleotide diversity even the analyzed accessions were collected from the limited area. This was in contrast with *V. trinervia* which accessions that were collected from widely separated locations showed lower nucleotide diversity. The cultivated accessions of *V. mungo* from Thailand, India, and Australia were cluster together and separated from wild accessions, suggesting that it is probably monophyletic. The distance between Thai

wild and Indian cutivated and wild accessions revealed that Thai wild accessions were truly native to Thailand.

Based on the distribution of the Asian *Vigna*, Thailand appears to be the center of diversity of section *Angulares* of the subgenus *Ceratotropis* (Tomooka et al., 2002a). These Asian *Vign*a, particularly the north of Thailand, can furnish germplasm that is useful for understanding both speciation and domestication in this agriculturally important group and analysis of the Asian *Vigna* germplasm from this area is expected to reveal insights into evolution of section *Angulares*.

3.6 References

Ba, F. S., Pasquet, R. S. and Gepts, P. (2004). Genetic diversity in cowpea [*V. unguiculata* (L.) Walp.] as revealed by RAPD markers. **Genet. Res. Crop Evol.** 51: 539-550.

Chainuwat, C., Potan, N. and Worasan, T. (1988). Mungbean and black gram production and development in Thailand. In **"Mungbean" Proc. 2nd International Symposium** (pp 657-668). Shanhua, Taiwan: AVRDC.

Chandel, K. P. S., Lester, R. N. and Starling, R. J. (1984). The wild ancestors of urd and mung beans *(Vigna mungo* (L.) Hepper and *V. radiata* (L.) Wilczek. **J. Linn. Soc.** 89: 85-96.

Coulibaly, S., Pasquet, R. S., Papa, R. and Gepts, P. (2002). AFLP analysis of the phenetic organization and genetic diversity of *Vigna unguiculata* L. Walp. reveals extensive gene flow between wild and domesticated types. **Theor. Appl. Genet.** 104: 358-366.

Dellaporta, S. L., Wood, J. and Hicks, J. B. (1983). A plant DNA mini preparation: version II. **Plant Mol. Biol. Rep.** 1: 19-21.

Felsenstein, J. (1992). Phylogenies from restriction sites, a maximum likelihood approach. **Evolution** 46: 557-574.

Hamrick, J. L. and Godt, M. J. W., (1990). Allozyme diversity in plant species. In A. H. D. Brown, M. T. Clegg, A. L. Kahler and B. S. Weir (eds.). **Plant Population Genetics, Breeding, and Genetic Resources** (pp 43-63). Massachusetts: Sinauer Associated Sunderland.

Innan, H., Terauchi, R., Kahl, G. and Tajima, F. (1999). A method for estimating nucleotide diversity from AFLP data. **Genetics** 151: 1157-1164.

Konarev, A. V., Tomooka, N. and Vaughan, D. A. (2002). Proteinase inhibitor polymorphism in the genus *Vigna* subgenus *Ceratotropis* and its biosystematic implications. **Euphytica** 123:165-177.

Kuroda, Y., Urairong, H. and Sato, Y. I. (2002). Population genetic structure of wild rice (*Oryza rufipogon*) in mainland Southeast Asia as revealed by microsatellite polymorphism. **Tropics** 12: 159-170.

Maréchal, R., Mascherpa, J. and Stainier, F. (1978). Etude taxonomique d'un groupe complexe d'espèces des genres *Phaseolus* et *Vigna* (Palilionaceae) sur la base données morphologiques et polliniques, traitées par l'analyse informatique. **Boissiera** 28: 1-273.

Nei, M. (1973). Analysis of genetic diversity in subdivided populations. **Proc. Natl. Acad. Sci. USA** 70: 3321-3323.

Ng, N. Q. and Maréchal. (1985). Cowpea taxonomy, origin and germplasm. In S. R. Singh and K. O. Rachie (eds.). **Cowpea Research, Production and Utilization** (pp 11-22). Chichester, UK: John Wiley and Sons.

Niyomdham, C. (1992). Notes on Thai and Indo-Chinese Phaseoleae (Leguminosae-Papilionodeae). **Nordic J. Bot.** 12: 339-346.

Panaud, O., Chen, X. and McCouch, S. R. (1996). Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). **Mol. Gen. Genet.** 2: 252-238.

Saravanakumar, P., Kaga, A., Tomooka, N. and Vaughan, D. A. (2004). AFLP and RAPD analyses of intra- and inter specific variation in some *Vigna* subgenus *Ceratotropis* (Leguminosae) species. **Aust. J. Bot.** (52: 417-424).

Shannon, C. E. and Weaver, W. (1949). **The mathematical theory of communication.** Urbana, USA: University of Illinois Press.

Srinives, P. and Yang, C. Y. (1988). Utilization of mungbean germplasm in Thailand. In **Mungbean, Proc. 2nd International Symposium** (pp 71-80). Shanhua, Taiwan: AVRDC.

Tateishi, Y. (1985). **A Revision of the Azuki Bean Group, the Subgenus *Ceratotropis* of the Genus *Vigna* (Leguminosae).** PhD thesis, Tohoku University.

Tateishi, Y. and Maxted, N. (2002). New species and new combinations in *Vigna* subgenus *Ceratotropis* (Piper) Verdcourt (Leguminosae, Phaseoleae). **Kew Bulletin** 57: 625-633.

Tomooka, N. (1991). Genetic diversity and landrace differentiation of mungbean *Vigna radiata* (L.) Wilczek, and evaluation of its wild relatives (the subgenus

Ceratotropis) as breeding materials. **Tech. Bull. Trop. Agr. Res. Center, Japan** No. 28. 64 p.

Tomooka, N., Chotechuen, S., Boonkerd, N., Taengsan, B., Nupllean, S., Vaughan, D., Egawa, Y., Tokoyama, T. and Tateishi, Y. (1997). Collection of seed and nodule samples from wild subgenus *Ceratotropis* species (genus *Vigna*) in central and northern Thailand. In **Annual Report on Exploration and Introduction of Plant Genetic Resources** 13: 189-206. National Institute of Agrobiological Sciences. (in Japanese with English summary).

Tomooka, N., Srinives, P., Boonmalisan, D., Chotechuen, S., Taengsan, B. and Ornnaichart, P. (2000). Field survey of high temperature tolerant Asian *Vigna* species in Thailand. In **Annual Report on Exploration and Introduction of Plant Genetic Resources** 16: 171-186. National Institute of Agrobiological Sciences. (in Japanese with English summary).

Tomooka, N., Vaughan, D. A., Moss, H. and Maxted, N. (2002a). **The Asian *Vigna*:Genus *Vigna* subgenus *Ceratotropis* genetic resources.** The Natherlands: Kluwer Academic Publishers. 270 p.

Tomooka, N., Maxted, N., Thavarasook, C. and Jayasuriya, A. H. M. (2002b). Two new species, sectional designations and new combinations in *Vigna* subgenus *Ceratotropis* (Piper) Verdcourt (Leguminosae). **Kew Bulletin** 57: 613-624.

Tomooka, N., Yoon, M. S., Doi, K., Kaga, A. and Vaughan, D. A. (2002c). AFLP analysis of a *Vigna* subgenus *Ceratotropis* core collection. **Genet. Res. Crop Evol.** 49: 521-530.

Tomooka, N., Abe, K., Thein, M. S., Twat, W., Maw, J. B., Vaughan, D. A. and Kaga, (2003). Collaborative exploration and collection of cultivated and wild legume

species in Myanmar, 15th Oct.-15th Nov. (2002). In **Annual Report for Exploration and Introduction of Plant Genetic Resources, National Institute of Agrobiological Sciences** 19: 67-83. (in Japanese with English summary).

Xu, R. Q., Tomooka, N., Vaughan, D. A. and Doi, K. (2000a). The *Vigna angularis* complex: Genetic variation and relationships revealed by RAPD analysis, and their implications for in situ conservation and domestication. **Genet. Res. Crop Evol.** 47: 123-134.

Xu, R. Q., Tomooka, N. and Vaughan, D. A. (2000b). AFLP markers for characterizing the azuki bean (*Vigna angularis*) complex. **Crop Sci.** 40: 808-815.

Yoon, M. S., Doi, K., Kaga, A., Tomooka, N. and Vaughan, D. A. (2000). Analysis of genetic diversity in the *Vigna minima* complex and related species in East Asia. **J. Plant Res.** 113: 375-386.

Zong, X. X., Kaga, A., Tomooka, N., Wang, X. W., Han, O. K. and Vaughan, D. A. (2003). The genetic diversity of the *Vigna angularis* complex in Asia. **Genome** 46: 647-658.

CHAPTER IV

COLLECTION OF *Vigna* species SUBGENUS *Ceratotropis* IN CHIANG MAI AND MAE HONG SON PROVINCES

4.1 Abstract

Wild *Vigna* species (subgenus *Ceratotropis*) of 19 populations were collected along the roads in Chiang Mai and Mae Hong Son provinces during 9-12 December 2002. In some populations more than one species was found growing adjacent to each other but in slightly different habitat. A total of 117 individual seed samples were collected and identified. They were *V. hirtella* (46 samples), *V. minima* (46 samples), *V. tenuicaulis* (14 samples), and *V. umbellata* (11 samples). The seed samples were used for analyzing genetic diversity by molecular marker technique.

4.2 Introduction

Most of the previous explorations of wild *Vigna* species in Thailand as mentioned in Chapter II did not show the details of routes and locations of collection except the explorations of Tomooka et al. in 1997 and 2000. These two reports (Tomooka et al., 1997, 2000) depicted the passport data of each collected accession including for example species, route, and location. These data were useful for the following exploration. Therefore, this study followed their journey but more than one plant in

each site were looked for. The suitable time for exploration and collecting seeds was generally around October to December because these wild *Vigna* species were photosensitive which flowered and set pod only once a year during this time. The objective of this exploration was to collect and conserve genetic germplasm of *Vigna* species in subgenus *Ceratotropis* collected from Chiang Mai and Mae Hong Son and these collected samples were used as the materials for analyzing intra-population genetic diversity in the following section.

4.3 Materials and Methods

The exploration was during 9-12 December 2002 starting from route number 1095 from Chiang Mai to Mae Hong Son and route number 107 from Chiang Mai to Fang district. The car was stopped every 10 km and then *Vigna* in subgenus *Ceratotropis* around that area were looked for by observing its key morphological characters as mentioned in Chapter II. After the plants were found, their location was measured by using GPS (global positioning system) which indicated latitude, longitude and altitude. The data were recorded in passport data sheet. Herbarium specimens, root nodules and seed samples were collected separately from each plant. The location of each population and each plant were drawn on site map. Some of the herbarium specimens would be kept in a gene bank at Pathum Thani Rice Research Center and some of them would be sent to Kew, England. Species of collected plants were identified according to the method of Tomooka et al. (2002). Then seed size was measured including width and length of seed and of hilum.

4.4 Results and Discussion

From this exploration, 19 populations were collected. Thirteen of which were on route 1095 and other six were on route 107 (Table 4.1). The samples collected composed of four *Vigna* species which were *V. hirtella*, *V. minima*, *V. tenuicaulis*, and *V. umbellata*. One hundred and seventeen seed samples and nine root nodules were collected. Seed and hilum sizes of each accession were shown in Table 4.2 and the passport data were in Table 4.3.

Table 4.1 Date, routes, species and numbers of collected seeds.

Date	Route	Site	Species and no. of seeds collected
9	Route 1095 from Chiang Mai to Mae Hong Son	1-4	*V. hirtella* 10, *V. tenuicaulis* 11
10	Route 1095 started at km 65 from Chiang Mai to Mae Hong Son	5-8	*V. hirtella* 11, *V. minima* 17, *V. tenuicaulis* 1
11	Route 1095 from Mae Hong Son back to Chiang Mai	9-13	*V. hirtella* 13, *V. minima* 21
12	Route 107 from Chiang Mai to Fang district	14-19	*V. hirtella* 12, *V. minima* 8, *V. tenuicaulis* 2, *V. umbellata* 11

Site 1 (altitude 869 m): only one plant of *V. umbelata* was found at the mountain slope.

Site 2 (altitude 775 m): two groups of each *V. hirtella* and *V. tenuicaulis* grew on the way to Mae Lak waterfall. *V. hirtella* grew as small plants under the shadow of trees and grew at the higher area than *V. tenuicaulis*. *V. hirtella* growing at this site

had white mark at leaf base which was not found at other sites. *V. tenuicaulis* grew in lighter area mixing with other plants.

Site 3 (altitude 1365 m): a few small *V. tenuicaulis* were found on the roadside.

Site 4 (altitude 1049 m): *V. hirtella* and *V. tenuicaulis* were found growing alternately. This might be due to the wind or animals. The plants growing here were very small because of shallow soil surface.

Site 5 (altitude 1130 m): *V. hirtella* and *V. minima* were found on both sides of the road. One side was pine forest, sandy soil, the plants found here were very small and had yellowish leaves. The other side was shading area and the plants found here were stronger.

Site 6 (altitude 786 m): here was the humid forest which was lower than the road. There were three *V. minima* plants winding with other trees and growing separately.

Site 7 (altitude 1108 m): *V. hirtella* grew on the roadside but their pods had not matured yet, so only a few samples were collected. *V. hirtella* found here were infected by powdery mildew.

Site 8 (altitude 842 m): *V. hirtella* and *V. tenuicaulis* plants were found at the area closing to orchard. Cultivated rice bean (*V. umbellata*) plants were found scattering in this area. Thus, this area was expected to be rice bean field in the past but it was stopped planting long time ago, however, there was no wild rice bean found in adjacent forest. This might be implied that the rice bean cultivated here might be introduced from other places far away from here.

Site 9 (altitude 841 m): this population had many *V. minima* growing in the deep humid forest. Most of them were found along the narrow way that someone had

cleared. Because this forest was cluttered so it was difficult to explore inside. It was expected to have more *V. minima* and might be other *Vigna* species.

Site 10 (altitude 507 m): *V. minima* plants found here were over matured and some pods shrank, so a few seeds were collected.

Site 11 (altitude 738 m): *V. hirtella* and *V. minima* were found on the opposite side of the road. *V. hirtella* plants were found in the shading area under the trees while *V. minima* grew at the light side. On the hill at the same side as *V. hirtella* found, there were two more *V. minima* plants found but quite apart from *V. hirtella*.

Site 12 (altitude 575 m): this population was at the earthen dyke around rice field close to Mae Hong Son Rice Research Station. At here, many *V. hirtella* were found; however, they had high opportunity to be destroyed.

Site 13-15 (altitude 558-594 m): *V. hirtella* and *V. minima* were found at site 13-14 and site 15 respectively. A few of them grew a long the roadside close to each other. Some of them were in the flowering stage, so only a few seeds collected.

Site 16 (altitude 569 m): *V. hirtella* and *V. minima* were found growing nearby a small village. A few *V. hirtella* plants were on the roadside whereas many *V. minima* grew scattering in the bamboo forest on the hill but they had not set pods yet.

Site 17 (altitude 682 m): there were very diverse species here because 4 species were found, which were *V. hirtella, V. tenuicaulis, V. minima* and *V. umbellata* growing mix together on the roadside. The outstanding species was *V. umbellata* because of their plenty yellow flowers; however, most of them had not yet set pods, so a few seeds were collected.

Site 18-19 (altitude 494-512 m): there were not many *V. umbellate* plants on the roadside. At site 18, they grew mixing with giant sleeping grass close to the rice field. At site 19, they grew mixing with grasses.

Besides *Ceratotropis* species found, there was a plant that looked like *Ceratotropis* except its red flowers. It had not set pod then; however, its herbarium specimen was collected. The identification by Tomooka revealed that this species was *Dumbaria*.

From this exploration, the difference of habitat of each species was found. *V. hirtella* and *V. tenuicaulis* grew mostly in dry and light shading area. *V. minima* grew in humid and high shading area in the forest, whereas *V. umbellata* grew in open area and was tolerant to the disturbance better than other species because it grew very well at high disturbance area such as on the roadside while other species could not. The infection of powdery mildew with *V. hirtella* found at site 7 showed that this species was not suitable as the source of powdery mildew resistant gene. At some locations, especially on the roadside, we could not collect seeds because the plants had just flowered.

The identification of species based on their flowers or stipule morphological characters at the collection site was easily confused because of the environmental effect on their morphological characters. Therefore, the identification based on seed morphology was more precise. However, seeds of *V. hirtella* and *V. tenuicaulis* were similar, so it was necessary to be identified by DNA analysis.

4.5 Conclusion

There were many *Vigna* species in Thailand even the exploration took just two routes. Thus, Thailand might be a center of *Vigna* species. However, the locations that could be explored were just the roadside and the forest where sometimes we could not go in deep. The plants growing especially on roadsides were prone to be destroyed and may become extinct. Therefore, it is necessary to conserve these *Vigna* species which could be used as genetic source for mungbean improvement in the future.

4.6 References

Tomooka, N., Chotechuen, S., Boonkerd, N., Taengsan, B., Nupllean, S., Vaughan, D., Egawa, Y., Tokoyama, T. and Tateishi, Y. (1997). Collection of seed and nodule samples from wild subgenus *Ceratotropis* species (genus *Vigna*) in central and northern Thailand. In **Annual Report on Exploration and Introduction of Plant Genetic Resources** 13: 189-206. National Institute of Agrobiological Sciences (in Japanese with English summary).

Tomooka, N., Srinives, P., Boonmalisan, D., Chotechuen, S., Taengsan, B. and Ornnaichart, P. (2000). Field survey of high temperature tolerant Asian *Vigna* species in Thailand. In **Annual Report on Exploration and Introduction of Plant Genetic Resources** 16: 171-186. National Institute of Agrobiological Sciences. (in Japanese with English summary).

Tomooka, N., Vaughan, D. A., Moss H. and Maxted N. (2002). **The Asian *Vigna*: Genus *Vigna* subgenus *Ceratotropis* genetic resources.** The Netherlands: Kluwer Academic. 270 p.

Table 4.2 Average seed and hilum size of each accession.

No.	Accession no.	Species	Hilum length (mm)	Hilum width (mm)	Seed width (mm)	Seed length (mm)	Seed thickness (mm)
1	w02-02-01	*V. hirtella*	1.485	0.564	1.740	2.795	2.215
2	w02-02-02	*V. hirtella*	1.520	0.608	1.735	3.030	2.370
3	w02-02-03	*V. hirtella*	1.510	0.589	1.805	2.900	2.175
4	w02-02-04	*V. hirtella*	1.490	0.606	1.605	2.700	2.305
5	w02-02-05	*V. hirtella*	1.580	0.565	1.680	3.090	2.420
6	w02-02-06	*V. hirtella*	1.360	0.566	1.755	2.865	2.360
7	w02-02-07	*V. tenuicaulis*	1.165	0.458	1.530	2.190	1.895
8	w02-02-08	*V. tenuicaulis*	1.240	0.475	1.860	2.750	2.060
9	w02-02-09	*V. tenuicaulis*	1.145	0.448	1.835	2.620	1.940
10	w02-02-10	*V. tenuicaulis*	0.862	0.369	1.635	1.955	1.790
11	w02-02-11	*V. tenuicaulis*	1.125	0.454	1.875	2.660	2.080
12	w02-02-12	*V. tenuicaulis*	0.950	0.383	1.325	2.295	1.610
13	w02-02-13	*V. hirtella*	1.295	0.636	1.705	2.865	2.205
14	w02-03-01	*V. tenuicaulis*	0.927	0.341	1.740	2.205	1.785
15	w02-04-01	*V. tenuicaulis*	1.012	0.331	1.415	2.355	1.735
16	w02-04-02	*V. hirtella*	1.470	0.582	1.895	2.800	2.105
17	w02-04-03	*V. tenuicaulis*	1.140	0.361	1.510	2.430	1.525
18	w02-04-04	*V. hirtella*	1.460	0.562	1.865	2.580	2.065
19	w02-04-05	*V. tenuicaulis*	0.949	0.331	1.250	2.095	1.440
20	w02-04-06	*V. hirtella*	1.480	0.529	1.355	2.565	2.105
21	w02-04-07	*V. tenuicaulis*	0.871	0.403	1.795	2.240	1.895
22	w02-05-01	*V. hirtella*	1.580	0.582	1.660	2.740	2.115
23	w02-05-02	*V. hirtella*	1.605	0.617	1.795	3.010	2.340
24	w02-05-04	*V. minima*	2.600	0.918	1.945	4.160	2.600
25	w02-05-05	*V. minima*	2.630	0.922	2.220	3.370	3.340
26	w02-05-06	*V. minima*	2.635	1.041	2.270	3.710	2.835
27	w02-05-07	*V. minima*	2.855	1.160	2.220	3.780	2.440
28	w02-05-08	*V. hirtella*	1.305	0.395	1.880	2.995	1.890
29	w02-05-09	*V. minima*	2.435	0.863	1.885	3.255	3.255
30	w02-05-10	*V. minima*	2.040	0.736	2.195	3.155	2.560
31	w02-05-11	*V. minima*	2.440	0.698	1.845	3.835	2.450
32	w02-05-12	*V. hirtella*	1.490	0.444	1.535	3.285	1.860
33	w02-05-13	*V. hirtella*	1.280	0.433	1.685	2.780	1.980
34	w02-05-16	*V. hirtella*	1.420	0.433	1.665	2.895	1.870

Table 4.2 Average seed and hilum size of each accession (continued).

No.	Accession no.	Species	Hilum length (mm)	Hilum width (mm)	Seed width (mm)	Seed length (mm)	Seed thickness (mm)
35	w02-05-18	*V. minima*	2.695	0.948	1.985	4.025	2.740
36	w02-05-19	*V. minima*	2.810	0.939	2.105	4.390	2.890
37	w02-05-20	*V. minima*	2.690	0.957	2.000	4.105	2.655
38	w02-05-21	*V. minima*	2.430	0.844	1.890	4.160	2.460
39	w02-06-01	*V. minima*	3.100	1.040	2.255	3.765	2.700
40	w02-06-02	*V. minima*	3.510	0.951	2.105	4.525	2.670
41	w02-06-03	*V. minima*	3.180	0.828	1.930	3.895	2.475
42	w02-06-04	*V. minima*	2.930	0.835	2.030	4.045	2.740
43	w02-06-05	*V. minima*	2.685	0.911	1.805	3.255	2.460
44	w02-06-06	*V. minima*	3.190	0.943	1.820	3.700	2.820
45	w02-07-01	*V. hirtella*	1.380	0.569	1.485	2.780	2.135
46	w02-07-02	*V. hirtella*	1.315	0.550	1.770	2.755	2.070
47	w02-08-01	*V. hirtella*	1.150	0.443	1.370	2.365	1.830
48	w02-08-02	*V. hirtella*	1.465	0.458	1.740	2.815	2.085
49	w02-08-03	*V. hirtella*	1.475	0.579	1.945	2.875	2.315
50	w02-08-04	*V. tenuicaulis*	1.355	0.503	1.920	2.595	2.285
51	w02-09-01	*V. minima*	3.055	0.941	2.275	4.180	2.905
52	w02-09-02	*V. minima*	3.000	0.837	2.105	4.045	2.745
53	w02-09-03	*V. minima*	2.975	0.866	2.165	4.005	2.775
54	w02-09-04	*V. minima*	3.025	0.827	2.040	3.125	3.335
55	w02-09-05	*V. minima*	2.665	0.705	1.745	3.990	2.850
56	w02-09-06	*V. minima*	2.695	0.747	2.090	3.870	2.575
57	w02-09-07	*V. minima*	2.495	0.781	1.905	3.410	2.350
58	w02-09-08	*V. minima*	2.535	0.584	1.655	3.195	1.950
59	w02-09-09	*V. minima*	3.160	0.843	2.100	3.965	2.375
60	w02-09-10	*V. minima*	2.075	0.870	2.050	4.230	2.700
61	w02-09-11	*V. minima*	2.885	0.782	1.810	3.905	2.365
62	w02-09-12	*V. minima*	2.935	0.854	1.860	3.860	2.330
63	w02-09-13	*V. minima*	3.005	0.829	2.065	4.135	2.320
64	w02-10-01	*V. minima*	2.680	0.893	1.755	3.805	2.445
65	w02-10-02	*V. minima*	2.935	1.100	2.175	3.700	2.700
66	w02-11-01	*V. minima*	2.795	0.964	2.270	4.065	2.875
67	w02-11-02	*V. minima*	3.530	1.245	2.480	5.075	2.600
68	w02-11-03	*V. minima*	3.760	1.240	2.310	4.945	2.885

Table 4.2 Average seed and hilum size of each accessions (continued).

No.	Accession no.	Species	Hilum length (mm)	Hilum width (mm)	Seed width (mm)	Seed length (mm)	Seed thickness (mm)
69	w02-11-04	*V. minima*	3.615	1.090	2.390	4.840	2.890
70	w02-11-05	*V. minima*	3.425	1.125	2.285	4.325	2.785
71	w02-11-07	*V. hirtella*	1.435	0.573	1.670	2.750	1.895
72	w02-11-08	*V. hirtella*	1.825	0.564	1.920	3.455	2.345
73	w02-11-09	*V. hirtella*	1.760	0.562	1.655	3.235	2.120
74	w02-11-10	*V. hirtella*	1.925	0.573	2.060	3.475	2.485
75	w02-11-11	*V. hirtella*	1.630	0.523	1.610	3.235	2.090
76	w02-11-12	*V. minima*	3.530	0.856	2.265	4.255	2.740
77	w02-12-01	*V. hirtella*	1.235	0.514	1.505	2.485	1.755
78	w02-12-02	*V. hirtella*	1.265	0.484	1.475	2.535	1.655
79	w02-12-03	*V. hirtella*	1.365	0.483	1.750	2.990	2.280
80	w02-12-04	*V. hirtella*	1.435	0.520	1.630	2.625	2.025
81	w02-12-05	*V. hirtella*	1.315	0.429	1.410	2.735	1.800
82	w02-12-07	*V. hirtella*	1.500	0.557	1.745	2.560	1.945
83	w02-12-08	*V. hirtella*	1.360	0.465	1.745	3.010	2.035
84	w02-13-01	*V.hirtella*	1.525	0.552	1.885	2.725	2.070
85	w02-14-01	*V.hirtella*	1.690	0.680	1.545	3.155	2.080
86	w02-14-02	*V.hirtella*	2.150	0.615	1.585	3.665	2.210
87	w02-14-03	*V.hirtella*	1.980	0.681	1.425	3.520	2.055
88	w02-14-04	*V.hirtella*	1.760	0.615	1.730	3.245	2.290
89	w02-14-05	*V.hirtella*	1.980	0.761	1.660	3.465	2.180
90	w02-14-07	*V.hirtella*	1.875	0.638	1.640	3.410	2.050
91	w02-15-01	*V. minima*	1.605	0.940	1.790	4.010	2.645
92	w02-15-02	*V. minima*	1.445	0.945	1.715	3.955	2.470
93	w02-15-03	*V. minima*	1.230	0.733	1.780	3.010	2.295
94	w02-17-02	*V. minima*	2.285	0.927	1.970	3.760	2.805
95	w02-17-03	*V. tenuicaulis*	1.425	0.376	1.755	2.145	2.095
96	w02-17-04	*V. tenuicaulis*	1.344	0.377	1.945	2.295	2.145
97	w02-17-05	*V. minima*	2.265	1.035	2.070	4.190	2.780
98	w02-17-06	*V. hirtella*	1.830	0.720	1.745	3.380	2.140
99	w02-17-07	*V. minima*	2.215	1.045	2.075	4.045	2.785
100	w02-17-08	*V. minima*	2.050	0.960	1.910	3.735	2.615
101	w02-17-09	*V. minima*	2.310	0.985	2.130	4.285	2.625
102	w02-17-10	*V. hirtella*	1.660	0.692	1.910	3.005	2.240

Table 4.2 Average seed and hilum size of each accessions (continued).

No.	Accession no.	Species	Hilum length (mm)	Hilum width (mm)	Seed width (mm)	Seed length (mm)	Seed thickness (mm)
103	w02-17-11	*V. hirtella*	1.635	0.617	1.850	3.090	2.305
104	w02-17-11a	*V. hirtella*	1.825	0.621	1.775	3.135	2.165
105	w02-17-12	*V. umbellata*	1.865	0.905	1.595	3.920	2.130
106	w02-17-13	*V. hirtella*	1.595	0.611	1.645	3.015	2.215
107	w02-17-14	*V. hirtella*	0.815	0.597	1.935	3.375	2.545
108	w02-17-15	*V. umbellata*	1.135	1.045	2.025	3.375	2.485
109	w02-18-01	*V. umbellata*	0.845	0.800	1.730	3.555	2.205
110	w02-18-02	*V. umbellata*	0.860	0.919	1.780	3.580	2.290
111	w02-18-03	*V. umbellata*	0.880	0.804	1.595	3.745	2.335
112	w02-19-01	*V. umbellata*	0.935	0.924	1.825	3.565	2.230
113	w02-19-02	*V. umbellata*	0.915	0.784	1.700	3.820	2.360
114	w02-19-03	*V. umbellata*	0.950	0.833	1.855	3.870	2.305
115	w02-19-04	*V. umbellata*	0.810	0.847	1.770	3.735	2.230
116	w02-19-05	*V. umbellata*	1.080	0.845	1.860	4.035	2.250
117	w02-19-06	*V. umbellata*	0.825	0.768	1.715	3.410	2.165

Table 4.3 Passport data of accessions collected.

Site	Coll. Date	Coll. No.	Species	Status	Collection site
1	9/12	w02-01	*V. umbellata*	wild	left to Mae Hong Son, route 1095, 25 km W of Mae Taeng, Chiang Mai province
2	9/12	w02-02	*V. tenuicaulis* and *V. hirtella*	wild	Mae Lark water fall (left to Mae Hong Son), route 1095, 36 km W of Mae Taeng, Chiang Mai province
3	9/12	w02-03	*V. tenuicailis*	wild	left to Mae Hong Son, route 1095, 65.5 km W of Mae Taeng, Chiang Mai province
4	9/12	w02-04	*V. hirtella* and *V. tenuicaulis*	wild	route 1095, 55 km W of Mae Taeng, Chiang Mai province
5	10/12	w02-05	*V. hirtella and V. minima*	wild	route 1095, 75 km W of Mae Taeng, Chiang Mai province
6	10/12	w02-06	*V. minima*	wild	route 1095, 111 km W of Mae Taeng, Chiang Mai province
7	10/12	w02-07	*V. hirtella*	wild	route 1095, 118 km W of Mae Teang, Chiang Mai province
8	10/12	w02-08	*V. hirtella* and *V. tenuicaulis*	weedy	route 1095, 133.2 km W of Mae Taeng, Chiang Mai province
9	11/12	w02-09	*V. minima*	wild	route 1095, right to Mae Hong Son, 186 km W of Mae Taeng, Mae Hong Son province
10	11/12	w02-10	*V. minima*	wild	route 1095, right to Mae Hong Son, 176 km W of Mae Taeng, Mae Hong Son province
11	11/12	w02-11	*V. hirtella* and *V. minima*	weedy	route 1095, 164.8-164.9 km W of Mae Taeng, Mae Hong Son province
12	11/12	w02-12	*V. hirtella*	weedy	route 1095, 175 km W of Mae Taeng, near Rice Experimantal Station of Mae Hong Son, Mae Hong Son province
13	11/12	w02-13	*V. hirtella*	wild	route 1095, right to Mae Hong Son, 146 km W of Mae Taeng, Mae Hong Son province
14	12/12	w02-14	*V. hirtella*	weedy	left to Fang route 107, 300 km of Chiang Mai, Chiang Mai province
15	12/12	w02-15	*V. minima*	weedy	right to Fang, route 107, 102.375 km N of Chiang Mai, Chiang Mai province
16	12/12	w02-16	*V. hirtella* and *V. minima*	weedy	Ban Hua To, right to Fang, route 107, 103.15 km N of Chiang Mai, Chiang Mai province
17	12/12	w02-17	*V. hirtella*, *V. minima*, *V. tenuicailis*, and *V. umbellata*	wild	route 107, 110.675-111.275 km N of Chiang Mai, Chiang Mai province
18	12/12	w02-18	*V. umbellata*	weedy	left to Mae Ai, route 1089, 2.1 km N of Fang district, 150 km N of chiang Mai, Chiang Mai province
19	12/12	w02-19	*V. umbellata*	weedy	route 107, 86.65-86-675 km N of Chiang Mai, Chiang Mai province

Table 4.3 Passport data of accessions collected (continued).

Site	Coll. No.	Latitude Longitude	Altitude (m)	Habitat	Shading	Disturbance	Growth stage	Seed
1	w02-01	N19-7-21.3 E98-44-32.3	869	roadside	light	high	flowering	no
2	w02-02	N19-9-14.4 E98-41-33	775	forest	medium	high	mature	yes
3	w02-03	N19-15 E98-36	1365	roadside	open	high	mature	yes
4	w02-04	N19-15-0.7 E98-39-4.9	1049	roadside	open	high	mature	yes
5	w02-05	N19-16-8.6 E98-32-21.3	1130	forest	light	medium	flowering	yes
6	w02-06	N19-26-8.8 E98-22-34.6	786	forest	heavy	high	mature	yes
7	w02-07	N19-26-17.2 E98-20-24.2	1108	roadside	light	medium	flowering	yes
8	w02-08	N19-28-6 E98-17-47.1	842	roadside	open	high	mature	yes
9	w02-09	N19-26-49 E98-0-33.9	841	forest	medium	low	mature	yes
10	w02-10	N19-29-21.1 E98-3-9	507	forest	medium	low	past maturity	yes
11	w02-11	N19-33-48.8 E98-10-20.6	738	forest and roadside	heavy and open	high	mature	yes
12	w02-12	N19-32-35.8 E98-12-52	575	grassland	open	high	flowering	yes
13	w02-13	N19-31-59.8 E98-13-42.4	594	roadside	open	high	flowering	yes
14	w02-14	N19-30-49.3 E99-4-39	561	roadside	open	high	flowering	yes
15	w02-15	N19-34-9.2 E99-4-47.1	558	roadside	open	high	mature	yes
16	w02-16	N19-34-16.8 E99-5-7.9	569	forest and roadside	light and open	high	flowering	no
17	w02-17	N19-35-58.9 E99-6-59.1	682	roadside	open	high	flowering and mature	yes
18	w02-18	N19-54-49.7 E99-11-51.1	494	roadside	open	high	flowering	yes
19	w02-19	N19-28-45.2 E99-0-51	512	roadside	open	high	mature	yes

Table 4.3 Passport data of accessions collected (continued).

Site	Coll. No.	No.of Herbarium	Thailand[1]	Rhizobium	Remark
1	w02-01	2	yes	no	
2	w02-02	3	yes	yes	some pods were steriled narrow leave with mark
3	w02-03	1	yes	no	
4	w02-04	7	yes	yes	
5	w02-05	6	yes	no	pod disease and bruchid
6	w02-06	0	no	yes	leaf and pod disease, large nodule, bruchid
7	w02-07	4	yes	no	powdery mildew, pest
8	w02-08	4	yes	no	there are *V. umbellata* (cv.) and African cowpea around here,pod disease
9	w02-09	5	yes	yes	sandy loam soil, semi open forest, pod/seed disease, leaf pest, and bruchid
10	w02-10	1	no	no	red silty clay soil, pod disease
11	w02-11	8	yes	no	red silty clay soil, pod disease, pest (bruchid)
12	w02-12	2	yes	no	pod disease
13	w02-13	0	no	no	leaf disease and pest
14	w02-14	5	yes	no	leaf disease
15	w02-15	1	yes	yes	pod disease and pest
16	w02-16	3	yes	yes	no seed collected
17	w02-17	9	yes	no	*V. umbellata* were flowering but *V. hirtella* and *V. minima* mature, pest is bruchid
18	w02-18	6	yes	yes	
19	w02-19	4	yes	no	leaf and pod disease

[1] Herbarium specimens deposited at Pathum Thani Rice Research Center, Thailand

CHAPTER V

SSR ANALYSIS OF SYMPATRIC *Vigna* species OF NORTHERN THAILAND

5.1 Abstract

The heterozygosity in *Vigna* population and the relationship among species were studied based on 20 microsatellite loci. In a total of 95 individuals of 15 populations, four *Vigna* species were identified as *V. hirtella* Ridley, *V. minima* (Roxb.) Ohwi & Ohashi, *V. tenuicaulis* N. Tomooka & Maxted, and *V. umbellata* (Thunb.) Ohwi & Ohashi. Low level of heterozygous loci was revealed, however, the maximum number of heterozygous loci per plant was found in *V. hirtella* (0.74). One of *V. hirtella* accessions showed high heterozygosity sharing alleles between *V. hirtella* and *V. minima*. This represented a hybrid between these two species. Phenetic distance whithin species was lowest in *V. umbellata* (0.049) while the highest was within *V. hirtella* (0.124). Among all species, *V. hirtella* and *V. tenuicaulis* were closest (0.140) which formed *V. hirtella/ V. tenuicaulis* complex in clustering analysis but one accession could not be clarified. The highest distinctness (0.155) was found between *V. minima* and *V. tenuicaulis* and between *V. minima* and *V. umbellata*. This study shows that northern Thailand is a center of diversity of *Vigna* species belonging to section *Angulares*. Outcrossing was very low among species analyzed, however, interspecific crossing could occur.

5.2 Introduction

Studying with population genetic diversity in *Vigna* can illustrate the process and mechanism of evolution and also provide information useful for biological conservation (Schaal et al., 1991), and gene flow studies. Recent studies of Asian *Vigna* have revealed that *V. hirtella* appeared to be a species complex and populations from lower elevation were distinct based on AFLP profile from highland populations (Seehalak et al., in print). In addition, the relationship between *V. tenuicaulis* and *V. hirtella* is poorly understood. In northern Thailand these species and others occur in close proximity often at the same site. It is unclear what barriers to outcrossing exist between these species and their habitat preferences.

Population genetic diversity study within *Vigna* species has used various molecular techniques such as restriction fragment length polymorphism (RFLP), chloroplast DNA, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) (Yoon et al., 2000), inter simple sequence repeat (ISSR; Ajibade et al., 2000), and simple sequence repeat (SSR; Wang et al., 2004). Since SSR markers have a number of advantages including high polymorphism, codominance, high reproducibility, rapid and simple genotyping assays (Li et al., 2001), and hypervariability. Therefore, they have been successfully used for genomic mapping, DNA fingerprinting, marker-assisted selection (Yu et al., 2000), population and evolutionary studies (Zhou et al., 2003), and gene flow studies (Wang et al., 2004). However, SSR markers used for studying intra-population of the Asian *Vigna* were limited to populations of the azuki bean complex in Japan (Wang et al., 2004). There has been no analyses of the intra-population genetic diversity of tropical species

of Asian *Vigna.* This present study reveals the intra-population genetic diversity of Asian *Vigna* collected from Chiang Mai and Mae Hong Son provinces of Thailand.

5.3 Materials and Methods

Plant Materials

The plant materials used in this study consisted of 95 accessions collected from 15 populations, ten of which were sampled along route 1095 between Chiang Mai and Mae Hong Son on the 9th and 10th of December 2002 and the other 5 populations were sampled from route 107 between Chiang Mai and Fang district on 11th December 2002. Seeds were collected from individual plants spaced more than one meter apart in each population and the herbarium specimens were also collected. The identity of each individual was confirmed based on seed and floral characteristics of the plants grown from collected seeds in the field at Suranaree University of Technology, Nakhon Ratchasima. The accessions used for this analysis are listed in Table 5.1.

Table 5.1 Populations analyzed.

Population number	Latitude	Longitude	Altitude (m)	Species composition	No. individuals	Site description
Road from Chiang Mai to Mae Hong Son						
2	19-09-14N	98-41-33E	775	*V. hirtella*	6	Along paths in woodland semi cultivated area
				V. tenuicaulis	6	
4	19-15-01N	98-39-05E	1049	*V. hirtella*	3	Roadside ditch, mainly sunny
				V. tenuicaulis	3	
5	19-16-09N	98-32-21E	1130	*V. minima*	7	Forested area near road
				V. hirtella	5	
6	19-26-09N	98-22-35E	786	*V. minima*	5	Forested area near road
7	19-26-17N	98-20-24E	1108	*V. hirtella*	2	Open roadside slope
8	19-28-06N	98-17-47E	842	*V. hirtella* *V. tenuicaulis*	3 1	Shady roadside and open areas This plant seems to be represent a plant morphologically *V. tenuicaulis* but by SSR analysis *V. hirtella*
9	19-26-49N	98-00-34	841	*V. minima*	13	Forested area near road
10	19-29-21N	98-03-09E	507	*V. minima*	2	Shady roadside
11	19-33-49N	98-10-21	738	*V. hirtella*	4	Shady roadside
				V. minima	4	Forested area
12	19-32-36N	98-12-52E	575	*V. hirtella*	6	Open roadside

Table 5.1 Populations analyzed (continued).

Population number	Latitude	Longitude	Altitude (m)	Species composition	No. individuals	Site description
Road from Chiang Mai to Fang						
14	19-30-49N	99-04-39E	561	*V. hirtella*	4	Shady roadside slope
15	19-34-09N	99-04-47E	558	*V. minima*	2	Shady roadside
17	19-35-59N	99-06-59E	682	*V. hirtella*	5	Roadside semi-shade
				V. tenuicaulis	2	Open
				V. minima	5	Under bamboo-full shade
18	19-54-50N	99-11-51E	494	*V. umbellata*	2	Roadside
19	19-28-45N	99-00-51E	512	*V. umbellata*	5	Roadside

DNA Extraction

Total genomic DNA was extracted from seeds of individual plants. The number of seeds used varied depending on seed size of each species. Five seeds of *V. minima* and ten each of *V. hirtella*, *V. tenuicaulis*, and *V. umbellata* were used. The seeds were soaked in distilled water overnight in a 2-ml tube and then transferred into a new tube filled with 400 µl of extraction buffer [200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA, 0.5% SDS (sodiumdodecylsulfate), 250 ng/:l of proteinase K]. Seeds were ground by grinding machine under 25/sec velocity for 30 seconds and incubated at 37°C for 1 hour. After that 400 µl of 2xCTAB buffer [2% CTAB (cetyltrimethylammonium bromide), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% w/v PVP (polyvinylpyrolidone) MW 40,000] and 800 µl of chloroform: isoamyl alcohol (24:1) containing 3% phenol were added and mixed by using rotator, then centrifuged at 12000 rpm for 10 min at room temperature. Supernatant was transferred to 1.5 ml tube filled with 700 µl isopropanol and incubated at room temperature for 10 min to precipitate DNA. The samples were centrifuged at 12000 rpm for 5 min and the supernatant was discarded followed by washing the DNA pellet with 500 µl of 70% ethanol and then incubated for 5 min in a rotator. Centrifugation at 12000 rpm for 2 min was done and the supernatant was removed. Two hundred µl of TE-RNase (100 ng/µl) were added, and then incubated at 37°C for 1 hour. DNA concentration was estimated and standardized against known concentration of λ DNA on 1% (w/v) agarose gel.

Microsatellite Analysis

Thirty-eight SSR primers created from azuki bean DNA sequences (Wang et al., 2004) were screened for amplification if they could reveal polymorphism among *Vigna* species collected in northern Thailand. Twenty microsatellite primers, that gave clear banding patterns in the expected molecular weight range in selected *Vigna* accessions, were selected for population analysis. The information of these primers is given in Table 5.2. To detect polymorphism, PCR was performed in 10 µl reaction mixture containing 0.5 ng of template DNA, 1X buffer, 0.2 mM each of dNTP mixture, 2.0 mM $MgCl_2$, 0.15 pmol primer and 0.02 unit of KOD+ DNA polymerase (TOYOBO, Japan). The amplification of each primer was performed in Gene Amp PCR system 9700 (Perkin Elmer, Norwalk, CT, USA) with an initial cycle at 94°C for 2 min followed by 35 cycles at 94°C for 30 sec and 68°C for 30 sec and followed by 68°C for 3 min. Amplified products were run in 6% acrylamide gel and the banding patterns were visualized according to the silver staining method described by Panuad et al. (1996).

Data Analysis

SSR bands were scored as present (1) or absent (0). The data were analyzed by two methods. The first method used NTSYSpc version 2.10y software (Rohlf, 1998) to quantify paire-wise genetic similarity based on Jaccard's coefficient and the cluster was carried out by unweighted pair-group method with arithmetic mean (UPGMA) (Sokal and Michener 1958). The second method used the computer program Populations 1.2.28 (Langella, 1999). The relationships among and within *Vigna* populations were estimated from the SSR data using the neighbor-joining (NJ)

clustering method on the basis of Nei (Satou and Nei, 1987) unbiased genetic distance. This method was used to construct the unrooted cladogram.

Table 5.2 Primers used, number of alleles and heterozygous plant detected from each primer.

Primers	Linkage group in *V. angularis*	No. of alleles	No. of heterozygous plant
CEDG002	11	18	11
CEDG006	2	18	1
CEDG008	5	13	4
CEDG013	1 (ang x riu)	16	1
CEDG016	8	11	1
CEDG018	5	12	3
CEDG019	1	6	0
CEDG027	5 (ang x riu)	18	1
CEDG029	2	9	1
CEDG030	8	10	0
CEDG032	1	10	3
CEDG033	8	28	6
CEDG034	umknown	14	2
CEDG035	8	14	2
CEDG036	4	15	1
CEDG037	6	13	1
CEDG040	8	9	0
CEDG041	7	10	3
CEDG042	11	10	2
CEDG043	3	15	0
Total		269	
Average		13.45	

Phenetic distance within and between species was estimated by Mantel-Struct 1.0 (Miller, 1999) and used Simple Matching (Sokal and Michener, 1958) as an index for calculating similarity coefficient.

5.4 Results and Discussion

Twenty out of 38 screened SSR primers were selected for analyzing *Vigna* species from northern Thailand (Table 5.2). These primers are located on 9 of the 11 linkage groups of the *V. angularis* (azuki bean) genome map. A total of 269 alleles were detected from 20 primers. The number of alleles per locus ranged from 6 to 28, with an average of 13.45 alleles. The maximum number of alleles was obtained from CEDG033. Primer CEDG019 gave the lowest number of alleles. The number of heterozygous plant discerned from each primer ranged from 0 to 11. Primer that gave the maximum number of heterozygous plants was CEDG002 while CEDG019, CEDG030, CEDG040, and CEDG043 gave none. This could be implied that primer CEDG002 probably amplified the sequence that had high mutation frequency. Sample number 14/2 showed the highest number of heterozygous loci, which was 13 loci (data not shown). The number of alleles per plant compared between species was highest in *V. tenuicaulis* (5.17, Table 5.3). The maximum number of heterozygous loci per plant was found in *V. hirtella* (0.74). This might reflect a relatively higher level of outcrossing in this species than others analyzed. Moreover, 10 alleles out of 28 heterozygous loci found in *V. hirtella* were from *V. minima*, particularly sample 14/2. Of 20 loci recorded from 20 primers analyzed for this individual, 13 loci were heterozygous, far more than any other individuals analyzed and all loci composed of alleles that were also found in *V. minima*. This strongly supported the view that this

sample represented a hybrid between *V. hirtella* and *V. minima* and SSR marker had high efficiency in detecting heterozygous loci. This hybrid reflected the natural outcrossing between *V. hirtella* and *V. minima* and could be useful in facilitating interspecific hybridization.

Table 5.3 SSR comparison among species.

Species	No. plants (populations analyzed)	No. of alleles	No. of heterozygous loci detected	alleles/plant	alleles/ population	heterozygous loci/plant	heterozygous loci/population
V. hirtella	38(9)	149	28	3.92	16.56	0.74	3.11
V. minima	38(7)	120	10	3.16	17.14	0.26	1.43
V. tenuicailis	12(4)	62	2	5.17	15.50	0.17	0.50
V. umbellata	7(2)	36	3	5.14	18.00	0.43	1.50

The results of phenetic distance within species calculated by Mantel test constructed using simple matching coefficient showed that there was geographic differentiation. In *V. minima* species, population 11 and 17 diverged from the others (Table 5.4). From Table 5.5 *V. hirtella* in population 14 showed high distance against the other populations of *V. hirtella* and *V. tenuicaulis*. The lowest phenetic distance between species was found between *V. hirtella* and *V. tenuicaulis* (0.14, Table 5.6) whereas the highest (0.155) was found between *V. minima* and *V. tenuicaulis* and between *V. minima* and *V. umbellata*. Distance within *V. umbellata* was lowest (0.049), which agreed with the previous study that *V. umbellata* collected in Thailand had very low diversity. The greatest variation (0.124) was found within *V. hirtella* as it was classified in three groups and one subgroup in the overall dendrogram (Figure 5.1).

The dendrogram prepared through the UPGMA cluster analysis was shown in Figure 5.1. The accessions of the same species in the same populations were mostly grouped together. However, there were some accessions that could not be clearly separated, indicating their genetic similarity. At 0.91 similarity coefficient six clusters were grouped. There were *V. minima*, *V. umbellata*, *V. hirtella*/ *V. tenuicaulis* complex and *V. hirtella* showing three groups and one subgroup.

This dendrogram showed that *V. minima* and *V. hirtella* samples collected along the route from Chiang Mai to Fang (population 14-19) were different from the samples collected from the route Chiang Mai to Mae Hong Son (population 2-13).

Table 5.4 Phenetic distance between populations within *V. minima* constructed using Simple Matching Coefficient.

Population	5	6	9	11	17
5	0.139				
6	0.253	0.114			
9	0.253	0.261	0.109		
11	0.329	0.295	0.303	0.095	
17	0.308	0.307	0.285	0.314	0.022

Table 5.5 Phenetic distance between population within *V. hirtella* and *V. tenuicaulis* constructed using Simple Matching Coefficient.

Population	Hir-pop2	Hir-pop4	Hir-pop5	Hir-pop8	Hir-pop11	Hir-pop12	Hir-pop14	Hir-pop17	Ten-pop2
Hir-pop2	0.085								
Hir-pop4	0.193	0.009							
Hir-pop5	0.247	0.217	0.110						
Hir-pop8	0.252	0.264	0.118	0.067					
Hir-pop11	0.242	0.262	0.157	0.119	0.027				
Hir-pop12	0.261	0.265	0.139	0.118	0.128	0.042			
Hir-pop14	0.274	0.270	0.278	0.275	0.273	0.276	0.044		
Hir-pop17	0.246	0.251	0.240	0.231	0.231	0.248	0.137	0.032	
Ten-pop2	0.241	0.266	0.230	0.197	0.206	0.219	0.270	0.247	0.043
Ten-pop4	0.246	0.275	0.251	0.230	0.228	0.249	0.290	0.258	0.166

Table 5.6 Phenetic distance between species constructed using Simple Matching Coefficient.

Species	*V. minima*	*V. hirtella*	*V. tenuicaulis*	*V. umbellata*
V. minima	0.104			
V. hirtella	0.148	0.124		
V. tenuicaulis	0.155	0.140	0.083	
V. umbellata	0.155	0.151	0.154	0.049

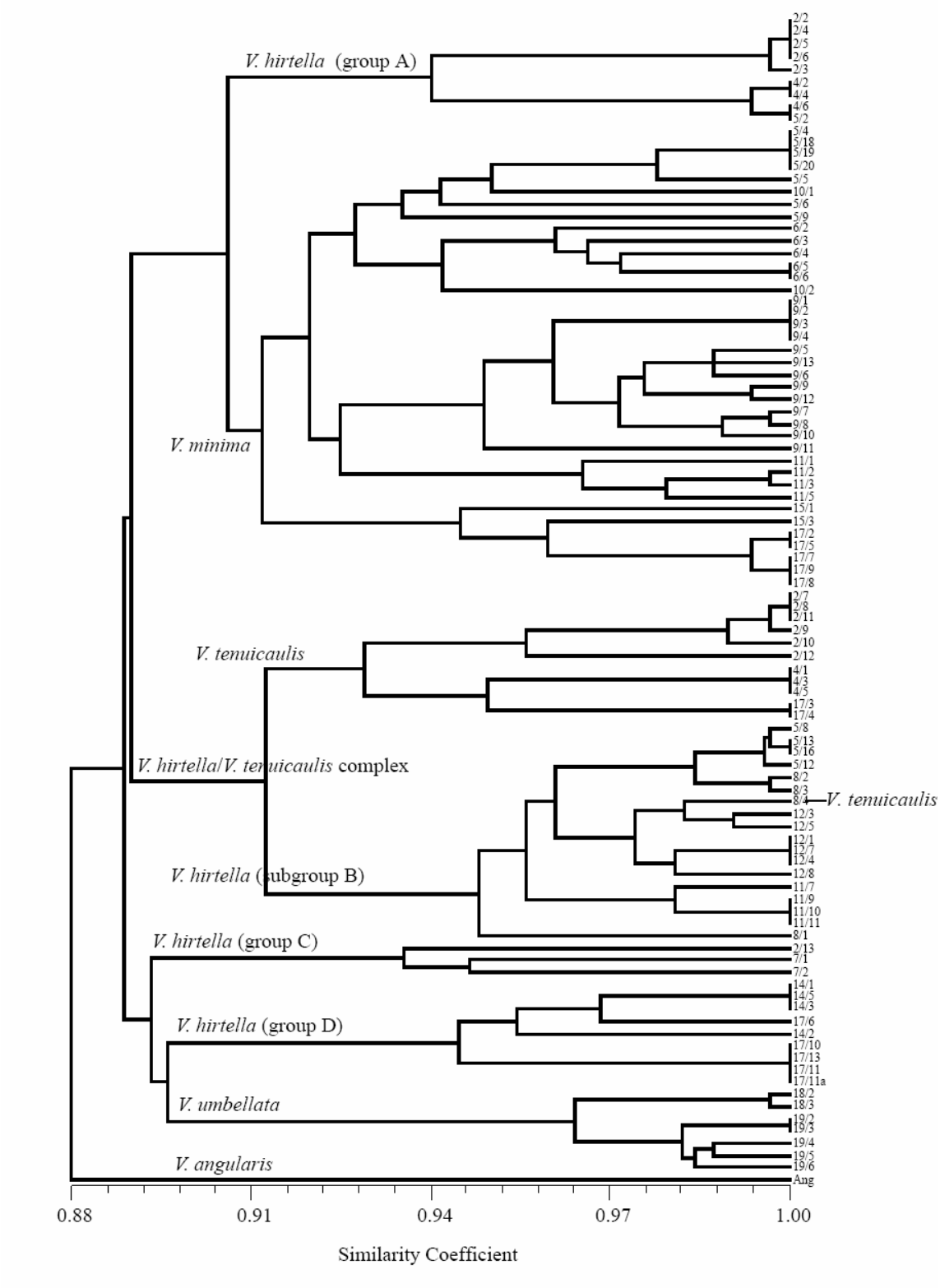


Figure 5.1 UPGMA dendrogram of all accessions based on Jaccard's similarity Coefficient.

Among 149 alleles found in 38 individuals of *V. hirtella* collected, 32 were only found in 3 individuals constituting group C. This showed the necessity for thorough sampling to capture rare alleles. *V. hirtella* displayed the greatest variation in habitat and also had the greatest genetic variation revealed by SSR analysis (Table 5.6). This species generally occurred in either full or semi-shade area. The habitats generally showed an intermediate level of disturbance. The species grew over a wide range of altitudes (561-1130 m). However, there did seem to be a degree of spatial differentiation. Thus *V. hirtella* from Chiang Mai to Mae Hong Son road had a different SSR profile from populations collected toward the Chiang Mai to Fang road (overall dendrogram, Figure 5.1).

V. hirtella group A which was closely related to *V. minima*, consisted of 9 individuals from 3 populations located at the altitude ranged 775-1130 m. Subgroup B in the *V. hirtella/V. tenuicaulis* complex consisted of 17 individuals from 4 populations located at the altitude ranging from 575 to 1130m. Group C, which was closely related to *V. umbellata* consisted of 3 individuals from 2 populations located at the altitude ranging from 775 to 1108 m. These two groups and one subgroup were collected on Chiang Mai-Mae Hong Son road while group D, which was also closely related to *V. umbellata* and consisted of 9 individuals from 2 populations at the 561-682 m altitude, was collected on Chiang Mai-Fang road. This showed that the difference among *V. hirtella* groups was not affected by the altitude. According to the results of AFLP analysis in chapter 3, *V. hirtella* was divided into 2 subgroups; *V. hirtella* (a) was close to *V. umbellata* while *V. hirtella* (b) was close to *V. tenuicaulis*. Therefore, *V. hirtella* group C and D in this analysis might be *V. hirtella* (a) and subgroup B might be *V. hirtella* (b) in the previous analysis. However, this result

showed that *V. hirtella* was close to *V. minima*, which has not been previously reported. The three individuals in group C had many alleles that were not found in other individuals analyzed, especially accession 2/13 that was very different from its population. From 20 loci analyzed here only one heterozygous locus was found in this plant while there was none in other individuals analyzed in this population.

In *V. hirtella*/ *V. tenuicaulis* complex, these two species were clearly separated except the sample number 8/4. This sample was identified as *V. tenuicaulis* based on morphology but it was placed as *V. hirtella* after SSR analysis. Thus, the morphology of this accession might be changed by the environment or the number of SSR markers used in this study was not enough to distinguish completely between *V. hirtella* and *V. tenuicaulis*. If this is the case the use of more SSR primers may be useful.

In six of 15 locations collected more than one *Vigna* species grew in close proximity. At site 17 four species were found growing in close proximity (Figure 5.2). However, each species grew in different habitats, especially *V. minima* in northern Thailand growing most distinctively. This *V. minima* occurred generally in forests where there was slight disturbance (e.g. beside forest paths). It was typically found in shaded areas in the under-story of dry deciduous forests such as teak and pine where there was a low level of competition from other herbaceous plants. It was common and occurred over a wide range of altitudes (507-1130 m). *V. tenuicaulis* and *V. umbellata* grew generally in open area, but *V. umbellata* grew more spread than *V. tenuicalis* whereas *V. hirtella* grew in semi-shaded area. Since each of these four *Vigna* species was found in a wide range of altitudes and there was no difference on the grounds of altitude. It may be a transitional zone; therefore difference in altitudinal variation was not revealed. The altitude ranges for these collections were:

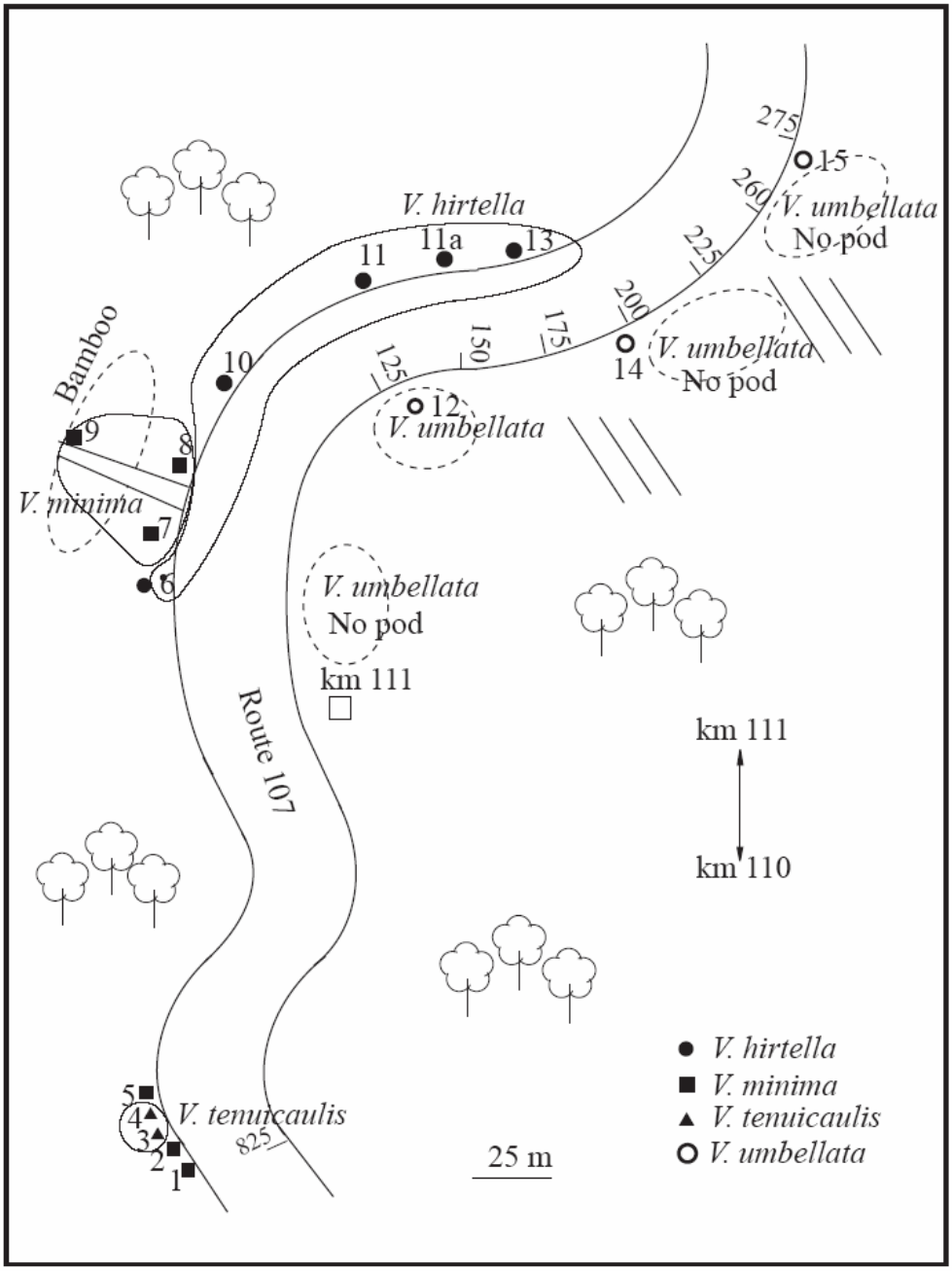


Figure 5.2 Sketch map of site 17. *V. hirtella* (●); *V. minima* (■); *V. tenuicaulis* (▲); *V. umbellata* (O).

V. minima 749 m (507-1130 m mean of 7 sites); *V. hirtella* 829 m (561-1130 m mean of 9 sites); *V. tenuicaulis* 837 m (682-1049 m mean of 4 sites); and *V. umbellata* 503 m (507-1130 m mean of 2 sites).

V. tenuicaulis was usually found growing in full sun and disturbed habitats at the edge of the road. It grew over a wide range of altitudes (682-1049 m).

V. umbellata was later flowering than other species collected. At site 17 it was flowering profusely but no pods with seeds could be collected. Its habitat was similar to *V. tenuicaulis*, being in full sun and highly disturbed habitats. It often occurred in larger roadside populations than *V. tenuicaulis*, suggesting that it was more vigorous (aggressive) than *V. tenuicaulis*.

However, all three species may be found in these different niches growing a few meters apart, thus there might be some barriers to interspecific crossing among them.

An unrooted neighbor-joining tree of samples from population 2 was presented in Figure 5.3. Samples collected from this population were divided into 3 groups. *V. hirtella* group consisting of sample 2/4-2/6, *V. tenuicaulis* group consisting of sample 2/7-2/12, and one *V. hirtella* accession 2/13. This result was similar to UPGMA analysis and was in agreement with the collected habitat where *V. hirtella* group (2/4-2/6) was found in semi-shaded area, whereas *V. tenuicaulis* group (2/7-2/12) grew in the open area and relatively far from *V. hirtella*. For the accession number 2/13, it was very different from both previous groups. It was collected far away from other samples in this population (Figure 5.4), which may represent individual dispersed by birds or vehicles or there could be some more plants close to this accession growing deep inside the forest.

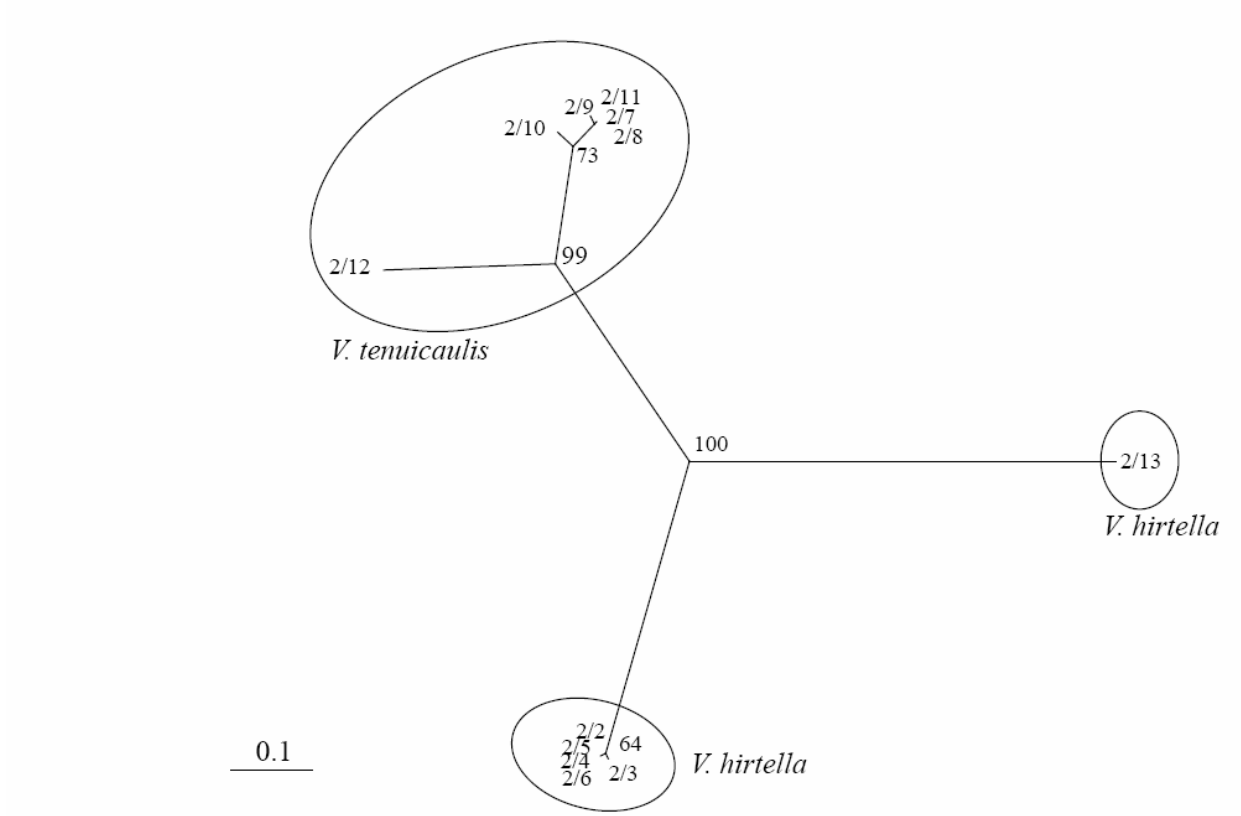


Figure 5.3 An unrooted neighbor-joining tree showing the genetic relationship between individuals of population 2. The number at the node is a bootstrap value higher than 50%.

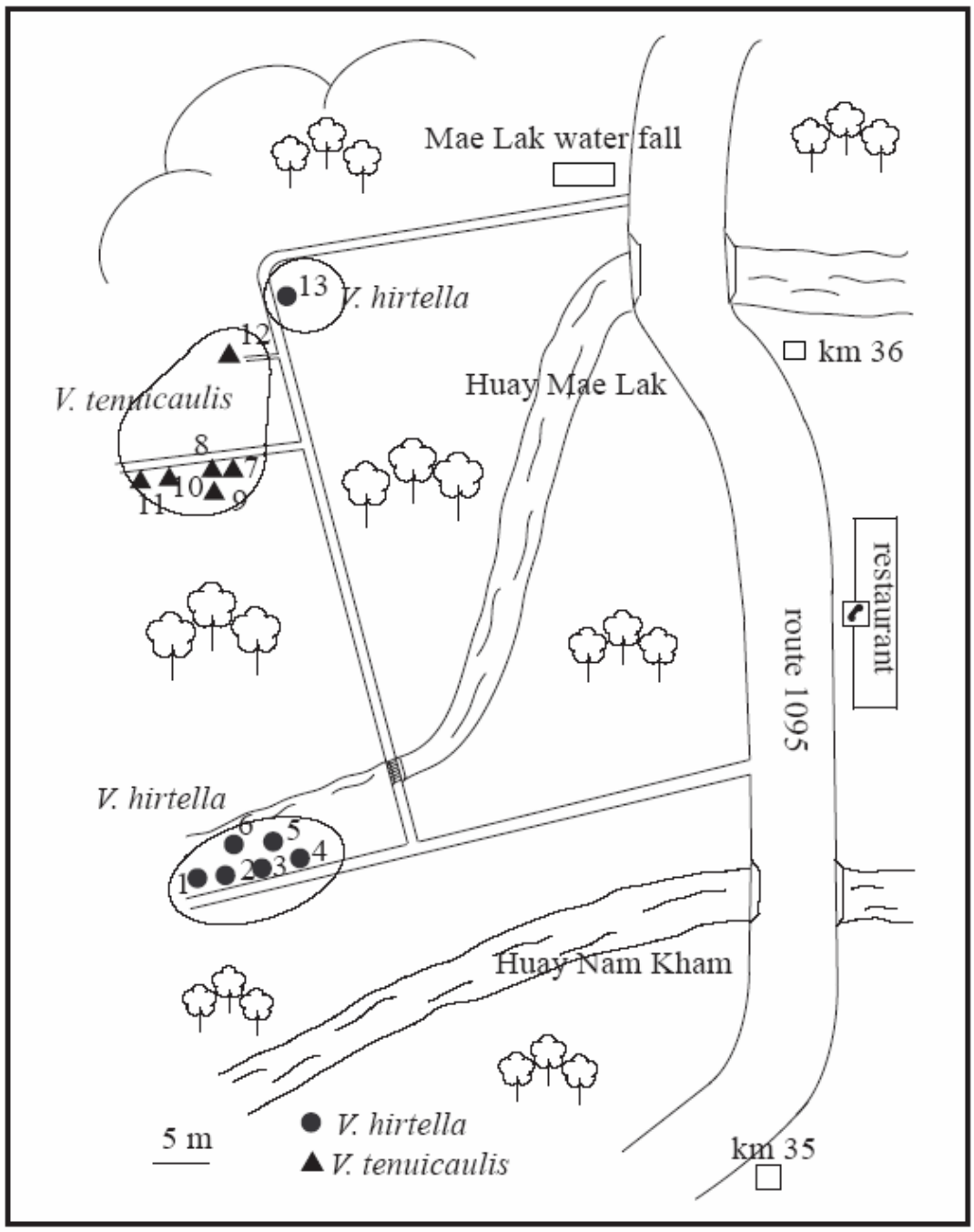


Figure 5.4 Sketch map of site 2. *V. hirtella* (●) and *V. tenuicaulis* (▲).

This study has shown that the microsatellite pattern of individuals collected from different sites across northern Thailand is rather complex. Analysis revealed that *V. minima*, *V. tenuicaulis*, and *V. umbellata* individuals form species-specific clusters. However, *V. hirtella* is best described as complex of species or species aggregate. The results of SSR analysis suggested that the evolutionary trend was from forest dwelling *V. minima* to the species of more open and disturbed habitats, i.e. *V. tenuicalis* and *V. umbellata*. *V. hirtella* occupied a transition zone between the forest and open habitats. These relationships are diagrammatically shown in Figure 5.5.

5.5 Conclusion

Northern Thailand is a center of species diversity for *Vigna* species of section *Angulares*, subgenus *Ceratotropis*. Many sites have several sympatric species. Analysis of individuals within populations reveals a low level of heterozygous loci, indicative of a low level of outcrossing. However, two individuals with alleles commonly found in two different species suggest occasional interspecific crossing had occurred. This suggests *Vigna* in northern Thailand have characteristics similar to legume species complexes reported from other regions [e.g. the *Vicia sativa* complex from Europe (Potokina et al., 2000)].

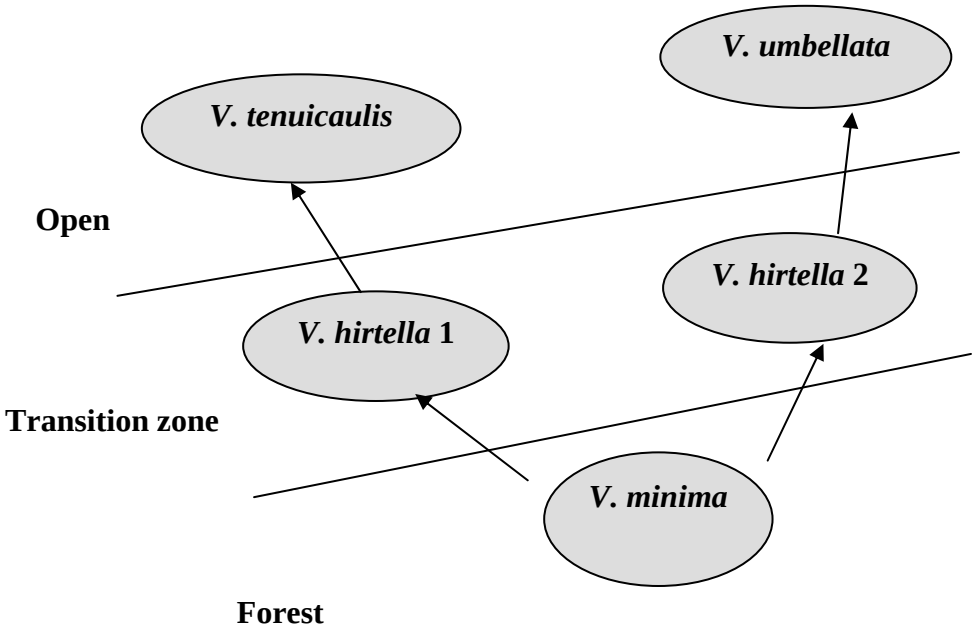


Figure 5.5 Relationship among *Vigna* species of northern Thailand.

5.6 References

Ajibade, S. R., Weeden, N. F. and Chite, S. M. (2000). Inter simple sequence repeat analysis of genetic relationships in the genus *Vigna*. **Euphytica** 111: 47-55.

Langella O. (1999). POPULATION 1.2.28: Population genetic software. Department of populations, genetics and evolution, CNPS, France.

Li, C. D., Fatokun, C. A., Ubi, B., Singh B. B. and Scoles, G. J. (2001). Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. **Crop Sci.** 41: 189-197.

Miller, M. P. (1999). Mantel-Struct 1.0: A program for the detection of population structure through the use of Mantel tests. USA: Department of biological sciences, Northern Arizona University,.

Panaud, O., Chen, X. and McCouch, S. R. (1996). Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). **Mol. Gen. Gent.** 2: 252-238.

Potokina, E., Vaughan, D. A., Eggi, E. E. and Tomooka, N. (2000). Population diversity of the *Vicia sativa* agg. (Fabaceae) in the flora of the former USSR deduced from RAPD and seed protein analyses. **Gent. Resour. Crop Evol.** 47: 171-183.

Rohlf, F.S. (1998). NTSYSpc: Numerical taxonomy and multivariate analysis systems version 2.10y. New York, USA: Exeter Publications.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. **Mol. Biol. Evol.** 4: 406-425.

Schaal, B. A., Leverich, W. J. and Rogstad, S. H. (1991). Comparison of methods for assessing genetic variation in plant conservation biology. In D.A. Falk and K.E. Holsinger (eds.). **Genetics and Conservation of Rare Plants**. New York: Oxford University Press. 304 p.

Seehalak, W. Tomooka, N., Waranyuwat, A., Thipyapong, P., Laosuwan, P., Kaga, A. and Vaughan, D. A. (2006). Genetic diversity of the *Vigna* germplasm from Thailand and neighboring regions revealed by AFLP analysis. **Genet. Resour. Crop Evol.** (in print).

Sokal, R. R. and Michener, C. D. (1958). A statistical method for evaluating systematic relationships. **Univ. Kan. Sci. Bull.** 38: 1409-1438.

Wang, X., Kaga, A., Tomooka, N. and Vaughan, D. A. (2004). The development of SSR markers by new method in plant and their application to gene flow studies in azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi]. **Theor. Appl. Genet.** 109: 352-360.

Yoon, M. S., Doi, K., Kaga, A., Tomooka, N. and Vaughan, D. A. (2000). Analysis of the genetic diversity in the *Vigna minima* complex and related species in east Asia. **J. Plant Res.** 113: 375-386.

Yu, K., Park, S. J., Poysa, S. and Gepts, P. (2000). Integration of simple sequence repeat (SSR) markers into a molecular linkage map of common bean (*Phaseolus vulagaris* L.). **J. Hered.** 91: 429-434.

Zhou, H. F., Xie, Z. W. and Ge, S. (2003). Microsatellite analysis of genetic diversity and population genetic structure of a wild rice (*Oryza rufipogon* Griff.) in China. **Theor. Appl. Genet.** 107: 332-339.

CHAPTER VI

INTERSPECIFIC HYBRIDIZATION BETWEEN *Vigna radiata* AND *Vigna* spp.

6.1 Abstract

The crosses between mungbean (*Vigna radiata*) and *Vigna* spp. including *V. aconitifolia*, *V. mungo*, *V. radiata* var. *sublobata*, *V. stipulacea*, and *V. trinervia* were made to transfer certain characters of these species into mungbean and to evaluate their genetic relationships. It was found that the cross and reciprocal cross between *V. radiata* and its wild relative (*V. radiata* var. *sublobata*) was successful. Their hybrids grew vigorously and fertile. Mature hybrid pods were obtained from the crosses between *V. radiata* var. *sublobata* x *V. trinervia*, *V. mungo* x *V. radiata* var. *sublobata*, *V. mungo* x *V. radiata*, *V. radiata* var. *sublobata* x *V. mungo*, and *V. radiata* x *V. mungo* but their seeds were shrunken and abortive. The hybrid pods from the crosses between *V. radiata* x *V. stipulacea*, *V. radiata* var. *sublobata* x *V. stipulacea*, *V. mungo* x *V. stipulacea*, *V. stipulacea* x *V. mungo*, and *V. stipulacea* x *V. radiata* var. *sublobata* were set but they dropped before reaching maturity. Embryo culture was used to rescue some hybrids and two hybrid plants from *V. mungo* x *V. stipulacea* were obtained. However, these two seedlings died after transferring from test tubes to vermiculite. The F_1 hybrids of *V. radiata* var. *sublobata* x *V. radiata*

exhibited intermediate characteristics between their parents and they were confirmed by ISSR markers that they were truly hybrids.

6.2 Introduction

Interspecific hybridization or crossing of different species is an important method for plant breeding program, for example, to incorporate the desirable trait (s) from one species to another, to increase genetic variation, and to clarify the taxonomic relationship. The intercrossing among members of *Vigna* species, if possible, can enhance the success of mungbean breeding program. However, very often that the efforts were not successful due to difficulties in crossing, and even though successful, the resultant pods were abortive and sometimes, F_1 hybrids were sterile. The previous experiments showed no pod setting from the crosses between *V. radiata* and *V. minima*, *V. trinervia*, wild *V. umbellata* (Egawa et al., 1996), and *V. aconitifolia* (Ngampongsai et al., 1995). The hybrid plants obtained from embryo rescue were from the crosses between *V. radiata* and *V. angularis*, *V. mungo*, *V. umbellata* (Smatt, 1985, quoted in Ngampongsai et al., 1995), *V. grandiflora* (Egawa et al., 1991), and *V. glabrescens*, but the hybrids were sterile (Ngampongsai et al., 1995). High percentage of pod setting and viable seeds were observed in the cross between *V. radiata* var. *sublobata* x *V. trinervia* (Egawa et al., 1996).

Nowadays, in addition to morphological observation, many molecular markers are the powerful tools for plant breeders. The objective of this study is to use ISSR marker to verify whether or not the hybrids are true. This marker was used because it can differentiate between parents and hybrids, and detect multiloci polymorphisms more rapidly and easily as compared with other markers (Kumar et al., 2001).

The objectives of this study were to evaluate cross compatibility between *V. radiata* and *Vigna* spp. and to confirm the hybrids using ISSR marker and morphological comparison between hybrids and respective parents.

6.3 Materials and Methods

Plant Materials

Five *Vigna* species were used in this experiment. *V. radiata* variety SUT 1, which has many prominent characters including up-lifted pods, few sets of pod maturation, grabrous pods, large seed size, high yield and high resistance to Cercospora leaf spot and powdery mildew. The species selected for crossing with *V. radiata* were *V. aconitifolia*, *V. mungo*, *V. radiata* var. *sublobata*, *V. stipulacea*, and *V. trinervia*.

V. aconitifolia was highly resistant to drought and high temperature. *V. mungo* (black gram) has prominent characters including large amount of sulfur-containing amino acids, plant vigor, resistance to diseases and insects, and tolerance to adverse environmental conditions. *V. radiata* var. *sublobata* (wild relative of mungbean) is resistant to bruchid (Ngampongsai, 2000). No useful traits of *V. stipulacea* have been reported. However, the field observation of this species showed resistance to disease and drought. According to the genetic diversity analysis of *V. trinervia*, it was positioned between section *Angulares* and *Ceratotropis* (Tomooka et al., 2002a). Therefore, this species might be used as the bridge to facilitate gene transfer from other species into *V. radiata*.

All of them were grown at SUT farm. During the flowering period, the young flowers of maternal parent were emasculated the day before blooming and were

pollinated with the pollen of paternal parent in the subsequent morning. The pollinated flowers were covered with cotton or parafilm bag. The fertilized pods were divided into two parts. For the first part, their embryos were rescued in 8-10 days after pollination. For the second part, fertilized pods were allowed to grow naturally. The number of fertilized pod settings and number of F_1 plants were recorded. F_1 plants were grown in comparison with their respective parents at SUT farm and their morphology was observed.

Embryo Rescue

After fertilization, the embryos were rescued according to the method described by Ngampongsai (2000). At 8-10 days after pollination, the immature fertilized pods were cleaned with detergent and tap water, rinsed in 70% (v/v) ethanol for 5 min, disinfected with 15% (v/v) Clorox® solution [10% (w/w) Sodium Hypochlorite] with 2-3 drops of Tween20 for 15 min in laminar flow hood, followed by 3 rinses of sterilized distilled water every 5 min and dried in Petri dishes. The immature embryos were excised from pods, cultured in culture tubes containing White's agar medium supplemented with 200 mg/l of yeast extract (see Appendix Table 2A) and kept in culture room at 25ºC, 26000 Lux of light intensity and 8-hr daylight. The seedlings were transferred to sterilized vermiculite and kept in a growth chamber for acclimatization.

DNA Extraction

Young leaf samples taken 0.1 g each from F_1 plant and its parents were ground and DNA was extracted using a method of Owens (2003). Six hundreds µl of

extraction buffer [3% CTAB (cetyltrimethyl ammonium bromide), 1.4M NaCl, 20mM EDTA pH 8.0, 0.1 Tris-HCl pH 8.0, 2% PVP MW 40,000, and 0.2% β-mercaptoethanol] was mixed and incubated at 65ºC for 30 min. One volume (600 µl) of 24:1 chloroform: isoamyl alcohol was mixed, and then centrifuged at 13,000 rpm for 15 min. The supernatant was transferred into a new 1.5 ml tube. After that, 0.5 volume of 5M NaCl and 1 volume of cold isopropanol was added and incubated at -20ºC for 20 min, and centrifuged at 13,000 rpm for 15 min. The supernatant was decanted and added with 1 ml of cold 70% (v/v) ethanol over the pellet. The tube was centrifuged again and decanted. The pellet was allowed to dry and added with TE (10 mM Tris-Cl, 1 mM EDTA) to resuspend the pellet. After the pellet dissolved, RNaseA (1 mg/ml) in the amount of 11.1% of the final volume of TE was added and incubated at 37ºC for 30 min. DNA concentration was quantified by agarose gel electrophoresis comparing with known concentrations of λ DNA.

ISSR Analysis

Ten ISSR primers obtained from the University of British Columbia (Table 6.1; Ajibade et al., 2000) were screened with DNA from *V. mungo*, *V. radiata*, and *V. radiata* var. *sublobata*. The primers which showed different banding patterns between maternal and paternal parents were used for evaluating whether F_1 plants were hybrids or self pollinated.

PCR reactions (20 µl) consisted of 100 ng template DNA, 1x buffer, 250 µM each dNTP, 0.4 µM primer, 3.5 mM $MgCl_2$, and 1 unit *Taq* polymerase. The reaction mixture was subjected to amplification in *Px2* Thermal Cycler (Thermo Hybaid, Flanklin, MA). Cycling parameters were initial denaturation step at 94°C for 5 min,

followed by 94°C, 1 min, 50°C, 1 min and 72°C, 4 min. This cycle was repeated 35 times, followed by 10 min extension at 72°C. The amplification products were separated in 2% agarose gel, using 1X TBE buffer. Gels were stained with ethidium bromide and DNA bands were visualized on UV transluminator. DNA banding pattern from hybrid plants gave both maternal and paternal parent patterns but the DNA banding of self pollinated plant should be similar to that of the maternal parent.

Table 6.1 List of ISSR primers screened and their nucleotide sequences.

UBC No.	Sequences
808	5'- AGA GAG AGA GAG AGA GC
825	5'- ACA CAC ACA CAC ACA CT
826	5'- ACA CAC ACA CAC ACA CC
834	5'- AGA GAG AGA GAG AGA GYT
835	5'- AGA GAG AGA GAG AGA GYC
836	5'- AGA GAG AGA GAG AGA GYA
841	5'- GAG AGA GAG AGA GAG AYC
847	5'- CAC ACA CAC ACA CAC ARC
855	5'- ACA CAC ACA CAC ACA CYT
856	5'- ACA CAC ACA CAC ACA CYA

Note: Primers were obtained from the University of British Columbia. Y = pyrimidines, R= purines

6.4 Results and Discussions

The results of each cross combination are shown in Table 6.2. Among 22 cross combinations twelve of them were compatible. Percentage of pod setting ranged from 0 to 26.32. The highest percentage of pod setting (26.32) was found in the cross between *V. radiata* var. *sublobata* and *V. trinervia*. However, this was due to the low number of pollinated flowers.

Although the hybrid pods were set, most of them fell 3-5 days after pollination before they were old enough to be rescued. Moreover, most of the mature hybrid pods had shrunken seeds (Figure 6.1) which resulted in a low number of hybrid plants obtained. In the case of embryo culture, twelve hybrid pods from five cross combinations were rescued; four pods from *V. mungo* x *V. radiata* var. *sublobata*, three from *V. mungo* x *V. stipulacea*, two each from crosses *V. radiata* x *V. stipulacea* and *V. radiata* var. *sublobata* x *V. stipulacea*, and one from *V. mungo* x *V. radiata*.

Table 6.2 The results of crosses among different species of *Vigna*.

Cross combinations	No. pollinated flowers	No. pod set	% pod setting	No. mature pods	No. of plants obtained from mature seed	No. rescued pods	No. plantlets obtained from rescue	Notes
V. radiata* and *V. radiata* var. *sublobata								
V. radiata x *V. radiata* var. *sublobata*	184	10	5.43	5	6			
V. radiata var. *sublobata* x *V. radiata*	181	5	2.76	3	9			
V. radiata* and *V. mungo								
V. radiata x *V. mungo*	345	17	4.93	7				Hybrid seeds were abortive
V. mungo x *V. radiata*	363	51	14.05	34		1		Hybrid seeds were abortive
V. radiata var. *sublobata* x *V. mungo*	243	31	12.76	9				Hybrid seeds were abortive
V. mungo x *V. radiata* var. *sublobata*	140	23	16.42	11		4		Hybrid seeds were abortive
V. radiata, V. mungo* and *V. aconitifolia								
V. radiata x *V. aconitifolia*	31							
V. aconitifolia x *V. radiata*	122							
V. mungo x *V. aconitifolia*	20							
V. aconitifolia x *V. mungo*	41							

Table 6.2 The results of crosses among different species of *Vigna* (continued).

Cross combinations	No. pollinated flowers	No. pod set	% pod setting	No. mature pods	No. of plants obtained from mature seed	No. rescued pods	No. plantlets obtained from rescue	Notes
V. radiata* and *V. stipulacea								
V. radiata x *V. stipulacea*	364	68	18.68			2		Hybrid pods dropped 3-5 DAP[a]
V. stipulacea x *V. radiata*	238							
V. radiata var. *sublobata* x *V. stipulacea*	99	13	13.13			2		Hybrid pods dropped 3-5 DAP
V. stipulacea x V. radiata var. *sublobata*	108	3	2.78					Hybrid pods dropped 3-5 DAP
V. mungo* and *V. stipulacea								
V. mungo x *V. stipulacea*	186	15	8.06			3	2	
V. stipulacea x *V. mungo*	145	6	4.14					Hybrid pods dropped 3-5 DAP
V. radiata* and *V. trinervia								
V. radiata x *V. trinervia*	18							
V. trinervia x *V. radiata*	21							
V. radiata var. *sublobata* x *V. trinervia*	19	5	26.32	1				Hybrid seeds were abortive
V. trinervia x V. radiata var. *sublobata*	1							

Table 6.2 The results of crosses among different species of *Vigna* (continued).

Cross combinations	No. pollinated flowers	No. pod set	% pod setting	No. mature pods	No. of plants obtained from mature seed	No. rescued pods	No. plantlets obtained from rescue	Notes
V. mungo* and *V. trinervia								
V. mungo x *V. trinervia*	16							
V. trinervia x *V. mungo*	11							

[a] DAP = days after pollination

Figure 6.1 An example of shrunken F_1 seeds of *V. radiata* var. *sublobata* x *V. mungo.*

1) Cross-compatibility of *V. radiata* and its wild relative (*V. radiata* var. *sublobata*)

When *V. radiata* was used as maternal parent, percentage of pod setting was higher than using its wild relative. The hybrid plants obtained from these crosses grew vigorously and fertile. Different varieties were used [CN36 (*V. radiata*) and TC1966 (*V. radiata* var. *sublobata*)] in a similar experiment by Ngampongsai (2000), and a higher percentage of pod setting (26.1%) was observed. This showed the effect of different varieties on cross compatibility.

The morphological characteristics of F_1 as compared with their parents are shown in Table 6.3 and Figures 6.3-6.11. From the morphological observation of hybrid plants derived from *V. radiata* var. *sublobata* x *V. radiata*, the amount of their stem pubescence, plant size, and F_1 pod length were intermediate between their parents. These indicated that these characters were controlled by additive genes. The hybrids gave dark brown seed coat and climbing growth habit similar to *V. radiata* var. *sublobata*, indicating that these traits were controlled by dominant gene (s).

Table 6.3 The morphological characteristics of hybrids and their respective parents.

Species	Seedling color	Stem pubescence	Stem color	Days to flowering	Climbing	Pod length (F_1)	Pod pubescence (F_1)	Seed coat color (F_1)
V. radiata	Green	None	Green	35	No	11.6	No	Green
V. radiata var. *sublobata*	Red	High	Red	44	Yes	3.2	Yes	Dark Brown
V. radiata x *V. radiata* var. *sublobata*, F_1	Red	Medium	Red	35	Yes	7.5	Yes	Dark Brown
V. radiata var. *sublobata* x *V. radiata*, F_1	Red	Medium	Red	41	Yes	7.3	Yes	Dark Brown

a b c

Figure 6.2 Seedling of *V. radiata* var. *sublobata* (a), *V. radiata* (b), and *V. radiata* var. *sublobata* x *V. radiata* (c). Note on the color of hypocotyl.

a b

c

Figure 6.3 Plant morphology of *V. radiata* var. *sublobata* (a), *V. radiata* (b), and *V. radiata* var. *sublobata* x *V. radiata* (c). Note on growth habit.

a

b

c

Figure 6.4 Stem pubescence and leaf vein color of *V. radiata* var. *sublobata* (a), *V. radiata* (b), and *V. radiata* var. *sublobata* x *V. radiata* (c).

a

b

c

Figure 6.5 Stipule of *V. radiata* var. *sublobata* (a), *V. radiata* (b), and *V. radiata* var. *sublobata* x *V. radiata* (c).

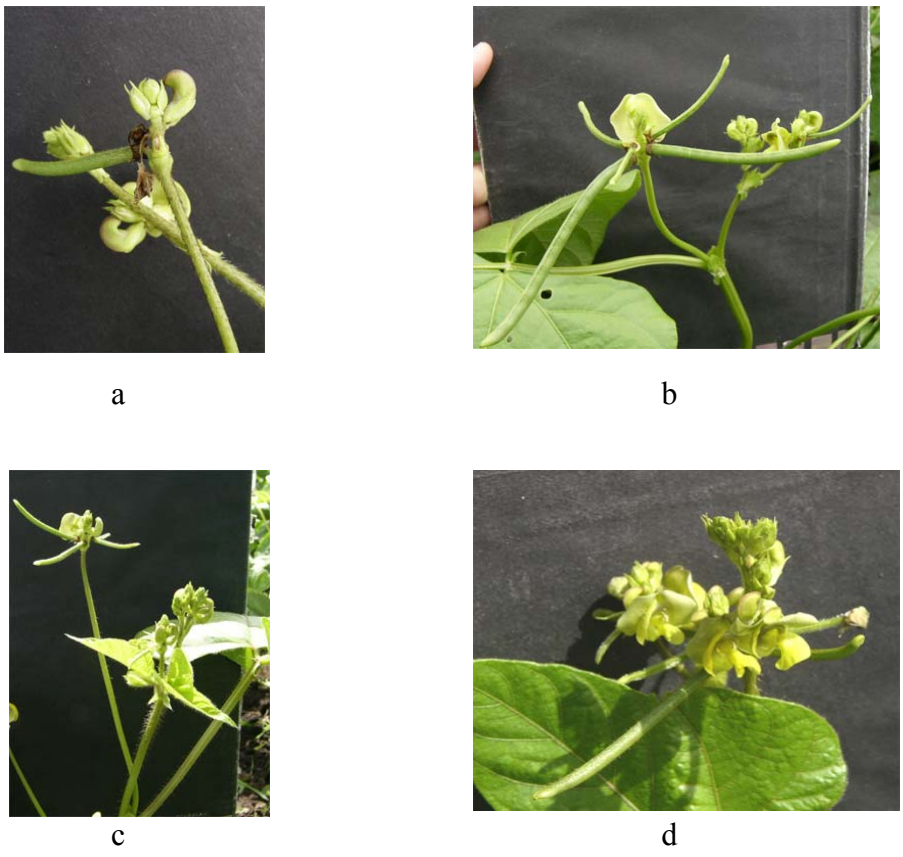


Figure 6.6 Inflorescence of *V. radiata* var. *sublobata* (a), *V. radiata* (b), and *V. radiata* var. *sublobata* x *V. radiata* (c and d).

Figure 6.7 Young flowers of *V. radiata* var. *sublobata* (a), *V. radiata* (b), and F_1 of *V. radiata* var. *sublobata* x *V. radiata* (c).

Figure 6.8 Flowers of *V. radiata* var. *sublobata* (a), *V. radiata* (b), and F_1 of *V. radiata* var. *sublobata* x *V. radiata* (c).

Figure 6.9 Pod length of *V. radiata* var. *sublobata* (a), F_1 of *V. radiata* var. *sublobata* x *V. radiata* (b), and *V. radiata* (c).

Figure 6.10 Seed size of *V. radiata* var. *sublobata* (a), F_1 of *V. radiata* var. *sublobata* x *V. radiata* (b), and *V. radiata* (c).

2) Cross compatibility of *V. radiata* and *V. mungo*

The hybrid pods could be obtained by both using *V. radiata* as maternal or paternal parent crossed with *V. mungo*. However, the percentage of pod setting was higher when *V. mungo* was maternal parent. In this study the cross between *V. radiata* x *V. mungo* gave 4.93% pod setting (345 pollinated flowers; Table 6.2) which was slightly lower than that obtained by Ngampongsai et al. (1995) who obtained 5.6% pod setting (124 pollinated flowers) from crossing between the variety CN36 (*V. radiata*) x PL2 (*V. mungo*). Later, Ngampongsai (2000) repeated the experiment and obtained a higher percentage of pod setting (24.1%, 207 pollinated flowers) and the F_1 seeds could germinate both by nature and embryo rescue. The lower percentage of pod setting obtained from this study, even though higher number of pollinated flowers were used, indicated that the level of cross compatibility depended on the varieties used and/or on environmental condition.

When the wild relative of *V. radiata* (*V. radiata* var. *sublobata*) was used to cross with *V. mungo*, a higher number of pods was set. However, no hybrid plant was obtained from both crosses as the hybrid seeds were abortive and the embryos could not germinate from rescuing.

The relationships among members of *Ceratotropis* species, identified by AFLP analysis (Tomooka et al., 2002b), showed that *V. radiata* was closest to its wild relative (*V. radiata* var. *sublobata*) followed by *V. mungo*. Therefore, the cross between *V radiata* and its wild relative was successful and their hybrids were fertile. However, only the pods were obtained from the cross between *V. radiata* and *V. mungo* but their seeds were abortive. From AFLP analysis, *V. mungo* was closer to *V. radiata* than its wild relative. However, the cross compatibility showed that percentage of pod setting from the cross between *V. radiata* var. *sublobata* and *V. mungo* (12.76%) was higher than that from the cross between *V. radiata* and *V. mungo* (4.93%).

3) Cross-compatibility of *V. radiata*, *V. mungo* and *V. aconitifolia*

These cross combinations among *V. radiata*, *V. mungo* and *V. aconitifolia* were not successful neither using *V. aconitifolia* as maternal nor paternal parent. These results were similar to previous studies made by Ngampongsai et al. (1995) and Ngampongsai (2000).

4) Cross-compatibility of *V. radiata* and *V. stipulacea*

Hybrid pods were obtained only when *V. radiata* was used as maternal parent crossed with *V. stipulacea* but they dropped 3-5 days after pollination which were too young to be rescued.

Crossing of *V. radiata* var. *sublobata* x *V. stipulacea* and their reciprocals gave hybrid pods but the percentage, when using *V. radiata* var. *sublobata* as maternal parent, was less than the respective reciprocal. However, their hybrid pods also dropped before reaching maturity and rescuing embryos was not successful.

5) Cross-compatibility of *V. mungo* and *V. stipulacea*

The pods were set when using either *V. mungo* as maternal or paternal parent crossed with *V. stipulacea* but the higher percentage was obtained when it was the maternal parent. Two hybrid plants were obtained from rescuing the embryos of *V. mungo* x *V. stipulacea* (Figure 6.2). The shoots appeared 4 days after rescuing. As the embryos were very small at the time of culture, the plant development was slow and the root system was weak. All the seedlings eventually died after they were transferred to sterilized vermiculite.

Figure 6.11 F_1 hybrid plant from *V. mungo* x *V. stipulacea*, 72 days after rescuing the embryo.

V. radiata and *V. mungo* are in section *Ceratotropis* while *V. aconitifolia* and *V. stipulacea* are in section *Aconitifolia*. This is a reason why crossing among these species were difficult. According to the result of AFLP analysis by Tomooka et al. (2002b), *V. radiata* and *V. mungo* were closer to *V. aconitifolia* than *V. stipulacea*. However, the results in Table 6.2 showed that crossing of *V. radiata* and *V. mungo* with *V. stipulacea* gave a higher percentage of pod setting than with *V. aconitifolia*.

6) Cross-compatibility of *V. radiata* and *V. trinervia*

The number of pollinated flowers of the cross between *V. radiata* and *V. trinervia* was low because *V. trinervia* did not flower. This species does not always respond to short day treatment and the factors that initiate flowering are not known (Tomooka et al., 2002a).

No pod setting was observed from the cross between *V. radiata* and *V. trinervia.* The pod could set but no plant was obtained when crossing was made between *V. radiata* var. *sublobata* and *V. trinervia*. However, Egawa et al. (1996) could get hybrid plants from this cross but they were highly sterile.

7) Cross-compatibility of *V. mungo* and *V. trinervia*

There was no pod set obtained from *V. mungo* and *V. trinervia* cross combination neither using *V. mungo* as maternal nor paternal parent.

ISSR analysis

After screening 10 ISSR primers using DNA of *V. radiata* and *V. radiata* var. *sublobata*, six primers showed polymorphisms between *V. radiata* and *V. radiata* var. *sublobata*. Two primers were selected for analyzing hybrids. Primers 825 and 841 were selected for analyzing the hybrids between *V. radiata* and *V. radiata* var. *sublobata*.

Banding patterns of *V. radiata* var. *sublobata* x *V. radiata* hybrids showed combined patterns of their maternal and paternal parents, confirming that they were true hybrids (Figure 6.12).

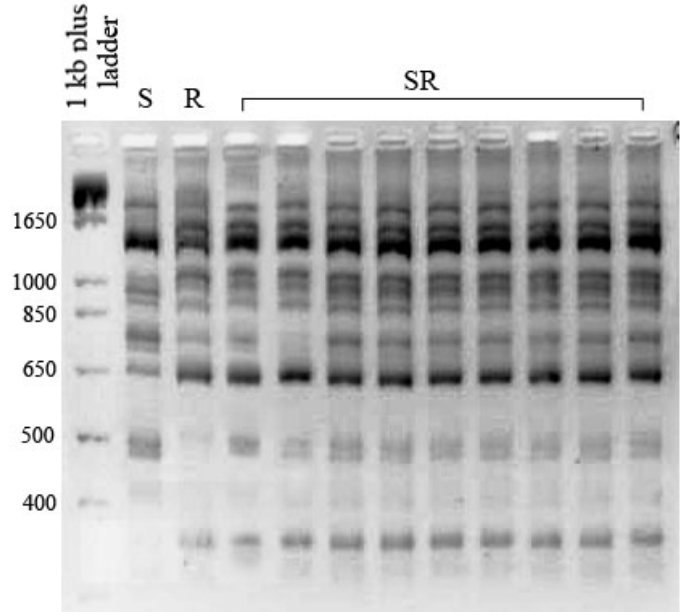


Figure 6.12 ISSR polymorphism among *V. radiata* var. *sublobata* (S), *V. radiata* (R), and *V. radiata* var. *sublobata* x *V. radiata* (SR) amplified by primer 841.

The cross-incompatibility among these *Vigna* species or the low number of pod set might be caused by the improper pollination technique employed in this study or/and pre- or post-zygotic barriers. For the pre-zygotic barriers, fertilization is prevented by gametic incompatibility which is an incapacity of pollen to germinate on the style of the pistil or to form a viable zygote with an egg of another species (Solbrig, 1970, quoted in Stoskopf, 1993). It is associated with the delay of pollen tubes to enter into the ovary as in the cross between *V. umbellata* and *V. minima* (Gopinathan et al., 1986) or they might develop abnormally as in the cross between *V. unguiculata* and *V. vexillata* (Barone et al., 1992). This gametic incompatibility is governed by a complex multiple allelic system of S-genes which prevents gametic union (syngamy) of gemetes carrying identical S-alleles as described by Stoskopf (1993).

Even though the fertilization occurred, the post-zygotic barriers might prevent embryo and endosperm from developing (Gopinathan et al., 1986). This appeared in the cross between cowpea and *V. vexillata* in which embryos stopped developing at the globular stage and the embryo and endosperm nuclei degenerated 5-8 DAP resulting in seed abortion (Barone et al., 1992).

In the case of hybrid weakness as seen from the hybrid between *V. mungo* and *V. stipulacea*, Hardley and Openshaw (1980, quoted in Stoskopf, 1993) suggested that the causes for such weakness were the disharmony between the genomes, genome and cytoplasm of the parental species, and/or genotype of the F_1 zygote and the genotypes of endosperm or the maternal tissue. Moreover, hybrid weakness may be associated with improper timing of critical processes such as cell division, cell organization, and differentiation and may result from weak genetic messages from the two parental genomes. For the shrivelling of seed, Skovmand et al. (1984, quoted in Stoskopf, 1993) suggested that this was caused by the differences in the amount of terminal heterochromatin found on the parent chromosomes. As the amount of heterochromatin slowly declined to about 50% of chromosome, grain plumpness improved.

6.5 Conclusion

This study found that the cross between *V. radiata* and *V. radiata* var. *sublobata* was successful. The hybrids obtained from these parents grew well and were fertile. Their characters including pod length, seed size and the amount of stem pubescence were intermediate between their parents. The red color of seedling and stem and the

dark brown color of seed coat of these hybrids were dominantly inherited traits. The unsuccessful interspecific crossings indicated the difficulty of transferring genes from one species to another. Thus, genetic engineering might be necessary to facilitate conventional mungbean breeding.

6.6 References

Ajibade, S. R., Weeden, N. F. and Chite, S. M. (2000). Inter simple sequence repeat analysis of genetic relationships in the genus *Vigna*. **Euphytica** 111: 47-55.

Barone, A., Del Giudice, A. and Ng, N. Q. (1992). Barriers to interspecific hybridization between *Vigna unguiculata* and *Vigna vexillata*. **Sex. Plant Repro.** 5: 195-200.

Egawa, Y., Chotechuen, S., Tomooka, N., Ngampongsai, S., Thavarasook, C. and Kitbamroong, C. (1996). Cross-compatibility among the subgenus *Ceratotropis* species of the genus *Vigna*. In Y. Egawa and S. Chotechuen (eds.). **Phylogenetic Differentiation of Mungbean germplasm (Subgenus *Ceratotropis* of the Genus *Vigna*) and Evaluation for Breeding Program** (pp 9-18). Japan International Research Center for Agricultural Sciences (JIRCAS), Japan and Chai Nat Field Crops Research Center, Thailand.

Egawa, Y., Tomooka, N., Lairungreang, C., Nakeeraks, P. and Thavarasook, C. (1991). The possibility to use *V. mungo* (black gram) and *V. grandiflora* for mungbean breeding. In N. Tomooka, C. Lairungreang, P. Nakeeraks, Y. Egawa, and C. Thavarasook (eds.). **Mungbean and the Genetic Resources: The Subgenus *Ceratotropis* in Ecological Studies on Tropical Food Legumes in Relation to Adaptation to Cropping Systems in Thailand** (pp 39-43). Tropical

Agriculture Research Center, Japan and Chai Nat Field Crops Research Center, Thailand.

Gopinathan, M. C., Babu, C. R. and Shivanna, K. R. (1986). Interspecific hybridization between rice bean (*Vigna umbellata*) and its wild relative (*V. minima*): Fertility-sterility relationships. **Euphytica** 35: 1017-1022.

Hadley, H. H. and Openshaw, S. J. (1980). Interspecific and intergeneric hybridization. In W. R. Fehr and H. H. Hadley (eds.). **Hybridization of Crop Plants (**pp 133-159). Madison, Wisconsin: American Society of Agronomy-Crop Science Society of America. Quoted in N. C. Stoskopf. (1993). **Plant Breeding: Theory and Practice**. Colorado, USA: Westview Press. 531 p.

Kumar, L. D., Kathievel, M., Rao, G. V. and Nagaraju, J. (2001). DNA profiling of disputed chilli samples (*Capsicum annum*) using ISSR-PCR and FISSR-PCR marker assays. **Forensic Sci. Int.** 116: 63-68.

Ngampongsai, S. (2000). Interspecific hybridization of mungbean |*Vigna radiata* (L.) Wilczek| and related *Vigna* species using embryo rescue techniques. In **Proc. National Mungbean Research Conf. VIII** (pp 1-6). Nakhon Pathom: Kasetsart University.

Ngampongsai, S., Pichitporn, S., Promkum, W., Srinives, P. and Egawa, Y. (1995). Embryo culture of interspecific hybrids among *Vigna* species. In **Proc. Sixth Mungbean Workshop** (pp 91-102). Nakhon Ratchasima: Suranaree University of Technology. (in Thai).

Owens, C. L. (2003). SNP detection and genotyping in *Vitis*. **Acta Hort.** 603: 139-140.

Skovmand, B., Fox, P. N. and Villareal, R. L. (1984). Triticale in commercial agriculture: Progress and promise. **Adv. Agron.** 37: 1-45. Quoted in N. C. Stoskopf. (1993). **Plant Breeding: Theory and Practice**. Colorado, USA: Westview Press. 531 p.

Smatt, J. (1985). Evolution of grain legumes. III. Pulses in the genus *Vigna*. **Expt. Agric.** 21: 87-100. Quoted in S. Ngampongsai, S. Pichitporn, W. Promkum, P. Srinives and Y. Egawa. (1995). Embryo culture of interspecific hybrids among *Vigna* species. In **Proc. Sixth Mungbean Workshop** (pp 91-102). Nakhon Ratchasima: Suranaree University of Technology.

Solbrig, O. T. (1970). Principles and Methods of Plant Biosystematics. Macmillan Co., Collier-Macmillan Co., Toronto, pp.108-109. Quoted in Stoskopf, N. C. (1993). **Plant Breeding: Theory and Practice**. Colorado, USA: Westview Press. 531 p.

Stoskopf, N. C. (1993). **Plant Breeding: Theory and Practice**. Colorado, USA: Westview Press. 531 p.

Tomooka, N., Vaughan, D. A., Moss H. and Maxted N. (2002a). **The Asian *Vigna*: Genus *Vigna* subgenus *Ceratotropi*s genetic resources.** The Netherlands: Kluwer Academic. 270 p.

Tomooka, N., Yoon, M. S., Doi, K., Kaga, A. and Vaughan, D. A. (2002b). AFLP analysis of a *Vigna* subgenus *Ceratotropis* core collection. **Gen. Res. Crop Evol.** 49: 521-530.

CHAPTER VII

CONCLUSION AND RECOMMENDATION

Conclusion

1. The AFLP analysis of the Asian *Vigna* in Thailand and neighboring region revealed that:
   - every species under study showed geographic differentiation,
   - *V. umbellata* was domesticated in northern Thailand and it was hypothesized to be a recently evolved species from *V. exilis*,
   - *V. hirtella* was divided into two distant subgroups which might be of different species. Thus, the morphological difference of these two subgroups require further study in more details,
   - *V. grandiflora* was confirmed to be a distinct species from *V. radiata*,
   - cultivated *V. mungo* is probably monophyletic,
   - Thai wild *V. mungo* accessions were truly native to Thailand.
2. From the exploration of Asian *Vigna* in Chiang Mai and Mae Hong Son provinces, it was found that:
   - four species were collected including *V. hirtella*, *V. minima*, *V. tenuicaulis*, and *V. umbellata*,
   - each species grew in different habitats which *V. hirtella* occurred generally in semi-shaded areas, *V. minima* occurred in shaded areas, while *V. tenuicaulis* and *V. umbellata* grew in open areas.

3. SSR analysis of sympatric *Vigna* species of northern Thailand showed that *V. hirtella* had the maximum number of heterozygous loci per plant in which some loci were from *V. minima*. This exhibited the natural interspecific crossing between *V. hirtella* and *V. minima*.
4. Interspecific hybridization attempts among *Vigna radiata* and *Vigna* spp. were unsuccessful. The fertile hybrid plants obtained were only from the cross between *V. radiata* and its wild relative (*V. radiata* var. *sublobata*).

Recommendation

This study showed that there were many *Vigna* species distributing in various parts of Thailand. However, there has been no report about the exploration in southern Thailand where the climate and environment were different from the other parts. The other *Vigna* species which were not found in the other parts of Thailand might be found in this region.

The sympatric species found during the exploration suggested that seed samples at the same site should be separately collected.

The morphology of *Vigna* species should be studied in more details to identify the useful traits for plant breeding program.

The mechanism of unsuccessful interspecific crossing among *Vigna* species should be further studied which will be useful for overcoming the barriers. Other varieties of cultivated mungbean and blackgram should be crossed with *Vigna* spp to increase genetic variability. The techniques that might be used to facilitate interspecific crossing or trait transferring among the *Vigna* species are, for example, hormone application, protoplast fusion, or genetic engineering techniques.

APPENDIX

Appendix Table 1A. Materials used in the AFLP analysis.

Code	JP no.[a]	Species name (status)	Origin	Province	Latitude	Longitude	Altitude (m)
Set 1. subgenus Ceratotropis section *Angulares* (except *V. trinervia*)							
Um002	210639	*Vigna umbellata* (wild A)	Thailand	Kanchanaburi	N14-44	E98-34	200
Um004	210640	*Vigna umbellata* (wild A)	Thailand	Kanchanaburi	N14-40	E98-23	867
Um005	210642	*Vigna umbellata* (wild A)	Thailand	Kanchanaburi	N14-44	E98-38	195
Um045	210672	*Vigna umbellata* (wild A)	Thailand	Phitsanulok	N16-52	E100-38	165
Um047	210674	*Vigna umbellata* (wild A)	Thailand	Phetchabun	N16-40	E101-05	860
Um048	210675	*Vigna umbellata* (wild A)	Thailand	Phetchabun	N16-40	E101-06	970
Um049	210676	*Vigna umbellata* (wild A)	Thailand	Phetchabun	N16-32	E101-02	715
Um050	210677	*Vigna umbellata* (wild A)	Thailand	Phetchabun	N16-25	E101-11	165
Um052	210679	*Vigna umbellata* (wild A)	Thailand	Phetchabun	N16-32	E101-18	235
Um070	108525	*Vigna umbellata* (wild A)	Thailand	Tak	N16-48	E98-57	510
Um074	108529	*Vigna umbellata* (wild A)	Thailand	Tak	N16-46	E98-51	380
Um088	108547	*Vigna umbellata* (wild A)	Thailand	Lampang	N18-30	E99-20	390
Um089	108548	*Vigna umbellata* (wild A)	Thailand	Lampang	N18-31	E99-31	400
Um102	108563	*Vigna umbellata* (wild A)	Thailand	Chiang Mai	N19-27	E99-00	510
Um103	108564	*Vigna umbellata* (wild A)	Thailand	Chiang Mai	N19-28	E99-00	510
Um157	210797	*Vigna umbellata* (wild A)	Myanmar	Mandalay	N21-52	E96-10	100
Um158	210802	*Vigna umbellata* (wild A)	Myanmar	Mandalay	N21-58	E96-23	855
Um159	210803	*Vigna umbellata* (wild A)	Myanmar	Mandalay	N21-51	E96-21	860
Um012	210647	*Vigna umbellata* (wild C)	Thailand	Ratchaburi	N13-18	E99-34	260
Um027	210662	*Vigna umbellata* (wild C)	Thailand	Rayong	N12-50	E101-44	100
Um030	210665	*Vigna umbellata* (wild B)	Thailand	Uttaradit	N17-50	E100-02	350
Um032	210666	*Vigna umbellata* (wild B)	Thailand	Uttaradit	N17-50	E100-02	350
Um033	210667	*Vigna umbellata* (wild B)	Thailand	Phrae	N17-56	E100-04	240
Um034	207985	*Vigna umbellata* (wild A)	Thailand	Phrae	N18-13	E100-17	260
Um036	210668	*Vigna umbellata* (wild A)	Thailand	Phrae	N18-22	E100-24	300
Um037	211786	*Vigna umbellata* (wild B)	Thailand	Phrae	N18-22	E100-24	300
Um066	108521	*Vigna umbellata* (wild A)	Thailand	Tak	N16-48	E99-03	109
Um069	108524	*Vigna umbellata* (wild A)	Thailand	Tak	N16-48	E99-03	109
Um073	108528	*Vigna umbellata* (wild A)	Thailand	Tak	N16-47	E98-53	405
Um075	108530	*Vigna umbellata* (wild A)	Thailand	Tak	N16-44	E98-34	220
Um077	108532	*Vigna umbellata* (wild A)	Thailand	Tak	N16-47	E98-37	235
Um080	108536	*Vigna umbellata* (wild B)	Thailand	Tak	N16-58	E98-41	340
Um082	108539	*Vigna umbellata* (wild C)	Thailand	Lampang	N17-36	E99-13	210
Um085	108543	*Vigna umbellata* (wild B)	Thailand	Lampang	N18-29	E99-30	370
Um129	109670	*Vigna umbellata* (wild B)	Thailand	Phitsanulok	N16-47	E100-51	150
Um132	109677	*Vigna umbellata* (wild A)	Thailand	Nan	N18-47	E100-44	420
Um138	109679	*Vigna umbellata* (wild A)	Thailand	Nan	N18-47	E100-44	420
Um139	109676	*Vigna umbellata (wild A)*	Thailand	Nan	N18-47	E100-44	420
Um140	109678	*Vigna umbellata (wild B)*	Thailand	Nan	N18-47	E100-44	420
Um086	108544	*Vigna umbellata (cult.)*	Thailand	Lampang	N18-29	E99-30	370

Appendix Table 1A. Materials used in this study (continued).

Code	JP no.[a]	Species name (status)	Origin	Province	Latitude	Longitude	Altitude (m)
Um087	108545	*Vigna umbellata* (cult.)	Thailand	Lampang	N18-29	E99-31	371
Um114	108550	*Vigna umbellata* (cult.)	Thailand	Chiang Rai	N19-11	E99-30	620
Um115	105863	*Vigna umbellata* (cult.)	Thailand	Mae Hong Son	N18-18	E97056	750
Um116	105883	*Vigna umbellata* (cult.)	Thailand	Loei (seed dealer)	N17-22	E101-16	410
Um117	105885	*Vigna umbellata* (cult.)	Thailand	Loei	N17-22	E101-16	650
Um118	105870	*Vigna umbellata* (cult.)	Thailand	Chiang Mai	N19-05	E98-51	420
Um121	105880	*Vigna umbellata* (cult.)	Thailand	Loei	N17-34	E100-54	710
Um122	105855	*Vigna umbellata* (cult.)	Thailand	Uthaitani (Uthaitani market)	N15-22	E100-01	80
Um123	105859	*Vigna umbellata* (cult.)	Thailand	Tak (Mae Sot market)	N16-42	E98-34	400
Um124	105875	*Vigna umbellata* (cult.)	Thailand	Chiang Mai (Chiang Mai market)	N19-54	E99-49	500
Um126	105879	*Vigna umbellata* (cult.)	Thailand	Nan (Nan market)	N18-46	E100-46	450
Um127	105871	*Vigna umbellata* (cult.)	Thailand	Chiang Mai (Chom Thong market)	N18-24	E98-40	360
Um141	110837	*Vigna umbellata* (cult.)	Thailand	Pha Yao	N19-18	E100-09	240
Um143	105825	*Vigna umbellata* (cult.)	Thailand	Mae Hong Son (market)	N19-17	E97-58	450
Um145	105822	*Vigna umbellata* (cult.)	Thailand	Chiang Rai	N20-11	E99-34	420
Um175	100311	*Vigna umbellata* (cult.)	Nepal	Phabgdwam Pakhribas V.P.	N27-20	E87-42	1580
Um176	99485	*Vigna umbellata* (cult.)	Japan	Nagasaki	N34-28	E129-20	20
Ex006	207983	*Vigna exilis* (wild)	Thailand	Kanchanaburi	N14-01	E99-14	110
Ex009	205884	*Vigna exilis* (wild)	Thailand	Ratchaburi	N13-34	E99-46	150
Ex011	210646	*Vigna exilis* (wild)	Thailand	Ratchaburi	N13-35	E99-40	130
Ex013	210648	*Vigna exilis* (wild)	Thailand	Ratchaburi	N13-22	E99-47	105
Ex015	210650	*Vigna exilis* (wild)	Thailand	Petchaburi	N13-06	E99-55	80
Ex016	210651	*Vigna exilis* (wild)	Thailand	Petchaburi	N10-33	E104-42	130
Ex018	210653	*Vigna exilis* (wild)	Thailand	Petchaburi	N12-57	E99-54	110
Ex019	210654	*Vigna exilis* (wild)	Thailand	Petchaburi	N12-51	E99-56	60
Ex060	210684	*Vigna exilis* (wild)	Thailand	Sara Buri	N14-38	E101-08	315
Te003	205883	*Vigna tenuicaulis* (wild)	Thailand	Kanchanaburi	N14-42	E98-28	662
Hi021	205885	*Vigna hirtella* (wild)	Thailand	Nakorn Ratchasima	N14-25	E101-25	750
Te042	210671	*Vigna tenuicaulis* (wild)	Thailand	Nan	N19-05	E101-09	625
Te091	108551	*Vigna tenuicaulis* (wild)	Thailand	Chiang Mai	N19-07	E98-41	760
Te092	108552	*Vigna tenuicaulis* (wild)	Thailand	Chiang Mai	N19-07	E98-41	760
Te093	108553	*Vigna tenuicaulis (wild)*	Thailand	Chiang Mai	N19-07	E98-41	760
Te095	108555	*Vigna tenuicaulis (wild)*	Thailand	Chiang Mai	N19-15	E98-36	1365
Hi101	108562	*Vigna hirtella (wild)*	Thailand	Mae Hong Son	N19-26	E98-24	1085
Hi105	108566	*Vigna hirtella (wild)*	Thailand	Chiang Mai	N19-33	E100-06	535
Hi111	108515	*Vigna hirtella (wild)*	Thailand	Chiang Rai	N20-18	E99-50	1075
Hi112	108542	*Vigna hirtella (wild)*	Thailand	Chiang Rai	N20-19	E99-50	1130

Appendix Table 1A. Materials used in this study (continued)

Code	JP no.[a]	Species name (status)	Origin	Province	Latitude	Longitude	Altitude (m)
Te160	109682	*Vigna tenuicaulis* (wild)	Thailand	Chiang Rai	N19-52	E99-49	460
Te161	217444	*Vigna tenuicaulis* (wild)	Myanmar	Shan	N22-59	E97-45	735
Te162	217486	*Vigna tenuicaulis* (wild)	Myanmar	Chin	N22-55	E93-40	1590
Hi169	217435	*Vigna hirtella* (wild)	Myanmar	Shan	N20-43	E97-03	1360
Hi170	217491	*Vigna hirtella* (wild)	Myanmar	Chin	N23-23	E93-39	1567
Hi171	109681	*Vigna hirtella* (wild)	Thailand	Chiang Mai	N19-04	E99-23	530
Mi020	210655	*Vigna minima* (wild)	Thailand	Sara Buri	N14-26	E100-54	90
Mi038	210669	*Vigna minima* (wild)	Thailand	Nan	N18-3	E100-57	340
Mi041	205886	*Vigna minima* (wild)	Thailand	Nan	N19-05	E101-09	625
Mi044	205888	*Vigna minima* (wild)	Thailand	Nan	N19-07	E101-09	850
Mi056	205890	*Vigna minima* (wild)	Thailand	Sakhon Nakhon	N16-58	E103-59	310
Mi057	210682	*Vigna minima* (wild)	Thailand	Mukdahan	N16-50	E104-08	305
Mi058	210683	*Vigna minima* (wild)	Thailand	Mukdahan	N16-19	E104-31	235
Mi059	205891	*Vigna minima* (wild)	Thailand	Surin	N14-26	E103-41	305
Mi104	101829	*Vigna minima* (wild)	Thailand	Chiang Mai	N19-33	E99-06	535
Mi107	108568	*Vigna minima* (wild)	Thailand	Chiang Mai	N19-37	E99-09	665
Mi166	210806	*Vigna minima* (wild)	Myanmar	Kalaw	N20-39	E96-33	1230
Mi168	210824	*Vigna minima* (wild)	Myanmar	Pa-an	N16-51	E97-41	15
MiDKL	107869	*Vigna minima* (wild)	Thailand	Chai Nat	N15-15	E99-59	150
Set 2. genus *Ceratotropis* section *Ceratotropis, V. trinervia and V. unguiculata*							
Tr022	210657	*Vigna trinervia* (wild)	Thailand	Chantaburi	N12-52	E102-16	250
Tr023	210658	*Vigna trinervia* (wild)	Thailand	Chantaburi	N12-52	E102-16	250
Tr028	210663	*Vigna trinervia* (wild)	Thailand	Rayong	N12-47	E101-28	150
Tr090	108549	*Vigna trinervia* (wild)	Thailand	Chiang Mai	N19-02	E99-18	545
Tr163	108840	*Vigna trinervia* (wild)	Malaysia	Pahang	N04-03	E102-18	50
Mu110	1085123	*Vigna mungo* (wild)	Thailand	Chiang Rai	N20-17	E100-01	410
Mu146	219130	*Vigna mungo* (wild)	Thailand	Chiang Mai	N19-23	E98-59	450
Mu147	107878	*Vigna mungo* (wild)	Thailand	Chiang Mai	N19-07	E98-46	520
Mu148	218939	*Vigna mungo* (wild)	Thailand	Chiang Mai	N19-50	E99-11	640
Mu149	219131	*Vigna mungo* (wild)	Thailand	Chiang Mai	N20-01	E99-17	460
Mu172	212357	*Vigna mungo* (cult.)	India	India	-	-	-
Mu173	212358	*Vigna mungo* (cult.)	Australia	Australia	-	-	-
Mu174	212362	*Vigna mungo (cult.)*	India	India	-	-	-
Mu177	219132	*Vigna mungo (cult.)*	Thailand	Breeding line	-	-	-
Mu178	106710	*Vigna mungo (cult.)*	Thailand	Breeding line	-	-	-
Mu179	109668	*Vigna mungo (cult.)*	Thailand	Phetchabun	N16-17	E101-04	160
Mu184	107874	*Vigna mungo (wild)*	India	India	-	-	-
Mu185	107873	*Vigna mungo (wild)*	India	India	-	-	-
Gr029	207984	*Vigna grandiflora (wild)*	Thailand	Chai Nat	N15-11	E100-06	46
Gr061	108509	*Vigna grandiflora (wild)*	Thailand	Nakhon Sawan	N15-34	E100-03	50
GrBan	107862	*Vigna grandiflora (wild)*	Thailand	Photsanulok	N16-45	E100-07	260

Appendix Table 1A. Materials used in this study (continued)

Code	JP no.[a]	Species name (status)	Origin	Province	Latitude	Longitude	Altitude (m)
Ra182	110830	*Vigna radiata* (cult.)	Thailand	Released variety	-	-	-
Un051	210678	*Vigna unguiculata* (wild)	Thailand	Phetchabun	N16-25	E101-11	165
Un190	105881	*Vigna unguiculata* (cult.)	Thailand	Loei	N17-35	E100-53	450

[a] JP no. is the number of the acession in the Genebank of the ministry of Agriculture, Forestry and Fisheries, Japan

Appendix Table 2A. Ingredients in White's medium (White, 1963) pH 5.7-5.8

Macronutrient	mg/l
KNO_3	80
$MgSO_4.7H_2O$	720
$NaH_2PO_4.H_2O$	16.5
$Ca(NO_3)_2.4H_2O$	300
Na_2SO_4	200
KCl	65
Micronutrient	mg/l
KI	0.75
H_3BO_3	1.5
$MnSO_4.4H_2O$	7
$Fe_2(SO_4)_3$	2.5
$ZnSO_4.7H_2O$	3
Vitamins and amino acid	mg/l
Glycine	3
Cystein	1
Thiamine HCl	0.1
Pyridoxine HCl	0.1
Nicotinic acid	0.5
Ca d-pantothenic	1.0
Sucrose	20 g
Yeast extract	200 mg

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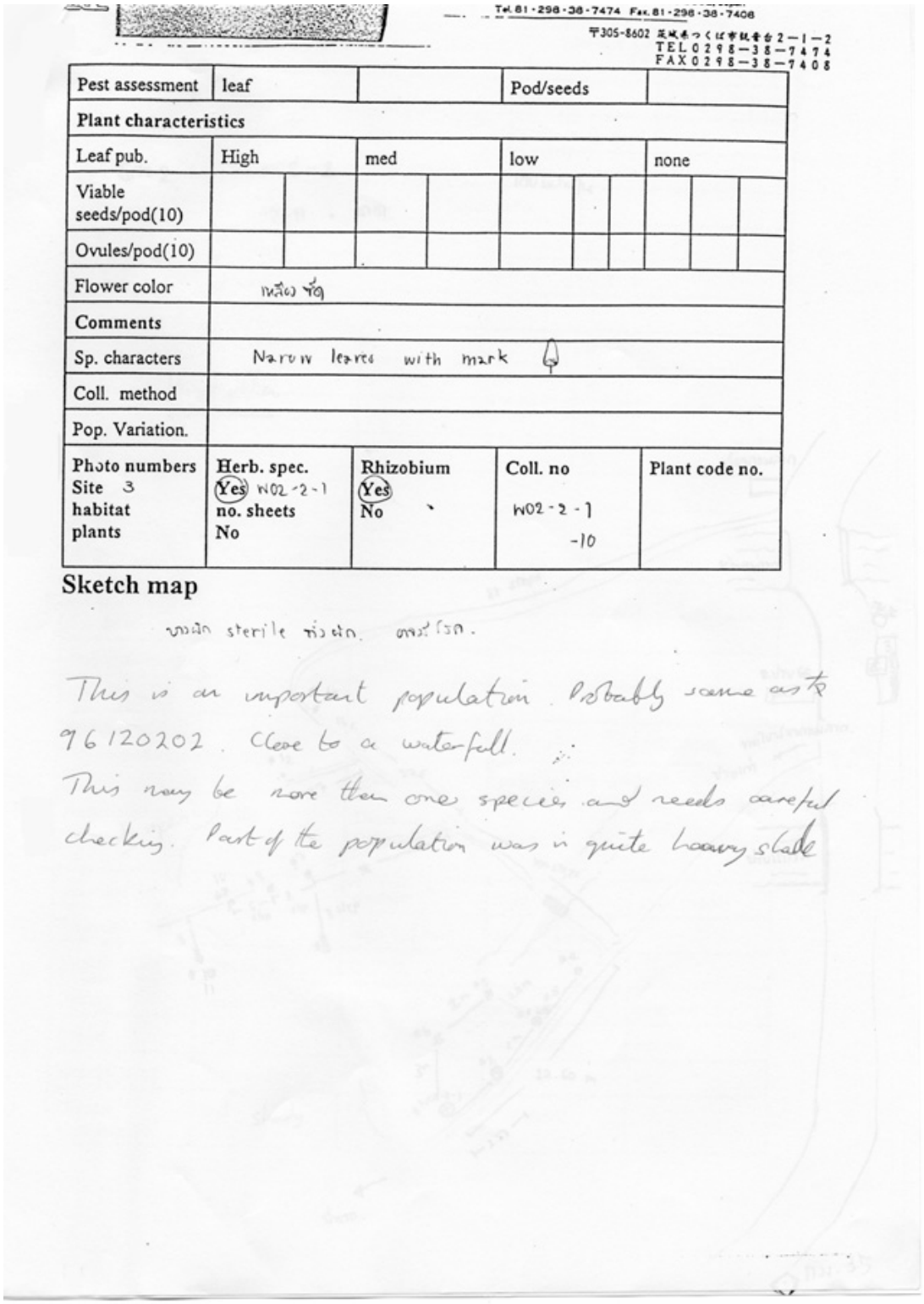
Passport data				Date	d 9	m12	y02
Collectors				Collecting no.	W02-2-1		
Site No	2			Plant no codes			
Scientific name		Local name					
Location							
GIS coordinate	19° 09' 14.4" N 098° 41' 33.0" E						
Map reference	\						
Address	Land holder	J		E			
	Village	J		E			
	Nearest town	J		E			
	Prefecture	J		E			
Site							
Topography	Mountains \	hills	plain	other specify			
Altitude	775 mt						
Slope (degree)							
Land use							
Soil type(map)	clay	Geology(map)					
Climate(map)							
Habitat Associated vegetation type		Forest	bushes low bushes	cultivated			
		grassland	other specify ต้นไม้ใหญ่ ประปราย				
Associated plants specific		Dominant sp. Other spp.					
Shading(%)	heavy	semi shade. medium \	light	open (none)			
Degree of disturbance		high \	med	low	none		
Population	Size (m^2)						
State	vegetative	flowering	mature \	past maturity			
Status	wild \	weedy	cultivated	mixed			
Introgression	yes(extent)		no				
Disease assessment	leaf		Pods/seed	\ sterile ประปราย			



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Pest assessment	leaf				Pod/seeds					
Plant characteristics										
Leaf pub.	High		med		low			none		
Viable seeds/pod(10)										
Ovules/pod(10)										
Flower color	เหลือง									
Comments										
Sp. characters	Narrow leaves with mark									
Coll. method										
Pop. Variation.										

Photo numbers Site 3 habitat plants	**Herb. spec.** (Yes) W02-2-1 **no. sheets** No	**Rhizobium** (Yes) No	**Coll. no** W02-2-1 -10	**Plant code no.**

Sketch map

มีแต่ sterile ไม่มีดอก ดอกร่วง.

This is an important population. Probably same as to 96120202. Close to a waterfall.
This may be more than one species and needs careful checking. Part of the population was in quite heavy shade

Appendix Figure 1A. The example of passport data sheet.

BIOGRAPHY

Worapa Seehalak was born on September 28, 1978 in Uttaradit province. She received Bachelor degree of science with first class honor in 1999 from Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University. In 2001, she was granted the scholarship from the Thailand Research Fund under the Royal Golden Jubilee Program to pursue a Ph. D. degree in the School of Crop Production Technology, Suranaree University of Technology under the supervision of Prof. Dr. Aree Waranyuwat. At the RGJ-Ph. D. Congress VI in 2005, she received Certificate for outstanding research (poster) presentation. Her publications are:

1. Seehalak, W., Chaitieng, B., Vaughan, D. A. and Waranyuwat, A. (2004). Exploration and collection of wild *Vigna* species in subgenus *Ceratotropis* from Chiang Mai and Mae Hong Son provinces. In **Proc. Ninth Mungbean Workshop** (pp. 21-29). Lampang: Lampang Wiang Thong Hotel. (in Thai).
2. Seehalak, W., Tomooka, N., Waranyuwat, A., Thipyapong, P., Laosuwan, P., Kaga, A. and Vaughan, D. A. (2006). Genetic diversity of the *Vigna* germplasm from Thailand and neighboring regions revealed by AFLP analysis. **Genet. Resour. Crop Evol.** (in press).
3. Seehalak, W., Tomooka, N., Waranyuwat, A., Thipyapong, P., Laosuwan, P., Kaga, A., Chaitieng, B. and Vaughan, D. A. SSR analysis of sympatric *Vigna* species in northern Thailand. **Genet. Res. Crop. Evol.** (in preparation).