MORPHOMETRICS AND MOLECULAR SYSTEMATICS OF THE
GENUS *Afgekia* Craib (FABACEAE)

Mr. Yotsawate Sirichamorn

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Botany
Department of Botany
Faculty of Science
Chulalongkorn University
Academic Year 2006

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MORPHOMETRICS AND MOLECULAR SYSTEMATICS OF THE GENUS *Tarebia* Gould (PROSOBRANCHIA: THIARIDAE)

Mr. Somsak Panha

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biology
Department of Biology
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มอร์โฟเมทริกซ์และอนุกรมวิธานเชิงโมเลกุลของพืชสกุล *Afgekia* Craib

(FABACEAE)

นายยศเวท สิริจามร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2549
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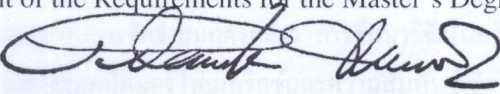|	
Thesis Title	**MORPHOMETRICS AND MOLECULAR SYSTEMATICS OF THE GENUS** *Afgekia* **Craib (FABACEAE)**
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Filed of Study	Botany
Thesis Advisor	Associate Professor Thaweesakdi Boonkerd, Ph.D.
Thesis Co-advisor	Tosak Seelanan, Ph.D.

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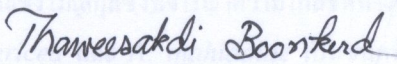
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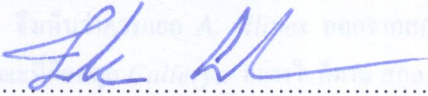
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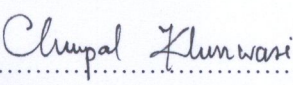
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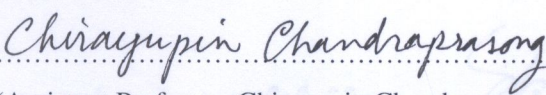
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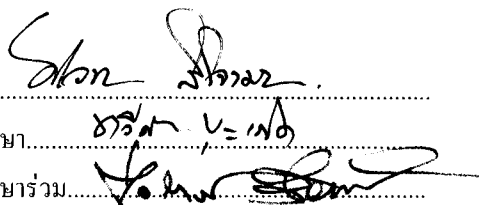(Assistant Professor. Chirayupin Chandraprasong)

ยศเวท สิริจามร : มอร์โฟเมทริกซ์และอนุกรมวิธานเชิงโมเลกุลของพืชสกุล *Afgekia* Craib (FABACEAE) (MORPHOMETRICS AND MOLECULAR SYSTEMATICS OF THE GENUS *Afgekia* Craib (FABACEAE)) อ. ที่ปรึกษา: รศ. ดร. ทวีศักดิ์ บุญเกิด, อ. ที่ปรึกษาร่วม: อ. ดร. ต่อศักดิ์ สีลานันท์, 74 หน้า.

สกุล *Afgekia* Craib เป็นพืชสกุลขนาดเล็กของวงศ์ Fabaceae เผ่า Millettieae ปัจจุบันมี 3 ชนิด คือ ถั่วแปบช้างหรือกันภัย (*A. sericea* Craib) กันภัยมหิดล (*A. mahidolae* Burtt et Chermsirivathana) และกันภัยพายัพหรือเครือเลือด (*A. filipes* (Dunn) Geesink) จากการศึกษา เบื้องต้น พบว่า *A. sericea* และ *A. mahidolae* มีสัณฐานวิทยาที่คล้ายคลึงกันมาก ในขณะที่ *A. filipes* มี ลักษณะหลายประการที่แตกต่างออกไปจากพืชสองชนิดแรกของสกุล *Afgekia* ประกอบกับมีลักษณะบาง ประการที่ร่วมกับสกุล *Callerya* Endl. ทำให้เกิดข้อสงสัยถึงสถานะภาพทางอนุกรมวิธานและความ เหมาะสมของระบบการจัดจำแนกดังกล่าว การศึกษาวิจัยในครั้งนี้จึงมุ่งเน้นการศึกษาถึงความสัมพันธ์ทางด้าน สัณฐานวิทยาด้วยเทคนิคมอร์โฟเมทริกซ์และความสัมพันธ์เชิงโมเลกุลด้วยเทคนิค RAPD และการศึกษา ลำดับนิวคลีโอไทด์บริเวณส่วน ITS ในนิวเคลียส และ *trnL-F* IGS spacer ในคลอโรพลาสต์ ตามลำดับ ผล จากการศึกษามอร์โฟเมทริกซ์ ด้วยวิธีการวิเคราะห์การจัดกลุ่มและการวิเคราะห์การจัดจำแนก โดยใช้ลักษณะ เชิงปริมาณ 29 ลักษณะ และลักษณะเชิงคุณภาพ 1 ลักษณะ พบว่าในการวิเคราะห์การจัดกลุ่มที่ค่าระยะห่างทาง อนุกรมวิธานประมาณ 0.76 พบว่า *A. sericea* และ *A. mahidolae* รวมอยู่ในกลุ่มเดียวกัน ในขณะที่ *A. filipes* แยกกลุ่มออกไป ทำนองเดียวกันกับผลการวิเคราะห์การจัดจำแนก พบว่า *A. sericea* และ *A. mahidolae* รวมอยู่ในกลุ่มเดียวกันและแยกจาก *A. filipes* ตามแกนจัดจำแนกที่ I เช่นเดียวกันกับผลจาก การศึกษาอนุกรมวิธานเชิงโมเลกุลซึ่งพิจารณาความใกล้ชิดทางพันธุกรรมและสายสัมพันธ์ทางวิวัฒนาการ ต่าง ก็สนับสนุนว่า *A. sericea* และ *A. mahidolae* เป็นชนิดที่มีความใกล้ชิดกันมากและมีบรรพบุรุษร่วมกัน ส่วน *A. filipes* นั้นไม่ได้เป็นส่วนหนึ่งของสกุล *Afgekia* ข้อสรุปจากการศึกษาสัณฐานวิทยา และ อนุกรมวิธานเชิงโมเลกุล จึงเห็นว่าควรแยก *A. filipes* ออกจากสกุล *Afgekia* โดยสกุลที่เหมาะสมที่สุด สำหรับ *A. filipes* ในขณะนี้คือสกุล *Callerya* อย่างไรก็ตาม สกุล *Callerya* เอง ก็ควรมีการศึกษาทบทวน ใหม่อีกครั้งเพื่อกำหนดขอบเขตและนิยามของสกุลให้ชัดเจนขึ้น

ภาควิชา..........พฤกษศาสตร์.................. ลายมือชื่อนิสิต...

สาขาวิชา..........พฤกษศาสตร์.................. ลายมือชื่ออาจารย์ที่ปรึกษา...

ปีการศึกษา..........2549.................. ลายมือชื่ออาจารย์ที่ปรึกษาร่วม..

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KEY WORDS: *Afgekia* Craib / MORPHOMETRICS / RAPD / *trnL-F* / IGS / ITS / FABACEAE/ MILLETTIEAE

>   YOTSAWATE SIRICHAMORN: MORPHOMETRICS AND MOLECULAR
>   SYSTEMATICS OF THE GENUS *Afgekia* Craib (FABACEAE)
>   THESIS ADVISOR : ASSOC. PROF. THAWEESAKDI BOONKERD, Ph.D.;
>   THESIS CO-ADVISOR : TOSAK SEELANAN, Ph.D., 74 pp.

   Afgekia Craib is a small genus of the tribe Millettieae in the family Fabaceae. According to the recent classification system, there are three species within this genus. They are *A. sericea* Craib, *A. mahidolae* Burtt et Chermsirivathana and *A. filipes* (Dunn) Geesink. *Afgekia sericea* and *A. mahidolae* are morphologically similar, while *A. filipes*, is rather different. In addition, this species also has some characters similar to those found in its closely related genus of *Afgekia*, i.e. *Callerya* Endl.. These discrepancy leads to uncertain taxonomic status and unsuitability of species treatment in this genus. Therefore, this research aims to explore the taxonomic status of the species in the genus *Afgekia* based on morphological and molecular data, which obtained from morphometrics, RAPD and DNA sequencing analyses in nuclear ITS and chloroplast *trnL-F* IGS repectively. In morphometrics study, 29 quantitative and 1 qualitative characters were analyzed by means of Cluster Analysis and Canonical Discriminant Analysis. The result showed that at average taxonomic distance 0.76, *A. sericea* and *A. mahidolae* were placed in the same group. While *A. filipes* was distinctly separated into another group. From the result of Canonical Discriminant Analyses, *A. sericea* and *A. mahidolae* were placed in the same groups on canonical axis 1 and separated from *A. filipes*. Likewise, the results of molecular systematics from both RAPD and DNA sequencing analysis agreed with the result from morphometric study. A close genetic similarity between *A. sericea* and *A. mahidolae* was observed. These two species were sister taxon which derived from the same ancestor. In contrast, *A. filipes*, is not in a section of *Afgekia* and should not belong to this genus. The most suitable genus to place *A. filipes* is likely to be *Callerya*. However, the genus *Callerya* itself requires further investigation for clearer delimitation and proper definition of the genus.

Department……...Botany……………….Student's signature…*Yotsawate Sirichamorn*
Field of study…...Botany……………… Advisor's signature…...*Thaweesakdi Boonkerd*
Academic year…..2006………….…..…Co-advisor's signature…...…………………...

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

It is generally accepted that Plant Taxonomy is a basis for all plant researches. However, modern Plant Taxonomy do need to have evidences from the other branches of Plant Science as criteria for plant identification as well as classification. From the last two decades there were many taxa have been revised based mainly on morphological and molecular data (Judd et al, 1999). These new sources of evidences have made it possible to construct phylogenetic relationships among related taxa. Recent phylogenetic analyses have not only strengthened many traditional classifications but also provided insight into the fundamental changes of our understanding in plant evolution. At present, the nature of systematic botany has been changed. Classification must ultimately reflect phylogeny (the actual evolutionary relationships) is now widely accepted (Hoch & Stephenson, 1995).

The pea family, Fabaceae is the third largest family of the flowering plants, after the Asteraceae and Orchidaceae (Polhill & Raven, 1981). Like many other families, its evolutionary trend and taxonomic status are still unstable and waiting for revisions. Especially in the Tribe Millettieae, there are many complex problems in classification. Until 1978, when the first international Legume Conference was held, members of Millettieae were transfered to the other tribes, such as Galegeae, Dalbergieae and Tephrosieae *sensu stricto* (Hu, 2000). The modern concept of the tribe Millettieae (formerly Tephrosieae) was firstly established by Polhill (1971), and further refined by Geesink (1981, 1984). However, there are still no unique characters to distinguish Millettieae from taxa of the other tribes, and they can only be poorly defined as a "non Dalbergieae-Brongniartieae-Robinieae-Phaseoleae" group (Geesink, 1984).

An example of taxonomic problems is found in the genus *Afgekia* Craib of the tribe Millettieae, *Afgekia* is a small genus of perennial climber native to Thailand and South East Asia. This genus was established by W.G. Craib in 1927 and was named after the initials of Arthur Francis George Kerr (1877-1942), an Irish-born plant collector who worked in Thailand in the early twentieth century. According to Geesink (1984), there are three species within this genus. All of them grew naturally in Thailand, but not in common habitat. They are called Kan phai or Thua peap chang, *Afgekia sericea* Craib; Kan-phai Mahidol, *A. mahidolae* Burtt et Chermsirivathana and Kan-phai payub, *A. filipes* (Dunn) Geesink. The first two species are rather similar in many aspects, but, there are still some differences.

Afgekia sericea and *A. mahidolae*, share morphological, cytological, ecological and genetic similarity. For example, the general habit, the size and shape of their stems; leaves, flowers, pods and seeds are similar. They have the long terminal raceme and flowering at the same period, i.e. May to October (Phan Kê Lôc & Vidal, 2001; Boonkerd, 2001). Moreover, they have the same number of their chromosome (Prathepha, 1994) and the recent data from RAPD analysis also reveal their genetic similarity of those two species (Prathepha, 1999).

The third species, *Afgekia filipes*, is quite remote from the first two species. Previously, it was named, *Adinobotrys filipes* and then was transferred to the genus *Padbruggea* Miq. and then to the genus *Afgekia*, viz. *A. filipes*. (Geesink, 1984). This species commonly occurs in hill evergreen forest of Southern China, Myanmar, Northern and north-western of Thailand. Geesink (1984) had transferred this species to the genus *Afgekia* by using the character of seed which having fleshy and elongated funiculus corresponding to the fleshy and elongated hilum. In addition, this species also has long pedicels and no bracteoles like the other two previous recognized species. However, there are some morphological characters of this species that are quite different from the former two species. In contrast, *Afgekia filipes* shares some characters with its related genus, *Callerya* Endl. and *Millettia* Wight & Arnott, for example, glabrous anthers, general habit, calyx shape, pseudo-axillary panicle inflorescence and the presence of reddish brown pigments in young leaves.

The discrepancy from the above mentioned information leads to uncertain taxonomic status and raised the question of the suitability of species treatment in this genus.

Aim of the thesis

This research intends to explore the taxonomic status of the species in the genus *Afgekia* based on morphometric and molecular data analyses. The results obtained from this research will also be served as new additional data for phylogenetic relationship in the tribe Millettieae.

CHAPTER II
LITERATURE REVIEW

2.1 Taxonomic History of the genus *Afgekia* Craib

The genus *Afgekia* Craib is belonged to the pea family, Fabaceae and tribe Millettieae sensu Geesink (1984), which is one of the most complex problems in Legume systematics. The genus was firstly established by W. G. Craib in 1927, in honor of Dr. A. F. G. Kerr, a distinguished Irish-born plant taxonomist who worked for Flora of Thailand Project in the early twentieth century. *Afgekia sericea* Craib is a type species, it was firstly found from Nakhon Rachasima in 1927 (Craib, 1931) and was recognized as an endemic species to Thailand since then. However, it was recently reported from Ha Giang, Vietnam (Solomon, 2007)

Afgekia was a monotypic genus until 1967, the second species, *Afgekia mahidolae* Burtt and C. Chermsirivathana was found on a limestone hill in Kanchanaburi Province (Burtt and Chermsirivathana, 1971). This plant was named in honor of Her Royal Highness, the Princess Mother, Somdej Phra Sri Nakarindra Baromraj Chonni, whose interest in Thai flora is well known. Nowadays, this plant became the symbolic plant of Mahidol University. *Afgekia mahidolae* has some morphological characters similar to *A. sericea* but slightly differ in some aspects. For example, the flowers of *A. sericea* are purplish pink. The standard is pinkish ivory with pink patches on both sides at the base and a yellow patch in the middle. The wings are purplish pink and having two basal appendage on the upper margin. In contrast, the flower of *A. mahidolae* is purple. The standard is purple with yellow central patches. The wings are deep purple and it clearly differs from *A. sericea* by having one basal appendage and hairs at the top of the style.

The third species, *A. filipes* (Dunn) Geesink has a long taxonomic history. Previously, *Adinobotrys filipes* Dunn was transferred to *Padbruggea filipes* by W. G. Craib in 1928. Then, in 1984, R. Geesink had revised the tribe Millettieae, he considered the genus *Padbruggea* as a synonym of the genus *Callerya*. Since the general habit, the shape of the calyx and the glabrous anthers of *Padbruggea filipes* are indeed similar to certain species of *Callerya*. It differs only in the absence of bracteoles and in having long pedicels. However, Geesink studied seeds of this plant and found that the seed also has an elongated fleshy funicle with a corresponding elongated hilum which is a typical character of the Afgekias, but this character is much more distinct in the previous recognized species of *Afgekia.* He concluded that the characters in which this species

differs from the species of *Callerya* are all the characters of *Afgekia*. Therefore, he transferred this species to the genus *Afgekia* and this classification system has been used since then (Geesink, 1984).

In 2005, Deng and Qin had made a new combination in the genus *Afgekia*, i.e. *A. filipes* (Dunn) Geesink var. *tomentosa* (Z. Wei) Y.F. Deng and H.N. Qin. This new combination is transferred from *Whitfordiodendron filipes* Dunn var. *tomentosa* Z. Wei. They concluded that *A. filipes* var. *tomentosa* differs from var. *filipes* mainly in its coriaceous leaflets, and the pubescence of the inflorescence axis, the hairs are bright brown in color (Deng and Qin, 2005).

In addition, the key to genus from the morphological study of Geesink also implies the heterogeneity in this genus, i.e., *A. filipes* is morphologically similar to the genus *Callerya* (*Padbruggea*), while *A. sericea* and of *A. mahidolae* is morphologically related to the genus *Wisteria* Nutt. (Geesink, 1984).

2.2 Ecology and Utility Review

Afgekia sericea occurs naturally in the primary forest at low and medium altitudes, climbing on tree, or in thickets covered small shrubs, usually at or near the margin of the dry dipterocarp forest (Fig. 2.2 A, B). In Thailand, it is apparently confined to the Korat Plateau of north eastern of Thailand. It becomes a rare species due to fragmentation of its natural habitats by increasing need of land for agricultural purposes, housing and road construction. (Boonkerd, 1992, 2001; Prathepha, 1999 and Chourykaew et al., 2002) So far, only one population is in the protected area of the Sakaerat Research Experiment Station in Nakhon Ratchasima Province. During dry summer months, *A. sericea* is usually experienced forest fires, resulted in wilting and subsequently dry out of young branches and young pods. However, the basal stems are still survived, at the early beginning of the rainy season, new branches are produced from these stems, subsequently develop inflorescence (Boonkerd, 1992). Flowering period normally starts from mid of May to the end of October. Premature pods can be seen by the end of October and mature pods are found during March and April (Chourykaew et al., 2002).

Likewise, *A. mahidolae* occurs naturally in the low altitudes and low relative humidity forest and rather restrict to limestone hills (Fig. 2.2 C, D). So far, this species has a sporadical distribution in western part of Thailand, especially in Sai Yok district, Kanchanaburi Province. The above ground plant-parts become drooping and die in early dry season, but, in the early next rainy season, the new branches can be produced from

the remaining underground stem. Flowering period of *A. mahidolae*, and *A. sericea* are consistent, starting from mid of May to the end of October. It became rare and endanger species due to its small population size and geographical distribution of this species is restricted in limestone hill. (Prathepha, 1999).

Afgekia sericea and *A. mahidolae* are occasionally used as ornamental plants in Thailand because their showy beautiful inflorescence and their auspicious Thai name, i.e. Kan phai that means "protection against danger" in Thai. Seeds and roots of *A. sericea* are sometimes pharmaceutically used for Anorexia and child's Nutrient deficiency. (Chuakul et al. (eds), 1995) For *A. mahidolae*, this species became the symbolic plant of Mahidol University, Thailand in 1999 and was cultivated throughout the area of the University and its campus.

Of the three species in the genus *Afgekia*, *A. filipes* has the most wide distribution. It occurs naturally in southern China, Myanmar, Laos, Vietnam and northern Thailand (Phan Kê Lôc & Vidal, 2001), usually in the hill evergreen forest. It is a canopy liana which is difficult to notice (Fig. 2.2 E, F). Flowering period of *A. filipes* normally starts from mid of February to May. Mature pods are found during November to January. Seedlings of *A. filipes* are commonly found on the forest ground underneath the liana, without problems of seed germination or destruction by natural pest or pathogen. This species also has no or little problems of natural habitat destruction by human's activities, as was found in the first two species.

So far, *A. filipes* is rarely been utilized by man. However, it is cultivated as ornamental plants at Royal Agricultural Station Angkhang, Doi Angkhang, Chiang Mai province.

Pollination Syndrome in *Afgekia*

As mentioned earlier, *A. sericea* and *A. mahidolae* have been recognized as rare species (Boonkerd, 1992 and Prathepha, 1999). The amount of fruit setting in *A. sericea* is rather small, despite its long inflorescence with 100-400 florets (Boonkerd, 1992). As far as it is known, none or 1- 2 mature pods per inflorescence can be observed. The problem of pod setting under natural habitat is usually related to drought, as has been point out by Boonkerd (1987). However, it is surprising that *A. sericea* grown in non-natural habitat like Bangkok never produces a pod, despite plenty number of complete flowers and sufficient supply of water. It is found that plants grow in their natural habitat

produce flowers mainly during the rainy season while plants raised in Bangkok produce flowers almost all year round. The failure of fruit setting may be due to, at least in part that there may be some problems occur with the pollination of this species, for example unsuccessful pollen germination (Boonkerd, 1992). It was found that pollinators play an important role for successful fruit setting. There are nineteen insects and one bird species found to be visitors but only twelve might take part in the pollination, of which *Megachile velutina* Smith is likely a main pollinator for *A. sericea* (Chourykaew et al., 2002). In addition, bagging experiments yielded no fruit setting, while open pollination that allowed insect to visit flowers, resulted in fruit setting. It might be concluded that *A. sericea* is likely a cross-pollinating species and is self-incompatible. Thus, possible causes of low fruit setting might be the existence of self-incompatibility and young fruit abortion (Chourykaew et al., 2002).

2.3 Numerical Taxonomy

Presently, taxonomic evidences for establishment of classification and phylogeny are gathered from various sources. Since all parts of plant at all stages of its development can provide taxonomic characters, so taxonomic data must be assembled from many diverse disciplines i.e., comparative anatomy, embryology, palynology, cytogenetics, chemistry and so on (Jones and Luchsinger, 1987).

Numerical Taxonomy is the application of standard statistics and standard mathematics to solve the taxonomic problems (Clifford and Stephenson, 1975). The main objective of numerical methods is to simplify and portray degrees of relationship or similarity among any groups of organisms. This method utilizes many equally weighted characters and employs clustering and similar algorithms to yield objective grouping (Sneath and Sokal, 1973).

Sneath and Sokal (1973) summarized the following advantages of the numerical methods:

(a) The ability of the methods to construct a system of classification using various characters from different sources together, such as morphology, physiology, anatomy, cytology, genetics, chemistry, ecology and paleobotany;

(b) The ability of the methods to carry out efficiently by less highly skilled workers, since large portions of the taxonomic process are being done by a computer;

(c) The ability of the methods to provide greater discrimination along the spectrum of taxonomic differences and to be more sensitive in delimiting taxa. Thus they should give better classifications and keys than can be obtained by conventional methods.

Numerical taxonomy is actually an extension of Adansonian classification (Sneath and Sokal, 1973). It obtains principles in making classification established by Adanson (1727-1806). The followings are summary of Adanson's opinions on classification quoted by Sneath and Sokal (1973):

• The greater the content of information in the taxa of a classification and the more characters on which it is based, the better a given classification will be.

• Every character is of equal weight in creating natural taxa.

• Overall similarity between any two entities is a function of their individual similarities in each of the many characters in which they are being compared.

• Distinct taxa can be recognized because correlation of characters differs in the groups or organisms under study.

• Phylogenetic inferences can be made from the taxonomic structures of a group and from character correlation, given certain assumptions about evolutionary pathways and mechanisms.

• Taxonomy is viewed and practiced as an empirical (practical) science.

• Classifications are based on phenetic similarity.

Methods in Numerical Taxonomy

Numerical taxonomy requires many steps in practice:

1. selection of taxa for study (those are usually called Operational Taxonomic Units or OTUs) which are simply the starting point units in numerical taxonomy; they might be individual organisms, populations, species, genera, etc;

2. selection of characters;

3. description and/or measurement of character states;

4. comparision of states to (a) determine a measure of overall similarity between each pair of OTUs, and (b) determine the taxonomic structure, i.e. the detection of possible groups and subgroups among all OTUs;

5. ranking of all OTUs into the categories of the taxonomic hierarchy.

The Numerical Techniques

Cluster Analysis (CA)

The aim of this numerical technique is to place individual specimens into groups (Boonkerd et al., 2002). Moreover, cluster analysis is also used to identify a small number of groups such that elements belonging to a given group are, in some sense, more similar to each other than to elements belonging to other groups. Consequently, cluster analysis can be considered as another technique for data reduction (Dillon and Goldstein, 1984).

1. Steps of Cluster Analysis

Cluster analysis is a two-step process. The first step, a symmetric matrix of similarity or dissimilarity among objects will be calculated. The second step, a hierarchical grouping of these objects will be generated, and represented these in a cluster diagram, dendogram or phenogram. In each step, decisions must be made regarding the type of coefficient or clustering technique used; as such, there are a large number of ways a given data set may be analyzed.

2. Steps of Clustering Algorithms

In general, there are 5 specific steps in clustering:

1. Given a matrix of pairwise similarities/dissimilarities, find mm (d_{ij});

2. Define the depth of a branch between those OTUs as either d_{ij} or ($d_{ij}/2$);

3. Establish a new cluster containing OTUs i and j (i and j merged into a single OTU);

4. Define the distance from this cluster to all other OTUs/clusters by recomputing symmetric matrix with 1 less OTU;

5. Go back to step 1 and repeat.

This process is repeated until only one OTU remains. Alternative clustering methods differ primarily in how new distances will be defined in step 4. UPGMA clustering defines the distance of an OTU to a newly-established cluster as the arithmetic average of all component distances. Single linkage clustering uses the minimum distance among the component distances as the distance of an OUT from the new cluster, while complete linkage clustering uses the maximum of the component distances. Single and complete linkage represent extremes along a continuum, while UPGMA is in the middle.
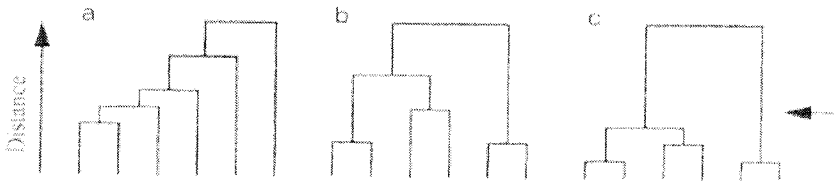
Figure 2.1 Dendrogram representative of (a) single-linkage, (b) UPGMA-lingkage and (c) complete-lingkage clustering

3. Clustering

Once we have a symmetric matrix of similarity/dissimilarity between all pairs of OTUs, we can then group these into sets of similar objects. Most commonly- used clustering algorithm is sequential, agglomerative, hierarchical, and non- overlapping; this type of clustering is known as SAHN clustering. Clustering is very straightforward, and proceeds by iteratively grouping the most similar OTUs and then recalculating the similarities/dissimilarities among those that remain. In this way, we generate a direct solution that is based on local optima.

Two procedures are involved in every step in the clustering procedure:

(1) joining of the most-similar OTUs to form clusters; and

(2) re-computation of the similarity/dissimilarity between newly-established clusters and potential new members. New members are admitted to clusters in a pair-group manner, which generates a bifurcating tree (phenogram, dendogram); alternatively, admission may be variable-group, which allows more than two OTUs at a node.

4. Dendrogram

A dendrogram is a diagrammatic illustration of relationships based on degree of similarity in which the OTUs are linked together at various levels of resemblance. It shows progressive inclusion of entity or groups of entities into larger groups so that the result is a set of hierarchically arranged groups within groups.

Groups are represented by stems. The inclusion of several groups into one group occurs at a node, and is depicted by a crossbar. The height at which each node occurs is known as its rank, and is based on a measure of resemblance.

The dendrogram is provided with a quantitative scale of the overall dissimilarity between groups. The most similar groups fuse first, followed by others fusing at

successively lower levels of similarities. The degree of resemblance of the clusters and the extent to which they are distinct from other is displayed very clearly.

Canonical Discriminant Analysis (CDA)

The concepts of CDA can be described as following.

The differences between the groups can be established statistically by means of a test of the probability of the null hypothesis that all the groups of objects have the same centroid (multivariate mean). If there is only one measured variable, this is equivalent to the one-way analysis of variance for comparing two or more groups.

The relationships between the groups can be assessed visually by means of a scatter plot in which the positions of the individuals or the group means or both are plotted on axes known as canonical axes (discriminant functions) which depend on the original observations and are chosen by the analysis to best represent the differences between the groups.

Canonical discriminant analysis (CDA) is sometimes known simply as "canonical analysis".

1. Principles of Canomcal Discriminant Analysis

This separation of groups (for two groups) is accomplished by finding a linear combination of the original variables for which the F value between groups is maximized.

In the cases of more than two groups, we get a set of canonical discriminant functions (canonical variates), or multiple axes that separate groups as sets of groups. Assuming that there are more variables than groups, there will be (m-1) canonical variates, where m is the number of groups. It is important to see the difference between Fisher discriminant functions and canonical discriminnat functions: discriminant functions connect pairs of centroids, while canonical variates summarize the major axes of among-group variation.

Canonical discriminant functions fit the equation:

$$Z_1 = a_{11}x_1 + a_{12}x_2 + a_{13}x_3 + \ldots + a_{1n}x_n$$

where the a_{ij} values are coefficients derived from Eigen analysis of the matrix between group variation, and the Z values are scores (coordinates) along the derived axis. If we bisect this axis, we can use the scores to assign individuals to groups at each end of the axis.

2. The step in Canonical Discriminant Analysis

    1). Transform the variables so that the pooled within-class covariance matrix is an identity matrix.

    2). Compute class means on the transformed variables.

    3). Discriminant function is created from data. The values of the discriminant function should be differed as much as possible between the groups, or so that for the discriminant scores are a maximum.

    4). Predicting or classifying new case using the discriminant function from step 3.

3. The Goal of Canonical Discriminant Analysis

    1). To find the axis of greatest discrimination between groups identified a priori.

    2). To test whether the means of those groups along that axis are significantly different.

    3). To attempt to assign individual specimen to a group.

In discriminant analysis there is an implied assumption that the groups are known a *priori*; that is, all of the observations are assumed to be correctly classified at the outset. In many research settings, however, the social scientist is unsure of the natural groupings that might be present. In such cases, the researcher may have no choice but to rely on the available measurements for the purpose of deciding whether the observations fall into constituent groups, and if so, to delineate the groups. Thus, in discriminant analysis we begin with a priori well-defined groups and ask how the given groups differ, whereas in cluster analysis we begin with groups that are initially undifferentiated and ask whether a given group can be partitioned into subgroups that differ in some meaningful way (Dillon and Goldstein, 1984).

Numerical studies have been performed on wide variety of organisms and these approaches have shown to be useful in evaluating and refining the existing classification. For the genus *Afgekia*, Boonkerd (2001), studied the Morphometric relationships among three population of *A. sericea*, which grows wild in N.E. Thailand. In this study, fifteen characters of both vegetative and reproductive parts were examined in 400 specimens using univariate and multivariate analyses. The result showed that cluster analysis and canonical discriminant analysis did not result in recognizable grouping of OTUs on a population basis. However, the standard size of the typical papilionaceous flower-form of the bean family tends to be useful as a diagnostic character for population-diversity study.

Moreover, the numerical approaches by means of morphometric were also carried out on various types of Leguminosae to clarify the complexity of their taxonomic status as listed below.

The morphometric study of seed were carried out in *Cytisophyllum, Cytisus, Chamaecytisus* and *Genista* from 43 populations and belonging to 22 species (Gonzalez-Anders and Ortiz 1995). This study consisted of quantitative characters and one qualitative character, testa color. Aims to find out if seed morphology alone can be used to clarify the confusion in taxonomic status of the group. The result showed that the characters responsible for maximum separation were the seed length, the maximum projected area, the color and the width of the aril. The results support the genera grouping established by Bisby, 1981, based in other morphological and biochemical studies.

Boonkerd, Saengmanee and Baum (2002), studied the variety of *Bauhinia pottsii* G. Don in Thailand on the basis of morphometric multivariate analysis. In this study, quantitative and qualitative morphological characters were examined in 200 specimens using multivariate and univariate analyses to determine the taxonomic relationship among the four varieties of *B. pottsii*. The result showed that some variation in qualitative characters was found between the varieties which separated them as previously defined in Flora of Thailand. Forty-three quantitative characters were subjected to cluster analysis to allow an objective classification into groups. The groups were subsequently evaluated by a canonical discriminant analysis. It was also found that these characters collectively support the four varieties as defined by qualitative characters. Among quantitative

characters, petal-claw length and ovary-stalk length together with some qualitative characters are useful for key construction to separate the four varieties.

Boonkerd, Pechsri and Baum (2005), performed a phenetic study of *Cassia* L. sensu lato. This big genus was subdivided in to 3 genera namely *Cassia* s. str., *Chamaecrista* and *Senna* (Irwin and Barneby, 1981). In order to investigate this new classification, 508 specimens of 18 taxa of the genus *Cassia* s.l. grown in Thailand were analyzed using cluster analysis and canonical discriminant analysis. In this study, the total 32 vegetative and reproductive morphological characters were employed. The result of cluster analysis showed that *Cassia* s.l. can be segregated into four groups, respectively viz. *Chamaecrista*, *Senna alata*, *Senna* and *Cassia* s. str. From a canonical discriminant analysis using the four-cluster grouping as a priori groups, it can be concluded that *Cassia* s. str., *Senna*, and *Chamaecrista* are indeed distinct taxa. The three most important characters that separate the three genera are filament length, fruit length, and ovary stalk length. Among the three genera, it was also found that *Senna* is rather a heterogeneous taxon.

Acacia aroma, *A. macracantha*, *A. caven*, and *A. furcatispina* were studied using morphometric techniques by Casiva et al. (2002). They used a correlation between species for morphometric analysis to obtain a phenogram that represented phenetic similarities among species. The results of cluster and principal component analyses agree with those obtained from molecular data. It was found that *Acacia aroma* and *A. macracantha* had the highest similarity, while *A. furcatispina* is the most differentiated species.

Recently, *Vicia* L. ser. *Vicia*, which includes the *Vicia sativa* aggregate, was examined using morhometric analyses (van de Wouw, Maxted, and Ford-Lloyd (2003). This study attempts to investigate the taxonomic relationships within the *Vicia sativa* aggregate by novel investigations along with a synthesis of the results obtained by previous researchers. Cluster analysis on the basis of 53 morphological characters justified the delimitation of four species within the series and six subspecies within the *Vicia sativa* aggregate. The morphometric data were coded and used in a phylogenetic analysis to study the relationships between the taxa. The phylogenetic analysis, using 33 variables, showed *Vicia pyrenaica* closely related to *Vicia sativa* subsp. *amphicarpa*.

2.4 Cytological Study Review

Cytology refers to the study of the cell. The information about the chromosomes i.e, chromosome number, shape, or pairing at meiosis is widely used for classification purposes (Jones and Luchsinger, 1987). Chromosome number, by itself, may be a useful systematic character. Similar chromosome numbers may indicate close relationship; different chromosome numbers often create some reproductive isolation through reduce fertility of hybrids, chromosome size, the position of the centromere, special banding patterns, and other features may also be systematically informative.

The cytological study on the genus *Afgekia* was performed by Prathepha, (1994). The result from his study showed that *Afgekia sericea* and *A. mahidolae* have the same chromosome number of 2n = 16 and are known as diploids. This chromosome number is the same number as most of the genus *Callerya* and *Wisteria* (Hu et al., 2002) The chromosomes are small in size which *A. sericea* (3.8 and 6.3 μm, 4.7±0.8) is slightly smaller than that of *A. mahidolae* (3.8 and 7.5 μm, 5.4±1.0). Meiotic studies in both species show 8 bivalents at metaphase I and the configuration is in the same pattern which exhibits seven ring bivalents and a rod bivalent.

2.5 Molecular Study Review

2.5.1 Random Amplification of Polymorphic DNA (RAPD)

The development of the polymerase chain reaction (PCR) has a significant impact in almost all areas of molecular biology (Saiki et al.,1988) and modifications of the basic procedure have allowed the development of numerous assays for detecting variation at the nucleotide level (Korner and Livak, 1989).

Randomly amplified polymorphic DNA (RAPD) is an example of PCR application used to accomplish the study of genetic polymorphism among diverse populations of organisms. RAPD is a technique that relies on the generation of amplification products for a given nucleic acid using an amplification-based scanning technique driven by arbitrary priming oligonucleotides. The result is the generation of amplification products (amplicons) that represent a multiplicity of anonymous sites that are characteristic of the studied genome (Dassanayake and Samaranayake, 2003).

A single primer approx 10 bp in size (40-70% G-C content) is generally used in RAPD technique. For the amplification of the target region, the distance between priming regions has to be not more than 3 to 4 Kb. (Caetano-Anolles, Bassam and Gresshoff, 1992.) Depending on the number of inverted complementary priming sites in an individual's genome, and lengths of the intervening DNA sequence, a given primer may amplify from 0 to 30 products (Prathepha, 1999). Because RAPD-PCR primers are not designed to amplify a specific target sequence, the amplified loci are anonymous and presumably scattered throughout the genome. RAPD loci carry the advantages that

1) RAPDs have considerable appeal for surveys of genomic variation and the identification of DNA-based characters for systematics since they are relatively inexpensive, randomly sample a potentially large number of loci and sequence information is not necessary for primer design (Huff et at., 1993; Williams et at., 1993), thus the technology can be easily applied to any taxon (Avise, 1994).

2) many of the loci may be acting as neutral markers (Prathepha, 1999).

Unfortunately, the technical simplicity of. the RAPDs procedure has obscured the difficulties of understanding product banding patterns which have led to criticisms of this approach (Harris, 1999).

Problems and limitation with RAPD

The Problems and limitation of RAPDs may conveniently be summarized as practical problems associated with technology and intellectual problems associated with the RAPD phenomenon, some of which are discussed at more length below.

1) Reproducibility

Reproducibility is an acknowledged problem in RAPD analyses and strict procedural standardization is required to obtain reproducible results (e.g. Williams et al., 1993; Jones et al., 1997). Factors that influence RAPD reproducibility include: (i) primer and primer concentration; (ii) Taq polymerase source; (iii) magnesium ion concentration; (iv) template concentration; (v) thermocycler; (vi) temperature profile (Williams et al., 1993; Staub et at., 1996). However, in strictly controlled situations within a single laboratory RAPD reproducibility problems can be overcome, although problems still exist with respect to transferring markers between laboratories, particularly if different gel systems or thermocyclers are routinely in use. Staub et al. (1996) suggested that for

reproducible PCR results it may be necessary to optimize reactions for specific lots of PCR reagents.

2) Primer structure.

There are 1,048,576 possible combinations of ten bases for RAPD primers. However, the majority of RAPD primers, rather than having random nucleotide contents, are constrained to have 60-70% GC (24,576 possible combinations) (Williams et al., 1993). It is known that GC content is not evenly distributed in plant genomes (Li and Graur, 1991), thus the RAPD technique may preferentially screen GC-rich regions. The high GC content of RAPD primers appears to be necessary for successful low temperature annealing that allows a degree of imperfect priming (Welsh and McClelland, 1994), and is consistent with the observation that the number of amplified PCR products is positively correlated with the GC content of the RAPD primer (Caetano-Anollés et al., 1991; Bucci and Menozzi, 1995, cited in Harris, 1999). Mismatch offered by GC-rich RAPD primers means that there is possible that different primers bind at the same or overlapping priming sites and that nested priming may occur. Thus products from different primers may be either identical or interdependent.

3) Dominance

Direct analysis of ethidium bromide-stained gels in RAPD analysis makes the implicit assumption that RAPD markers are dominant, these markers are scored either present (+) or absent (−), and therefore dominant homozygotes (++) and heterozygotes (+−) are indistinguishable from each other. Dominance of RAPD markers means that the estimation of genetic diversity and partitioning can only be made indirectly.

4) Homology

Homology between RAPD products is most frequently based on the assumption that products that migrate to the same position on a gel are identical by descent, and is similar to the scoring of restriction fragment data (Bremer, 1991). Products of the same size may be non-homologous due to: (i) limitations in gel resolution; and (ii) size convergence, due to deletions and insertions between primer sites and primer site loss/gain (Williams et al., 1993; Smith et al., 1994, cited in Harris, 1999). Furthermore, primer site distribution may result in products that display partial sequence

similarity and hence are not independent (Smith et al., 1994, cited in Harris, 1999). Such problems are presumably correlated with genetic distance, becoming more significant for comparisons between distantly related species than within species or between closely related species.

5) Allelic variation

Direct visualization of RAPD products assumes that there are only two alleles (+ or −) per locus. Two models have been applied for understanding the systematic consequences of allelic variation at a locus, the 'allele-as-character' model (independent allele model) and the 'locus-as-character' model (Buth, 1984, cited in Harris, 1999). The 'allele-as-character' model treats individual alleles and their presence or absence as binary characters. Unfortunately alleles in this model are not independent since they must sum to one; if one allele decreases in frequency then the other must increase. Thus the 'loss' of a RAPD product is not an independent event but must involve either the occurrence of a new allele or the change in frequency of an existing allele. Similar RAPD phenotypes may therefore mask different transformation events.

The failure of the 'allele-as-character' model has been recognized and corrections have been tried using a 'locus-as-character' model, where different allelic combinations are used as the character's states (Buth, 1984, cited in Harris, 1999). This model requires positive allele detection, although evidence exists that RAPDs may be multi-allelic (Haymer, 1994, cited in Harris, 1999), while population genetic models assume that RAPD loci are di-allelic (Lynch and Mulligan, 1994, cited in Harris, 1999), and allele 'loss' may be the result of different events. Thus there is no simple way of identifying alleles or their combinations at different loci, meaning that the 'allele-as-character' model is not suitable for the analysis of RAPD data.

In spite of the fact that this technique has some problems or limitations, RAPD plays many important roles in Fabaceae systematics. Samec and Nasinec (1996), used RAPD technique for the identification and classification of *Pisum sativum* L. genotypes. Each genotype was clearly identified and separated from the others. The results showed that RAPD technology is a rapid, precise and sensitive technique for identification of pea genotypes. In the same year, Liu (1996), had published the study of using RAPD analysis on genetic diversity and relationships among *Lablab purpureus* genotypes. He found a high level of genetic variation in this species but this was mainly restricted to the

difference between cultivated and wild forms. Kongkiatngam et al. (1996), examined genetic variation among cultivars of red clover (*Trifolium pratense* L.) by RAPD markers. The amplification patterns obtained from two primers could distinguish all 15 red clover cultivars. Cluster analysis based on these genetic distances separated these 15 cultivars into three groups, with two of the groups consisting of a single Japanese cultivar each, while the third group included cultivars from European, North American, and Japanese origins. Kaga et al. (1996) also investigated species relationships in the subgenus *Ceratotropis* of the genus *Vigna* by using RAPD analysis. In this study, the accessions used were separated into two main groups with an average of 70% differences. Within the main groups, five subgroups were recognized, which are completely agreement with taxonomic species.

Acharya, Mukherjee, and Panda (2004) used RAPD technique to study genome relationship among nine species of the tribe Millettieae, excluding the genus *Afgekia*. The results supported the clear grouping of species under their respective genera, inter- and intra-generic classification and phylogeny and also merger of *Pongamia* with *Millettia*. This also elevated *Tephrosia purpurea* var. *pumila* to the rank of a species (*T. pumila*) based on morphological characters which is also supported through this study of molecular markers.

The application of RAPD analysis to detect the genetic variation of the genus *Afgekia* was studied by Prathepha in 1999. He determined phylogenetic relationship between *A. sericea* and *A. mahidolae* and evaluated population genetic structure of natural populations of these two species. He also combined the RAPD information with the data from his previous karyotypic (Prathepha, 1994), ecological and morphological study (Prathepha, 1990). He concluded from the results of this study that *A. sericea* and *A. mahidolae* are monophyletically related species which became differentiated as a result of Allopatric speciation. This assumption was also supported by the paleogeological data of Thailand.

2.5.2 DNA Sequencing Analysis

In the past, taxonomic data was generally collected from morphological variation. They are useful for the delimitation of genera and species but have overlapped at higher taxa (e.g. tribal level). Molecular data have revolutionized our view of

phylogenetic relationships, although not for the reasons initially suggested. Early proponents of molecular systematics claimed that molecular data were more likely to reflect the true phylogeny than morphological data, ostensibly because they reflected gene-level changes, which were thought to be less subject to convergence and parallelism than were morphological traits. As a result, molecular data are now widely used for generating phylogenetic hypotheses (Judd et al., 1999).

The chloroplast encoded *trnL-F* region

Chloroplast (cp) DNA genome is a circular molecule and subdivided into two single copy regions, the large single copy (LSC) region and the small single copy (SSC) region, which are separated by inverted repeats. The LSC region is slightly less conserved in sequence than the rest of the chloroplast genome (Clegg, Learn and Golenberg, 1991); hence it is potentially more useful for studies at lower taxonomic levels. Interspecific cpDNA polymorphisms are predominant in the form of length variation due to insertion/deletion mutations (McCauley, 1995) and variation in copy number of mononucleotide microsatellite repeats (Powell, Morgante and Andre, 1995). Sequences from noncoding regions of the cpDNA genome are often used in systematics because such regions tend to evolve relatively rapidly, for example the *trnL-F* intergenic spacer (Gielly and Taberlet, 1994), the *atpB-rbcL* intergenic spacer (Hodges and Arnold, 1994), the *rbcL*-psal intergenic region (Morton and Clegg, 1993).

The cpDNA *trnT-L-F* region in land plants consists of the transfer RNA genes *trnT*[UGU], *trnL*[UAA] and *trnF*[GAA] arranged in tandem, separated by non-coding spacer regions. It is positioned in the large single copy region, approximately 8 kbp downstream of *rbcL*. The *trnL* gene of cyanobacteria and a number of chloroplast genomes, including that of all land plants, contain a group-I intron positioned between the U and the A of the UAA anticodon loop. This intron is inferred by phylogenetic analysis to have been present in the cyanobacterial ancestor of the plastid lineages of Rhodophyta, Chlorophyta and Gluacocystophyta (Besendahl et at., 2000).

The succession of conserved *trn* genes and the apparent absence of gene rearrangements in the *trnL-F* region facilitated the design of plant universal primers by Taberlet et at., (1991), in particular the *trnL* intron and *trnL-F* spacer (collectively, the *trnL-F* region) has become one of the most widely used chloroplast markers for phylogenetic analysis in plants (Quandt et at., 2004).

Internal Transcribed Spacer (ITS) Region

Nuclear ribosomal DNA (nrDNA) is organized as individual chromosomal units that are repeated thousands of times in the most of higher plant genomes. Each of these units contains the three genes that encode the 1 8S, 5.8S and 26S ribosomal RNA subunits, as well as several different spacer DNA regions. The nucleotide sequence variation found in both of the internal transcribed spacer regions (ITS-1 and ITS-2) is used extensively for the systematic analysis of closely related taxa because it has the rapid rate of evolutionary change, useful for assessing relationships at lower taxonomic levels as species levels. (Baldwin et al, 1995). The ITS sequences have proven to be a valuable source of characters to address phylogenetic relationships among closely related species in different plant families (Francisco-Ortega et at., 2001). The ITS sequences have also proven powerful in revealing hybridization and reticulate evolution (e.g. Sang, Crawford and Stuessy, 1997)

Many researches related to phylogenetic study of the tribe Millettieae based on data from DNA sequencing were carried out. For example, the phylogenetic systematics of the tribe Millettieae were examined based on the study of the chloroplast *trnK/matK* sequence (Hu et al., 2000) and ribosomal ITS regions (Hu et al., 2002). In these researches, one or two species of the genus *Afgekia* were include and the result showed the molecular relation to the genus *Callerya* and *Wisteria*. The three genera, *Afgekia*, *Callerya* and *Wisteria* were placed in the same clade called "Callerya group" where all the legumes in this group lack one copy of the inverted repeat (IR) in the chloroplast genome (Hu, 2000; Hu and Chang, 2003). The result from ITS phylogeny also strongly suggested that *A. filipes* is closely related to the Australian *Callerya* viz *C. magasperma* (F. Muell.) Schot. and *C. australis* (Endl.) Schot (Hu et al., 2002).

In conclusion, it is clearly found that there are many studies from various branches of science such as morphology, cytology, ecology, genetics or molecular data, directly or indirectly related to the genus *Afgekia*. Unfortunately, the three species of the genus *Afgekia* have never been studied at the same time. Therefore, the taxonomic status of the species in this genus is still obscure and worth investigating.
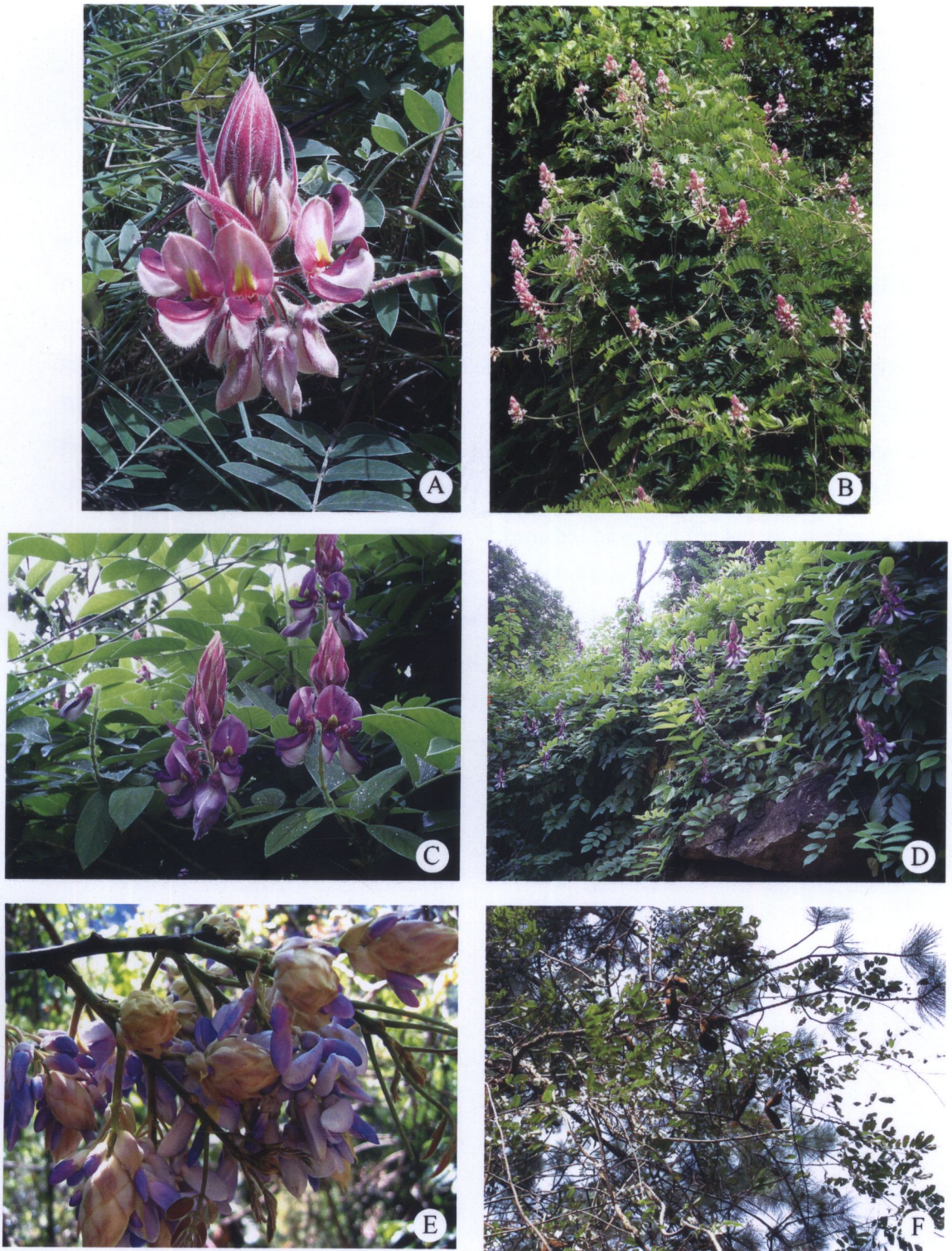
Figure 2.1 Afgekias's flowers and their natural habits and habitats. A, B: *A. sericea*, C,D: *A. mahidolae*, and E,F: *A. filipes*.

CHAPTER III

MATERIAL AND METHODS

3.1 Morphometric Study

Plant Material

A complete specimen with a branch, an inflorescence and pods, of *Afgekia sericea*, *Afgekia mahidolae* and *Afgekia filipes* were collected during flowering and fruiting periods from the known natural habitats (Table 3.1) as was labeled on the herbarium specimens deposited at Kasin Suvatabandhu Herbarium (BCU), Department of Botany, Faculty of Science, Chulalongkorn University; Forest Herbarium Thailand (BKF), National Park, Wildlife and Plant Conservation Department (DNP); Bangkok Herbarium (BK), Department of Agriculture; Suanluang Rama IX Herbarium, Rama IX Royal Botanic Garden, Bangkok and Queen Sirikit Botanic Garden Herbarium (QBG), Chiang Mai province. The specimens were determined based on the key to species in the Flore du Cambodge du Laos et du Viêtnam, Vol. 30, (Phan Kê Lôc & Vidal, 2001). Then they were confirmed by comparing to the voucher specimens. These collected specimens were used for all morphological study and morphometric analyses. In addition, young leaves of these 3 species and *Callerya atropurpurea* (Wallich) Schot and *Millettia brandisiana* Kurz were also collected for further Molecular Study.

Table 3.1 Species of the genus *Afgekia* and their collected locality.

Species	Locality	No. of specimens
A. sericea	Sakaerat Environmental Research Station Nakhon Rachasima	17
	Pak Thong Chai District, Nakhon Rachasima	5
	Phanom Rung Historical Park, Buri Ram	7
	Hin Ngarm National Park, Thep Sathit District, Chaiyaphume	8
	Total	<u>37</u>
A. mahidolae	Sai Yok District, Kanchanaburi	40
	Mahidol University, Salaya Campus, Nakhon Pathom (cultivated)	10
	Total	<u>50</u>
A. filipes	Doi Ang Khang, Chiang Mai	23
	Doi Suthep-Pui National Park Station, Chiang Mai	5
	Ruesi Valley, Doi Suthep-Pui National Park, Chiang Mai	4
	Total	<u>32</u>
	Total	<u>119</u>

Character Measurements

In total, 29 quantitative and 1 qualitative character of both vegetative and reproductive structures of the collected specimens were studied and used for multivariate analyses (Table 3.2 and Figure 3.1). The measurement of macroscopic quantitative character was carried out using a standard ruler or a digital caliper. While the microscopic quantitative characters were measured with an aid of the light microscope equipped with 10X lens coupled to micrometer disc and 10X objectives.

Morphological Characters	Abbreviation
1. Number of leaflets	NOL
2. Rachis length (cm)	RL
3. Rachis Diameter (mm)	RD
4. Petiole length (cm)	PETIL
5. Petiole Diameter (mm)	PETID
6. Leaflet length (cm)	LL
7. Leaflet width (cm)	LW
8. Average distance between each leaflet (cm)	ADBL
9. Stipule length (mm)	STIL
10. Stipule width (mm)	STIW
11. Peduncle length (cm)	PEDUL
12. Inflorescent length (cm)	INFL
13. Bract length (cm)	BL
14. Bract width (cm)	BW
15. Pedicel length (cm)	PEDIL
16. Calyx tube length (mm)	CALT
17. Calyx lobe length (mm)	CALL
18. Standard length (cm)	STANL
19. Standard width (cm)	STANW
20. Wing length (cm)	WL
21. Wing width (cm)	WW
22. Keel length (cm)	KL
23. Keel width (cm)	KW
24. Filament length (cm)	FIL
25. Anther length (mm)	ANTL
26. Anther width (mm)	ANTW
27. Pod length (cm)	PODL
28. Pod width (cm)	PODW
29. Pod thickness (mm)	PODT
30. Leaf Hairiness: 0 = glabrous, 1 = pubescence	

Table 3.2 Twenty nine quantitative morphological characters and 1 qualitative character used in multivariate analyses of *Afgekia* with their methods of scoring.
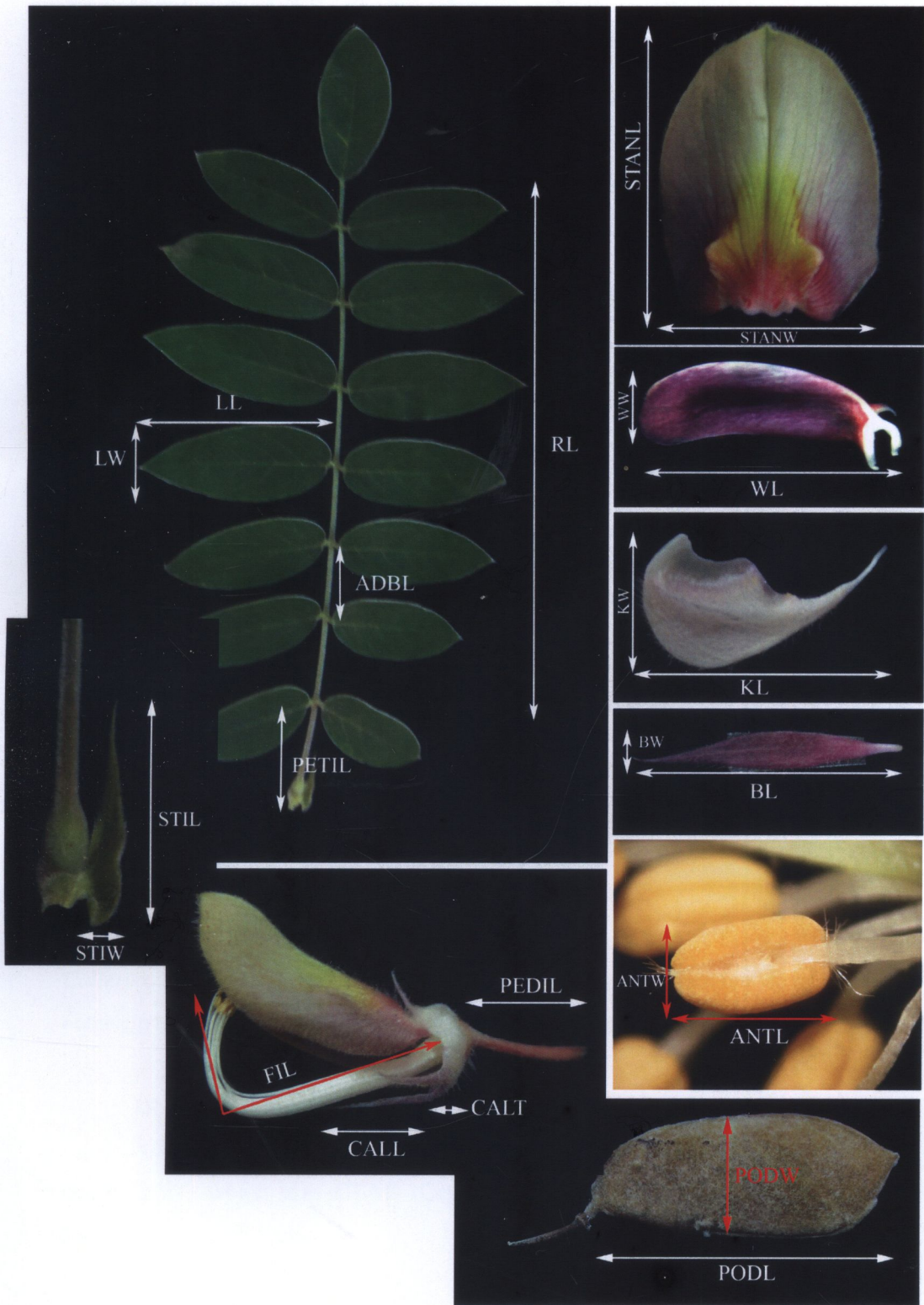
Figure 3.1 Some examples of vegetative and reproductive parts measurement of *Afgekia*.

Data analysis

119 specimens were used for all analyses. Cluster analysis (CA) and canonical discriminant analysis (CDA) were performed to determine the pattern of grouping of the collected specimens (OTUs).

In cluster analyses when mixed characters, i.e. quantitative and qualitative morphological characters were measured altogether, Gower similarity coefficient was calculated and clustered by the group-average method as suggested by Gower (1971). For these proposes a statistic package, MVSP (Kovach Computing Services, MVSP Plus, version 3.1) was used.

Canonical discriminant analysis was performed with SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Data (characters) with non-normal distribution were transformed using square roots or natural logarithms (Clifford & Stephenson, 1975). Then, stepwise discriminant analysis was used to select a subset of characters that maximized differences among the groups determined by clusters analysis. To characterize mean differences among the species, Canonical discriminant analysis was used to acquire insight into group differences and to estimate character weights from correlations between canonical variables and original variables. Correct classification rates were used as indicators of separation among the groups.

To summarize the range of variation between and within the segregated groups on each character, univariate analysis was performed. Boxplots of the most important characters were carried out using SPSSpc-FW (Anonymous, 1999).

3.2 Molecular Study

Plant materials

Afgekia species and its collecting localities are showed in Table 3.3. In this study, *Callerya atropurpurea* (Wallich) Schot and *Millettia brandisiana* Kurz are used as outgroup taxa.

DNA Extraction

Young leaves were used for DNA extraction by a modified method of Doyle and Doyle (1987) which uses CTAB (Cetyltrimethylammonium bromind) as the nonionic detergent for nuclei lysis.

The following procedure had been used In this study:

For preparation, set the microcentrifuge tube filled with 500 µl of modified CTAB extaction buffer and, preheat CTAB buffer in water-bath to 65 °C

1. Add liquid nitrogen to a pestle containing 0.1g of leaf material and grind to powder with a mortar.

2. Transfer powder to extraction buffer in a microcentrifuge tube, vertex 10 second, and incubate at 65°C for 20-30 minutes, mixing every 10 minutes.

3. Add 500 µl of Chloroform : Isoamylalcohol mixture, invert gently 5 times and incubate by gently shaking at room temperature.

4. Centrifuge at 10,000 rpm at 4 °C for 10 minutes

5. Transfer supernatant into a new microcentrifuge tube

6. Add 0.1 volume of 3M Sodium acetate and mix then, add 0.6 volume iced-cold (-20°C) Isopropanol, mix by inverting.

7. Let the tube stand at -20 °C for 30 minutes

8. Centrifuge at 10,000 rpm at 4 °C for 10 minutes

9. Gently discard the supernatant (be careful not to disturb DNA pellet)

10. Add 500 µl of iced-cold 75% ethanol, gently invert the tube several times

11. Centrifuge at 10,000 rpm for 5 minutes, discard the supernatant and allow the pellet to air-dry

12. Dissolve DNA in 200 µl of sterile TE buffer and store DNA solution at -20 °C until use.

To increase the purity of DNA sample, optional steps for some samples are listed below:

1. Add 1 µl of Rnase (20 µg/ml in concentration) to digest RNA.

2. Incubate the solution at 37 °C for 30-60 minutes.

3. Add 200 µl of Phenol:Chloroform mixture, invert gently.

4. Centrifuge at 10,000 rpm at 4 °C for 10 minutes

5. Add 500 µl of Chloroform : Isoamylalcohol mixture, invert gently.

6. Centrifuge at 10,000 rpm at 4 °C for 10 minutes

7. Transfer supernatant into a new microcentrifuge tube

8. Add 0.1 volume of 3M sodium Acetate, mix, then, add 2 volume iced-cold (-20°C) absolute ethanol, invert gently

9. Let the tube stand at -20 °C for 30 minutes

10. Centrifuge at 10,000 rpm at 4 °C for 10 minutes

11. Gently discard the supernatant (be careful not to disturb DNA pellet)

12. Add 500 μl of iced-cold 75 % ethanol, gently invert the tube several times and allow the pellet to air-dry

13. Dissolve the DNA in 50-100 μl of sterile TE buffer and store DNA solution at -20 °C until use.

RAPD Analysis

Genetic variation at the molecular level among three species of the genus *Afgekia* and outgroups was detected by RAPD markers. The materials used in this analysis are showed in Table 3.3.

Screening of primers

Two hundred and thirty one primers of arbitrary ten-oligonucleotide sequence (Pacific Science Company, LTD., Thailand and Genset Oligos) were screened for selecting appropriate primers which gave polymorphic, scorable, reproducible DNA product (bands).

RAPD-PCR condition and electrophoresis

Amplification was carried out in a 25 μl volume in 0.2 ml PCR microtube using a PTC-100 Peltier Thermal cycler. The reaction mixture contained 200 μM of dNTPs, 1x Taq DNA polymerase buffer (QIAGEN, Leusden, Netherlands), 1.5 μM $MgCl_2$, 5 picomole primer, 50 ng DNA, and 0.5 unit of Taq DNA Polymerase (QIAGEN, Leusden, Netherlands). The amplification cycle was performed as follows: initial 3 minutes at 94°C denaturation; 40 cycles of 1 minutes at 94°C, 1 minutes at 36°C annealing, 2 minutes at

72 °C; and 5 minutes at 72 °C extension. The amplified products were instantly run on gel or stored at 4° C overnight before electrophoresis.

Amplified fragments were separated in 2.5 % agarose gel using 1x TAE buffer and were visualized and photographed using a gel documentation analysis set (BIO RAD), after staining with Ethidium bromide.

Data Analysis

Only clear-appearing bands of RAPD products were scored to estimate the genetic variations/relationships among the *Afgekia* species and outgroup taxa. The presence or absence of bands was coded in binary (1, 0) respectively. A SIMQUAL module which computes genetic similarity coefficients for qualitative data was calculated according to Dice (1945). The similarity matrix was employed to construct a dendrogram by the Unweighted Pair Group Method using Arithmetic Averages (UPGMA), using the SAHN-clustering and TREE programs from the NTSYS-pc, version 2.10m (Rohlf, 2000). In addition, RAPD bands were also grouped by distance analysis using a pair-wise genetic similarity according to the index of Nei and Li (1979). Phylogram was generated by the Neighbor-Joining (NJ) analyses done with PAUP* version 4.0b10 (Swofford, 2002). Bootstrap analysis was performed with 500 replicates under the Neighbor-Joining/UPGMA (Felsenstein, 1985). From this analysis, Neighbor-joining bootstrap values were computed and put in each branch of phylogram.

Table 3.3 Sampling sites and numbers of DNA samples of *Afgekia* and outgroup taxa used in RAPD analysis.

Species	Populations	No. of Individual	Abbreviations
Afgekia sericea	Sakaerat Environmental Research Station Nakhon Rachasima	5	SK
	Pak Thong Chai District, Nakhon Rachasima	5	PT
	Phanom Rung Historical Park, Buri Ram	5	P
	Hin Ngarm National Park, Thep Sathit District, Chaiyaphume	5	C
Afgekia mahidolae	Sai Yok District, Karnchanaburi	6	K
	Mahidol University, Salaya Campus, Nakhon Pathom (cultivated)	6	MU

Table 3.3 (continue)

Species	Populations	No. of Individual	Abbreviations
Afgekia filipes	Doi Ang-Khang, Chiang Mai	5	AK
	Doi Suthep-Pui National Park station, Doi Suthep-Pui National Park, Chiang Mai	5	ST
	Ruesi Valley, Doi Suthep-pui National Park, Chiang Mai	5	RS
Callerya atropurpurea	Ta Sae District, Chumporn	2	T
	Ron Phibun District, Nakhon Si Thammarat	1	R
	Khoa Nan National Park, Nakhon Si Thammarat	1	KN
Millettia brandisiana	Satit School of Kasetsart University, Bangkok (cultivated)	3	-

DNA Sequencing Analysis

The ITS fragment was amplified using the primer combinations ITS1/ITS4, 'ITS1' (5'-TCCGTAGGTGAACCTGCGG-3') and 'ITS4' (5'-TCCTCCGCTTATTG ATATGC-3') (White et al., 1990). The thermal cycling protocol comprised of 30 cycles, each with 1 minute 30 second of denaturation at 94 °C, 2 minutes of annealing at 49-50 °C, and 3 minutes of extension at 72 °C, concluding with an additional extension of 10 minutes at 72 °C after the final cycle.

The *trnL-F* intergenic spacer was amplified using the primer combinations e/f, 'e' (5'-GGTTCAAGTCCCTCTATCCC-3') and 'f' (5'-ATTTGAACTGGTGACACGAG-3') (Taberlet et al., 1991). The thermal cycling protocol comprised of 30 cycles, each with 1 minute 30 second of denaturation at 94 °C, 2 minutes of annealing at 49-50 °C and 3 minutes of extension at 72 °C concluding with an additional extension of 10 minutes at 72 °C after the final cycle.

PCR reactions were set up in 50 µl reactions, usually containing 1x PCR buffer, 0.2 mM dNTPs in equimolar ratio, 0.2 pmol primer, 0.5 U of Taq DNA polymerase (QIAGEN, Leusden, Netherlands), 50-100 ng of genomic DNA and 2.5 mM $MgCl_2$. Amplification was carried out using the PTC-100 Thermal Cycler (MJ Research).

Double stranded PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Leusden, Netherlands) according to manufacturer's instructions, and the purified products were eluted in 30-50 µl of Buffer EB (10 mM Tris-HCl pH 8.5). Purified DNA sequencing was directly done by Genome Research Centre (Hong Kong).

Data Analysis

Upon completion of DNA sequencing, base calling was verified by examining fluorographs in Chromas version 1.45 (McCarthy, 1997), and corrections were made as necessary. Sequence alignment was done manually using the GeneDoc version 2.6.002 (Nicholas and Nicholas, 1997).

The nucleotide sequences of ITS and *trnL-F* IGS were analyzed separately. For analizing ITS region, Phylogenetic analyses were conducted using the parsimony method in PAUP* version 4.0b10 (Swofford, 2002) using maximum parsimony (MP) with the factory settings of the branch-and-bound search option activated. Gaps were treated as missing. Bootstrap analysis was performed with 100 replicates under the branch-and-bound search (Felsenstein 1985). For this analysis, nucleotide sequence of *Callerya australis* (Endl.) Schot. (ID: AF467024), *Callerya megasperma* (F.Muell.) Schot. (ID: AF467028), *Wisteria sinensis* (Sims) Sweet (ID: AF467503) and *Millettia pulchra* (Benth.) Kurz (ID: AF467479) were obtained from NCBI GenBank sequence database. Those species were also used as outgroup taxa.

For analizing *trnL-F* IGS region, like ITS, Phylogenetic analyses were conducted using the parsimony method in PAUP* version 4.0b10 (Swofford, 2002). Due to the small number of taxa, an exhaustive search with the Fitch parsimony criterion was employed to search for the most parsimonious (MP) tree. Characters were equally weighted. Bootstrap (Felsenstein, 1985) was used to obtain a measure of support for each branch. 100 bootstrap replications were performed under branch-and-bound search.

CHAPTER IV

RESULTS

4.1 Specimen Collections

A total of 119 specimens were collected and included in the overall analyses. Of these, 37 specimens belonged to *A. sericea*, 50 specimens belong to *A. mahidolae* and 32 specimens belong to *A. filipes*, respectively. These specimens (except for 10 specimens of *A. mahidolae*) were gathered from their natural habitats during July 2005 to February 2006. Slightly morphological variations among populations of the same species are observed. For example, the specimens of *A. sericea* collected from Phanom Rung Historical Park, Buri Ram Province showed more darker colour of floral parts than the other populations of the same species or, the *A. sericea* specimes collected from Sakaerat Environmental Research Station, Nakorn Rachasima Province showed the larger pods. Moreover, it was also found that some mature specimens of *A. filipes* have coriaceous leaflets, especially for those found at Doi Angkhang, Chiang Mai Province. This finding is in agreement with Deng and Qin (2004).

4.2 General Morphology Study

1) Stem

All species of *Afgekia* are perennial woody climbers. The distance between nodes is quite long. Lenticels and bark can be observed in old stems. Young stem are densely covered with white or silvery pubescent hairs (Fig. 4.1 C3), except for *A. filipes*, which its stem is covered with golden-brown hairs or sometimes glabrous. Stem are round in cross section. The stem of *A. filipes* is much larger than the former two species. In addition, it is found that the red sap will appear if the stem of this species is cut or injured. (Fig. 4.1 C2).

2) Leaves

The leaves of all *Afgekia* species are imparipinnate with 4-9 pairs of leaflets. Leaflet is opposite in arrangement. In *A. sericea*, there are 7-9 pairs of leaflets. The shape of leaflet is oblong or lanceolate. The texture is chartaceous. The upper surface is green and pubescent while the lower surface pale green, more densely covered with silvery pubescent hairs than upper surface (Fig. 4.1 A1). In *A. mahidolae*, the leaflet characters

are similar to *A. sericea*, however, it has only 4-5 pairs of leaflets and less silky pubescent (Fig. 4.1 B1-B3). The stipule in both species is light green, free lateral, persistent and pubescent. The shape of stipule is lanceolate. In contrast, the leaf of *A. filipes* is glabrous when mature or slightly covered with golden-brown hairs. The texture of leaflet is chartaceous or subcoriaceous (sometimes coriaceous). The colour of young leaves is usually reddish brown or brownish green (Fig. 4.1 C1) and gradually turn to green during the maturation. The young leaf or bud is always covered with golden-brown hairs. The stipule of this species is minute, dark green, free lateral and pubescent. The shape of stipule is triangular. Different from the former two species, the stipule of *A. filipes* is caducous.

3) Flowers and Inflorescences

The inflorescences of *A. sericea* and *A. mahidolae* are long terminal or axillary raceme (Fig. 4.2 A1, B1). It can be up to 1 meter long in *A. sericea*. In *A. filipes,* the character of inflorescences is distinctly different. It is the axillary raceme which usually bear or cluster together at adjacent position and formed a panicle-liked structure. The position of these inflorescences is usually near the terminal of a branch (Fig. 4.2 C3). The peduncles of all species are silky pubescent, covered with short hairs. The flowers of all species are commonly papilionaceous form, containing 1 standard petal, 2 wing petals and 2 keel petals. In *A. filipes*, the flowers are fragrant while *A. sericea* and *A. mahidolae* are odorless. The floral parts of *A. sericea* and *A. mahidolae* are usually covered with hairs, while *A. filipes* is usually glabrous. The flower of *A. sericea* is pinkish ivory. Standard is pinkish-ivory with pink patches on both sides at the base and a yellow patch in the middle (Fig. 4.2 A2). Abaxial side of standard is pubescent. Wings are purplish pink with 2 wing's basal appendages (Fig. 4.2E). Keels are pale ivory, pubescent and broadly falcate. The flower of *A. mahidolae* is purple (Fig. 4.2 B2-B3). Standard is purple with central yellow patch (Fig. 4.2 B2). Abaxial side of standard is pubescent. Wing petal is deep purple with only one wing's basal appendage (Fig 4.2E). Keels are white or pale ivory, pubescent and broadly falcate. In *A. filipes,* Standard is pale violet with central yellow patch and glabrous. Wing petals are violet; without basal appendage and keels are white, glabrous and broadly falcate. Bracts of all species are showy, they are usually longer than the corresponding flower buds and also broader in *A. filipes*. Bracteoles are absent except in *A. filipes* which found the reduced hair-liked bracteoles (Fig. 4.2 G). The

characters of calyx in *A. sericea* and *A. mahidolae* is similar, with 5 long and acute calyx lobes, basally fused along their margins, forming a calyx tube. Calyx lobes arranged in bilabiate form, i.e. the two upper lobes and three lower lobes. The upper two calyx lobes are shorter than the other three. In the fromer two species, calyx lobes are long and acuminate at apex. On the other hands, the calyx of *A. filipes* is different because the calyx lobes are short, triangular and usually obtuse at tip. The numbers of and the characters of stamens in this genus is quite similar, but, differ in the anther character and stamen fusion. All of them has 10 monadelphous (the upper filament adnate to the other 9 found in *A. sericea* and *A. mahidolae*) or pseudomonadelphous (the upper filament only slightly adherent to the other 9 found in *A. filipes*) stamens. All stamens are fertile with white filaments and yellow anthers. *A. sericea* and *A. mahidolae* have the anthers with a basal tuft of hairs (Fig. 4.2 D), whereas *A. filipes* has glabrous anthers. The ovaries of all species are pubescent. In *A. mahidolae*, the top of the ovary style is covered with hairs (Fig. 4.2 F), while the other species are glabrous.

4) Pods and Seeds

A. sericea and *A. mahidolae* has similar pod's characterisics. The pods of both species are pale green and pubescent when young and then, turn to pale brown and woody when mature, tardily dehiscent. The shape of pod is oblong, mucronate at apex, inflated, and sutures without wings. The texture of pod is usually smooth or slightly wrinkled. There are usually 2 seeds per pod. The seed of both species are lenticular, brown to dark brown in colour. Funicle is swollen, fleshy and elongated. Corresponding hilum 1/2 the circumference of the seed, strap-shaped.

The pod of *A. filipes* are much larger than the first two species. It is pale green and pubescent when young and then, turn to pale brown when mature, tardily dehiscent. The shape of pod is oblong, rounded to ellipsoid, inflated, and sutures without wings. Pod texture is usually wrinkled. Pod peel is woody and thick when mature, usually contains only one seed per pod. The seed is brown or dark brown in colour, elliptic in shape. Fimicle is swollen, fleshy and elongated; corresponding to elongated hilum which less distinct than in *A. sericea* and *A. mahidolae*. (Fig. 4.3)

Figure 4.1 Variations in Leaves and stem of three species of the genus *Afgekia*. A1-A2: *A. sericea*; A1: Stem and leaves, showed 7-9 pairs of leaflets per a leaf, A2: the lower surface of a leaf, covered densely with white-sivery hairs. B1-B3: *A. mahidolae*; B1: Stem and leaves, showed 4-5 pairs of leaflets per a leaf, B2: the lower surface of a leaf, less pubescent than *A. sericea*, B3: young vegetative parts, usually covered with white pubescent hairs, and C1-C2: *A. filipes*; C1: Stem with young reddish or brownish green leaves, C2: red sap in cut stem.

Figure 4.2 Flowers, inflorescence and pods of three species of the genus *Afgekia*. A1-A3: *A. sericea*; B1-B3: *A. mahidolae*; and C1-C3: *A. filipes*; D: anther with basal tuft of hairs found in *A. sericea* and *A. mahidolae*, E: wing petal of *A. sericea* (left) and *A. mahidolae* (right) shows different in colour and the numbers of basal appendage; F: reduced bracteole (red arrow) photographed from dry specimen of *A. filipes*; G: *A. sericea* (left) and *A. mahidolae* (right) show glabrous and hairly style respectively.
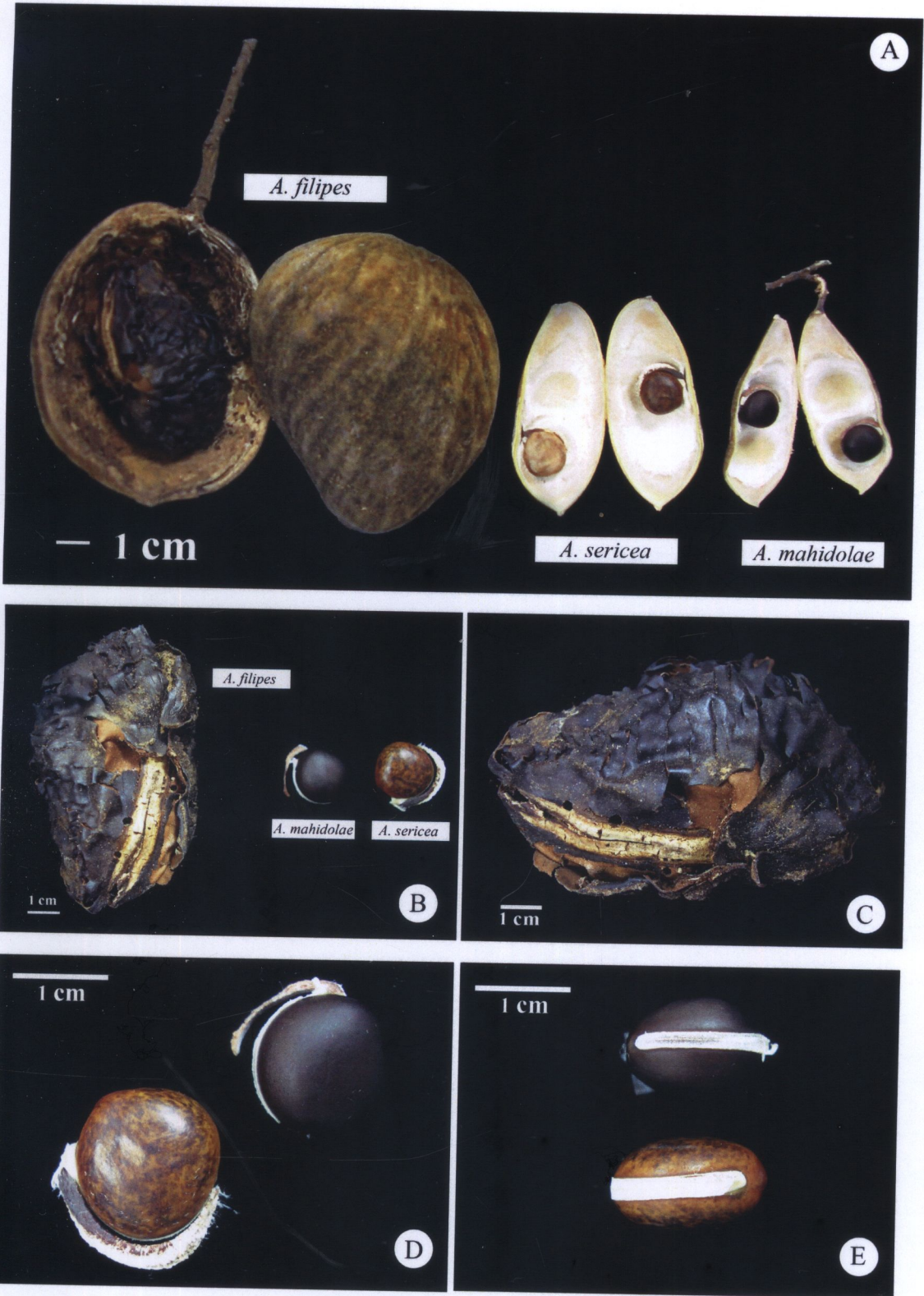
Figure 4.3 Dry pods and seeds of *Afgekia* species. A: pod with seeds, B: seed, C: seed of *A. filipes*, show fleshy and elongate hilum, D: Seed of *A. sericea* and *A. mahidolae*, show fleshy and elongate funicle corresponding to E: fleshy and elongate strap-shaped hilum.

From the result of general morphology study mentioned above, it can be summarized the morphological difference among those three species of *Afgekia* as show in Table 4.1 below. The character with the superscript " * " are the characters also found in the related genus, *Callerya*. The character with the superscript " ᴹ " are the characters also found in the related genus, *Millettia*.

Morhological Characters	Species		
	A. sericea	*A. mahidolae*	*A. filipes*
Vegetative parts			
Habit	Lianas, claimbing low and medium canopy	Lianas, claimbing low and medium canopy	Lianas, claimbing high canopy
Stem size	Small or medium	Small or medium	Large
Young Stem hairiness	Densely covered with white or silvery pubescent hairs	Densely covered with white or silvery pubescent hairs	Covered with golden-brown hairs or sometimes glabrous
Red sap in woody stem	absent	absent	Present*
Leaf hairiness	Lower surface covered densely with silvery pubescent hairs	Lower surface covered with white pubescent hairs	Young leaf usually covered with golden-brown hairs Mature leaf usually glabrous
Reddish-brown pigments in young leaf	absent	absent	Present* ᴹ
Stipule shape	Lanceolate with long acute apex	Lanceolate with long acute apex	Triangular
Reproductive parts			
Inflorescences	Long terminal or axillary raceme	Long terminal or axillary raceme	Axillary racemes, bear at adjacent position near the terminal of a branch and form panicle-like structure
Flower fragrance	odorless	odorless	Fragrant*
Bract size compared with corresponding flower bud	Longer but narrower	Longer but narrower	Longer and broader
Bracteoles	absent	absent	reduce
Calyx lobe shape	Long and acute	Long and acute	Short triangular*
Standard colour	Ivory with pink patches on both sides at base and yellow patch in the middle	Purple with yellow central patch	Pale violet with yellow central patch
Wings colour	Purplish pink	Deep purple	Violet
No. of wing's basal appendage	2	1	0
Style hairiness	absent	present	absent
Level of filament fusion	Monadelphous	pseudomonadelphous	pseudomonadelphous
Anther hairiness	present	present	absent*
Texture of pod peel	Smooth or slightly wrinkled	Smooth or slightly wrinkled	Wrinkled
Seed shape	Lenticular	Lenticular	Elliptic
Hilum	Strap-shaped,1/2 the circumference of the seed	Strap-shaped,1/2 the circumference of the seed	Strap-shaped, 1/6 to 1/8 the circumference of the seed

4.3 Morphometric Study

1. Cluster analysis

In cluster analysis, similarity (or distance) coefficient matrix which was the average taxonomic distance derived from 29 quantitative characters and 1 qualitative character was produced.

The dendrogram (Fig. 4.4) clearly separated the 119 specimens into either two or three groups at the 0.76 and 0.8 level of Gower Similarity Coefficient respectively (Figure 4.4). In the two-cluster grouping, specimens classified as group 1 consisted of all specimens of *A. sericea* and *A. mahidolae*. While, all specimens of *A. filipes* distinctly separated into another group without overlapping. In the three-cluster grouping, the group 1, which previously consisted the specimen of *A. sericea* and *A. mahidolae* in the two-cluster grouping, was divided in to 2 groups. The fisrt group was composed of all *A. sericea*'s specimen, and the second group was composed of all *A. mahidolae*'s specimen. The third group is the same as group 2 in the two-cluster grouping, consisted only *A. filipes*'s specimens.

Both the two and three-cluster groupings demonstrate a clear separation of *A. filipes*. On the other way, the dendrogram presents the close morphological relationships between *A. sericea* and *A. mahidolae*. At present, it is generally accepted that *A. sericea* and *A. mahidolae* are good species (Burtt and Chermsirivathana, 1971) and belong to the same genus, *Afgekia*. While *A. filipes* was transferred to *Afgekia* based mainly on seed characters (Geesink, 1984). The result from cluster analysis suggested that *A. filipes* might not be placed in the genus *Afgekia*.

2. Canonical discriminant analysis

In canonical discriminant analysis, 29 morphological characters were examined by stepwise discriminant analysis to determine which characters are important in discriminating among these three groups as suggested by cluster analyses. The linear discriminant function classification resulted showed 100% correctly classified. For this reason, the linear discriminant function (Table 4.2) could be used for identification of the *Afgekia*'s specimens. The nature of entries differnences was showed by the pooled within canonical structures (Table 4.3). Canonical variable 1 was 99.7 % correlated with the 29

characters and explained 88% of the total variance (Table 4.4). It was highly associated with character PODW, PODT, PODL, FIL, BW, RD, PEDUL, LL and PETIL. Canonical variable 2 was 97.9 % correlated with the 29 characters and explained 12% of the total variance. This axis was highly associated with BL, NOL, LW, ANTL, CALL, KL, PEDIL, STANL, INFL and STANW. The 10 variables, STIL, PETID, ANTW, CALT, WW, KW, WL, STIW, RL and ADBL were not used in the analysis according to the result of stepwise discriminant analysis (Table 4.3).

The ordination plot on the two canonical axes indicated that the three species of the genus *Afgekia* were distinct (Fig. 4.5). The three species were separated into 2 groups on canonical axis 1. *A. sericea* and *A. mahidolae* belong to the first group. *A. filipes*, on the other hands, was distinctly divided into another group. In canonical axis 2, three species were separated into three groups. Each group represented each species.

Table 4.2 Classification Function Coefficients of 3 categories/species base on 29 morphological characters. To identify a specimen of *Afgekia* multiply each character score by its coefficients in each column; compute the total for each column; the column with the highest total is the species to which the specimen belongs.

Characters	Species		
	A. sericea	*A. mahidolae*	*A. filipes*
NOL	125.389	89.726	102.297
RD	83.606	79.110	145.436
PETIL	59.327	92.320	97.495
LL	206.548	147.914	275.082
LW	-139.568	-65.690	-132.346
PEDUL	-37.476	-27.572	-68.901
INFL	5.234	-10.640	-9.553
BL	-7.413	-48.764	-59.414
BW	-17.846	-13.751	42.920
PEDIL	-64.740	-102.571	-70.741
CALL	6.003	1.967	-0.646
STANL	-20.432	28.612	12.329
STANW	31.321	24.473	83.788
KL	253.701	288.774	308.187
FIL	-1.339	1.896	-99.909
ANTL	198.386	155.347	180.156
PODL	278.501	290.444	402.048
PODW	237.185	206.628	443.844
PODT	111.363	109.492	177.955
(Constant)	-1400.627	-1331.964	-2300.126

Table 4.3 Pooled within canonical structure of 2 categories base on 29 morphological characters according to the result of canonical discriminate analysis

Characters	Discriminant function	
	1	2
PODW	0.535*	0.023
PODT	0.304*	0.033
PODL	0.275*	0.070
FIL	-0.260*	-0.114
STIL [a]	-0.194*	-0.072
BW	0.191*	-0.053
PETID [a]	0.185*	-0.062
RD	0.136*	0.024
PEDUL	-0.133*	0.000
LL	0.131*	-0.072
PETIL	0.103*	-0.032
ANTW [a]	0.067*	0.059
CALT [a]	-0.049*	0.005
WW [a]	-0.034*	0.005
BL	-0.114	0.283*
NOL	0.005	0.239*
LW	0.033	-0.238*
KW [a]	-0.147	0.228*
ANTL	-0.098	0.223*
WL [a]	-0.069	-0.219*
CALL	-0.207	0.217*
KL	-0.192	-0.204*
PEDIL	0.094	0.157*
STANL	-0.048	-0.143*
STIW [a]	-0.042	0.138*
RL [a]	0.001	0.130*
INFL	-0.076	0.104*
ADBL [a]	0.012	-0.074*
STANW	0.049	-0.051*

Note: The number with superscript * represent the largest absolute correlation between each variable and any discriminant function,

Character with superscript [a] indicated character not used in the analysis.

Table 4.4 Summary of canonical discriminate function of 3 categories/species base on 29 morphological characters

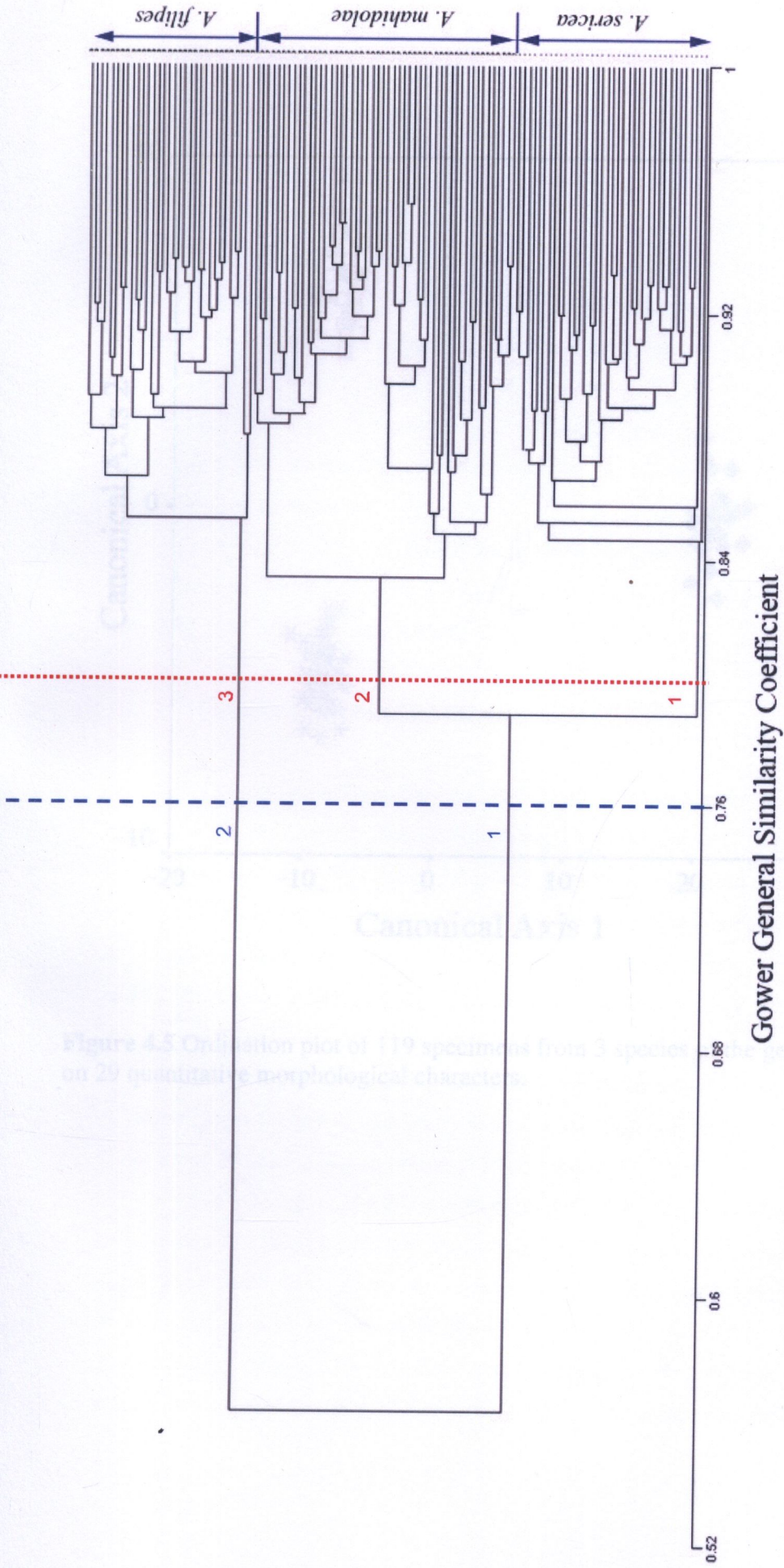| Function	Eigenvalue	% of variance	Comulative %	Canonical correlation	Wilk's lamda	Chi-square	Sig.
1	172.770	88.0	88.0	0.997	0.000	894.652	0.000
2	23.619	12.0	100.0	0.979	0.041	342.775	0.000

Figure 4.4 UPGMA phenogram base on Gower's general similarity coefficient calculated between means of 29 quantitative and 1 qualitative characters of the genus *Afgekia*.
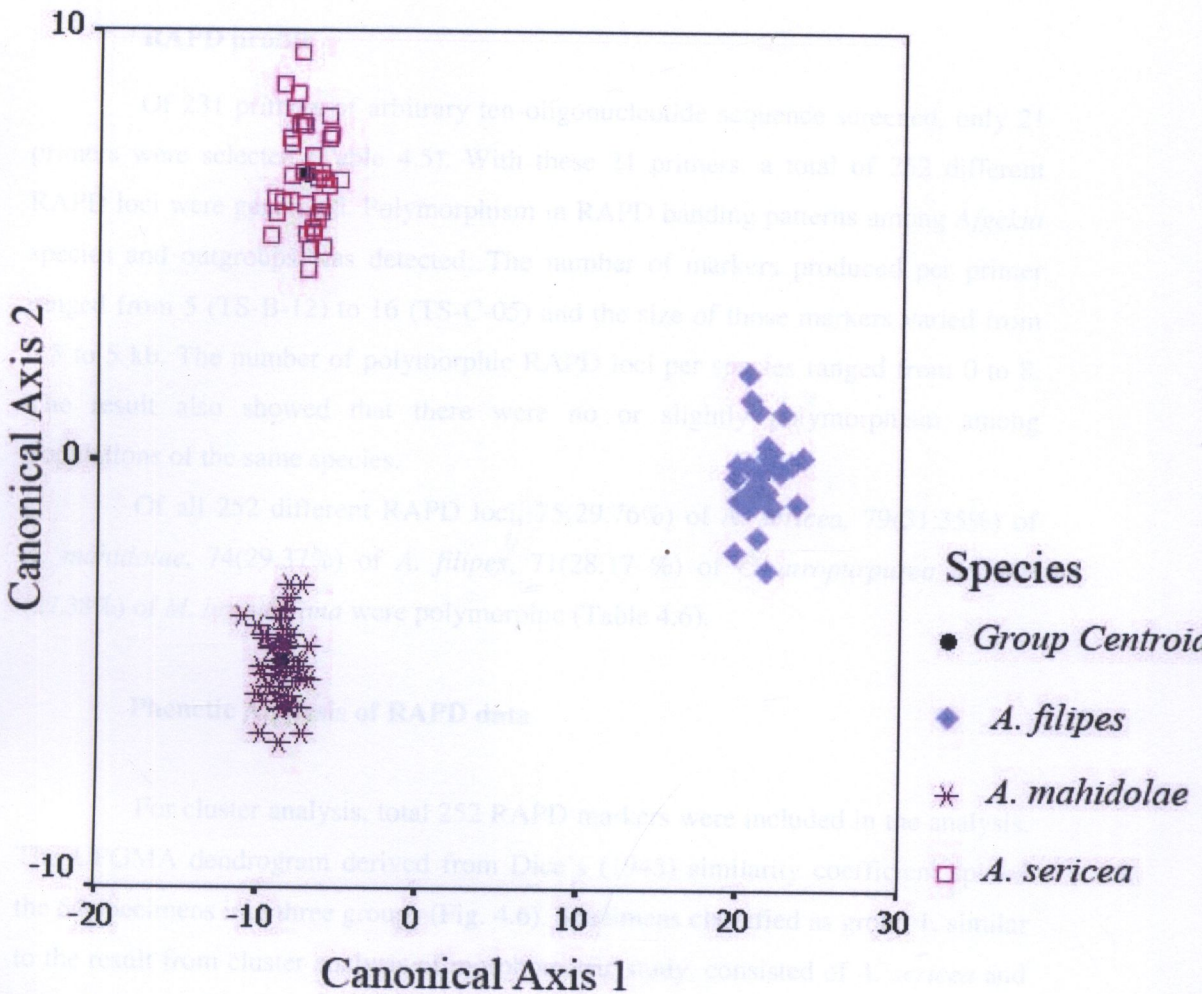
Figure 4.5 Ordination plot of 119 specimens from 3 species of the genus *Afgekia* base on 29 quantitative morphological characters.

4.4 Molecular Study

4.4.1 RAPD Analysis

RAPD profile

Of 231 primers of arbitrary ten-oligonucleotide sequence screened, only 21 primers were selected (Table 4.5). With these 21 primers, a total of 252 different RAPD loci were generated. Polymorphism in RAPD banding patterns among *Afgekia* species and outgroups was detected. The number of markers produced per primer ranged from 5 (TS-B-12) to 16 (TS-C-05) and the size of those markers varied from 0.5 to 5 kb. The number of polymorphic RAPD loci per species ranged from 0 to 8. The result also showed that there were no or slightly polymorphism among populations of the same species.

Of all 252 different RAPD loci, 75(29.76%) of *A. sericea*, 79(31.35%) of *A. mahidolae*, 74(29.37%) of *A. filipes*, 71(28.17 %) of *C. atropurpurea* and 69 (27.38%) of *M. brandisiana* were polymorphic (Table 4.6).

Phenetic Analysis of RAPD data

For cluster analysis, total 252 RAPD markers were included in the analysis. The UPGMA dendrogram derived from Dice's (1945) similarity coefficient spitted the 54 specimens into three groups (Fig. 4.6). Specimens classified as group 1, similar to the result from cluster analysis of morphometric study, consisted of *A. sericea* and *A. mahidolae*. Specimens classified as group 2 encompassed two species, *A. filipes*, and *C. atropurpurea*. While group 3 consisted only *M. brandisiana*.

The dendrogram demonstrates that *A. sericea* is closely related to *A. mahidolae* and this two species are clearly separated from *A. filipes*. In addition, *A. filipes* shows the genetic similarity to the genus *Callerya*. Thus, the genus *Afgekia* is not a well defined group according to RAPD data.

The phenogram from Neighbor-joining cluster analysis (Fig. 4.7) on the basis of Nei-Li (1979) coefficient showed that *A. sericea* and *A. mahidolae* formed a sister group with 91% Bootstrap support. The second group comprised of only *M. brandisiana* also formed sister group to the group consisted of *A. sericea* and *A. mahidolae*, though with relatively low Bootstrap support (59%). *A. filipes* was placed into another group and was sister to (the first group + second group) with

100% Bootstrap support. All samples of *C. atropurpurea* formed a group and were placed at the basal position. In this RAPD analysis, among populations of each species, no or slightly low genetic variations were found.

Table 4.5 Twenty one RAPD primers, primer sequences and numbers of amplified loci per primer.

Primer Code	Nucleotide sequence	No. of amplified loci
TS-C-05	5′ GATGACCGCC 3′	16
TS-B-17	5′ AGGGAACGAG 3′	15
TS-C-06	5′ GAACGGACTC 3′	13
TS-A-13	5′ CAGCACCCAC 3′	12
TS-B-12	5′ CCTTGACGCA 3′	5
TS-C-08	5′ TGGACCGGTG 3′	10
TS-C-02	5′ GTGAGGCGTC 3′	13
SPC_4	5′GGACTGGAGT 3′	14
SPC_10	5′ CTGCTGGGAC 3′	13
SPC_21	5′ CAGGCCCTTC 5′	8
SPC_24	5′ AATCGGGCTG 3′	11
SPC_27	5′ GAAACGGGTG 3′	9
SPC_53	5′ GGGGTGACGA 3′	13
SPC_58	5′ GAGAGCCAAC 3′	11
SPC_80	5′ ACTTCGCCAC 3′	10
SPC_82	5′ GGCACTGAGG 3′	14
SPC_105	5′ AGTCGTCCCC 3′	13
SPC_115	5′ AATGGCGCAG 3′	14
SPC_119	5′ CTGACCAGCC 3′	13
SPC_120	5′ GGGAGACATC 3′	12
SPC_121	5′ ACGGATCCTG 3′	13
Total		252

Table 4.6 Number and percentage of DNA polymorphism in *Afgekia* species and outgroups.

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
TS-C-05	16	A. sericea	5	31.25
		A. mahidolae	2	12.50
		A. filipes	8	50.00
		C. atropurpurea	4	25.00
		M. brandisiana	4	25.00
TS-B-17	15	A. sericea	2	13.33
		A. mahidolae	4	26.67
		A. filipes	4	26.67
		C. atropurpurea	2	13.33
		M. brandisiana	5	33.33
TS-C-06	13	A. sericea	6	46.15
		A. mahidolae	4	30.77
		A. filipes	2	25.38
		C. atropurpurea	4	30.77
		M. brandisiana	2	25.38
TS-A-13	12	A. sericea	5	41.67
		A. mahidolae	5	41.67
		A. filipes	7	58.33
		C. atropurpurea	2	16.67
		M. brandisiana	4	33.33
TS-B-12	5	A. sericea	1	20.00
		A. mahidolae	2	40.00
		A. filipes	1	20.00
		C. atropurpurea	1	20.00
		M. brandisiana	1	20.00
TS-C-08	10	A. sericea	3	30.00
		A. mahidolae	4	40.00
		A. filipes	1	10.00
		C. atropurpurea	3	30.00
		M. brandisiana	0	0.00
TS-C-02	13	A. sericea	1	7.69
		A. mahidolae	3	23.08
		A. filipes	3	23.08
		C. atropurpurea	6	46.15
		M. brandisiana	3	23.07
SPC_4	14	A. sericea	6	42.86
		A. mahidolae	3	21.43
		A. filipes	1	7.14
		C. atropurpurea	4	28.57
		M. brandisiana	3	21.43
SPC_10	13	A. sericea	4	30.77
		A. mahidolae	4	30.77
		A. filipes	2	15.38
		C. atropurpurea	2	15.38
		M. brandisiana	4	30.77

Table 4.6 (continue)

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
SPC_21	8	A. sericea	3	37.50
		A. mahidolae	4	50.00
		A. filipes	3	37.50
		C. atropurpurea	3	37.50
		M. brandisiana	1	12.50
SPC_24	11	A. sericea	3	27.27
		A. mahidolae	4	36.36
		A. filipes	3	27.27
		C. atropurpurea	3	27.27
		M. brandisiana	3	27.27
SPC_27	9	A. sericea	2	22.22
		A. mahidolae	2	22.22
		A. filipes	3	33.33
		C. atropurpurea	2	22.22
		M. brandisiana	3	33.33
SPC_53	13	A. sericea	2	15.38
		A. mahidolae	4	30.77
		A. filipes	3	23.08
		C. atropurpurea	3	23.08
		M. brandisiana	3	23.08
SPC_58	11	A. sericea	5	45.45
		A. mahidolae	5	45.45
		A. filipes	5	45.45
		C. atropurpurea	4	36.36
		M. brandisiana	6	54.54
SPC_80	10	A. sericea	5	50.00
		A. mahidolae	5	50.00
		A. filipes	3	30.00
		C. atropurpurea	4	40.00
		M. brandisiana	1	10.00
SPC_82	14	A. sericea	4	28.57
		A. mahidolae	5	35.71
		A. filipes	5	35.71
		C. atropurpurea	4	28.57
		M. brandisiana	4	28.57
SPC_105	13	A. sericea	5	38.46
		A. mahidolae	5	38.46
		A. filipes	2	15.38
		C. atropurpurea	4	30.77
		M. brandisiana	4	30.77
SPC_115	14	A. sericea	4	28.57
		A. mahidolae	5	35.71
		A. filipes	5	35.71
		C. atropurpurea	3	21.43
		M. brandisiana	5	35.71
SPC_119	13	A. sericea	4	30.77
		A. mahidolae	2	15.38
		A. filipes	4	30.77
		C. atropurpurea	4	30.77
		M. brandisiana	4	30.77

Table 4.6 (continue)

Primer code	No. of amplified loci (a)	Species	No. of polymorphic fragments (b)	% polymorphic (b/a) x 100
SPC_120	12	A. sericea	3	25.00
		A. mahidolae	4	33.33
		A. filipes	4	33.33
		C. atropurpurea	5	41.67
		M. brandisiana	5	41.67
SPC_121	13	A. sericea	2	15.38
		A. mahidolae	3	23.07
		A. filipes	5	38.46
		C. atropurpurea	4	30.77
		M. brandisiana	4	30.77
Total	252	A. sericea	75	29.76
		A. mahidolae	79	31.35
		A. filipes	74	29.37
		C. atropurpurea	71	28.17
		M. brandisiana	69	27.38

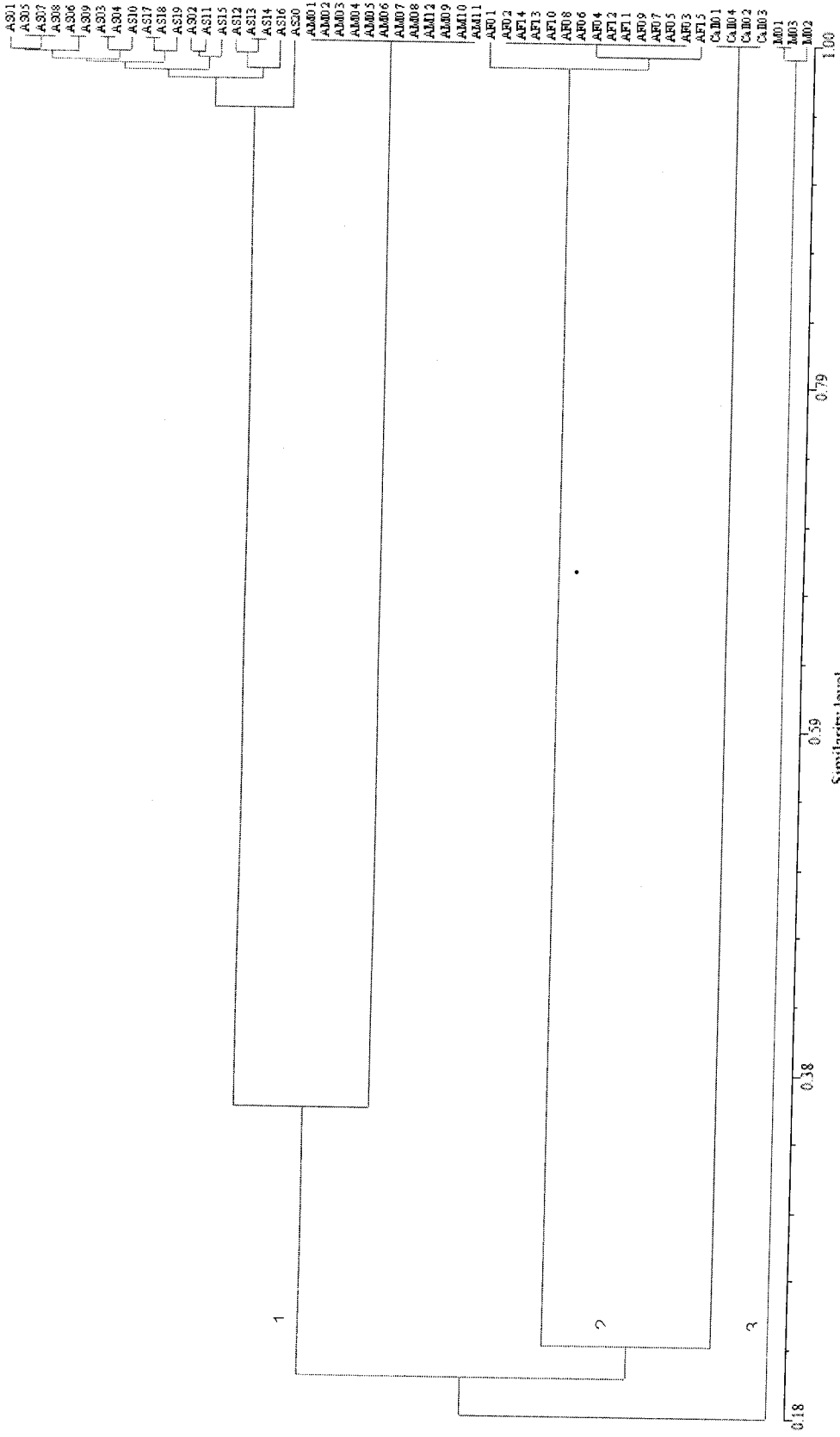**Figure 4.6** Dendrogram of 54 individuals of *Afgekia* species and outgroups generated by cluster analysis using UPGMA base on RAPD data. The abbreviation; AS= *A. sericea*, AM= *A. mahidolae*, AF= *A. filipes*, Call= *C. atropurpurea* and M= *M. brandisiana.*
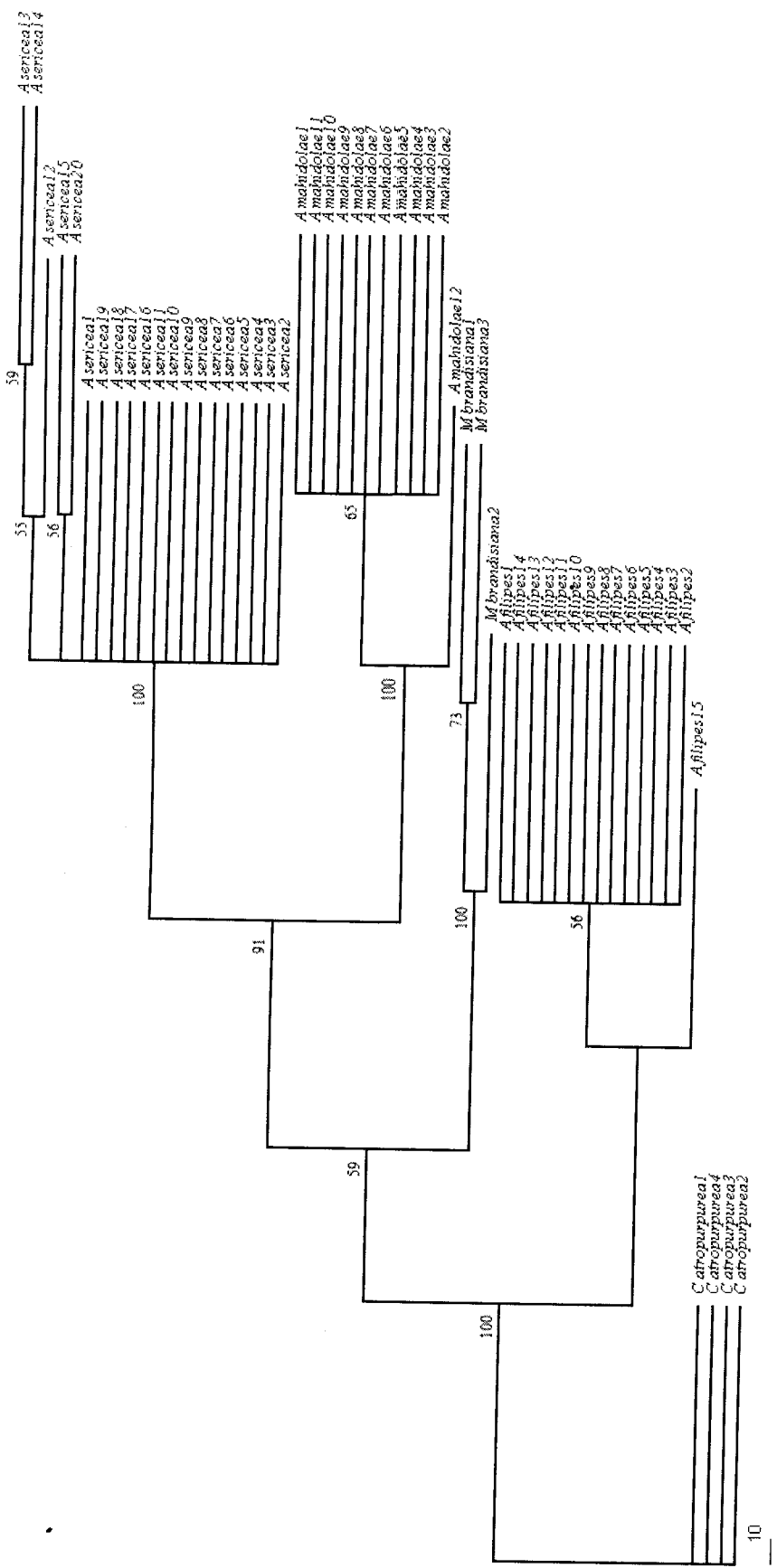
Figure 4.7 Phylogram of 54 individuals of *Afgekia* species and outgroups based on RAPD Neighbor-joining cluster analysis. Numbers above branches are bootstrap values.

53

Figure 4.8

Examples of RAPD band profiles of *Afgekia* and its outgroup taxa operated by primer SPC-58 (A, B) and primer TS-C-05 (C, D). Lane 1 (in A and C) and lane 2 (in B and D) are 1 kb DNA ladder (NEBiolab) respectively. Lane 17 (in A and C) and lane 21 (in B and D) are 100 bp DNA ladder(NEBiolab) respectively. The abbreviation above each picture indicated populations of each species according to Table 3.3. the alphabet 'N' is negative control.
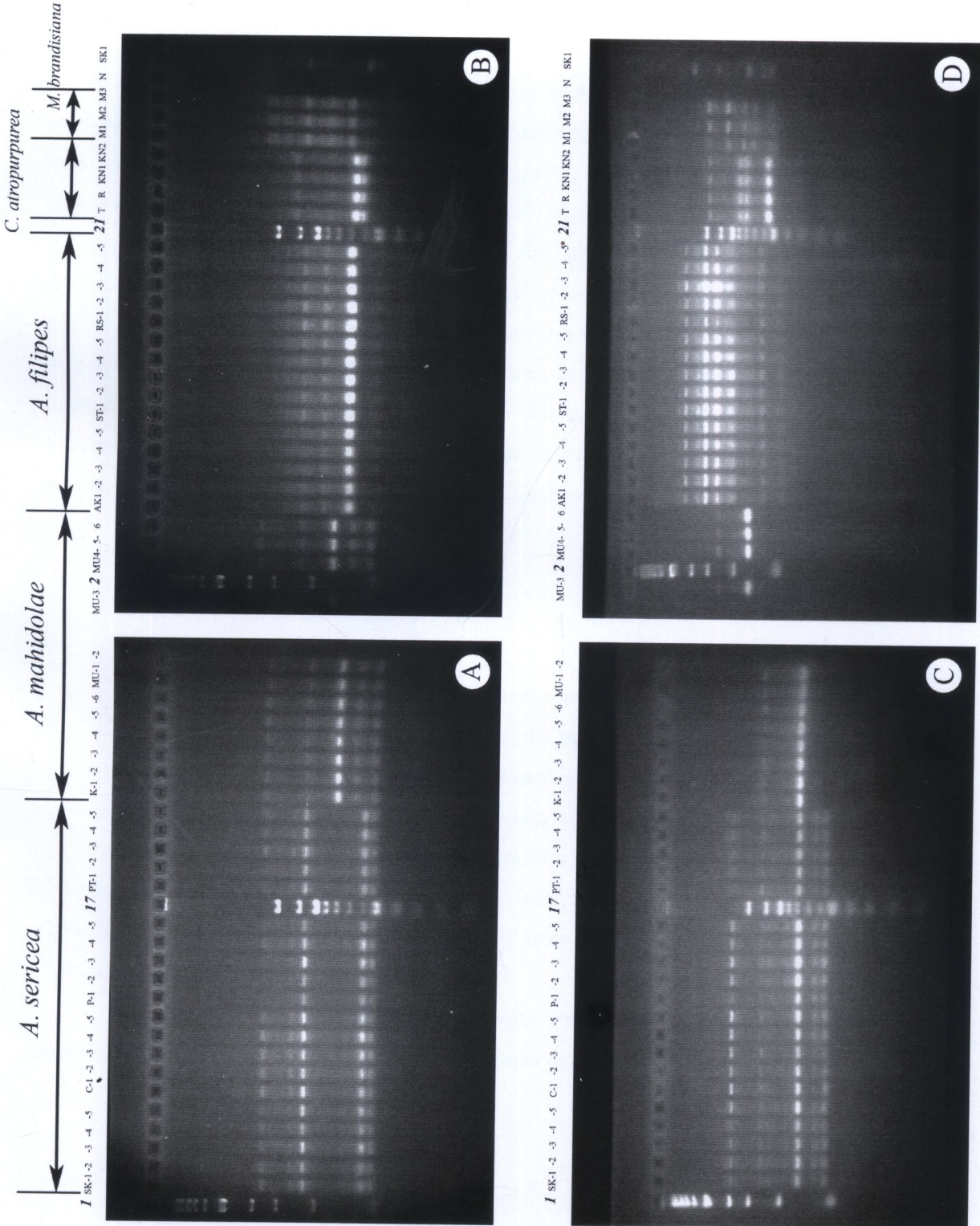
4.4.2 DNA Sequencing Analysis

ITS (Internal transcribed Spacer) Analysis

The length of ITS region ranged from 507 (*M. brandisiana*) to 679 bp (*A. filipes*). The aligned sequence of 15 taxa was consisted of 786 bp. Of the 786 characters, 326 were variable, of which 218 (27.7%) were potentially phylogenetically informative while 50 (6.4%) were singleton.

A branch and bound search produced only one most parsimonious tree of 397 steps with CI of 0.79 (excluding uninformative characters) and RI of 0.91 (Fig. 4.9). The most parsimonious tree resolved that the genus *Afgekia* was not monophyletic with 69% Bootstrap Support [BS]. The first clade consisted of a sister taxon, *A. mahidolae* and *A. sericea*, with 100% bootstrap support. This first clade was the sister taxon to the second clade, which comprised of 2 small subclades, namely the calde of *C. atropurpurea* and the clade of 2 *Millettia* species, with 95% bootstrap support. Within the second clade, *M. pulchra* formed the sister taxon to *M. brandisiana* with 100% bootstrap support in one subclade, and taxa of *C. atropurpurea* with 96% bootstrap support in another subclade. *A. filipes*, on the other hands, was remotely placed in its own clade, and formed sister to the first + the second clades with 69% bootstrap support.

trnL-F IGS (Intergenic Spacer) Analysis

The length of the *trnL-F* IGS region ranged from 346 (*M. brandisiana*) to 496 bp (*A. sericea*). Within the genus *Afgekia*, the length ranged from 402 (*A. filipes*) to 496 bp (*A. sericea*). The aligned *trnL-F* IGS sequence of 15 taxa was consisted of 628 bp. This non-coding region within *Afgekia* was quite variable and 20 indels ranging in size from 1 to 31 bp had to be inserted. Of all characters used, 87 were variable, of which 47 (7.48 %) were potentially phylogenetically informative while 9 (1.43 %) were singleton. At last, 5 taxa (1 taxa of *A. sericea*, 2 taxa of *A. mahidolae* and 2 taxa of *C. atropurpurea*) were excluded from the analysis.

The parsimony analyses resulted in 2 equally most parsimonious trees, with a length of 62 steps, CI = 0.92 (excluding uninformative characters) and RI = 0.95 (Fig.

4.10). The difference between these two topologies was the position of *A. filipes* and an outgroup taxon, *M. brandisiana*. The first topology showed that *M. brandisiana* was sister to the clade comprised of *A. sericea* and *A. mahidolae* with slightly weak bootsrap support (only 53%). In this topology, *A. filipes* was placed sister to the large clade consisted of *M. brandisiana*, *A. sericea* and *A. mahidolae*. Therefore, *Afgekia* is not monophyletic in the first topology. In the other topology, in contrast, the tree showed that genus *Afgekia* was monophyletic with slightly weak bootsrap support (only 58%). In this second topology, *A. mahidolae* was sister to *A. sericea* with 94% bootsrap support. *A. filipes* were placed as the sister taxon to the *A. malidole + A. sericea*.

Although the result from *trnL-F* IGS analysis could not provide conclusive evidence for the monophyly of *Afgekia* because of the contrary of the tree topologies, both topologies supported the close relationship between *A. sericea* and *A. mahidolae* while *A. filipes* were placed far away from them.
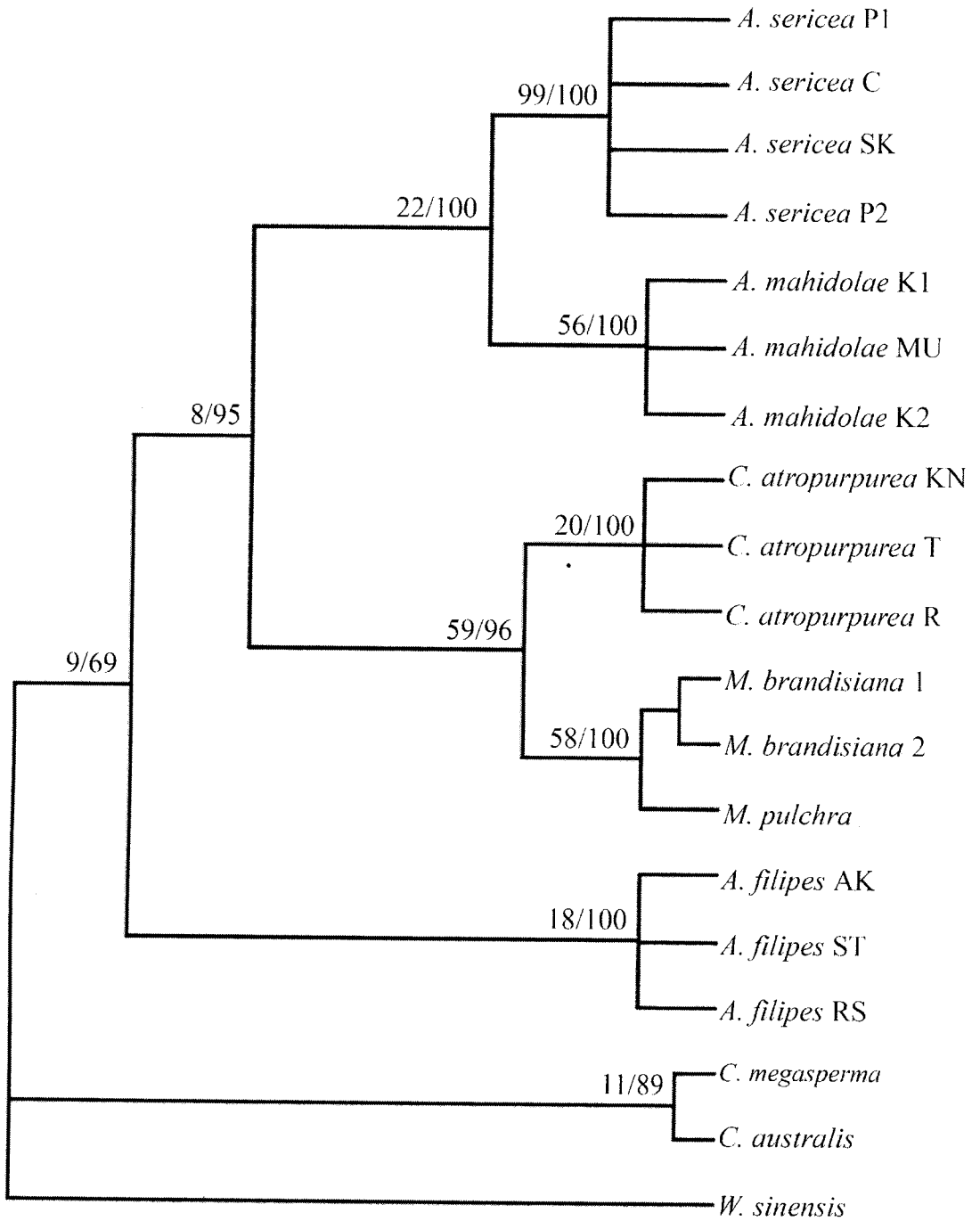
Figure 4.9 The single most parsimonious tree from the ITS analysis. Numbers in front of slash correspond to branch lengths; numbers after slash are bootstrap values from 100 replicates. The abbreviations after the specific epithets indicate populations of species according to Table 3.3.

Figure 4.10 The two equally most parsimonious trees of *Afgekia* and outgroup taxa from the *trnL-F* IGS analysis. Numbers in front of slash correspond to branch lengths; numbers after slash are bootstrap values from 100 replicates. The abbreviations after the specific epithets indicate populations of species according to Table 3.3.
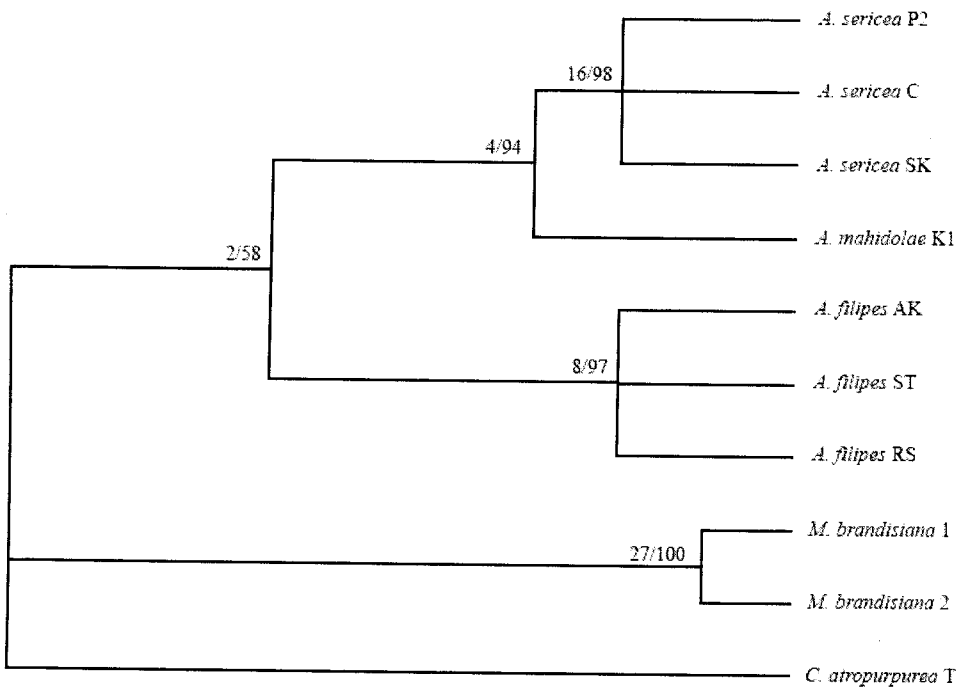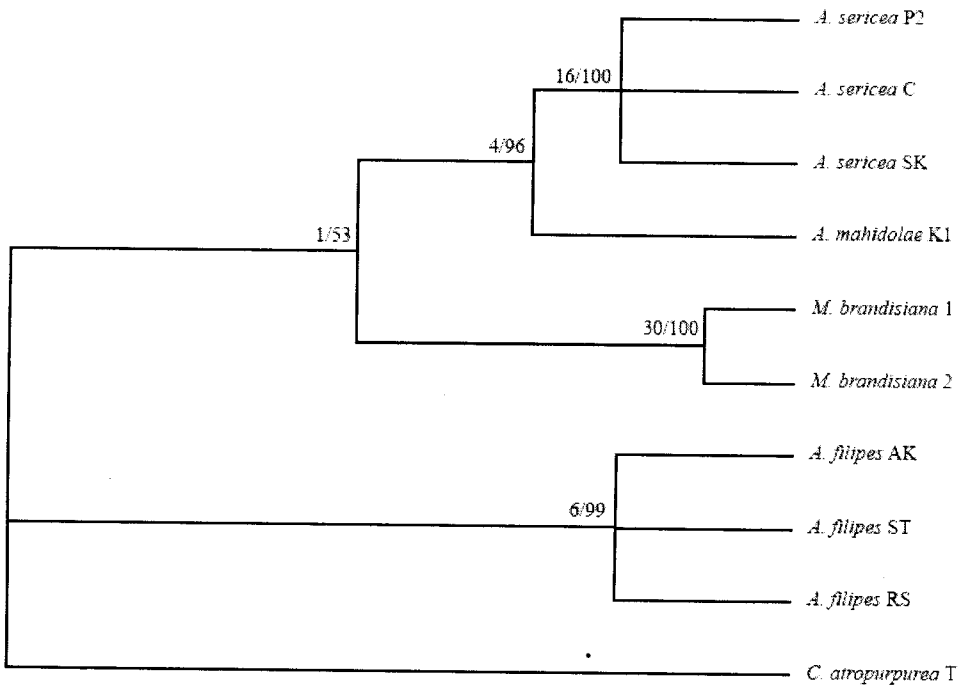
CHAPTER V

DISCUSSION

5.1 Morphological Evidences

It can be seen from Table 4.1 that *Afgekia filipes* and the previous recognized species of *Afgekia*, i.e. *A. sericea* and *A. mahidolae* have some distinct morphological features. The most distinguish character is the type of inflorescence. In general, *A. filipes* has paniculate-like inflorescence, i.e. each branch of this inflorescence type is not a true branching of a panicle but is a short axillary racemose inflorescence that units together at adjacent position near the terminal of a vegetative branch and formed a panicle-liked structure. In contrast, *A. sericea* and *A. mahidolae* have long axillary or terminal racemose inflorescence, without bearing or clustering at adjacent position. In the previous study of Geesink (1984), the different inflorescence types are the predominant structures used to subdivide the central genera of the tribe· Millettieae, viz. *Derris*, *Millettia*, *Lonchocarpus*, into smaller and clearer genera. For example, the genus *Callerya* was divided from *Millettia* on the account of it having true and thick terminal panicles (Schot, 1994). Moreover, from the taxonomic history of *Callerya* the inflorescence characteristics are also used to recognize the 2 synonymous genera of *Callerya*, viz. *Padburggea* and *Whitfordiodendron* (Dunn, 1911). The character of paniculate-like inflorescence found in *A. filipes* is found in neither *Callerya* nor *Millettia*. However, it requires more morphology characters than only the inflorescence type to indicate the suitable genus for this species.

Young leaves of *Afgekia. filipes* is usually reddish brown or brownish green and gradually turn to green at maturation. Those young leaves are nearly glabrous or sometimes slightly covered with golden-brown hairs, less pubescent than the two previous known species. As a climbing plant, it is found that the *Afgekia* species is a sun-loving plant and usually grow in fully exposed area. For this purpose, the plant usually obtains some morphological features that prevent the leaves from direct contact to strong sunlight or ultraviolet radiation (Mauseth, 2003), in this regards *A. sericea* and *A. mahidolae* have pubescent young leaves. In the other hand, young leaves of *A. filipes* is nearly glabrous, however this climbing plant has reddish brown pigments instead which contain some flavonoids or anthocyanin (Pourcel et al, 2007), this compounds was reported to protect the leaves from strong irradiation (Lumsden, 1997).The presence of

reddish-brown young leaves, interestingly, is also found in some species of *Callerya* and *Millettia*, the close relative of *Afgekia*.

The presence of blood-red sap in woody stem of *A. filipes* is another unique character. This character is occasionally found in the species of Fabaceae. Recently, Nielsen & Veillon (2004), had published a new species, *Callerya neocaledonica* I.C. Nielse & Veillon, collected from New Caledonia. They mentioned that this species is a large liana with blood-red sap in stems which is visible in only the old parts. This character is in common with *A. filipes* and is another evidence that indicates the close relationship between *A. filipes* and *Callerya*. The true roles of red sap of this species are still unknown. However, in most species of plants, sap containing various types of secondary metabolites such as terpenoids or other polyphenolic compounds plays a vital role in plant defense mechanism (Langenheim, 1993). This supports the observation from this research that *A. filipes* rarely has natural pests or pathogens.

As regards to flower, *Afgekia filipes* has fragrant flowers, whereas *A. sericea* and *A. mahidolae* are odorless. The fragrance of flower can attract pollinators to visit and resulted in successful pollination and fruit setting, (Weberling, 1992), in compensating to the dull color of bracts and flowers of this species. The fragrant flowers have been ascertained to be due to the presence of some volatile oils, a chemical substance unique to each plant group (Hill et al., 1972, cited in Gerlach and Schill1, 1988). However, this present research has no intention to investigate on chemotaxonomy. Any how, it is evident that *A. filipes* has a rather unique chemical compounds which is distinct from the previous known species of *Afgekia*. Moreover, *A. filipes* also exhibits some distinct morphological features of flowers. Anthers of *A. filipes* are glabrous while *A. sericea* and *A. mahidolae* have anthers with turf of hairs at the bases (Fig 4.2 D). *A. filipes* also has short triangular calyx lobes whilst *A. sericea* and *A. mahidolae* have long acute ones.

The diadelphous stamens with the upper filament wholly free is a common feature for most members of the Fabaceae, however there are some variations of this character among the genera (Geesink, 1984; Hu and Chang, 2003). It is found that the upper filament of *A. sericea* and *A. mahidolae* distinctly fuse to the other 9. While the upper filament of *A. filipes* is slightly adheres to the other 9. In *Callerya*, the upper filament is wholly free, whilst both free and united upper filament are found in *Millettia*. This is an additional distinctive character between *A. filipes* and the previous recognized species, *A. sericea* and *A. mahidolae*.

While *A. filipes* has many characters differ from the previous known *Afgekia* species, it seems likely that this plant species has many characters in common with the related genus *Callerya* and *Millettia* (Table 4.1). Geesink (1984) had transfered this species from the genus *Padbruggea* to the genus *Afgekia* based mainly on the characters of seed which having fleshy and elongate hilum corresponding to fleshy and elongate funicle. However, this character is not clearly distinct as compared to that found in *A. sericea* and *A. mahidolae*. The absence of bracteole is another character which Geesink decided to place this species in *Afgekia*. However, the result from morphological study showed that *A. filipes* has rather reduced bracteoles (Table 4.1), this result is agreed with the previous study by Wei (1994).

From Table 4.1 it also can be seen that *A. sericea* and *A. mahidolae* are morphologically similar. Both species can be separated base mainly on floral characters (Burtt and Chermsirivathana, 1971). Without flowers, it is hard to distinguish these two species. However, it was found that *A. mahidolae* has less number of leaflets and less hairiness on leaves than *A. sericea*. While the other vegetative characters of both species are hard to differentiate.

From all general morphological features mentioned above, it is evident to summarize that the *Afgekia* (sensu Geesink, 1984) is a heterogenous genus based mainly on qualitative characters. Since some characters are found only in *A. filipes*, and these characters are common with the other related genera. To re-delimit the genus *Afgekia* by excluding *A. filipes* may be a good alternative choice for the more clearer boundary and better understanding of the homogenous genus *Afgekia*.

5.2 Morphometric Analyses

The result of the morphometric study also supported a close relationship between *A. sericea* and *A. mahidolae*, since these two species were placed in the same group in the phenogram of cluster analysis at the 0.76 level of Gower similarity coefficient (Figure 4.4) and on canonical axis 1 of the ordination plot from the result of canonical discriminant analyses (Figure 4.5). It was evident that *A. filipes* was morphologically distant from *A. sericea* and *A. mahidolae* and was placed in its own group. There were nine important characters that were selected as important characters in giving the best separation of the groups (Table 4.3) on canonical axis 1, i.e. pod width, pod thickness, pod length, filament length, bract width, rachis diameter, peduncle length, leaflet length

and petiole length in descending order in a corresponding manner. In addition, on canonical axis 2, there were ten important characters, viz. bract length, number of leaflets, leaflet width, anther length, calyx lobe length, keel length, pedicel length, standard length, inflorescent length and standard width in descending order in a corresponding manner. It was found that canonical variable 1 was more important for segregating the three species, since canonical variable 1 is 99.7 % correlated with all the variables and the variance explained by it is 88 % (Table 4.4). Among the nine important characters of canonical variable 1, pod width, pod thickness, and pod length were the most three important characters that gave the best separation of the species. This conclusion agreed with the morphological observation. It is distinctly found that pod of *A. filipes* is large in size and the pericarp is hard, woody and rather thick as compared to pods of *A. sericea* and *A. mahidolae*. However, it is found that the pod of *A. sericea* is slightly larger than the pod of *A. mahidolae* (Figure 4.3 A).

In the light of both cluster and canonical discriminant analysis, it is evident that *A. filipes* should be classified into one taxon, preferably in its own/another genus separated from *Afgekia*.

5.3 Molecular Analysis

The results from all molecular analyses indicated in the same direction and were congruent with both general morphology study and morphometric analysis. In RAPD analysis, the UPGMA dendrogram derived from Dice's (1945) similarity coefficient supported the genetic similarity between two closely related species, *A. sericea* and *A. mahidolae*. Both species were placed in the same group. *A. filipes*, on the other hands, is genetically similar to *Callerya atropurpurea*, a member of the genus *Callerya*. The last group comprised only one outgroup taxon, *M. brandisiana*. This result was not surprisingly because all *Callerya* and *A. filipes* were at one time placed in the genus *Padbruggea* (Geesink, 1984) and previous molecular studies also indicated the close genetic relationships between *A. filipes* and *Callerya* (Hu et al, 2002; Hu and Chang, 2003). The result from RAPD analysis agreed with the morphological studies that separated *A. filipes* from *Afgekia*. This study, however, did not support the previous classification of Geesink whose proposed placing *A. filipes* in *Afgekia*.

The results from ITS sequencing analysis, in addition, showed that all species of *Afgekia* are not monophyletic. It also indicated the close phylogenetic relationship between *A. mahidolae* and *A. sericea* as both species were placed in the same clade with high bootstrap support up to 100%. *A. filipes* was placed in its own clade and was not part the former. Previous ITS studies (Hu et al., 2002) strongly suggested that *A. filipes* was closely related to the Australian *Callerya* viz *C. magasperma* and *C. australis*. This tropical Australian-Asian clade also formed a notable association with the genus *Wisteria*. Thus, the ITS sequences of those Australian *Callerya* and *Wisteria* (*W. sinensis*) were included in this analysis and the results showed that they were placed at the basal position of the tree, not sister to *A. filipes* as found in the previous study of Hu et al. (2002). This incongruent results might be due to the different sequence alignment or the choice of outgroup taxa in this analysis.

In contrast, the results of *trnL-F* IGS analyses provided 2 different views of relationships among three species of *Afgekia*. In one view, it was congruent with earlier analyses in this study, i.e. *A filipes* was placed outside the *A. sericea-A. mahidole* clade resulting paraphyly of the genus *Afgekia*. The other, however, the genus *Afgekia* was monophyletic with *A. filipes* as the basal clade of the genus. Although the latter view was in the same line of Geesink's (1984) classification, it constrasted not only results from morphology study, morphomemetric analyses, RAPD and ITS analyses in this study, but also previous studies of Hu et al. (2002) and Hu and Chang (2003). Because the monophyly of *Afgekia* had bootstrap support for only 58%, which quite low, thus it was suggested that *Afgekia* probably was not a monophyletic group. The exclusion of *A. filipes* from *Afgekia*, hence, will resolute this problem.

Surprising, that previous studies on morphology (Burtt and Chermsirivathana, 1971.) as well as this study and morphometric analyses results indicated that *A. sericea* and *A. mahidolae* are very morphologically similar to each other was not supported by molecular data. It was found that a large number of nucleotide substitution in ITS and *trnL-F* IGS sequences occurred between *A. sericea* and *A. mahidole* indicating that the morphological difference and genetic difference rates were not the same in these two species. Prathepha (1999) had discussed in his study that *A. sericea* and *A. mahidolae* become differentiated as a result of accumulation of chromosome rearrangements in allopatric populations of the ancestral species without changing the chromosome number. This hypothesis was supported from the past geological events in Thailand which clearly elucidated the origin and separation of these two species.

5.4 Taxonomic Implication

Thus far, in all analyses employing different types of characters in this study and previous studies (Hu et al., 2002; Hu Hu and Chang, 2003) conclusively suggested that *A. filipes* is not part of *Afgekia*. The characters used by Geesink (1984) may not be warranted to place this taxon within *Afgekia*. Therefore, it is recommended that this taxon should be removed from *Afgekia*. The question remains to which genus this taxon taxonomically belongs. The obstacle stems from that fact that previous studies indicated two genera, *Milletia* and *Callerya*, were polyphyletic. Therefore, it is difficult to ascertain that which of these two genera that *A. filipes* should be placed. From the results in this study as well as previous studies (Hu et al., 2002), *A. filipes* allied with *Callerya* but never with *Millettia*. As such, it seems conservative to transfer *A. filipes* to the genus *Callerya* for the time being until more concrete evidence indicate otherwise.

From all mentioned above, it indicated that *A. filipes* should not belong to *Afgekia* due to making *Afgekia* become monophyletic and homogeneous genus. Now, the question that what is the most suitable genus for *A. filipes* is raising. Base on morphological study, it is found that some characters found in *A. filipes* are also found in its closely related genera as showed in Table 4.1. The closest relative to be firstly attended is *Callerya* because *A. filipes* once were placed in the genus *Adinobotrys* and *Padbruggea*, which now were considered synonyms of *Callerya* according to Geesink (1984). The genus *Callerya* has a long taxonomic history. Once, it was only a forgotten synonym of *Millettia* until it was re-instated by Geesink (1984) and revised by Schot (1994). Geesink started to rearrange the subgroups of the polymorphic genus *Millettia* on account of their inflorescences into clearer circumscribed genera. As a consequence, *Padbruggea* and *Whitfordiodendron* (*Adinobotrys*) including *Millettia* section 'Eurybotryae' and 'Austromillettia' were united into the same genus *Callerya* which differ from *Millettia* due to the true paniculate inflorescences and the diadelphous stamens (Geesink, 1984 and Schot, 1994).

According to *Afgekia filipes*, this species has intermediate position. Craib (1928) agured that *Padbruggea* and *Whitfordiodendron* (*Adinobotrys*) were cogeneric because he found the resemblances of *Adinobotrys filipes* (now *Afgekia filipes*) in habit mostly *Padbruggea dasyphylla* (now *Callerya dasyphylla*), but has the generic character of *Adinobotrys*.

From the result of this study, the intermediate position of *Afgekia filipes* is still exist. This species has the seed characteristics belonging to *Afgekia*, but, some other characters belonging to *Callerya* (and sometime to *Millettia*). The molecular evidences from this and previous study (Hu et al, 2002) also revealed the genetic and phylogenetic similarity between *A. filipes* and *Callerya*, especially the Australian *Callerya* such as *C. megasperma* and *C. australis*. This similarity, surprisingly, is closer than other Asian *Callerya* such as *C. atropurpurea* or *C. cinerea*. This indicated the co-evolution of DNA sequence of *A. filipes* and Australian *Callerya* in spite of the different and completely separated habitat and distribution.

Geesink (1984) mentioned that *A. filipes* had many characters which indicated more primitive species than the other 2 species of *Afgekia*. *Callerya*, in the same way, exhibits more primitive characters than *Afgekia*, for example, the general habit (large liana to tree) or the fusing level of an upper filament to other 9 filaments (free). From the ITS study of Hu et al showed that *Callerya* were paraphyletic group and has the same ancestor with *Afgekia* and *Wisteria*. Therefore, because *A. filipes* has intermediate characters of *Callerya* and *Afgekia*, it might probable that this species was the evolutionary transition between those 2 genera. This is very interesting and may be a good candidate for evolutionary study of these groups in the future.

In conclude, the most probable and suitable genus to transfer *A. filipes* to is *Callerya* viz. *Callerya filipes* (Dunn) Sirichamorn due to its similar morphology such as general habitat, the presence of (reduce) bracteole, the shape of calyx lobe and glabrous anthers with slightly fusing upper filament. The character of inflorescence, unfortunately, does not match with *Callerya* because it was pseudopaniculated inflorescence according to Geesink (1984). However, in this study, it was also found that the inflorescence of *A. filipes* is only axillary racemes usually bearing at adjacent position near the terminal of a vegetative branch and forming a panicle-like inflorescence. This is not congruent with the definition of psedopaniculate inflorescence proposed by Geesink (1984) and the revision study of *Callerya* by Schot (1994). Thus, the definition of pseudopaniculated inflorescence proposed by Geesink (1984) is equivocal. From these reasons, the genus delimitation and definition of *Callerya* according to Geesink (1984) and Schot (1994) should be change. Obviously, the genus *Callerya* now requires a revision again.

CHAPTER VI

CONCLUSION

The genus *Afgekia* Craib is a small genus in the tribe Millettieae sensu Geesink of the Family Fabaceae. According to Geesink (1984), there are three species within this genus. All of them grew naturally in Thailand, but not in common habitat. They are *A. sericea* Craib, *A. mahidolae* Burtt et Chermsirivathana and *A. filipes* (Dunn) Geesink. The first two species are morphological, cytological and ecological similar. While *A. filipes* is quite remote from the first two species and shares some morphological characters to the related genus, *Callerya* Endl., Geesink (1984) transferred this species to the genus *Afgekia* base mainly on the seed character which shows elongated hilum and funicle and the absence of bracteoles. From the past up to now, there are many researches from various branches of science directly or indirectly related to the genus *Afgekia*. Unfortunately, no single paper has included all three species of the genus *Afgekia* in the study. Thus, the taxonomic status of the species in this genus is still obscure and worth investigating.

The result from general morphology study base mainly on qualitative characters showed that *A. filipes* had some characters which was not found in the other 2 species of *Afgekia*. In addition, these characters are similar to those found in some species of the related genera, *Callerya* and *Millettia*. For instances, the presence of blood-red sap in old stem which is found in *Callerya neocaledonica* I.C. Nielse & Veillon; reddish-brown young leaves which is found in many species of both *Callerya* and *Millettia*. Both characters are also observed in *A. filipes*. Another characters are the shape of calyx lobes, absence of anther hairiness or the level of upper filament fusion or general habit and habitat which are very similar to *Callerya*. Among those characters, the panicle-like inflorescence of *A. filipes* is the most distinguish character clearly different from *A. sericea* and *A. mahidolae* whose inflorescent are long, simple terminal or axillary raceme, never branching or jointing together.

Morphometric analyses were used to investigate the classification and taxonomic status and to determine the important morphological characters that contribute to discriminating all 3 *Afgekia* species. The results from both cluster analysis and canonical discriminant analysis, like the result from general morphology study, supported the morphological similarities between 2 closely related species, *A. sericea* and *A. mahidolae*, and contrasted *A. filipes* from other two species. According to Canonical Discriminant

Analysis, 19 characters are important characters in giving the best separation among 3 species on canonical axis 1 and 2. These include pod width, pod thickness, pod length, filament length, bract width, rachis diameter, peduncle length, leaflet length, petiole length, bract length, number of leaflets, leaflet width, anther length, calyx lobe legth, keel length, pedicel length, standard length, inflorescence length and standard width. Among those 19 characters, evidently, it was found the size of pod, i.e., pod width, pod thickness, and pod length were the most three important characters that gave the best separation among species. This conclusion agree with the morphological observation: *A. filipes* is naturally large in size and the pericarp is hard, woody and rather thick as compare with pods of *A. sericea* and *A. mahidolae*. It is also found that the pod of *A. sericea* is slightly larger than the pod of *A. mahidolae*.

Results from molecular analyses of both RAPD and DNA sequences are congruence with morphological analyses. Phenogram derived from RAPDs showed the close genetic similarity between *A. sericea* and *A. mahidolae*. These two species were clearly separated from *A. filipes*. In addition, dendrogram also showed that *A. filipes* was genetically similar to *C. atropurpurea*. Phylogenetic trees based on nuclear ITS and chloroplast *trnL-F* IGS regions indicated the same relationships among 3 *Afgekia* species. The most parsimonious trees revealed that *Afgekia* was not monophyletic. *A. sericea* always formed sister to *A. mahidolae* with very high boostrap support (100% for ITS and 95% for *trnL-F* IGS). *A. filipes*, on the other hands, were always not part of the *A. sericea-A. mahidolae* clade. Therefore, to make *Afgekia* monophyletic is to exclude *A. filipes* from the genus *Afgekia*. Moreover, the results from these molecular analyses also implied the non-monophyly of the genus *Callerya*.

In conclusion, all analyses in this study yielded congruent results. All indicated that *A. filipes* should not be placed in *Afgekia* due to its different morphological and genetic characteristics from other 2 species of *Afgekia*. These results were contrast to the widely used classification system by Geesink (1984). Seed characters, which Geesink used for transfering *Padbruggea filipes* to the genus *Afgekia*, is not taxonomically informative in the light from this study. The most suitable genus to transfer *A. filipes* is *Callerya*.

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