Report on

A study of genetic relationships among populations of two allied species in the genus *Afgekia* Craib (*Leguminosae*) in Thailand

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PREFACE

The final report of research project, *BRT 140006* consists of two main parts: 1) the four draft manuscripts submitted to various international journal of science for publication, and 2) general introduction and general conclusion of this work.

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บทคัดย่อ

ถั่วแปบช้าง (*Afgekia sericea* Craib) และ กันภัยมหิดล (*A. mahidolae* Burtt and Chermsiravathana) จากข้อมูลปัจจุบันพบเฉพาะในไทยเท่านั้น และนับเป็นพืชหายากของไทย ถั่วแปบช้างมีการกระจายพันธุ์ทางภาคตะวันออกเฉียงเหนือ ส่วนกันภัยมหิดลมีถิ่นอาศัยอยู่ที่เขาหินปูนของจังหวัดกาญจนบุรีของภาคตะวันตก ถิ่นอาศัยของพืชทั้งสองชนิดนี้แยกจากกันโดยชัดเจน โดยมีที่ราบลุ่มแม่น้ำเจ้าพระยาเป็นแนวขวางกั้น การศึกษาทางพันธุศาสตร์ของพืชทั้งสองชนิดนี้มีค่อนข้างน้อย ดังนั้นงานวิจัยนี้มีวัตถุประสงค์หลักอยู่สองประการ คือ ศึกษาความสัมพันธ์ทางพันธุกรรมโดยการวิเคราะห์โครโมโซมร่วมกับการใช้เทคนิค RAPD-PCR และศึกษาโครงสร้างทางพันธุกรรมในระดับประชากรของถั่วแปบช้างและกันภัยมหิดล โดยการประยุกต์ใช้เทคนิค RAPD-PCR

ข้อมูลจากการศึกษาโครโมโซมจาก 13 ประชากรของพืชทั้งสองชนิด พบว่าโครโมโซมของพืชทั้งสองชนิดมีขนาดเล็กและมีจำนวนโครโมโซมเท่ากันทุกประชากร (2N=16) จากการวิเคราะห์คาริโอไทป์ของพืชทั้งสองชนิดนี้ พบว่าโครโมโซมทั้ง 16 แท่ง แบ่งเป็น 2 แบบตามตำแหน่งของเซนโทรเมียร์คือเมทาเซนทริกและซับเมทาเซนทริก พืชทั้งสองชนิดมีคาริโอไทป์แบบสมมาตรแต่แตกต่างกันที่จำนวนคู่ของโครโมโซมทั้งสองแบบ และจากข้อมูลของ RAPD markers พบว่าพืชทั้งสองชนิดมีค่าความคล้ายคลึงทางพันธุกรรมในระดับสูง (S = 0.452) โครงสร้างทางพันธุกรรมของประชากรที่วิเคราะห์โดย Shannon's Information Index พบว่าค่าความหลากหลายทางพันธุกรรม (Hsp) ในถั่วแปบช้าง (Hsp=0.432) สูงกว่า กันภัยมหิดล (Hsp = 0.368) กันภัยมหิดลมีค่าความหลากหลายทางพันธุกรรมภายในประชากร (Hsp/Hpop = 0.61) สูงกว่าถั่วแปบช้าง (Hsp/Hpop = 0.52)

ประชากรของพืชทั้งสองชนิดนี้มีระดับความแตกต่างทางพันธุกรรมระหว่างประชากร (Nei's Gst) สูงมาก โดยถั่วแปบช้างและกันภัยมหิดลมีค่าความแตกต่างดังกล่าวเท่ากับ 0.426 และ 0.266 ตามลำดับ ซึ่งน่าจะเป็นผลจากการถ่ายทอดยีนระหว่างประชากร (Nm) ของพืชแต่ละชนิดน้อยมาก (Nm = 1.14, ถั่วแปบช้าง, Nm = 0.035, กันภัยมหิดล)

ผลจากการศึกษานี้แสดงให้เห็นว่า 1) ถั่วแปบช้างและกันภัยมหิดล มีความใกล้ชิดกันทางพันธุกรรม ดังนั้นพืชทั้งสองชนิดนี้น่าจะเกิดจากบรรพบุรุษร่วมกันโดยมีวิวัฒนาการเปลี่ยนแปลงเป็นสปีชีส์ใหม่ในถิ่นอาศัยที่แยกกัน และ 2) พืชทั้งสองชนิดมีความหลากหลายทางพันธุกรรมในระดับประชากรค่อนข้างสูง ดังนั้นในทางทฤษฎีแล้วปัจจัยทางพันธุกรรมมีอิทธิพลต่อการเปลี่ยนแปลงทางพันธุกรรมในประชากรเหล่านี้น้อยมาก แต่อย่างไรก็ตามปัจจัยอื่น ๆ อาจก่อให้เกิดการสูญพันธุ์ในพืชทั้งสองชนิดนี้ได้ ดังนั้นการที่จะทำให้พืชหายากของไทยทั้งสองชนิดนี้ไม่สูญพันธุ์ในเวลาอันใกล้อันเนื่องจากปัจจัยต่าง ๆ สามารถทำได้โดยการอนุรักษ์ถิ่นที่อยู่อาศัยของพืชทั้งสองชนิด

ABSTRACT

Afgekia sericea and *A. mahidolae* have been recorded only in Thailand. *A. sericea* is widely distributed throughout the northeastern region. In contrast, *A. mahidolae* is confined to open lime stone habitat, and is sporadically distributed in small populations in western region. The geographical range of these two species does not overlap, approximately 200 miles apart. These localities are separated by the whole width of the plains of Chao Phraya river. To date, there have been very few genetic analyses of these two species.

The objectives of the present study were to: 1) determine genetic relationships between these two species by means of chromosome studies and RAPD analysis; and 2) measure the genetic diversity and study the population structure of these two species using RAPD analysis.

Cytological investigation on *A. sericea* and *A. mahidolae* were carried out in 13 populations. All populations of these two species exhibited chromosome number of 2n = 16. Karyotypic analysis of both species showed a preponderance of median and submedian centromere, and these two species had symmetrical karyotypes. Variation in karyotype between *A. sericea* and *A. mahidolae* was observed in pairs of metacentric and submetacentric chromosomes. In addition, based on the presence vs. absence of RAPD markers illustrated that the two species are closely related species.

The genetic structure of *A. sericea* and *A. mahidolae* populations measured by Shannon's information index. *A sericea* exhibited genetic diversity for the species (Hsp) higher (Hsp = 0.4320 that those of *A. mahidolae* (Hsp = 0.368). *A. mahidolae* has genetic variability within populations (Hpop/Hsp) higher (Hpop/Hsp = 0.586) than those of *A. sericea* (Hpop/Hsp = 0.518). Furthermore, genetic divergence among populations of *A. sericea* and *A. mahidolae* populations, estimated by Nei' Gst value, were 0.426 and 0.266, respectively. These are consistent with low gene flow (Nm) occurred on among populations of each species.

To sum up, these results demonstrated that 1) *A. sericea* and *A. mahidolae* are closely related species, and the two species may arose as a result of allopatric speciation in different geographic population of the ancestral species; and 2) In terms

of genetic stochasticity, these two species have a relatively high genetic diversity in their populations. Thus these two species have significantly survival potential to present and future environmental changes. However, long-term survival of both species is dependent on their habitat conservation.

GENERAL INTRODUCTION

Biology of *Afgekia sericea* and *A. mahidolae*

The genus *Afgekia*, subfamily Papilionoideae, tribe Tephrosieae, was established by W.G. Craib in 1927 when he found *A. sericea* Craib at Nakhon Ratchasima province, northeastern Thailand. Further, in 1971 the second species, *A. mahidolae* are found at Kanchanaburi province (Burtt and Chermsirivathana, 1971). *Afgekia filipes* was described by Geesink (1984) who proposed to transfer *Adinobotrys filipes* Dunn to *Afgekia filipes*(Dunn)Geesink. Thus there are three related species recognized in the genus *Afgekia* i.e. *A. sericea, A. mahidolae* and *A. filipes* . The first two species are morphologically similar as shown in Fig. 1.1,while the third species is quite remote from them. The floral parts of a flower of *A. sericea* and *A. mahidolae* are slightly different. These two species are wild plant, woody climber with showy inflorescence. However, local people in Mahasarakham province have used *A. sericea* as a medicinal plant (Chualkul et al., 1994). The two species exhibit common circumstance on flowering phenology (Prathepha, 1990). Annually, both species flower during May to October. The entire flowering sequence, including seed maturity, is completed in the early dry season in Thailand. In early dry season, after seed production all parts of plant become drooping and die. However, in the early next wet season the new plant can develop from the alive parts of parents which are above and/or underground. Further, seed of the two species are produced a little number, even though a panicle or an inflorescence has quite long panicle length and containing with 200-300 flowers. These species commonly occur in Southern China,

Myanmar and Thailand. In Thailand, *A. sericea* is widely distributed throughout the northeastern region. In contrast, *A. mahidolae* is confined to open limestone habitat sporadically distributed in small populations in western part of Thailand. Thus these two species are truly allopatric which may represent a good candidate for studying species differentiation in the evolutionary process of this genus.

Recent studies on genetic variation of rare species have received increasing attention in recent year as have the evolutionary consequences of habitat fragmentation of plants (Baskauf, 1994).

Based on small population size of its geographical range, although the distribution is wide and occupy non-specific habitat, *A. sericea* has been recognized as a rare species. Whereas, *A. mahidolae* is also recognized as a rare species because of its small population size and the geographical distribution of this species is restricted in lime-stone hill.

Method in assessing genetic variability

To date, little is known about their genetics. The only published major study of chromosome number and meiotic behavior of *Afgekia* species first made by Prathepha (1994). The genetic relationship between *A. sericea* and *A. mahidolae* and genetic structure of these two species are undisputed. Thus, more studies in terms of genetic should be made for getting a possible explanation for genetic divergence and/or genetic relatedness of these two species. These answers can be garnered from

several kinds of data including morphology, cytogenetics and molecular markers. However, *Afgekia* species are not ideal material for cytogenetic analysis. The chromosomes are small, and distinctive cytogenetic feature are difficult to observe. The complete array of techniques used for analysis of DNA can be applied to the assessment of genetic variation in plants. These techniques may be derived into those that involve molecular marker methods and those based upon the comparison of gene sequences at specific loci. The type of molecular method used to measure genetic distance in plants will vary depending upon the magnitude of the genetic differences being assessed. A generalized comparison is given in Table 1.1. It appears that a combination of these methodologies will give a more precise image of genetic variation at a better efficiency. However, choice of different DNA markers are usually justified by the purpose of the study, available resources and even the research worker's personal preference. Technique such as RAPD analysis may be useful for distinguishing different genotypes within a plant species while sequence analysis of the ribosomal genes may allow species or higher level analysis.

Species and even populations or individuals within species often differ in diploid number and chromosome morphology and the value of chromosome data is that they may be used to distinguish evolutionary significant units (Wayne et al., 1992). Generally, the analysis of genetic variation or diversity in plants has been traditionally assessed by analysis of morphological traits or cytological analysis. For example, Fukuda (1989) demonstrated that *Trillium* species showed the variability in chromosome number and chromosome banding pattern within and among populations from North America and Asia. The assessment of phenotype may not be a reliable measure of genetic difference because of the influence of environment or gene

expression. The analysis of plant DNA allows the direct assessment of variation in genotype.

The molecular techniques of DNA sequencing and molecular marker analysis are now relatively routine techniques. The analysis of these data and its interpretation in the measurement of genetic relationships between different plants is more complex. The reliability of the conclusions of these studies is often dependent upon the rigor of the data analysis and interpretation. Phylogenetic studies and analysis of population genetics both require careful data interpretation. All methods require certain basic assumptions and have strengths and weakness (West and Faith, 1990). Mathematical method have been developed to allow correction for the errors in estimation of genetic distance associated with the scoring of complex gels such as those generated in RAPD analysis and with the difficulty of reproducing the DNA extraction and amplification (Lamboy, 1994).

Early approaches to genetic relationships and genetic variation in organisms has been measured most commonly with cytological and electrophoretic data that surveys of protein variation. Recently, DNA-based technique have become accessible, e.g. RFLP, SSR, DNA sequencing. These molecular techniques are increasingly being used to understand evolutionary process. DNA fingerprinting can provide information about relatedness among individuals in population. In addition molecular markers have provided information about levels of movement of individuals among populations and the distinctness of populations as evolutionary units.

Molecular biology is a rapidly expanding field that has become increasingly important in studies of population dynamics and population structure. The use of molecular method to study genetic differentiation within and among populations and explores how this knowledge can be applied in developing conservation and habitat management plans. Isozyme electrophoresis has been used to assess pattern of genetic variation in many species and is still the most widely used tool of population geneticists. However, isozyme studies have been criticized because they can only detect variation in gene encoding soluble enzymes. In contrast, the study of DNA variation permits examination of the entire genome.

To sum up, analysis of mitotic chromosomes of the *Afgekia* species may result in not only information on chromosome maker or their cytogenetic relationship of the two species but may find chromosome differentiation among populations. significantly, the use of small amounts of material and random primers make RAPD analysis an attractive tool for the study of genetic diversity in plant populations. The technique provides informative and reliable banding patterns that can be used to estimate the variability present in *Afgekia* species or in single populations. It is then possible to determine the distribution of variability in the plant analysed. Understanding the homogeneity/potential fragmentation of the gene pool is important in conservation and also provide essential information on population genetics as a prerequisite to planning recovery strategies for this genus. Consequently, the results from the experiments will help determine the current status of population dynamics, population structures, since genetic diversity improves survival potential by enhancing the adaptation of *Afgekia* species to present and future environmental

changes, the preservation of biodiversity and genetic diversity is a fundamental aim of conservation.

The objectives of this research

This study involves determining genetic relationships between *A. sericea* Criab and *A. mahidolae* Burtt and Chermsirivathana and also to evaluate population genetic structure of natural populations of these two species.

Research activities

1) Estimation genetic relationships of these two species through mitotic chromosome analysis and RAPD-PCR markers.
2) Determining genetic structure of natural populations of *A. sericea* and *A. mahidolae*

Table 1.1 Molecular techniques suitable for different levels of genetic distribution[a], modified from Henry (1997).

	Population genetics ⟶ Phylogenetic analysis					
	Individuals	Variety	Species	Genus	Family	Higher levels
SSR	**	***	*			
RAPD	**	***	**	*		
RFLP						
Nuclear genes	**	***	**	*		
Mitochondrial genes		*	**	**		
Chloroplast genes		**	***	**		
Sequencing						
ITS		**	***	**	*	
Ribosomal genes (18s)			*	**	***	***
Chloroplast genes				*	**	***

[a] Numbers of asterisks indicate increasing use of the techniques.

Figure 1. *Afgekia sericea* Craib, A; *A. mahidolae* Burtt and Chermsirivathana, B.

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Cytological and Morphological Characteristics of Two Closely Related Species of the Genus *Afgekia* Craib (Leguminosae) from Thailand.

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ABSTRACT

Analysis of mitotic karyotypes of samples collected from 13 natural populations of *Afgekia sericea* and *A. mahidolae* has revealed that each species exhibits a chromosome number of 2n=16. The chromosome complement of *A. sericea* consists of eight metacentric and eight submetacentric chromosomes, whereas that of *A. mahidolae* consists of 12 metacentric and four submetacentric chromosomes. Only slight differences in metaphase chromosomes morphology between the two species have been observed in this study, although they are geographically isolated. These data suggest that *A. sericea* and *A. mahidolae* arose as a result of allopatric speciation through the processes of allelic changes in different geographical populations of the ancestral species. This suggestion is supported by the geological history of Thailand.

Key words: mitotic karyotype, *Afgekia sericea* and *A. mahidolae*, speciation

INTRODUCTION

The genus *Afgekia*, subfamily Papilionoideae, tribe Tephrosieae, was established by W.G. Craib in 1927 when he first discovered *A. sericea* Craib at Nakhon Ratchasima province, northeastern Thailand. In 1971 the second species, *A. mahidolae* Burtt & Chermsirivathana, was found at Kanchanaburi province[1]. The name of this new species was dedicated, with permission, to Her Royal Highness, the King's Mother. Furthermore, *Afgekia filipes* was described by Geesink[2] who proposed to transfer *Adinobotrys filipes* Dunn to *Afgekia filipes* (Dunn) Geesink. Thus there are three related species recognized in the genus *Afgekia,* i.e., *A. sericea, A. mahidolae* and *A. filipes*. The first two species are morphologically similar while the third species is quite remote from them. Floristic characteristics of this genus have been described by Niyomdham[3]. The flowers occur in terminal paniculate inflorescences. The bract and bracteoles are large. The pods are dehiscent and woody with sculptured valves. The floral parts of *A. sericea* and *A. mahidolae* are slightly different[4] . *A. mahidolae* differs from *A. sericea* in having hair at the top of the style. These two species are wild, woody climbers with showy inflorescences. However, local people in Mahasarakham province have used *A. sericea* as a medicinal plant[5]. These species commonly occur in Southern China, Myanmar and Thailand. In Thailand, *A. sericea* is widely distributed throughout the northeastern region. In contrast, *A. mahidolae* is confined to open limestone habitat, distributed sporadically in small populations in western Thailand. Thus these two species are apparently allopatric and may therefore represent good candidates for studying the evolutionary process of species differentiation in this genus

This paper presents mitotic karyotypes and some morphological characteristics of *A. sericea* and *A. mahidolae* in the Thai populations.

MATERIALS AND METHODS

Seed specimens of *A. sericea* were collected from wild populations in Mahasarakham (MSK1:2/2/97, MSK2: 10/12/97, MSK3: 11/12/97, Chaiyaphum (CHP: 20/3/97, CHP2: 20/12/97), Nakhon Ratchasima (NSM1: 18/3/97, NSM2: 19/3/97, NSM3: 20/1/98), and Buriram (BRM1:23/12/97) provinces, northeastern Thailand. Seed samples of *A. mahidolae* were obtained from four localities in Kanchanaburi province, (KCB1: 8/2/97, KCB2: 8/2/97, KCB3: 9/2/97, KCB4: 10/2/97 western Thailand (Fig. 1). Seeds of those plants were cultivated in pots in the greenhouse at Mahasarakham University. Root tip cells were used for chromosome preparations using a technique described by Darlington and Cours[6], Chaiyasut[7] and Prathepha[8]. Fresh material was pretreated with α-bromonaphthalene for 28 hours at 4°C before it was fixed in 90% acetic acid for 30 min at room temperature. Then it was transferred to 70% ethyl alcohol and stored in a refrigerator at 4°C. To obtain good mitotic chromosomes, the root tips were hydrolyzed with 1N HCl for 12 min at 60°C before they were squashed in the standard Feulgen staining solution. Photomicrographs were recorded on Tmax 100 (TMX 135-36) Kodak film using an Olympus OBH-02 microscope. Chromosome measurements were made from five enlarged photomicrographs (x1000) of well-spread metaphase plates for each species. Metaphase chromosomes were selected and classified into different types according to centromere position as revealed by centromeric index value (CI): CI=long arm

length/ total length. The chromosome type based on CI value follows those described by Turpin and Lejeune[9] and Chaiyasut[7]. Chromosome size was also estimated and categorized into large (L), medium (M), and small (S) using a modified method of Vij et al.[10].

RESULTS AND DISCUSSION

Chromosome counts of the two species from samples collected from 13 populations have revealed mitotic karyotypes of 2n=16. This agrees with the count previously reported. All 16 metaphase chromosomes of *A. sericea* and *A. mahidolae* are small, ranging from 3.8-6.3 μm and 3.8-7.5 μm, resectively (Table 1, Fig. 2). Some small chromosomes of *A. sericea* lack a visible centromere. *Afgekia sericea* exibits four pairs of metacentric and four pairs of submetacentric chromosomes. The metacentric chromosomes include three large pairs and one medium pair while those of the submetacentric shape consist of one large, one small and two medium pairs (Fig. 2A and C). Thus the symmetrical karyotype formula of *A. sericea* is recognized as follows: $L_6^{m} + L_2^{sm} + M_2^{m} + M_4^{sm} + S_2^{sm}$.

Chromosome complement of *A. mahidolae* comprises 12 metacentric chromosomes (1 large and 5 medium pairs) and four submetacentric chromosomes (one large and one medium pairs) (Fig. 2B and D). Thus the symmetrical karyotype of *A. mahidolae* is formulated as follows: $L_2^{m} + L_2^{sm} + M_{10}^{m} + M_2^{sm}$ (Table 1).

Both species of the genus *Afgekia* exhibit typical chromosomal characteristics of woody plants in having small chromosomes[11]. This presents a technical problem in

comparative cytotaxonomy of the genus under investigation. Further, no chromosome marker could be observed in this study. However, chromosome number and morphology at metaphase-I are important to the knowledge of the origin and nature of speciation in the taxa[12]. Data from a previous study[8] reveal seven ring bivalents and one rod bivalent at metaphase-I which correspond with chromosome morphology of both species examined in this study. Based on Lewis' mode[13] of speciation, we hypothesized that *A. sericea* and *A. mahidolae* became differentiated as a result of accumulation of structural rearrangements of chromosomes in allopatric populations of the ancestral species without changing chromosome number, although minor differences in chromosome morphology between these two species were observed in this study.

Morphologically, the flowers of *A. sericea* are pink . The standard is pale yellow with pink patches on both sides at the base and a yellow patch in the middle. The wing petals are pink and have two basal appendages on the upper margin. In *A. mahidolae*, the flowers are purple. The standard is purple with a central patch of yellow (Table 2). The wing petals are purple and they clearly differ from *A. sericea* by having one basal appendage. However, the keel of both species are white but show different shape. The two species exibit common circumstance on flowering phenology[4]. Annually, both species flower from May to October and seed maturity is completed in the early dry season. In the early dry season, after seed production, all parts of the plant wilt and die. Nevertheless, early in the wet season, the new plant can develop from the woody climbers and the live parts which are underground of parental plants. Further, seeds of the two species are produced in small numbers, even though an inflorescence has a long panicle containing about 200-300 flowers.

However, the two closely related species might have become adapted to different habitats as suggested by the geological record.

The past geological events in Thailand[14] showed that in the early Cenozoic era, the large plain of the central region of Thailand was structurally formed by crustal movements. Due to block faulting as evidenced by several fault lines, a large tectonic basin was created, covering the area of the central region and the Gulf of Thailand. It is likely that the populations of *Afgekia* ancestral species became isolated and speciation occurred in the last few million years of the Cenozoic era. It is evident that during the Pleistocene epoch there were a series of glaciations that effected the climates worldwide including Thailand. Thus, *A. sericea* exhibits a wide range of distribution extending from the dividing line to several areas of the northeastern region, while *A. mahidolae* have been found only in some limestone mountains of the western region. In this context, geological isolation and environmental changes effectively promoted species differentiation of the isolated populations. Hence, we suggest that *A. sericea* and *A. mahidolae* arose as a results of allopatric speciation through some forms of genetic and morphological differentiation that took place in isolated populations of the ancestral species. Further detailed study on molecular systematics could shed some light on the time of separation of these two species of the genus *Afgekia* in Thialnd.

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Table 1. Mitotic karyotype features of *Afgekia sericea* and *A. mahidolae*.

Species	CRS(μm)	TL(μm)	Chr. no.	CI	Type	Size
A. sericea	3.8-6.3	79.5	1	0.62	sm	L
			2	0.58	m	L
			3	0.52	m	L
			4	0.50	m	L
			5	0.64	sm	M
			6	0.62	sm	M
			7	0.50	m	M
			8	0.67	sm	S
A. mahidolae	3.8-7.5	86.5	1	0.51	m	L
			2	0.68	sm	L
			3	0.52	m	M
			4	0.68	sm	M
			5	0.52	m	M
			6	0.58	m	M
			7	0.53	m	M
			8	0.52	m	M

CRS(μm)= range of chromosome size; TL(μm)=Total length of the haploid set;
CI= centromeric index; m= metacentric; sm= submetacentric: L= large;
M= medium; S= small.

Table 2. Comparisons of the morphological characteristics of *A. sericea* and *A. mahidolae*.

Species	Vegetative Characteristics	Floral Characteristics	Pollen morphology*
A. sericea	Habit: woody climber, Leaves: opposite, odd-pinnate, lancolate,cover with short soft hair.	Papilionoid flower, pink;Wing: 2 wing appendages; Style: absence of hair at the top of the style.	Shape:oblate spheroidal; Exine: microreticulate; Aperture type: tricolporate.
A. mahidolae	Habit: woody climber, Leaves: opposite, odd-pinnate, oval-elliptic,cover with short soft hair.	Papilionoid flower, purple;Wing: 1 wing appendage; Style: presence hair at the top of the style.	Shape:prolate spheroidal; Exine:reticulate; Aperture type: tricolporate.

* from Prathepha (1990)

Figure 1. Map of Thailand showing locations of five provinces where the 13 natural populations of *A. sericea* and *A.mahidolae* were sampled and used in this study. Large solid circle indicates the central plain, separating the northeastern and the western regions.

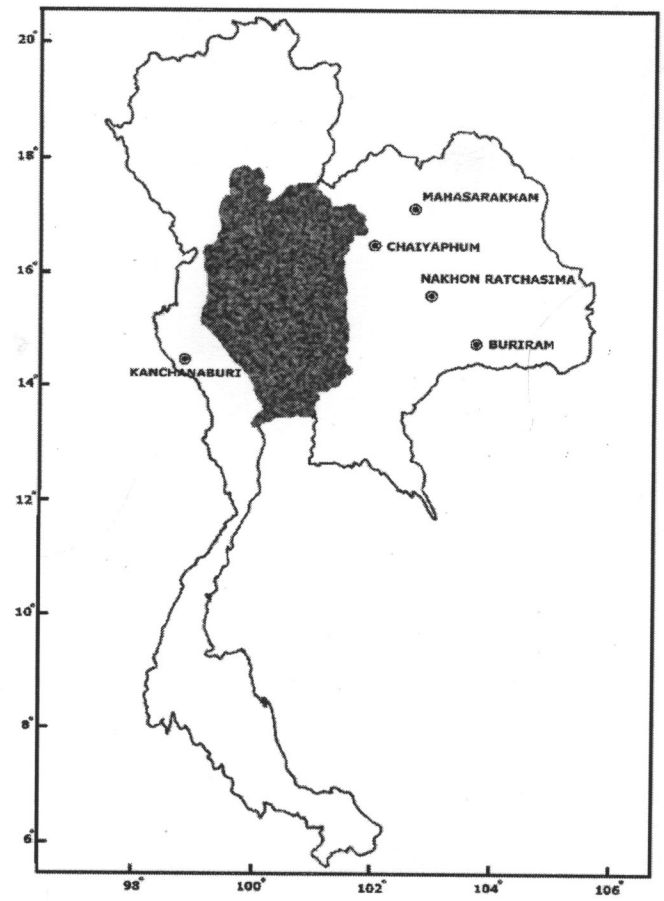
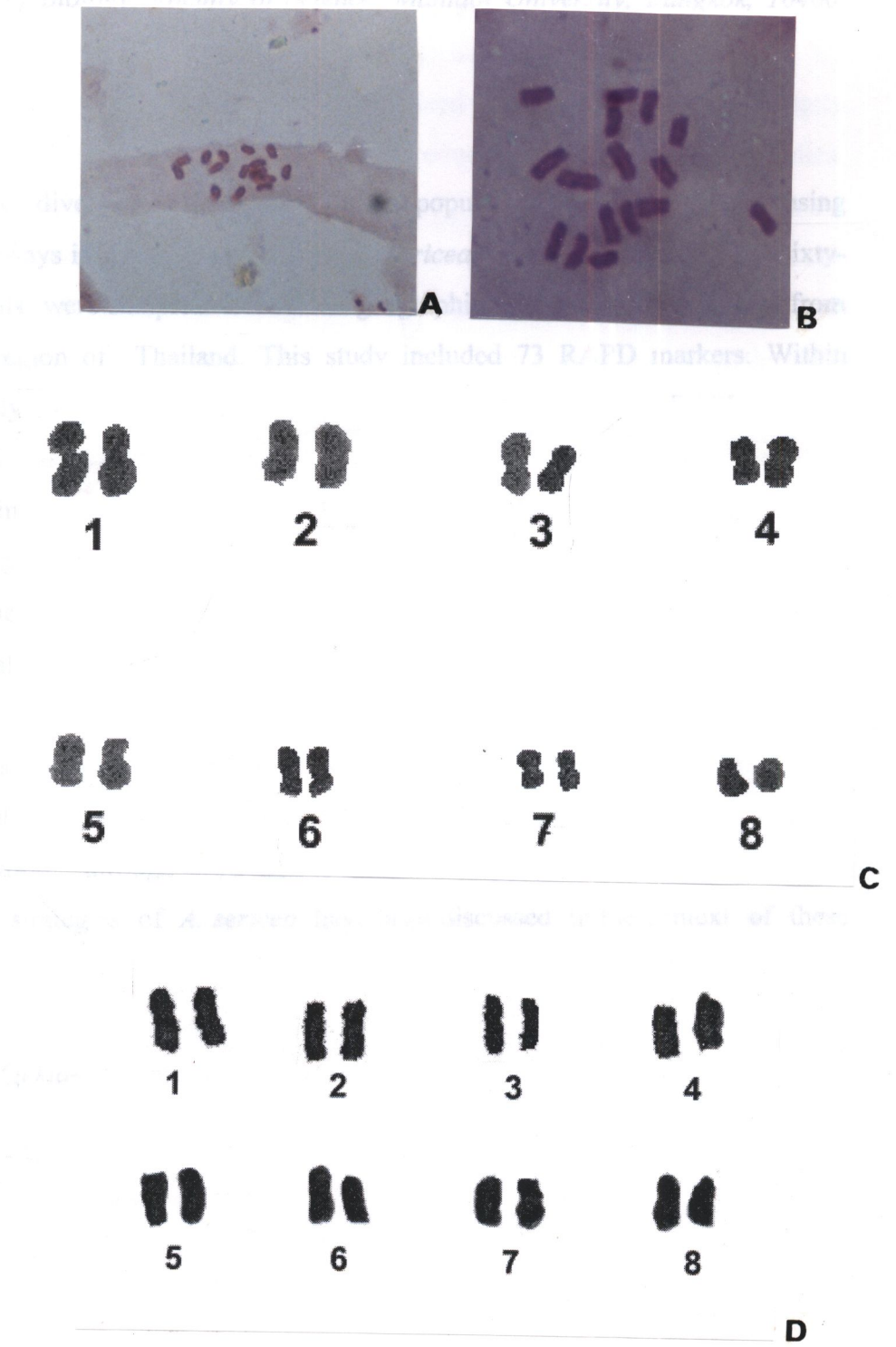


Figure 2. Mitotic chromosomes (2n=16) of (A) *A. sericea* and (B) *A. mahidolae*; C and D represent karyograms of these species, respectively.



Genetic differentiation in Thai populations of the rare species *Afgekia sericea* Craib (Leguminosae) revealed by RAPD-PCR assays

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Abstract

Genetic diversity within and among populations was investigated using RAPD-PCR assays in a rare species, *Afgekia sericea* Craib. Two hundred and sixty-nine individuals were sampled from nine geographically isolated populations from northeastern region of Thailand. This study included 73 RAPD markers. Within population polymorphism as measured by percentage of polymorphic RAPDs, varied between 38.4 and 60.3%. Genetic variability was measured using Shannon's information index and partitioned into between-and within-population components. Overall, the variation occurs among *A. sericea* populations was high (H_{sp}- H_{pop} / H_{sp} = 48.2%). The genetic diversity for the species (H_{sp}) was 0.432. Mean of within-population values (mean H_{pop}) for all populations was 0.224. The total genetic diversity was explained by high variation among populations (mean G_{st} = 0.426), which is consistent with low gene flow among populations (Nm = 0.35). High between-population genetic variation observed in this study could be explained by limited migration through seed and/or pollen dispersal among populations. Conservation strategies of *A. sericea* have been discussed in the context of these results.

Key words: *Afgekia*- Population structure- RAPD-PCR

Introduction

Afgekia sericea Craib is an endemic species to Thailand. Its geographic distribution is restricted to the northeastern region. Prathepha & Baimai (1998) reported that it has been found in four provinces of the northeastern region including Mahasarakham, Buriram, Chaiyaphum and Nakhon Ratchasima Provinces. *A. sericea* is a wild annual plant and woody climber with showy inflorescences. Local people of this region uesd this species as a medicinal plant (Chualkul et al., 1994).

A. sericea flowers from May to October. Seed maturity culminates in the early dry season (January or February). After seed production, the plant wilts and dies. Nevertheless, early in the wet season (May or June) , the new plant develops from the live portions of parental plants which remain underground (Prathepha, 1990). The seeds are few in number, even though each inflorescence has a long panicle, with 200-300 florets. Potential for seed dispersal, however, is relatively low, since the plant has large seeds, with an average mass of 1.4 g. From field observations, the plant's seed survival rate in its natural habitat is relatively low. Seed mortality is caused by a number of factors, including consumption of seeds endosperm by insects and fungi, as well as environmental factors. *A. sericea* is a diploid species with chromosome number of 2n=16 (Prathepha, 1994). It is the first plant of the genus *Afgekia* proposed by W.G. Craib (Burtt & Chermsirivathana, 1971). Natural populations of this plant are highly vulnerable to environmental changes such as those caused by human impact such as clearing for agriculture. Little is known about the genetics and the population structure of this species. Furthermore, the field of population genetic structure of rare species has recently received much attention, with increasing concern in the area of conservation biology (Bonnin et al., 1996). Moreover, good conservation policies must be based on a sound understanding of population genetics of the particular species(Rossetto, Weaver & Dixon, 1995). The preservation of biodiversity and genetic diversity is a fundamental aim of conservation, since genetic diversity ameliorates survival potential by enhancing the adaptation of a species to present and the future environmental changes. Consequently, the study of population genetics has been identified as one of the main priorities for conservation (Holsinger & Gottlieb, 1991).

Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique (Williams et al., 1990) has been used to characterize genetic diversity in populations of rare plants (e.g. Gustafsson & Gustafsson, 1994; Bonnin et al., 1996; Hsiao & Rieseberg, 1993). This assay is a relatively new technique that is sensitive, quick to perform, and can be applied to a large number of samples. This technique is a variant of the PCR in which a decamer oligonucleotide primer of arbitrary sequence is allowed to anneal at a relatively low temperature, priming the amplification of DNA fragments distributed at random in the genome. The random primers used in this technique can effectively scan a genome for inverted priming sites that are close enough to amplify the intervening sequences.

In this paper, we quantify within and between population genetic structure of *A. sericea* using RAPD assay. This information will be useful in evaluating the significance of different populaions as repositories of genetic diversity in *A. sericea.* Thus, the major objectives of this study are to: (1) quantify the amount and distribution of genetic diversity within and among subpopulations using genetic diversity measures, and (2) make conservation recommendations based on genetic data.

Materials and methods

Study areas

Seeds were collected from nine populations of *A. sericea* in four provinces of the northeastern region of Thailand: (i) Mahasarakham (MSK1, MSK2, MSK3); (ii) Chaiyaphum (CYP1, CYP2); (iii) Buriram (BRM); and (iv) Nakhon Ratchasima (NSM1, NSM2, NSM3) (Table 1). Their locations are shown in Figure 1. Samples were transported to and grown in a greenhouse of Mahasarakham University.

Extraction of DNA

Genomic DNA was extracted from fresh young leaves from 29-30 individuals per population, using the CTAB method following the method of Doyle & Doyle (1987), with minor modifications. Fresh young leaves (2 g) were ground using a mortar and

pestle . The grindate was added to 20 ml of extraction buffer (2% w/v) CTAB ; 100 mM Tris-Cl buffer (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, (1% w/v) PVP-40) and incubated at 60°C for 30 min. The homogenate was mixed with 24:1 chloroform:isoamyl alcohol (v/v) and mixed well by gentle inversion. Following centrifugation at 14,000 rpm for 5 min, the upper aqueous layer was tranferred to a fresh tube contaning 600 µl of cold isopropanol. The precipitaed DNA was with 400 µl of 75% ethanol with 10 mM ammonium acetate and was washed twice with 75% ethanol. The pellets were allowed to sit overnight at room temperature, and resuspended in TE buffer (10 mM Tris-Cl , pH 8.0, 1mM EDTA, pH 8.0) containing Rnase (10 mg/ml).

RAPD analysis

Ten-mer oligonucleotide primers used in this study (Table 2) were obtained from Operon Technologies. Amplification reactions were performed in 25 µl reaction volumes, with 16.9 µl steriled water (ddH_2O), 2.5 µl of 10X PCR reaction buffer (Promega), 1.9 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM dNTPs, 0.5 µl of 10 µM primer, 0.1 µl (0.04 units) of Taq DNA polymerase (Promega), and 1 µl of genomic DNA template (100 - 200 ng). Error was minimized by making one large (master mix) batch of all reagents for each primer except Taq DNA polymerase. PCR reactions were performed on a Hybaid Thermocycler, programmed for an initial melting step at 94°C for 4 min, followed by amplifications for 45 cycles, each consisting of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min. A final extension step at 72°C for 4 min was performed. A negative control reaction in which DNA was omitted was included with every run to verify the absence of contamination.

Fragment visualization

Amplified products were separated in 1% agarose gels (FMC BioProducts) in 0.5X TBE (Tris-Borate-EDTA) buffer. The gels were run for 2 hr at 75 V, stained with ethidium bromide (10 mg/ml), and photographed on a UV transluminator. To determine RAPD profiles, the size of each band was inferred by comparisons with a 1 kb ladder

(Promega) that used as the molecular weight marker (M) using a Gel Documentation System, GDS 8000 (UVP Inc., California, USA). Due to unclear genetic interpretation of RAPD bands, we chose analytical methods that minimized the number of genetic assumptions. Moreover, only the amplified products that consistenly appeared in two replications were scored as presence (1) / absence (0), for further analysis.

Data analysis

Bands observed by RAPD analysis were assigned a number in relation to their migration within the gel. Band with the highest molecular weight was assigned number 1 and so on in ascending order until the band of lowest molecular weight was assigned. It was assumed that bands of the same molecular weight in different individuals were identical. For each individual, the presence or absence of each band was determine and designated 1, present or 0, absent.

1. Measuring genetic diversity

The following three measures were used to quantify the amount of genetic variability within a population.

1.1 Genetic diversity was estimated by the Shannon-information index (Chakraborty & Rao, 1991) modified for RAPD analysis, which is defined as $H_0 = -\sum^{k}_{i=1} P_i \log_e P_i$. For each primer used, H_0 denotes the diversity of RAPD markers, in a population, k denotes the number of RAPD markers and P_i denotes the frequency of the i^{th} RAPD marker in a given population (or the proportional abundance of each banding pattern, or phenotype). For each population, H_0 was averaged over all the primers to determine the within-population diversity of RAPD markers. The average diversity of RAPD markers for all populations (H_{pop}) was calculated as the mean of H_0.

1.2 The percentage of polymorphic loci was the proportion of loci polymorphic within each population (Lin et al., 1997).

1.3 Genetic diversity (h) at each RAPD locus in each population was determined according to Nei's (1973) genetic diversity, which is defined as $h = 1 - \sum P_i^2$, where P_i is the frequency of the i^{th} allele (Peever & Milgroom, 1994). Genetic diversity within each population was calculated as the mean genetic diversity over all loci from all populations.

2. Genetic differentiation

Four methods were used to quantify the degree of differentiation among populations.

2.1 The genetic divergence among populations was estimated by calculating Nei's Gst value (Nei, 1987) as follows: $Gst = (Js - Jt) / (1 - Jt)$, in which $Js = \Sigma$ Avg (P_i^2) and $Jt = \Sigma [\text{Avg} (P_i)]^2$ and P_i is the frequency of the i^{th} allele.

2.2 Gene flow (*Nm*) represents the actual number of individuals exchanged among populations in each generation. We quantified gene flow using the indirect estimates of Crow & Aoki (1984) after Ellstrand & Elam (1993). In this model, the number of migrant individuals exchanged among demes each generation is $Nm = (1 / Gst - 1) / 4 [n / (n - 1)]^2$, in which n = total number of demes. Using the average value of *G*st over all loci.

2.3 Values for unbiased genetic identity (*I*), were estimated (Nei, 1978)

2.4 Genetic divergence among populations was also estimated using Nei's (1978) genetic distance for all pairs of populations and these values were used to construct dendrogram by the unweighted pair-group method with arithmetic averaging (UPGMA) using TREEVIEW, tree drawing software for Microsoft Windows (Page, 1996).

Most estimates in this study used the software POPGENE (Yeh & Boyle, 1997) to determine the population genetic structure of *A. sericea.*

Results and discussion

Although the RAPD-PCR techniques have been widely adopted, the method has received criticism. Based on issues of reproducibility of amplification profiles. Henry (1997) stated that the nature of the amplification process with short primers is such that many sites in the genome are potential templates and the profile obtained may be influenced by variation in the method used to prepare the DNA template , and the exact reaction composition and conditions used in PCR. This means that variation in the concentration of the primer or template can result in the amplification of different products (Muralidharam & Wakeland, 1993). Therefore, obtaining reliable results depends upon standardizing these conditions or identifying combinations of

conditions that give consistent results. Stardard primer, nucleotide, and magnesium concentrations, exact reproduction of temperature cycling conditions and DNA polymerase type and activity are essential (Henry, 1997). Moreover, standardization of DNA extraction and PCR steps may be necessary to achieve reproducible results (Yu & Pauls, 1994). Chalmers et al. (1992) state that RAPDs are a useful predictive tool to identify areas of maximum diversity and may be used to estimate levels of genetic variability in natural populations.

RAPD profile

A total of 73 different RAPD loci were generated by the 10 primers (Table 2). Variation in RAPD banding patterns among individuals of *A. sericea* has been detected (Figure 2a, b, c). The numbers of markers produced per primer ranged from 3 (OPK06) to 10 (OPB10). The number of polymorphic RAPD loci per population ranged from 28 (NSM 3) to 44 (CYP 2). Frequencies of alleles at 65 of 73 RAPD loci were significantly different among populations ($P < 0.001$). The gene diversity within each population (averaged over 73 loci) ranged from 0.112 in NSM3 to 0.184 in NSM1, and the mean gene diversity over all populations was 0.145 (Table 3).

Genetic differentiation among populations

Among *A. sericea* populations, genetic diversity varied considerably (Table 3 and 4). For instance, the percent polymorphic loci ranged from 38% (NSM3) to 60% (CYP 2). Shannon's information index of phenotypic diversity was used to partition the diversity into within- and between-population components (Table 5). *Hpop* provides a measure of the average diversity within each of the nine populations. OPB07 detect the most and OPB10 the least within-population variability. An examination of genetic diversity present within populations, (*Hpop* / *Hsp*), and between populations, (*Hsp* – *Hpop*) / *Hsp*, indicates that, on average, most of the diversity (51.8%) occurs within *A. sericea* populations. The distribution of variability between and within populations, however, varies between primers. For example, OPB10 detects most most variability between populations (81%), whereas primer OPB07 and OPK11 detect most variation within populations (70% and 69%, respectively). The genetic diversity within and among the nine populations revealed a high level of divergence (*Gst* value of 0.426)

(Table 6). This means that approximately 43 % of the genetic variation observed in this study was due to differentiation among populations of *A. sericea* compared with 57 % within them. Estimates of gene flow (*Nm*) (Table 6) ranged from 0.038 to 0.778. Over all populations, according to Crow & Aoki (1984), gene flow values (*Nm*) of less than 1 should be interpreted as little or no gene flow. Thus, the average of the values (*Nm* = 0.35) observed between populations in this study would indicate extremely rare migratory events.

Nei' s (1978), unbiased estimates of genetic identity (*I*) for all pairs of populations were high. Intra-specific *I* values for populations of *A. sericea* ranged from 0.6325 to 0.919 (Table 7). Phylogenetic trees constructed by the UPGMA method based on the Nei's unbiased genetic distance matrix are shown in Figure 3. From this phylogenetic tree it is clear that nine populations of *A. sericea* were divided into two major groups: one group of six populations (CYP1, NSM 2, NSM 3, MSK 1, MSK 2, and MSK 3), and a second group that consists of three populations (CYP2, NSM1 and BRM).

Genetic diversity

This is the first paper to report RAPD variation within and among natural populations of *A. sericea.* The population from CYP2 had the highest absolute level of polymorphism (44 out of 73 loci), while NSM3 population had the lowest (28 out of 73 loci). Chi-square tests for over all genetic structure were significant for 65 out of 73 loci. Over all *Gst* given by POPGENE was equal to 0.426, this value indicates strong differentiation among populations. Approximately, 48% of total genetic variability observed in this study occurred among populations. A much higher level of genetic differentiation was detected among *A. sericea* populations in this study, compared with most other plant species that have been studied (Huff et al., 1993; Russell et al., 1993, Huen et al., 1994; Link et al., 1995; Bonnin et al., 1996; Ashburner et al., 1997). We suggest that two factors may have caused the greater differentiation among populations: specialized habitat requirements and limited pollen and seed dispersal. First, two major habitat types, i.e., lower elevation populations (NSM 2, NSM 3, CYP 1, MSK1, MSK 2, and MSK 3) and higher elevation populations (CYP 2, NSM 1, and BRM) have been distinguished for *A. sericea* (Table 1). For example, the evidence support genetic variation between populations is that

RAPD markers amplified by OPK16, i.e., OPK16-479 bp locus was found in MSK1 and NSM1, but in CYP1, while OPK16-562 bp locus found in MSK1 and CYP1 and OPK16-691 bp locus was found in MSK1, but in CYP1. RAPD markers amplified by OPB20 exhibited OPB20-480 bp locus which were not found in higher altitude populations (NSM1, CYP1 and BRM). Another RAPD marker can be used as molecular marker between six lower elevation populations and three higher populations was the OPB11-379 bp locus and OPB11-480 bp locus. These RAPD markers were found in only the six lower altitude populations. Thus, we concluded that these RAPD markers may be closely associated with changes in a habitat gradient or/and elevation gradient. This conclusion is based on the assumption of ecological setting was the main restriction to gene flow that proposed by Hodes & Arnold (1994). The possible explanation for genetic divergence of populations is that seeds in the pod of *A. sericea* is the migrating unit. Because *A. sericea* has a low number of seeds per pod (1 - 3 seeds) and low number of produced pod (0 – 5 pods) per an inflorescence, and seeds are very large, we have suggested that seed migration is rare. Outcrossing via pollen between local individuals arising probably by immigration from nearby populations could not occur. Thus, high level of polymorphism was also observed within each population, varied between 0.1534 (NSM3) and 0.294 (NSM 1) (Table 3). There is evidence that low gene flow between populations plays a significant role in genetic differentiation among populations of *A. sericea*. Thus, in one sense the high level of genetic differentiation observed between populations of *A. sericea* is not surprising. The other evidence in support of this finding is that the populations sampled in this study were separated by many kilometers, this distance could be a factor accounting for this genetic distance. High G_{st} values of *A. sericea* populations found in this study may be caused by limited pollen and seed dispersal. Exactly, the mode of dispersal and mating system in *A. sericea* is not available for supporting this event. Although the two factors are associated with genetic structure within population of organisms.Thus, quantitative analyses of the mating system and mode of dispersal of this species should be made. According to Clegg (1980), methods of estimating mating system is dependent of the ability to detect heterozygotes. RAPDs are dominant so they cannot differentiate homozygotes and heterozygotes without a progeny test (McDonald, 1997, Lanham, 1996). Thus, in this context, RAPDs are limited. However, the genetic differentiation of this species

corresponds to the study of selfing plant species. Figures given in the literature for *Fst* values in nonhierachical analyses of population structure in selfing species are about 0.50 – 0.52 (Loveless & Hamrick, 1984); 0.51 in Hamrick (1989); 0.29 – 0.78 in Heywood (1991). These values are very close to the value *Gst* of 0.426 found in *A. sericea* populations by using POPGENE in this study.

Conservation considerations

Genetic diversity within populations is of great concern to conservation biologist because a paucity of genetic variation is thought to reduce the ability of populations to adapt to changing environments, thereby causing their extinction (Beardmore, 1983). Within population polymorphism among nine populations of *A. sericea*, as revealed by percentage of polymorphic RAPDs, varied between 38.4 % (NSM 3) and 60.3 % (CYP 2). Waugh & Powell (1992) stated that polymorphism as revealed by molecular markers could help to select priority areas for conservation and provide vital information for development of genetic sampling and improvement. Thus, location like NSM3 where genetic erosion (low percentage of polymorphic loci) is high should be given priority for *in situ* conservation. Furthermore, many conservation biologists have investigated genetic diversity in rare or endangered species (Maki et al., 1996). *Afgekia sericea* is particularly vulnerable to extinction. From nine populations surveyed in this study, only three (CYP2, NSM1, and BRM) are in areas with minimal human disturbance, and they are large populations relative to the six remainding populations. Of these three populations, NSM1(Skaerat Enviromental Research Station) is in protected area. Whereas the other two populations (CYP 2 and BRM) occur in mountainous areas where human impact is low. However, these two populations occur within a small geographic area, and remain vulnerable to local or natural catastrophes (e.g. drought, disease). Four populations (CYP1, MSK3, NSM2, and NSM3) occurred in roadside areas, and two populations (MSK1 and MSK2) are in cultural forests. All nine populations have low density. Moreover, the areal extent of the nine populations themselves is limited, approximately < 50 m^2 for six populations in the relatively flat areas and 100 – 500 m^2 for the three populations in mountainous areas. In addition, all nine populations were in poor condition, and no seedlings were observed during this study. According to the suggestions of Godt & Hamrick (1996), *Ex situ* efforts need to be undertaken to

preserve the widest range of genetic diversity within population by propagation of plant from seeds. Optimally, seeds from several individuals from each of the populations should be propagated in selected habitats. However, long-term survival of these species is dependent on habitat conservation and levels of genetic diversity within populations should be considered in determining conservation priorities. Since, low levels of genetic variation may constrain adaptation to changing environments, although plants may be phenotypically plastic and respond adequately without changes in gene frequencies (Foster & Sork, 1997). Thus, several efforts should be made to protect all sites, if possible, to prevent the loss of genetic diversity through genetic drift.

Acknowledements

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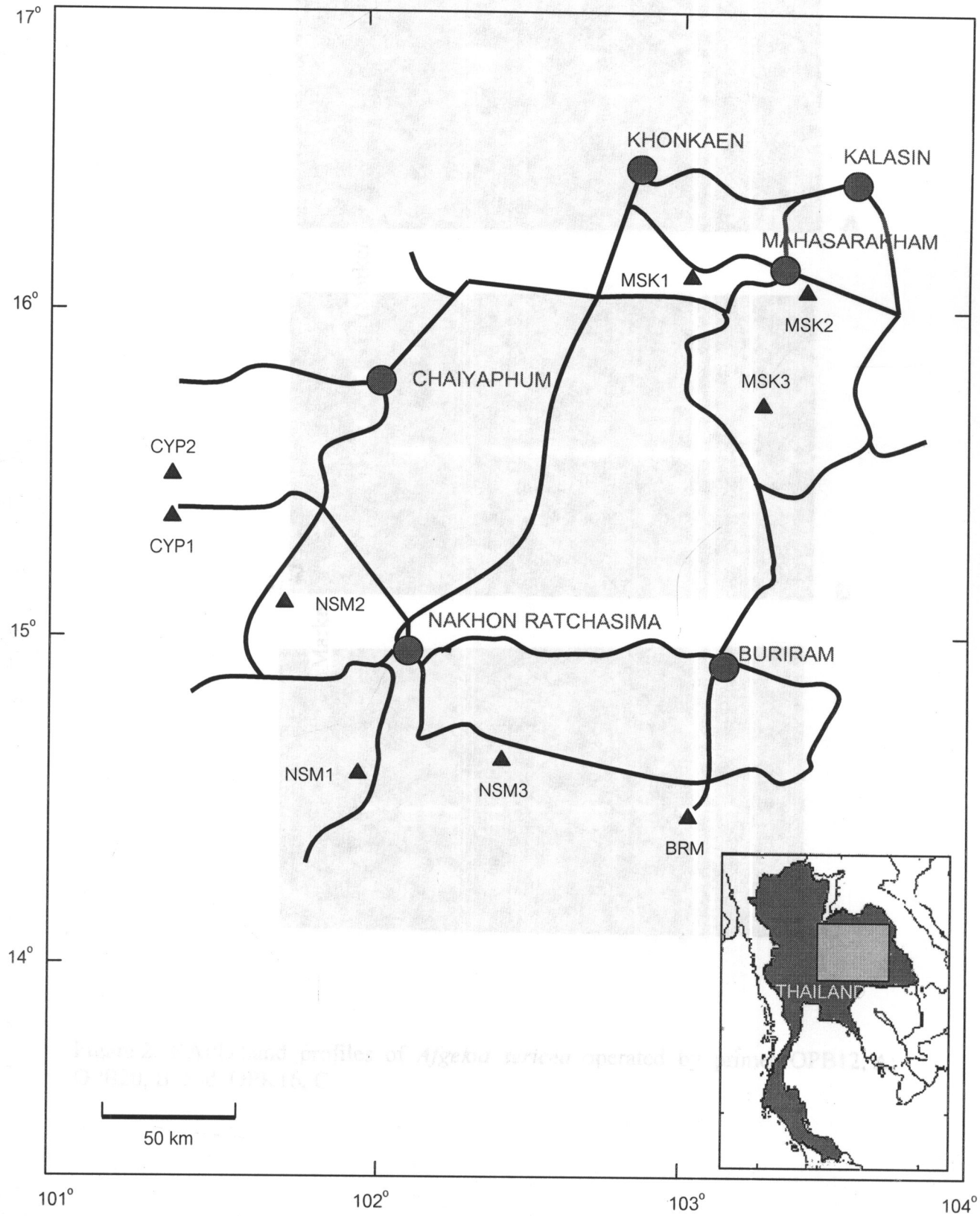

Figure 1. Map of Thailand showing four provinces where nine localities of *A. sericea* were collected in this study.

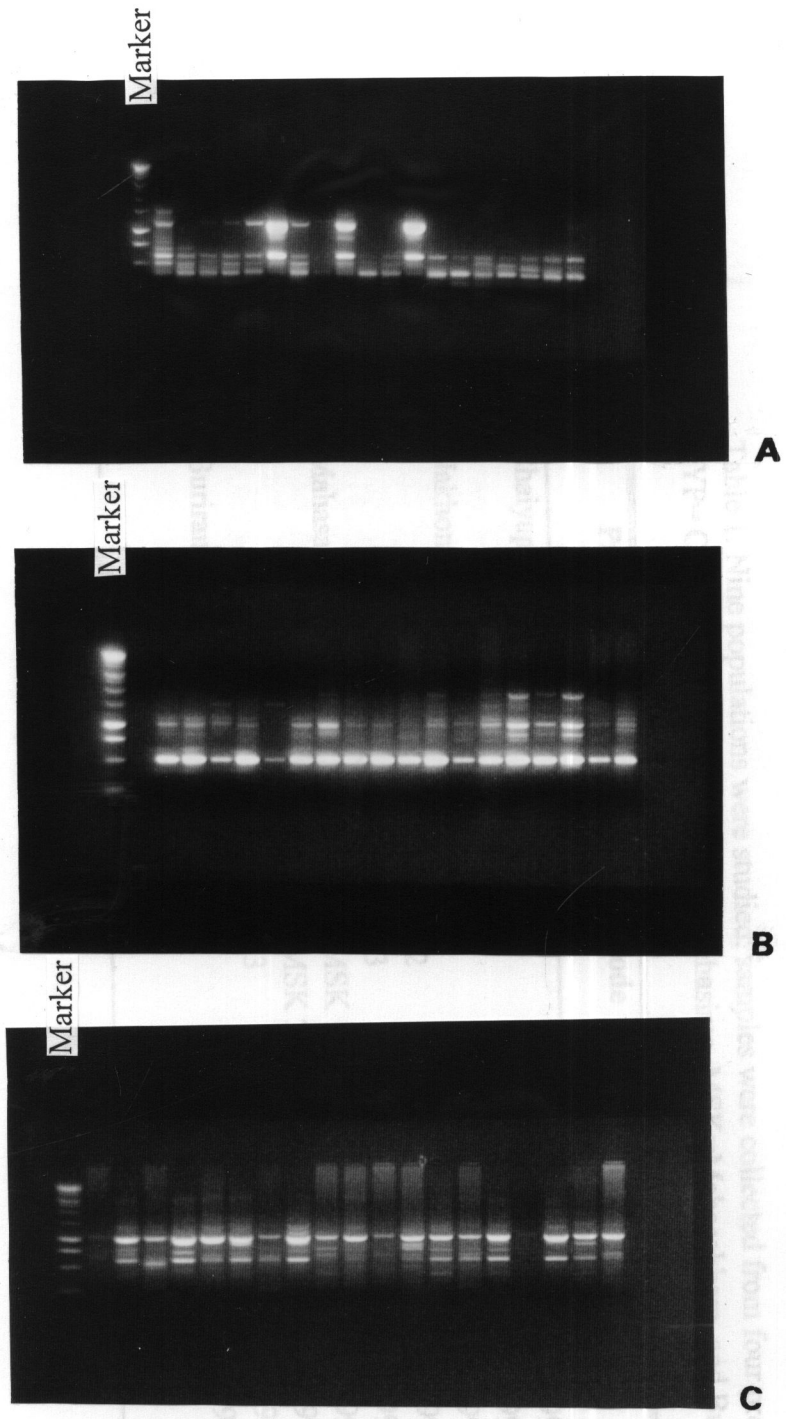

Figure 2. RAPD band profiles of *Afgekia sericea* operated by primer OPB12, A; OPB20, B and OPK16, C.

Table 1. Nine populations were studied, samples were collected from four provinces of northeastern Thailand. CYP= Chaiyaphum, NSM=Nakhon Ratchasima, MSK=Mahasakham, and Buriram=BRM.

Province	Habitat and code	Altitude (m)	Collection year
Chaiyaphum	Roadside; CYP 1	200	Mar., 1997
	Hill; CPY 2	600	Dec., 1997
Nakhon Ratchasima	Hill; NSM 1	682	Mar., 1997
	Roadside; NSM 2	200	Mar., 1997
	Roadside; NSM 3	200	Jan., 1998
Mahasarakham	Cultural forest; MSK 1	180	Feb., 1997
	Cultural forest; MSK 2	180	Dec., 1997
	Roadside; MSK 3	180	Dec., 1997
Buriram	Hill; BRM	700	Dec., 1997

Table 6.2. Random oligonucleotide primers used in this study.

Primer	Sequence
OPB05	5'TGCGCCCTTC3'
OPB07	5'GGTGACGCAG3'
OPB10	5'CTGCTGGGAC3'
OPB12	5'CCTTGACGCA3'
OPB20	5'GGACCCTTAC3'
OPK06	5'CACCTTTCCC3'
OPK11	5'AATGCCCCAG3'
OPK13	5'GGTTGTACCC3'
OPK15	5'CTCCTGCCAA3'
OPK16	5'GAGCGTCGAA3'

Table 3. Number of polymorphic loci, mean of effective number of alleles (ne), mean of gene diversity (h), and genetic diversity (Ho) estimated by the Shannon-information index.

Population	Number of polymorphic loci	Percentage of polymorphic loci	Effective number of alleles (ne)	Gene diversity (h)**	Genetic diversity (Ho)*
CYP1	36	49.3	1.199	0.129	0.201
CYP2	44	60.3	1.256	0.159	0.255
NSM1	42	57.5	1.321	0.184	0.294
NSM2	36	49.3	1.265	0.151	0.237
NSM3	28	38.4	1.186	0.112	0.153
MSK1	41	56.2	1.294	0.174	0.262
MSK2	31	42.5	1.213	0.131	0.197
MSK3	32	43.8	1.195	0.125	0.204
BRM	34	46.6	1.244	0.143	0.202
Mean	36	49.3	1.241	0.145	0.223

* Ho is the average, over all primers, of the Shannon's information index,
Ho = $-\Sigma^{k}_{i=1} P_i \log_e P_i$, where k is the number of RAPD markers for a particular primer,
and Pi denotes the frequency of the ith RAPD markers in a given population..

** $h = 1 - \Sigma^{k}_{i=1} P_i^{2}$

Table 4. Mean of genetic diversity (Ho) within populations of *A. sericea* from nine locations.

	Population								
Primer	**CYP1**	**CYP2**	**NSM1**	**NSM2**	**NSM3**	**MSK1**	**MSK2**	**MSK3**	**BRM**
	N=30	N=29	N=30	N=30	N=30	N=30	N=30	N=30	N=30
OPB05	0.0635	0.2073	0.4431	0.1298	0.2036	0.4860	0.2384	0.1845	0.1880
OPB07	0.4967	0.4266	0.2445	0.3347	0.3165	0.3207	0.3913	0.3772	0.5593
OPB10	0.0983	0.000	0.0751	0.0847	0.0405	0.0676	0.0920	0.1469	0.0624
OPB12	0.3137	0.1842	0.2927	0.1218	0.0951	0.0951	0.1248	0.0502	0.1774
OPB20	0.0389	0.2829	0.2516	0.2169	0.1679	0.0554	0.1954	0.0753	0.2103
OPK06	0.2755	0.2778	0.4100	0.2295	0.0000	0.3169	0.1904	0.3332	0.0494
OPK11	0.1864	0.4281	0.2424	0.5597	0.2196	0.4942	0.249	0.4074	0.1943
OPK13	0.2079	0.2587	0.5628	0.2983	0.2734	0.3024	0.1772	0.1040	0.2747
OPK15	0.2527	0.2811	0.2252	0.1467	0.1526	0.2728	0.1258	0.1163	0.2936
OPK16	0.2067	0.2047	0.1929	0.2511	0.0648	0.2092	0.1828	0.2468	0.0122
Mean	0.2140	0.2551	0.2940	0.2373	0.1534	0.2620	0.1967	0.2042	0.2022

Table 5. Components of genetic diversity in *A. sericea* from nine locations, partitioned into within and between populations for 10 random oligonucleotide primers.

Primer	Hpop	Hsp	Hpop / Hsp	(Hsp - Hpop) / Hsp
OPB05	0.244	0.556	0.439	0.561
OPB07	0.385	0.550	0.701	0.299
OPB10	0.075	0.395	0.191	0.809
OPB12	0.146	0.359	0.408	0.592
OPB20	0.166	0.277	0.599	0.401
OPK06	0.231	0.376	0.616	0.384
OPK11	0.331	0.479	0.692	0.308
OPK13	0.273	0.508	0.538	0.462
OPK15	0.207	0.527	0.393	0.607
OPK16	0.175	0.292	0.599	0.401
Mean	0.224	0.432	0.518	0.482

Table 6. Nei's genetic diversity values and estimates of gene flow between *A. sericea* populations. Total genetic diversity(Ht), genetic diversity within populations (Hs), and the proportion of total genetic diversity found among populations (Gst) were calculated for all variable loci. Nm indicates the number of migrants per generation and has been calculated using Crow and Aoki (1984) equation after Ellstrand and Elam (1993).

Primer	N	Hs	Ht	Gst	Nm
OPB05	269	0.161	0.377	0.529	0.176
OPB07	269	0.262	0.369	0.283	0.501
OPB10	269	0.047	0.235	0.840	0.038
OPB12	269	0.102	0.209	0.476	0.217
OPB20	269	0.123	0.200	0.203	0.778
OPK06	269	0.152	0.230	0.342	0.380
OPK11	269	0.223	0.223	0.296	0.471
OPK13	269	0.181	0.332	0.442	0.249
OPK15	269	0.211	0.250	0.586	0.140
OPK16	269	0.116	0.211	0.264	0.551
Mean		0.158	0.263	0.426	0.350

Table 7. Estimate values of genetic identity (I) (above diagonal), and genetic distance (D) (below diagonal) matrice of nine populations of *A. sericea*, based on Nei (1978)

Population	CYP1	CYP2	NSM1	NSM2	NSM3	MSK1	MSK2	MSK3	BRM
CYP1	-	0.8703	0.8439	0.8913	0.8616	0.8712	0.8740	0.8454	0.7060
CYP2	0.1389	-	0.8765	0.8359	0.8662	0.8251	0.8406	0.7635	0.6460
NSM1	0.1697	0.1318	-	0.8427	0.8806	0.8444	0.8413	0.8135	0.6304
NSM2	0.1151	0.1793	0.1711	-	0.9167	0.8717	0.8510	0.8458	0.6786
NSM3	0.1490	0.1437	0.1272	0.0810	-	0.8738	0.8459	0.8458	0.6766
MSK1	0.1379	0.1922	0.1691	0.1373	0.1349	-	0.8833	0.8838	0.7099
MSK2	0.1347	0.1737	0.1728	0.1613	0.1673	0.1241	-	0.8875	0.6742
MSK3	0.1679	0.2699	0.2064	0.1660	0.1674	0.1235	0.1193	-	0.7089
BRM	0.3481	0.4369	0.4615	0.3877	0.3906	0.3426	0.3942	0.3440	-

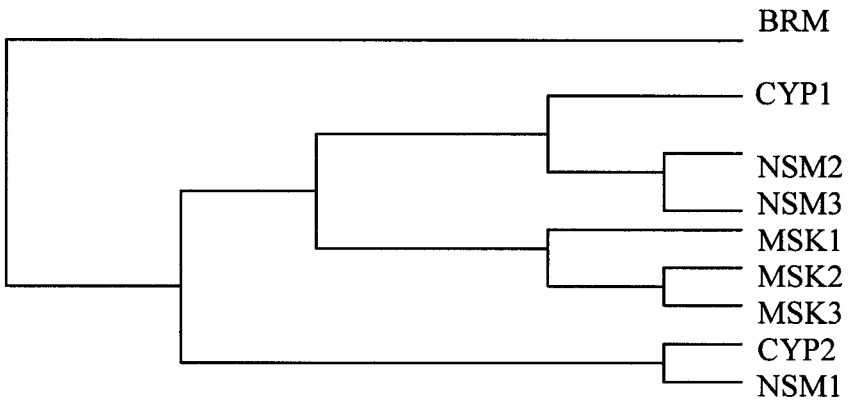

Figure 3. A dendrogram of nine *Afgekia sericea* populations constructed by UPGMA method.

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Genetic structure of the rare species, *Afgekia mahidolae* (Leguminosae) from western Thailand detected by RAPD-PCR

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ABSTRACT

Genetic variation in four populations of *Afgekia mahidolae* Burtt and Chermsirivathana, a rare species in western Thailand, was examined using RAPD-PCR analysis including 86 RAPD markers. It was found that within - population polymorphism measured by percentage of polymorphic RAPDs varied between 43.0% and 52.3%. Genetic variability was measured using Shannon's information index and partitioned into between- and within-population components. Overall, genetic variation within *A. mahidolae* populations was high (*Hpop / Hsp* = 59%). The mean of gene diversity (*h*) was 0.147. The genetic diversity for the species (*Hsp*) was 0.368 and those for within - population (*Hpop*) for all populations was 0.215. Population differentiation was high (*Gst = 0.266*), which is consistent with low gene flow between populations (*Nm* = 1.139). Theoretically, endemic species are typically depauperate in terms of genetic variation. Our results contradict this view, but support other previously published results. High within-population genetic variation in *A. mahidolae* populations observed in this study might be explained by the fact that founding individuals exhibited only sexual reproduction, with low gene flow between populations, and an absence of genetic drift.

ADDITIONAL KEY WORDS: - *Afgekia* – Population Differentiation - Genetic Divergence

INTRODUCTION

Afgekia mahidolae is dedicated, with permission, to Her Royal Highness, the King's Mother, whose interest in the Thai flora is v·ell known (Burtt & Chermsirivathana 1971). *Afgekia mahidolae* is a woody climber with terminal, recemose inflorescence, and flowers May to October. The entire flowering sequence, including seed maturity, is completed in the early dry season (usually February) after which, all parts of the plant wilt and die. Nevertheless, early in the wet season, new plants develop from the living portions of the parental plants which are above and/or underground (Prathepha, 1990), seed of this plant exhibits in small number, even though an inflorescence has long panicle containing about 80-200 florets. Seed dispersal is poor due to large seed (average of 1.4 g per seed). From field observations, the plant's seed survival rate was also found to be very low, since the biological and physical conditions play a major role in seed survival and seedling establishment. Cytologically, *A. mahidolae* exhibits metaphase karyotype of 2n=16 (Prathepha,1994).

This species is confined to limestone habitats. Therefore, it has small populations and narrow range of distribution. Although Burtt & Chermsirivathana (1971) suggested that eventually *A. mahidolae* would be found in southern Burma, to our knowledge, it have been documented only in Thailand. Hence, *A. mahidolae* is regarded as a rare and endangered species based on its restricted geographic range. The evolutionary potential of a species is dependent on the amount of genetic variation within populations (Hunter, 1996). Thus, the planning strategies for habitat restoration and conservation for rare species requires information on the genetic diversity of natural populations, and spatial pattern can be used to help avoid practices that degrade evolutionary potential of populations. We initiated the investigation on genetic structure of *A. mahidolae* from natural populations by using RAPD analysis.

RAPD assay is based on the amplification of nuclear DNA via the polymerase chain reaction (PCR), using short oligonucleotide primers of arbitrary sequence. Amplified DNA fragments are separated by gel electrophoresis to form banding

pattern and are inherited in a Mendelian fashion as dominant characters (Williams et al., 1990). As described by McDonald (1997), the RAPD technique has advantages over other methods. It has been successfully used to describe population genetics in many plant species (Chalmers *et al*, 1992; Huff *et al.*, 1993; Lynch & Milligan 1994; Gustafsson & Gustafsson, 1994). Unfortunately, RAPDs have certain drawbacks that must be weighed against their relative ease of use. The drawbacks include reproducibility of results, as well as comigration of bands in different individuals are homologous. However, problems of reproducibility associated with RAPD markers can be solved by the standardization of laboratory procedures (Nilsson *et al.*, 1997). Furthermore, there are several approaches to addressing this question, and they demonstrated that the fragments with a similar size are homologous to each other in their sequences (Kaga *et al.*, 1996; Bonnin *et al.*, 1996; Gandeboeuf *et al.*, 1997). RAPDs may prove useful in the study of rare plants for conservation purpose because allozyme variation is often minimal or nonexistent in small populations (Lesica *et al.*, 1988; Huen *et al.*, 1994; Rossetto *et al.*, 1995).

This paper presents the results of our study on genetic differentiation in natural populations of *A. mahidolae* in Thailand.

MATERIALS AND METHODS

Field collection

Seeds of *A. mahidolae* were collected from four populations in Kanchanaburi Province. Three of the populations occurred on the same limestone hill while the fourth population were obtained from another limestone hill, approximately 6 km apart. Seed samples were transported and grown in the greenhouse at Mahasarakham University for RAPD analysis.

Isolation of genomic DNA

Genomic DNA was extracted from the leaves of 19-20 individuals per population, using the CTAB method following the method of Doyle and Doyle (1987) with minor modifications. The leaves (2 g) were ground in a mortar with a pestle . The grindate was added to 20 ml of extraction buffer (2% (w/v) CTAB ; 100mM Tris-Cl buffer (pH 8.0), 20mM EDTA (pH 8.0), 1.4M NaCl), 1% (w/v) PVP-40) and incubated at 60°C for 30 min. The homogenate was added to 24:1 chloroform: isoamyl alcohol (v/v) and mixed well by gentle inversion. After centrifugation at 14000 rpm for 5 min, the upper aqueous layer was transferred to a fresh tube containing 600 µl of cold isopropanol. The precipitated DNA was added to 400 µl of 75% ethanol with 10 mM ammonium acetate and was washed twice with 75% ethanol. The pellets were allowed to sit overnight at room temperature, and were resuspended in TE buffer (10mM Tris-Cl (pH 8.0), 1mM EDTA (pH 8.0) containing RNase (10 mg/ml).

RAPD assay

Before the analyses were carried out we optimized the annealing temperature, the concentration of Mg^{2+}, the primer, the DNA template and polymerase. Finally, the experiment reaction mixtures were performed in a total of 25 µl reaction volume, containing 14.0 µl sterile water, 2.5 µl of 10X PCR reaction buffer (Promega), 3.0 µl of 25 mM $MgCl_2$, 2.5 µl of 4 mM dNTPs, 1.0 µl of 10 µM primer, 0.1 µl (0.5 units) of Taq DNA polymerase (Promega), and 1 µl of genomic DNA template (100 - 200 ng). Error was minimized by making one large (master mix) batch of all reagents for each primer of the experiment, except Taq DNA polymerase. PCR reactions were performed on a Hybaid thermocycler, programmed for an initial melting step at 94°C for 4 min, followed by 45 cycles, each cycle consisting of three steps of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min. A final extension step at 72°C for 4 min was

performed after the 45 cycles. A negative control reaction in which DNA was omitted was included with every run in order to verify the absence of contamination.

Amplified products were separated in 1% agarose gels (FMC BioProducts) in 0.5x TBE (Tris-Borate-EDTA) buffer. The gels were run for 2 hr at 75 V, stained with ethidium bromide (10 mg/ml), and photographed on a UV transilluminator. To determine RAPD profiles, the size of each band was inferred by comparisons with a 1 kb ladder (Promega), used as a molecular weight marker (M), using a Gel Documentation System, GDS 8000 (UVP Inc., California, USA). Only the amplified products that consistently appeared in two replications were scored as presence (1)/ absence (0), for further analysis.

Selection of primers and data analysis

Forty 10-mer primers (OPAS and OPAM Kit) from Operon Technologies were tested in different PCR reaction with the extracted plant DNAs for their consistent amplification of DNA fragments. Finally, ten primers producing the most clearest and most intense bands were selected out of forty for data analyses (Table 1). Bands observed by RAPD analysis were assigned a number in relation to their migration within the gel. Bands with the highest molecular weight were assigned number 1 and so on in ascending order until the band of lowest molecular weight was assigned. Each individual was scored for the presence or absence of amplification products considered to be relevant, and the data obtained were entered into a binary data matrix.

Statistical analysis

Genetic structure of *A. mahidolae* populations was estimated. Most estimates were generated using the software POPGENE kidly provided by Prof. F.C. Yeh. The genetic variability within population was determined using three measures. Firstly, the phenotypic frequencies detected with the primers in four populations were calculated, and estimates of population diversity were obtained using Shannon-information index (Chakraborty & Rao, 1991) modified for RAPD analysis, which is defined as

$H = -\sum^{k}_{i=1} P_i \log_e P_i$. For each primer used, H denotes the diversity of RAPD markers in a population, k denotes the number of RAPD markers, and P_i denotes the frequency of the i^{th} RAPD marker in a given population (or the proportional abundance of each banding pattern, or phenotype). H was calculated within species (H_{sp}) and within populations (H_{pop}). The proportion of diversity within populations is H_{pop}/H_{sp} and the proportion of diversity among populations is $(H_{sp} - H_{pop})/H_{sp}$ (Gustafsson & Gustafsson 1994). For each population, H_{pop} was averaged over all the primers to determine the within-population diversity of RAPD markers. The average diversity of RAPD markers for all populations (H_{pop}) was calculated as the mean of H_o. Secondly, the percentage of polymorphic loci, the proportion of loci polymorphic within each population (Lin et al., 1997). was estimated. Finally, gene diversity (h) at each RAPD locus in each population was determined according to Nei's (1973) gene diversity, which is defined as $h = 1 - \sum P_i^2$, where P_i is the frequency of the i^{th} allele (Peever & Milgroom, 1994). Gene diversity within each population was calculated as the mean gene diversity over all loci from all populations.

Population differentiation was determined using genetic divergence, gene flow, and genetic identity. Genetic divergence among populations was estimated by calculating Nei's Gst value (Nei, 1987) as follows: $Gst = (Js - Jt)/(1- Jt)$, in which $Js = \Sigma \text{ Avg } (P_i^2)$ and $Jt = \Sigma [\text{ Avg } (P_i)]^2$ and P_i is the frequency of the i^{th} allele. Gene flow (Nm) represents the actual number of individuals exchanged among populations in each generation. We quantified gene flow using the indirect estimates of Crow & Aoki (1984) after Ellstrand & Elam (1993). In this model, the number of migrant individuals exchanged among demes each generation is $Nm = (1 / Gst - 1) / 4 [n / (n - 1)]^2$, in which n = total number of demes. Using the average value of Gst over all loci. Values for unbiased genetic identity (I), were estimated (Nei, 1978), and genetic divergence among populations was also estimated using Nei's (1978) genetic distance for all pairs of populations. These values were used to construct dendrogram by the unweighted pair-group method with arithmetic averaging (UPGMA) using TREEVIEW, a tree drawing software for Microsoft Windows (Page, 1996).

RESULTS

RAPD products and genetic variability

Ten primers that produced strong, reproducible bands were selected for use in this study. Photographs of agarose gels containing bands produced by primer OPAS09 (KAN2) and OPAS08 (KAN3) are shown in Fig. 1. A total of 86 different RAPD markers were generated. Number of markers produced per primer ranged from 5 (OPAS10) to 11 (OPAS15), ranging in size from about 250 bp to 2 kb. For each population, the results are in the form of a matrix of presence-absence data for 86 RAPD bands for use in further analysis. The numbers of polymorphic RAPD loci per population ranged from 37 (KAN3) to 45 (KAN4) (Table 2). To evaluate whether the 86 RAPD loci frequencies differ between populations, Chi-square and likelihood ratio tests were performed for homogeneity of RAPD loci frequencies across populations.

Frequencies of alleles of 54 of 86 RAPD loci were statistically significant among populations ($P < 0.01$ or $P < 0.001$). This means that distribution of 32 of 86 PAPD loci were not homogeneous among populations. Gene diversity (h) in each population (averaged over 86 loci) varied between 0.139 in KAN1 to 0.155 in KAN2. The mean gene diversity over all populations was 0.147 (Table 2). The mean genetic diversity detected by the 10 primers for the species (H_{sp}) was 0.368 and 0.223 within populations(mean H_{pop}) (Table 3). We used Shannon's information index to measure the total genetic variability to be explained within- and between-population variability. The proportion of variation within populations (H_{pop}/H_{sp}) was 59% and among populations (H_{sp} - H_{pop}/ H_{sp}) was 41%. RAPD banding patterns gave interesting information of genetic variation in *A. mahidolae*. For instance, some individuals from KAN4, exhibited unique band (OPAS03-750 bp) that were not found in the other three populations. Similarly, the KAN3 population exhibited less variation within population when using the primer OPAS03. In addition, no polymorphism was detected by OPAS09 among individuals from KAN2. In contrast, high genetic variation was found in KAN4 when using OPAS14. Some intensive bands such as OPAS09-1457 bp and OPAS09-966 bp were found across all populations. Distribution of variability between

and within populations varied between primers. For example, primer OPAS12 detected the most variability between populations (65%), whereas primer OPAS09 detected the most variation within populations (84.5%) (Table 3).

Population divergence

To complete the analysis based on phenotypic frequencies Nei's estimate of similarity (Nei, 1987) was used to generate a similarity matrix (Table 5). Interpopulation genetic identity values of this species were consistently high. The mean genetic identity between *A. mahidolae* populations was 0.8339. The KAN1 and KAN3 populations exhibited the least variability, whereas the KAN3 and KAN4 populations exhibited the most. A dendrogram displaying hierarchical associations is given in Fig. 2. The dendrogram was generated by group-average clustering where the similarity between two groups is defined as the average similarity of all points of unit involving a member of each group. The KAN4 population appeared to form a distinct group. For overall population, the mean value of Gst (Nei, 1987) across all polymorphic loci of *A. mahidolae* is 0.266 (Table 4), and suggests that approximately 27% the total genetic diversity is partitioned into the among-population component. An indirect estimate of gene flow (Nm) ranged from 0.239 to 4.867. The mean Nm was 1.139 indicated very rare migration between *A. mahidolae* populations.

DISCUSSION

Genetic structure

A much higher level of genetic variability determined by Shannon's information index was detected within *A. mahidolae* populations (H_{pop}/H_{sp}) = 59%) (Table 3). Thus, most of the species' genetic diversity exists within each population then it is more likely to be able to evolve in response to a changing environment. Because of its high level of genetic diversity, the questions arise as to whether this species is predominantly an outcrossing species, unlike many self-compatible species of papilionoid legumes (Shivanna & Owens 1989). Previous research has shown that

inbreeding species have lower gene diversity (range 0.294 – 0.008, mean 0.125) than those of outbreeding species (range 0.328 – 0.174, mean 0.257) (Schoen & Brown 1991). The mean gene diversity of *A. mahidolae* (h = 0.147) is close to those of inbreeding species. Many papilionoid legumes species that are self-compatible species possess a stigma cuticle (membrane) covering the stigma, requiring rupture before pollen will germinate (Jusaitis, 1994). This context may support the fact that *A. mahidolae* flowers are produced in long inflorescences consisting of many flowers, But some individuals of *A. mahidolae* can produce pods containing large seeds as well as some flowers could set some pods per an inflorescence.

Current evidence suggests that effective population sizes of plant species are small and, in most cases, much smaller than the actual population sizes (Levin & Kerster, 1994; Schaal, 1980). The facts that some individuals of *A. mahidolae* in each population set fruits in each flowering season.Thus the effective population size of all populations may be less than the actual population size. Most of the diversity of this species was detected within populations, similar to those obtained by Hamrick (1989), who indicated that in outbreeding and woody perennial plants most variation occurred with populations based on isozyme analyses. Hamrick's results may not be directly correlated with the RAPD data found in *A. mahidolae* in this study. However, molecular markers like RAPD may show more polymorphic than enzymatic markers (Fritsch & Rieseberg, 1992; Liu & Furnier, 1993; Heun *et al.*, 1994).

It has been shown that overall geographically restricted species tend to have less genetic variability than the widespread species (Hamrick & Godt (1990); Karron (1987); Karron *et al.*(1988); Moran & Hopper (1983). Moreover, rare species are often expected to be genetically depauperate, perhaps as a results of founder effect, genetic drift, and/or inbreeding in small populations. In addition, some endemics seem to be adapted to a narrow set of environmental conditions, which might limit the genetic diversity maintained by selection (Baskauf *et al.*, 1994). However, Karron (1987) caution over drawing conclusion about associations when comparing population genetic information from unrelated taxa The author further suggested that closely related widespread congeners be used for comparison with rare species, as they often share ecological and historical traits. There is considerable evidence that bottlenecks and founder effects play a significant role in governing patterns of genetic variability in colonizing species (Barrett & Kohn 1991). As mentioned earlier, *A.*

mahidolae is restricted to limestone glade occurring in small populations. Hence, the levels of genetic variation within populations of A. mahidolae would be depedent on the amount of genetic variation in the founding individuals, the length of time the population remained small, and level of gene flow. Once populations of *A. mahidolae* were established, they could survive and expand by sexually produced seeds. Thus, *A. mahidolae* populations may exhibit the reliance on sexual reproduction for persistence in the limestone habitats resulting in the maintenance of high levels of population genetic diversity. Ledig & Conkle (1983) pointed out that it is uncertain whether breeding system is capable of maintaining variability in small populations in the absence of migration. Furthermore, the potential to maintain high levels of genetic variation in reduced and scattered populations is important because of its implications for genetic resource conservation. The small effective population sizes and low levels of gene flow imply that genetic drift will play important role in the genetics of *A. mahidolae* populations. Thus, studies of mating system and floral biology in this rare species will further provide useful information in population differentiation.

Population estimates for each sampling site of *A. mahidolae* ranged from 50 – 100 mature plants. Genetic drift should cause genetic variability among subpopulations very rapidly. The proportion of differences among populations, using Nei's Gst staistic, which is similar to Wright's Fst (Nei, 1973), was relatively high (mean Gst = 0.266). The high level of population differentiation observed for *A. mahidolae* corresponds to low gene flow among populations (mean Nm = 1.139). Moreover, A. mahidolae seems to have poor seed dispersal abilities due to its large seed. Yet, a requirement for colonization is seed dispersal after individuals establishment. Thus, we suggest that the origin of seeds establishing new populations for the two populations (KAN1 and KAN2) come from population KAN3 which occupies at higher elevation than the other populations. We also suggest that seed dispersal mechanism for colonization of this species was probably water flow from higher in elevation to lower other populations.

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Table 1. Random oligonucleotide primers used in this study.

Primer	Sequence
OPAS03	5'ACGGTTCCAC3'
OPAS04	5'GTCTTGGGCA3'
OPAS08	5'GGCTGCCAGT3'
OPAS09	5'TGGAGTCCCC3'
OPAS10	5'CCCGTCTACC3'
OPAS12	5'TGACCAGGCA3'
OPAS14	5'TCGCAGCGTT3'
OPAS15	5'CTGCAATGGG3'
OPAS18	5'GTTGCGCAGT3'
OPAS19	5'TGACAGCCCC3'

Table 2. Number of polymorphic loci, mean number of effective number of alleles(ne), and mean number of gene diversity(h) and genetic diversity(Ho) estimated by the Shannon-information index

Population	Number of polymorphic loci	Percentage of polymorphic loci	Effective number of alleles (ne)	Gene diversity (h)**	Genetic diversity (Ho)*
KAN1	39	45.4	1.23	0.139	0.213
KAN2	39	45.4	1.26	0.155	0.232
KAN3	37	43.0	1.24	0.142	0.215
KAN4	45	52.3	1.25	0.152	0.236
Mean	40	46.5	1.245	0.147	0.224

* Ho is the average, over all primers, of the Shannon's information index,
$Ho = -\Sigma^{k}_{i=1} P_i \log_e P_i$, where k is the number of RAPD markers for a particular primer,
and Pi denotes the frequency of the ith RAPD markers in a given population..

Table 3. Components of genetic diversity in *A. mahidolae* from four locations, partitioned into within and between populations for 10 random oligonucleotide primers.

Primer	Hpop	Hsp	Hpop / Hsp	(Hsp - Hpop) / Hsp
OPAS03	0.178	0.383	0.465	0.535
OPAS04	0.215	0.308	0.698	0.302
OPAS08	0.289	0.455	0.635	0.365
OPAS09	0.218	0.258	0.845	0.155
OPAS10	0.064	0.085	0.753	0.247
OPAS12	0.145	0.414	0.350	0.650
OPAS14	0.345	0.578	0.597	0.403
OPAS15	0.302	0.369	0.818	0.182
OPAS18	0.241	0.476	0.506	0.494
OPAS19	0.157	0.349	0.450	0.550
Mean	0.215	0.368	0.586	0.414

Table 4. Nei's genetic diversity values and estimates of gene flow between *A. mahidolae* populations. Total genetic diversity (Ht), genetic diversity within populations (Hs), and the proportion of total genetic diversity found among populations (Gst) were calculated for all variable loci. Nm indicates the number of migrants per generation and has been calculated using Crow and Aoki (1984) equation after Ellstrand and Elam (1993).

Primer	N	Hs	Ht	Gst	Nm
OPAS03	77	0.116	0.256	0.337	0.389
OPAS04	77	0.143	0.197	0.177	0.918
OPAS08	77	0.192	0.305	0.287	0.491
OPAS09	77	0.143	0.155	0.089	2.022
OPAS10	77	0.035	0.039	0.039	4.867
OPAS12	77	0.097	0.274	0.453	0.239
OPAS14	77	0.218	0.401	0.409	0.285
OPAS15	77	0.200	0.243	0.116	1.505
OPAS18	77	0.153	0.309	0.424	0.268
OPAS19	77	0.106	0.229	0.329	0.403
Mean		0.140	0.241	0.266	1.139

Table 5. Estimate values of Nei's unbiased measure of genetic identity (I) (above diagonal), and genetic distance (D) (below diagonal) for nine populations of *A. mahidolae* , based on Nei (1978).

Population	KAN1	KAN2	KAN3	KAN4
KAN1	***	0.8405	0.8631	0.8238
KAN2	0.1738	***	0.8415	0.8214
KAN3	0.1472	0.1725	***	0.8128
KAN4	0.1939	0.1967	0.2073	***

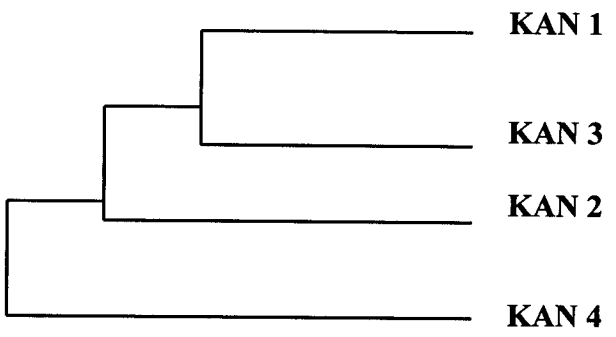

Figure 1. A dendrogram of four populations of *A. mahidolae* contructed by UPGMA method.

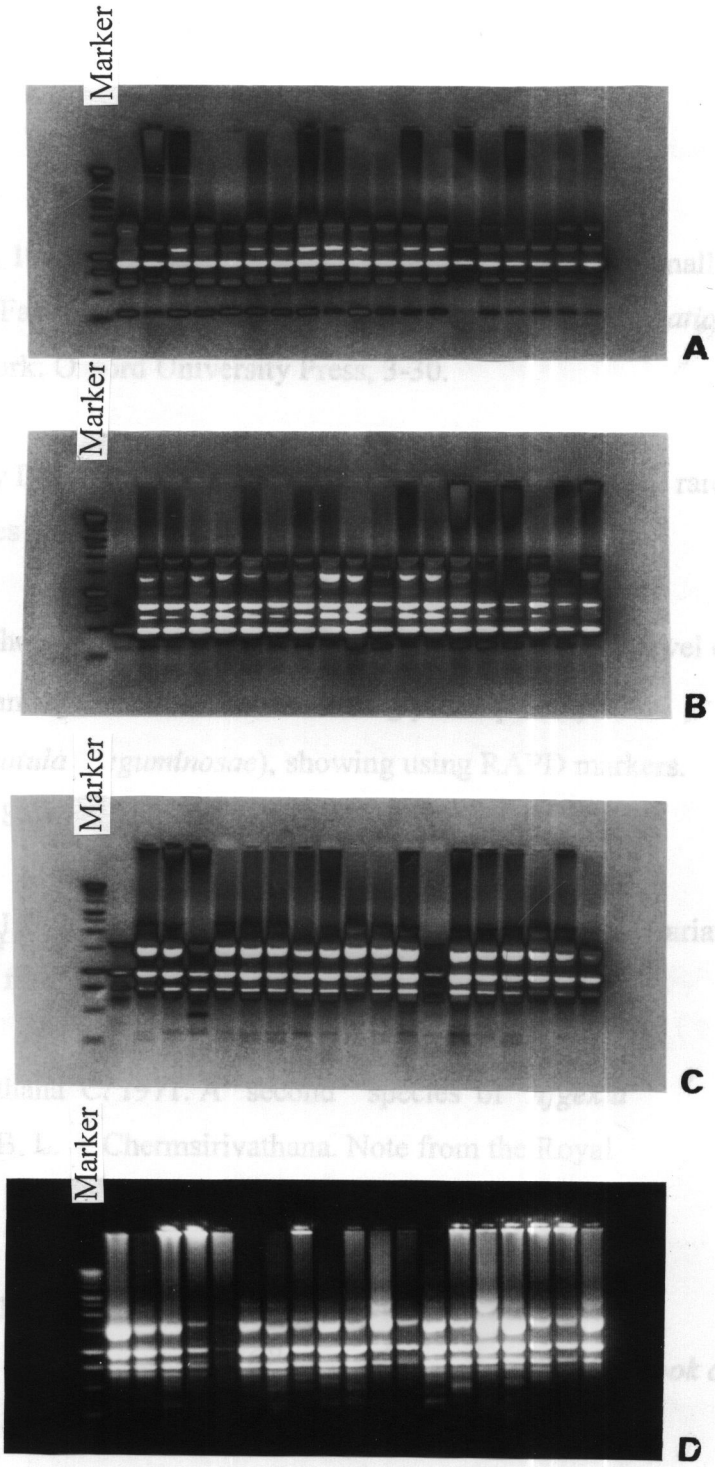

Figure 2. RAPD band profiles generated from DNA of *Afgekia mahidolae*. Population KAN3 operated by primer OPAS03, A; OPAS08, B; population KAN2 operated by primer OPAS09, C and population KAN4 operated by primer OPAS09, D.

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Genetic relationships in *Afgekia sericea* Craib and *Afgekia mahidolae* Burtt and Chermsirivathana (*Leguminosae*) from Thailand evaluated by RAPD-PCR fingerprint

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ABSTRACT

The potential used of RAPD markers for taxonomic studies in two closely related species, *Afgekia _sericea* and *A. mahidolae*, includes *Sesbania grandiflora* and *S. bispinosa* used as the outgroup ,was investigated. Nine selected random decamer primers were used to amplify multiple PCR products with genomic DNA as template. Random amplified polymorphic DNA (RAPD) 'fingerprints' were analyzed for 104 scorable amplification fragments. Based on the number of different RAPDs between each pair of species, cluster analysis by an unweighted pair group method with arithmetic averages (UPGMA) was performed to exploit genetic relationships. It was revealed that the molecular classification was in good agreement with the known lineage. The two closely related species were clearly distinguishable from the outgroup, indicating that similarities based on the presence vs. absence of RAPDs were reflections of genetic similarities. We suggest that RAPD 'fingerprint' is a useful for studying both classification and genetic relationships of closely related species.

Key words: *Afgekia sericea, Afgekia mahidolae*, RAPD fingerprint, genetic relationships, classification.

Introduction

Recent biosystematic treatments of the genus *Afgekia* Craib have focused on relationships between *Afgekia sericea* and *Afgekia mahidolae* based on morphological characteristics and cytological studies (Prathepha 1990; Prathepha and Baimai ,unpublished data). The genus *Afgekia* belongs to subfamily papilionoideae. The essential features of this subfamily are papilionoid flowers. The petals are imbricate in bud with the adaxial or standard outside the wing petals, and two inner most petal or keel housing the stamens and pistil. To date, these two species have been recorded only in Thailand. *A. sericea* exhibits a wide range of distribution extending from the dividing line between central and northeastern, Dong Phaya Yen and Phetchabun Ranges, to many areas of northeastern region. While, *Afgekia mahidolae* is found only in some limestone hills of the western. Habitats of these two species do not overlap. The two species are climbing herbs and morphologically similar. The plants exhibit inflorescence paniculate and the flowers occur in leaf-axils. The bract and bracteoles are large. Pod is dehiscent and woody with sculptured valves. The floral parts of the flower of the two species are slightly different. In *A. sericea*, the standard petal is pale yellow with pink patches on both sides at the base and a yellow patch in the middle. The wing petals are pink and have two basal appendages on the upper margin. For *A. mahidolae*, the standard is purple with central patch of yellow. The wing petals are purple and they clearly differ from *A. sericea* by having one basal appendage on the upper margin. Furthermore, *Afgekia mahidolae* differ from its relative in presence of hairs at the top of the style. The keel petals of both species are white, but exhibit different shape. The two species exhibit common circumstance on flowering phenology (Prathepha 1990). Annually, these two species flower from May to October. The entire flowering sequence including seed maturity is completed in February. At that time, after seed production, all parts of the plant wilt and die. Nevertheless, the new plant can develop from the live parts of parental plants are underground. Further, seeds of the two species are produced in small numbers, even though an inflorecence has a long panicle containing about 80-300 florets. From morphological and cytological evidences (unpbished data), we suggested that the two species arose as a result of allopatric

speciation through the processes of allelic changes and/or chromosomal rearrangements in different geographical populations of the ancestral species.

In recent years, molecular genetics has provided a number of powerful new methodologies with which to construct phylogenetic associations, while avoiding some of the limitations of classical phylogenetic characters. Of particular note is the RAPD (random amplified polymorphic DNA) procedure developed by Williams et al. (1990). RAPDs is new molecular marker for comparative analysis that is quick and easy to use, refractory to many environmental influences, and practically unlimited in number. The successful application of RAPD to taxonomic and evolutionary studies has been documented in a variety of plants (e.g. Demeke et al. 1992; Kresovich 1992; Kazan et al. 1993; Duneman et al. 1994; Campos et al. 1994; Samec and Nasinec 1996; Kaga et al. 1996; Hoey et al. 1996; Gandeboeuf et al. 1997). Comparisons of RAPD and RFLP in determining genetic similarity among *Brassica oleraceae* genotypes revealed that these marker systems provide equivalent levels of resolution for determining genetic relationships (dos Santos et al. 1994). Observed differences between the methods were attributed to sampling error rather than fundamental differences in how each approach reveals polymorphism. Gehrig et al. (1997) also documented that RAPD-PCR is a rapid and sensitive technique providing a useful tool for studying plant phylogenetic relationships at the infragenetic level. In this study we have applied RAPD-PCR to investigated genetic relationships between *Afgekia sericea* and *A. mahidolae,* in order to determine the genetic relatedness between the two taxa.

Materials and methods

Plant materials

Seeds of *A. sericea* and *A. mahidolae* were collected from their natural populations. Seeds from each species grown in a greenhouse to produce leaf material for DNA extraction. Ten individuals were examined from each species. Two outline papilionoid legumes, *Sesbania bispinosa* (Jacq.) W.F. Wight, and *S. grandiflora* (Linn.) Poir. used as outgroup that provide a basis for the calculation of the relative position of *Afgekia sericea* and *A. mahidolae*. Young unexpanded leaves of the outgroup were harvested from their natural habitats and stored at –20° C until further processing.

Isolation of genomic DNA

Genomic DNA was extracted from fresh young leaves, using the CTAB (cetyltriammonium bromide) method of Doyle and Doyle (1987) with minor modifications. Fresh young leaves (2 g) was ground in mortar with a pestle . Then, 20 ml of DNA extraction buffer (2% (w/v) CTAB; 100mM Tris-Cl buffer (pH 8.0), 20mM EDTA (pH 8.0), 1.4M NaCl), 1% (w/v) PVP-40) was added and the samples were incubated at 60°C for 30 min. The extracts were added with 24:1 chloroform:isoamyl alcohol (v/v) and mixed well by gentle inversion. After centrifugation at 14000 rpm for 5 min, the supernatant was collected into a fresh Eppendorf tube. The DNA was precipitated with isopropanol. Then, the precipitated DNA was added with 75% ethanol with 10 mM ammonium acetate and was washed twice with 75% ethanol. The pellets were allowed to sit overnight at room temperature, and resuspended in 500 µl of TE buffer (10mM Tris-Cl (pH 8.0), 1mM EDTA (pH 8.0) containing RNase (10 mg/ml).

RAPD assay

Amplification was performed in a 25 µl reaction volume, consisting of 2.5 µl of 10X PCR buffer (Promega), 3.0 µl of 25 mM $MgCl_2$, 2.5 µl of 4 mM dNTPs, 1 µl of 10 µM primer, 0.5 U of Taq DNA polymerase (Promega), 1 µl of DNA extracted solution (100 - 200 ng), and an appropriate volume of sterile distilled water to make up the final volume to 25 µl. PCR reactions were carried out with the Hybaid thermocycler, programmed for an initial melting step at 94°C for 4 min, followed by 45 cycles, each cycle consisting of three steps of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min. A final extension step at 72°C for 4 min was performed after the 45 cycles. A negative control reaction in which DNA was omitted was included with every run in order to verify the absence of contamination. Amplified products were separated in 1% agarose gels (FMC BioProducts) in 0.5X TBE (Tris-Borate-EDTA) buffer. The gels were run for 3 hr at 75 V, stained with ethidium bromide (10 mg/ml), and photographed on a UV transilluminator.

Selection of primers and data analysis

Forty 10-mer primers (Kit OPAS and OPAM) from Operon Technologies were tested in different PCR reaction with the investigated plants DNAs for their consistent amplification of DNA fragments. Finally, nine primers producing the most clear and intense bands were selected out of forty for data analyses (Table 1). RAPD fragments were inferred by comparisons with a 1 kb ladder (Promega), used as a molecular size marker (M), using a Gel Documentation System, GDS 8000 (UVP Inc., California, USA). RAPD fragments were identified by the OPERON primer code. RAPD fragments corresponding to a given primer were named according to the fragment size in base pairs. For example , the band OPAS05-1140 refers to a band 1140 base pairs (bp) long, amplified by primer OPAS05. RAPD fragments that found from ten individuals of each species were scored for the presence or absence, while intraspecific polymorphism were ignored . Only the amplified products that consistently appeared in two replications were scored as presence (1)/ absence (0), for further analysis.

Statistical analysis

For analysis of RAPD data, the relative density of the bands was not considered and comigrating bands on 1% agarose gel were interpreted as homologous. The presence or absence of bands was coded in binary (0, 1) form. Polymorphisms between species were calculated using the index of genetic distance (1 – S). S values were obtained by using the method that based on the theory of Nei and Li (1979). This method involved estimating the fraction of shared fragments (S) between two species, using the formula $S = 2m_{xy} / (m_x + m_y)$, where m_{xy} is the number of RAPD fragments shared by the two species and m_x and m_y represent the total number of RAPD fragments present in each species. S can take any value between 0 and 1, with 0 meaning that no common bands exist, and 1 meaning that the obtained patterns are identical. Based on the number of different bands between each pair of species. Dendrogram of four species were obtained by the UPGMA (Unweighted pair-group method using arithmetic mean) cluter analysis (Sneath and Sokal 1973), using SPSS/PC program package, release 4.0 (see Appendix I).

Results

RAPD profile

Nine primer used in this experiment illustrated that *A. sericea* and *A. mahidolae* can be distinguished by RAPD markers. The RAPD markers generated by some of primers are shown in Fig. 1. All primers yielded amplification products, and consistent and reproducible bands were scored for numerical analysis. Abundant polymorphism was detected with all primers used in this study, while weak and uncertain amplified products were ignored. Primers generated from 7 to 14 fragments ranging from 0.347 to 3.183 kb in size. Either monomorphic or species-specific bands were found with the nine primers. Eighty-three RAPD markers shown in Table 2 obtained from total number of bands found in ten individuals of *Afgekia* species. While, 104 RAPD markers found in both *Afgekia species* and *Sesbania species*. Therefore, this means that some RAPD markers were found in either *Afgekia* species or *Sesbania* species. For example, OPAS03-800, OPAS07-770, OPAS10-2112 and OPAS10-1911, and OPAS12-930 (indicated by arrows) found only in *Sesbania*

species (Figs. 2A-D). However, intraspecific polymorphism was found in RAPD profile of those species. Genetic similarity between *A. sericea* and *A. mahidolae* varied from 0.333 to 0.625 operated by OPAS10 and OPAS12, respectively (Table 3). The average genetic similarity value of the two species was 0.452.

Phenetic analysis of RAPD data

For cluster analysis, a total 104 RAPD markers were included in the analysis. Based on the number of different bands of each species, a command file included the data matrix for use in the cluster analysis is presented in the Appendix I. We used these values to construct a dendrogram using UPGMA method. Two distinct groups can be identified on the dendrogram obtained by this procedure (Fig. 3), one consists of *Afgekia* species and the other one includes *Sesbania* species.

Discussion

Among molecular genetic techniques, RAPD-PCR is easier and faster than other methods, but may have problems with the reproducibility between laboratories. However, we used optimal conditions to obtain reproducible results. Therefore, we suggest RAPD fragments that are polymorphic between *Afgekia sericea* and *A. mahidolae* could be used as RAPD markers to distinguish or could be useful as genetic markers for species identification. Although these two species are easy to differentiate on the basis of morphological characteristics. However, RAPD profiles of the two species showed a relative homogeneity that confirmed their close genetic similarity. Moreover, an advantage of the RAPD-PCR technique is that it produce a fingerprint of genome of the two species, thus providing additional information to complement traditional classification method such as morphological or biochemical analysis. In order to obtain a proper characterization of genetic relationships, Wilkie et al. (1993) recommended surveying at least 50 loci. Although the value of RAPDs for estimating genetic variation within and divergence among species is compromised by their prevailing dominant mode of inheritance, this is not a significant problem when using them strictly as genetic markers where they either are or are not present in particular

plants (Crawford et al. 1993). Therefore, a total 104 RAPD markers included in this experiment seems adequate to accurately represent the level of genetic relationships. Noli et al. (1997) stated that important sources of bias introduced when using RAPDs to survey genetic relationships are caused by scoring error and/or underestimation of the level of genetic diversity resulting from two problems. Firstly, the inability of RAPDs to reveal more than two alleic variants (presence or absence) at each locus. Other one is the coincidence of bands of similar size. Although previously studied stated that there are two methodological problems remain to be discussed. One is the evaluation of amplification product patterns on the electropherogram, since there is at present no better mathematical tool available for this purpose. Therefore, we have chosen the calculations proposed by Nei and Li (1979). Although originally proposed and usually applied to RFLP-fingerprint investigation.In deed, our results show the evaluation method of Nei and Li can reasonably be applied to RAPD, agree with the results of Gehrig et al. (1997). The other problem is the selection of a suitable outgroup from the species to be compared for the calculation of phylogenetic distance among the target species in the dendrogram. As noted already by Gehrig et al. (1997) , in RAPD derived dendrogram the outgroup can provide a basis for the calculation of the relative position of species to each other. However, since to our knowledge the lack of evident major clades in the papilionoideae make it difficult to select outgroup taxa from within the subfamily. Finally, both species of *Sesbania*, *S. grandiflora* (Linn.) Poir,, and *S. bispinosa* (Jacq.) W.F. Wight were chosen as outgroup. With respect to the classification system of the genus *Afgekia*, RAPD markers appear to have the potential to complement "classical" taxonomic studies. As regards the species *A. sericea* and *A. mahidolae*, the hypothesis of a common ancestry was recently put forward by Prathepha and Baimai (unpublished data) we used morphological and cytological evidences. The result of this investigation using RAPD analysis, that both species clustered together, could be interpreted as additional evidence for closely related to each other.

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Table 1. Nine nucleotide sequences of random primers used scored in RAPD analysis and number of RAPDs for *A. sericea* and *A. mahidolae*

Primer	Sequence (5' to 3')	No. of RAPDs scored
OPAS03	ACGGTTCCAC	6
OPAS04	GTCTTGGGCA	9
OPAS05	GTCACCTGCT	9
OPAS06	GGCGCGTTAG	14
OPAS07	GACGAGCAGG	12
OPAS09	TGGAGTCCCC	12
OPAS10	CCCGTCTACC	6
OPAS12	TGACCAGGCA	8
OPAS19	TGACAGCCCC	7

Table 2. Eighty-three RAPD markers (in bp) found in genomes of *A. sericea* and *A. mahidolae*

Primer	*A. sericea*	*A. mahidolae*	Primer	*A. sericea*	*A. mahidolae*
OPAS03	-	1801	**OPAS07**	3183	3183
	1574	-		2572	2572
	943	943		-	1130
	681	681		1019	1019
	525	-		837	-
	274	274		-	775
				682	682
OPAS04	2140	-		-	532
	1519	1819		480	-
	1500	1500		364	364
	1113	1113		268	-
	1009	1009		119	-
	902	-			
	-	884	**OPAS09**	1961	1961
	849	-		1634	1634
	-	560		-	1590
				-	1469
OPAS05	-	1383		1500	1500
	1317	-		1400	-
	1140	-		939	-
	-	1162		-	1000
	1047	1047		975	975
	1000	1000		860	860
	-	857		-	750
	785	785		681	681
	765	765			

Table 2. Continued

Primer	*A. sericea*	*A. mahidolae*	Primer	*A. sericea*	*A. mahidolae*
OPAS06	-	1651	**OPAS10**	1669	-
	-	1547		1650	-
	-	1252		1213	1213
	-	1174		940	940
	1062	1062		813	-
	894	894		750	-
	788	788			
	-	727			
	604	604	**OPAS12**	1576	-
	573	573		1349	1349
	499	499		1281	1281
	-	450		1116	1116
	403	403		931	-
	-	347		819	-
				732	732
OPAS19	1878	-		678	678
	1345	1345			
	-	1037			
	-	931			
	869	869			
	734	-			
	553	553			

Table 8.3. Genetic similarities (S) between *A. sericea* and *A. mahidolae* using the formula of Nei and Li (1979), $S = 2\ m_{xy} / (\ m_x + m_y\ )$, where m_{xy} is the number of amplified products shared by the two species, and m_x and m_y are the total number of amplified products *found in A. sericea* and *A. mahidolae*, respectively.

Primer	Genetic similarity (S)
OPAS03	0.429
OPAS04	0.444
OPAS05	0.444
OPAS06	0.5
OPAS07	0.417
OPAS09	0.5
OPAS10	0.333
OPAS12	0.625
OPAS19	0.375
Mean	0.4519

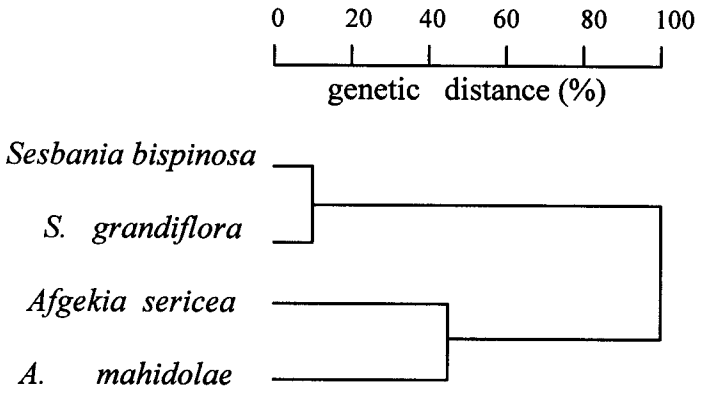

Figure 1. Relationships between of A. sericea and A. mahidolae revealed by UPGMA cluster analysis based on the number of different RAPDs.

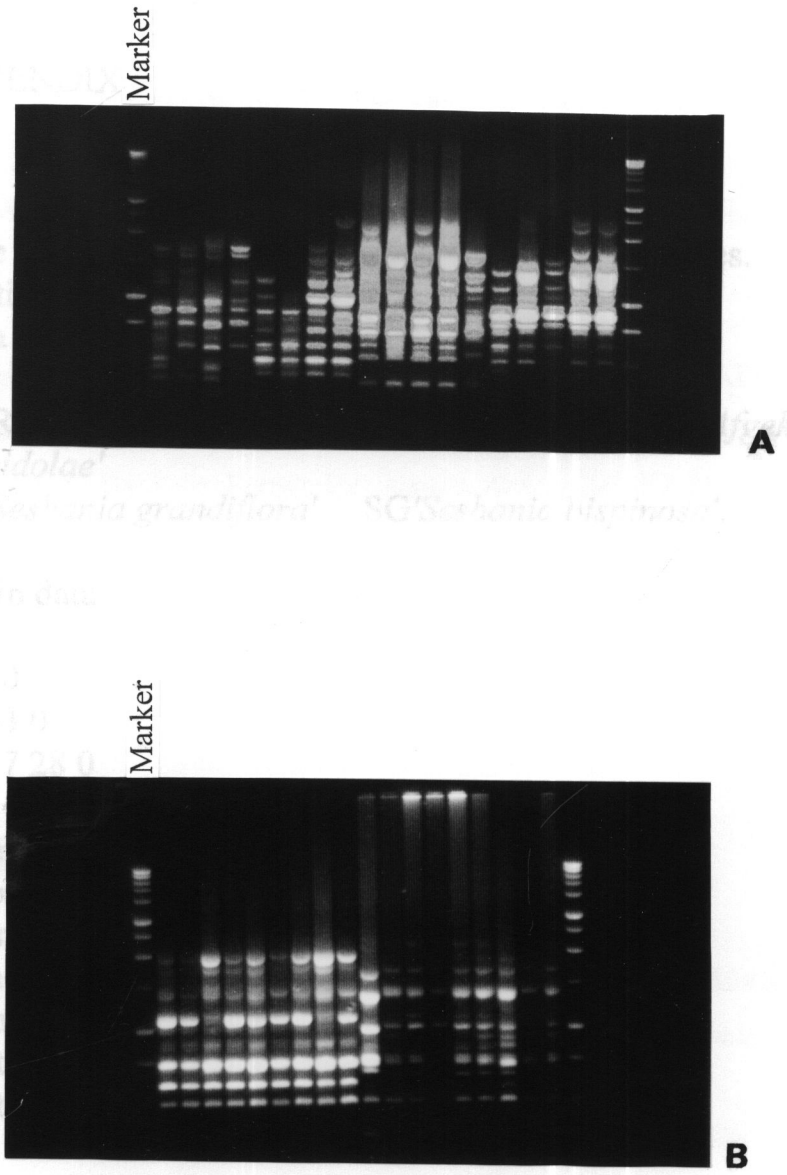

Figure 2. RAPD band profiles of *A. sericea* (lane 1-9) and *A. mahidolae* (lane 10-18) operated by primer OPAS07, A and OPAS19, B; M, molecular size marker (1kb ladder, Promega).

APPENDIX I

Title Phenetic RAPD data analysis of *Afgekia* species.
Subtitle cluster analysis.
Data list matrix free
/AS AM SB SG.
VARIABLE LABELS AS' *Afgekia sericea*' AM'*Afgekia mahidolae*'
SB'*Sesbania grandiflora*' SG'*Sesbania bispinosa*'.
n 4 .
Begin data
0
42 0
59 43 0
66 57 28 0
End data
set more = off
set printer = on
Cluster AS to SG
/Read = triangle
/Print=schedule cluster(1,3) distance
/Plot=vicicle(1,3) Dendrogram
/Method=BAVERAGE.

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GENERAL CONCLUSION AND DISCUSSION

Remedies of problems for RAPD-PCR

PCR methodology is known to be highly sensitive to the reaction condition (Samec and Nasinec, 1996). Since in routine species identification, the problems can arise with the RAPD pattern reproducibility and the RAPD evaluation of RAPD fingerprints when the assays are performed in various lab. The extraction of plant nucleic acid is fundamental requirement for genome characterization. However, the extraction of high molecular weight DNA is only one part of the task, as initial DNA extracts often contain large amounts of RNA, proteins, polysaccharides, tannins and pigments which are difficult to separate from the DNA . Most proteins can be removed by denaturation and precipitation from the extract, for example using chloroform and/or phenol. Treatment of the extract with heat-treated RNAse A will remove most of RNA. Polysaccharide-like contaminants are often more difficult to remove and, when present in high concentration, may cause the DNA extract to assume an almost gel-like constitution. Moreover, they may interfere in subsequent procedures even at relatively low concentrations. In this study, we chose the CTAB method as procedure for DNA extraction. This procedure has been successfully with a wide range of species of plants to give a good yield of reasonably high molecular weight DNA.

Staub et al. (1996) reported that DNA from young leaves provided the most consistent results when compared with old leaves. They suggested that this effect of tiisue age on RAPD phenotype may be due to elevated amounts of degradative enzymes and polysaccharides present in older tissue, which can alter DNA quality and quantity. Since DNAs were extracted from samples having the same fresh weight, there may have been variation in the amount of DNA in tissue of different ages due to differences in cell size between expanding vs. mature leaf tissue. With respect to the above reason, in this study DNAs were extract from unexpanded fresh leaf of plant materials for good yield.

The influence of the target concentration on RAPDs , reported by several authors (Schierwater and Ender, 1993), represent an additional drawback to the routine use of these molecular markers due to the DNA quantity previously required. However, Gallego and Martinez (1996) demonstrated that this problem is overcome since they found no differences in the RAPD profiles obtained over a template concentration ranging from 5 to 100 ng by their amplification reaction. Nevertheless, for reproducibility of RAPD assays, Stuab et al. (1996) suggested that it may be necessary to optimize reaction for specific lots of PCR reagents (i.e. 10X reaction buffer, $MgCl_2$ concentration stock solution and source of the DNA polymerase) from either commercial or in-house sources.

General conclusion

To date, *Afgekia sericea* and *A. mahidolae* have been recorded only in Thailand. The two species occupy clearly different habitats. *A. sericea* is widely distributed throughout the northeastern region, while *A. mahidolae* is confined to open limestone hill of western region. Although, *A. sericea* is widely distributed, but now, the remaining populations are very small. Similarly, because of *A. mahidolae* populations are small size and the geographic distribution of this species is restricted in lime-stone hill. With respect to the suggestion of Rabinowitz et al. (1986), the two species, therefore, are recognized as rare species.

In the present study, we initiated to study genetic relationships and genetic structure of *A. sericea* and *A. mahidolae* populations through chromosome and RAPD analyses.

Based on morphological and cytological data that found slight differences between the two taxa. Moreover, based on the presence vs. absence of RAPD markers illustrated that *A. sericea* and *A. mahidolae* are closely related species, and the two species may arose as a result of allopatric speciation in different geographical populations of the ancestral species. This conclusion supported by the high value of genetic similarity (S = 0.452) between the two species.

As formerly described, the two species were recognized as rare species, Beardmore (1996) suggested that genetic diversity within populations is of great concern to conservation biologist because paucity of genetic variation is thought to reduce the ability of population to adapt to changing environments, thereby causing their extinction. This conclusion corresponds to the fact that all populations of the two species are under the minimum effective population size of Franklin's recommendation (Franklin, 1980). He recommended that to maintain sufficient genetic variability for adaptation to changing environmental conditions, the minimum effective population size should be around 500. Thus a good conservation policies of rare species must be based on the sound understanding of the biology of the particular species and of the factors migrating against its natural survival. Moreover, the preservation of biodiversity and genetic diversity is a fundamental aim of conservation, since genetic diversity ameliorates survival potential by enhancing the adaptation of a species to present the future environmental changes (Rossetto et al., 1995).

The genetic structure of *A. sericea* and *A. mahidolae* populations measured by using Shannon's information index, Genetic variability within species (Hsp), *A. sericea* populations exhibited Hsp higher than those of *A. mahidolae*. In contrast, genetic variation within *A. mahidolae* populations (59%) were higher than those of *A. sericea* populations (52%). In addition, genetic divergence among populations of *A. sericea* and *A. mahidolae*, estimated by Nei Gst value, were high. *A. sericea* and *A. mahidolae* populations have Gst value of 0.426 and 0.266, respectively. These are consistent with low gene flow (Nm = 1.14 – 0.35) between populations of each species.

Afgekia mahidolae was recognized as a rare and endemic species to Thailand. Theoretically, the rare species are typically depauperate in terms of genetic variation. Our results contradict this view, but support other previously published results. Hunter (1996) recommended a species has a relatively high genetic variability among populations, it is necessary to maintain many different populations to maintain the species' genetic diversity. Thus, long-term survival of *A. sericea* and *A. mahidolae* populations is dependent on their habitat conservation (in situ conservation). Our results illustrated that the degree of genetic diversity in

populations of the two species are significantly survival potential to present and future environmental changes.