

PHYLOGENETIC RELATIONSHIP BASED ON
FLAVONOIDS AND ITS REGIONS STUDIES ON
SELECTED *BOESENBERGIA* SPECIES IN SABAH

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HUBUNGAN FILOGENI BERDASARKAN KAJIAN
FLAVONOID DAN 'ITS REGIONS' SPESIES
BOESENBERGIA YANG TERPILIH DARI SABAH

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DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

25 JUNE 2004



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ABSTRAK

HUBUNGAN FILOGENI BERDASARKAN KAJIAN FLAVONOID DAN 'ITS REGIONS' SPESIES *BOESENBERGIA* YANG TERPILIH DARI SABAH

Satu kaedah penyaringan mudah dengan HPLC menggunakan kolum analitikal fasa lawan dijalankan ke atas sembilan spesies dari genus *Boesenbergia* dan *Zingiber philipseae*. Kadar aliran kolum adalah 0.5mL/min dengan pengeluaran gradien menggunakan 0.5% asid formik akuas: Asetonitril (99:1) ke nisbah 0:100 dalam jangkamasa 25 minit. Pengesanan adalah di atas jarak gelombang ultra-lembayung ternampak antara 190nm ke 500nm dan jarak gelombang spesifik pada 280nm. Jenis kumpulan flavonoid dicam daripada spektra. Setiap species didapati mengandungi beberapa sebatian flavonoid daripada dua (*Boesenbergia* sp. 4) ke tujuh (*B. pulchella* var. *attenuata*) sebatian. Semua spesies *Boesenbergia* itu didapati mengandungi sebatian flavon/flavononol. Daripada sembilan spesies *Boesenbergia*, tujuh didapati mengandungi flavon glikosida kecuali *Boesenbergia* sp. 4 dan *Boesenbergia* sp. 5. Hanya empat species *Boesenbergia* didapati mengandungi sebatian isoflavon iaitu *Boesenbergia* sp. 1, *Boesenbergia aurantiaca*, *Boesenbergia aurantiaca* var. dan *Boesenbergia* sp. 5. Sementara itu, hanya spesies dari Semenanjung Malaysia, *Boesenbergia rotunda*, mengandungi sebatian kalkan yang tidak dapat dikesan dalam spesies-spesies dari Borneo. 19 penjujukan *Boesenbergia* dan penambahan lima kumpulan-luaran memberi jumlah sampel di dalam kajian filogeni kepada 24 spesies. Satu analisa penjujukan yang terdiri daripada 19 penjujukan ITS spesies *Boesenbergia* bersama-sama lima penjujukan kumpulan luaran telah dilakukan. Didapati 248 mempunyai maklumat parsimoni. *Tamijia flagellaris* digunakan untuk mengakar pokok filogeni dan perhubungan monofilitik genus *Hedychium* adalah 'sister group' kepada *Boesenbergia* (BS=100%). Analisis filogeni ke atas 'ITS region' memberi satu 'most parsimonious tree'. Daripada matriks lengkap data, pokok filogeni yang dihasilkan (panjang=162288 langkah) itu memberi indeks konsisten=0.6877; indeks pertahanan=0.7045 dan indeks konsisten terskala=0.4845. Hubungan antara spesies *Boesenbergia* yang dikaji adalah separa diselesaikan. Terdapat spesies yang mempunyai sokongan 'bootstrap' yang lemah ke atas dahan pokok filogeni tersebut. Dua kumpulan *Boesenbergia* kelihatan monofilitik ('Bootstrap'=97%). Kumpulan pertama itu, iaitu, BOESENBERGIA I mempunyai sokongan monofilitik dengan nilai 'bootstrap support' sebanyak 70%. Kumpulan kedua pula, iaitu BOESENBERGIA II terdiri daripada 'Borneo clade' I, 'Borneo clade' II, 'Borneo clade' III, 'Borneo clade' IV dan *B. rotunda* ('Bootstrap'=98%). Sebatian flavonoid dan data 'ITS region' memberi keputusan yang kongruen dalam pengumpulan di atas.

ABSTRACT

PHYLOGENETIC RELATIONSHIP BASED ON FLAVONOIDS AND ITS REGIONS STUDIES ON SELECTED BOESENBERGIA SPECIES IN SABAH

Nine species of *Boesenbergia* and one of *Zingiber*, namely, *Zingiber philipsea* were screened using HPLC equipped with a reverse-phased analytical column. The flow rate was 0.5mL/min and gradient elution with 0.5% aqueous Formic acid: Acetonitrile from 99:1 to 0:100 ratio in 25 minutes with detection over a wavelength range 190nm to 500nm and specific detection at 280nm. Types of flavonoids were viewed from the online spectra of the crude extract of each peak. Each species contained a few flavonoid compounds ranging from two (*Boesenbergia* sp. 4) to seven (*Boesenbergia pulchella* var. *attenuata*). All the *Boesenbergia* species were found to contain flavonone/flavonol compounds. From the nine species of *Boesenbergia*, seven were found to contain flavone glycosides except *Boesenbergia* sp. 4 and *Boesenbergia* sp. 5. Only four species of *Boesenbergia* were found to contain isoflavone compounds. They were *Boesenbergia* sp. 1, *Boesenbergia aurantiaca*, *Boesenbergia aurantiaca* var. and *Boesenbergia* sp. 5. Meanwhile, the only species from Peninsular Malaysia, *Boesenbergia rotunda*, was found to contain chalcone which was undetected in the Bornean species. An alignment was made of 19 ITS sequences of *Boesenbergia* species, plus five outgroup sequences. A total of 248 parsimony-informative positions were done. *Tamijia flagellaris* was used to root the tree and the monophyletic *Hedychium* species is the sister group to the *Boesenbergia* (Bootstrap=100%). Phylogenetic analysis of the ITS region resulted in the generation of a single most parsimonious tree. With the complete data matrix of the region, including uninformative characters, the resulting tree had a length (L) of 162288 steps with the following fit measures: Consistency Index=0.6877, Retention Index=0.7045 and Rescaled Consistency Index=0.4845. The phylogenetic tree partially resolved the relationship among species within the genus *Boesenbergia*. The species have some branches with weak bootstrap support, showing that some ambiguity still exist in the placement of species. Two group of *Boesenbergia* appeared monophyletic (Bootstrap= 97%). The first group is monophyletic supported by 70% bootstrap value (BOESENBERGIA I) and the second group constituted BOESENBERGIA CLADE II: Borneo clade I, Borneo clade II, Borneo clade III, Borneo clade IV and *B. rotunda* (Bootstrap=98%). The flavonoid and ITS region data gave congruent results in the groupings of the above.

LIST OF ABBREVIATIONS

BS	: Bootstrap
CI	: Consistency Index
DNA	: Deoxyribonucleic acid
HPLC	: High performance liquid chromatography
H ₂ O	: Water
ITS	: Internal transcribed spacer
MeOH	: Methanol
PCR	: Polymerase chain reaction
PDA	: Photodiode array
RC	: Rescaled Consistency Index
rDNA	: Ribosomal deoxyribonucleic acid
RI	: Retention Index
RNA	: Ribonucleic acid
rRNA	: Ribosomal ribonucleic acid
TLC	: Thin layer chromatography
TWR	: Tabin Wildlife Reserve
UV	: Ultraviolet
UV-vis	: Ultraviolet-visible



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Appendix C.4: Morphological characteristics of *Boesenbergia* species:

B. pulchella, *B. pulchella* var. *attenuata*, *B. pulcherrima*
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KEYWORDS

Phylogeny, Phylogenetic, *Boesenbergia*, Zingiberaceae, Flavonoids



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CHAPTER 1

INTRODUCTION

1.1 Phylogenetic reconstruction

Phylogenetic reconstruction is used as a model to obtain a better understanding of both patterns and processes of evolution (Soltis & Soltis, 2000). The organisms are arranged into groups on the basis of their evolutionary relationship, which involves the process of separation of lineages. This maybe caused by adaptation of the organism to extreme habitats or the change of environment (Judd, *et al.*, 1999). The continuous research on phylogeny will contribute to the knowledge for the exploration and exploitation of plants and for conservation of organisms or ecosystem (Hegnauer, 1988).

The premier and novel publication of the phylogenetic relationships of plant family was produced between 1962 and 1973 with updates from 1986 until today (Hegnauer, 1963; 1986a, 1986b; 1988; 1990). Hegnauer reviewed the literature on the distribution in the plant kingdom of secondary substances of many plant families based on their chemical profiles. The development of molecular systematics in later years such as using the plastid gene *rbcL* for phylogenetic studies had resulted in many cladograms on plant families. These cladograms can be used as a framework for further studies, using a combination of data from the various disciplines (Grayer, Chase & Simmonds, 1999).

The trend to discover new chemical compounds useful for plant systematics is rarely reported in the past twenty to thirty years. More phytochemical investigations today are based on bioactivity-led perspective. Computer databases of plant compounds in the future can help assess chemical relationships between taxa using

phylogenetic methods (Grayer, Chase & Simmonds, 1999), comparable to the usage of molecular databases like GenBank.

1.2 Genus *Boesenbergia* - an important genus?

The importance of *Boesenbergia rotunda* (L.) Mansf. in ginger phytochemistry has proved outstanding. *B. rotunda* was previously known as *Boesenbergia pandurata* (Robx.) Schltr. A number of intensive studies had been carried out on this plant in the last decade (Chu, *et al.*, 1998; Murakami, Ohigasi & Koshimizu, 1995; Tashiro, *et al.*, 1995; Tiwawech, *et al.*, 2000; Trakoontivakorn, *et al.*, 1999; Tuchinda, *et al.*, 2002). The flavonoid compounds in this plant are so notable that there are many on-going clinical studies in countries like Thailand and Japan. The relationships and medicinal values of other species in this genus remain unclear, however.

There is still a large uncharted territory of bioactive metabolites in plants of this genus. According to Harborne (2000), almost every plant species has a unique collection of secondary constituents distributed throughout its tissues. Currently, a few studies are done, mainly on the morphology and clinical properties of the genus *Boesenbergia*. Very little has been done, however, on *Boesenbergia*'s cytogenetic, genetics and phylogeny.

We do not know whether the chemicals in *Boesenbergia rotunda* have altered or evolved faster than the other species in the same genus. There could be a large variation between *B. rotunda* and other species. Therefore, the aims of this research are to conduct a study on the phytochemistry properties of a few *Boesenbergia* species and to correlate the evolution of chemical constituents and molecular genetics with existing morphological data.

The medicinal values from folk medicines are normally known to indigenous people. The knowledge of the usage of other species of *Boesenbergia*, however, remains unknown. For instance, as told by villagers in Lahad Datu, Sabah, roots of one *Boesenbergia* species in the Tabin limestone area are used to heal stomach

CHAPTER 2

LITERATURE REVIEW

2.1 The phylogeny of the family Zingiberaceae

Zingiberaceae is a very well known family of plants among botanists worldwide. It is a pantropical family of plants of about 53 genera and 1300 species with the center of diversity in South and Southeast Asia (Larsen, *et al.*, 1999, Humpreys, 1996; Kress, Prince & Williams, 2002).

The first report on phylogeny done on the order of Zingiberales by Kress (1990) showed the most parsimonious topology as (Musaceae (Strelitziaceae (Lowaceae (Heliconiaceae ((Zingiberaceae, Costaceae) (Cannaceae, Marantaceae)))))). The cladogram proposed the recognition of eight families, two superfamilies and five suborders within the Zingiberales (Figure 2.1).

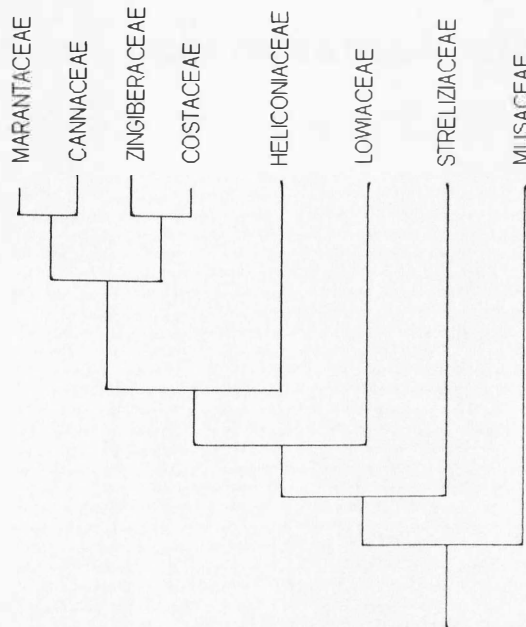
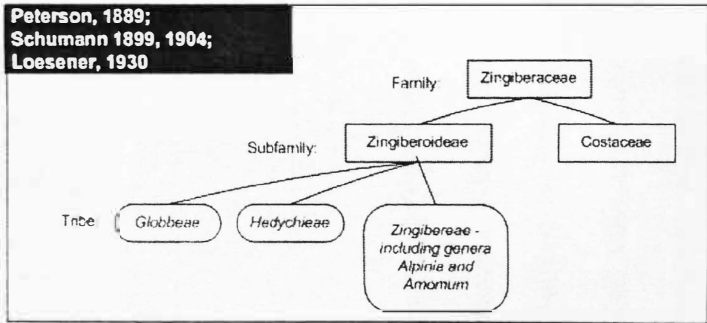


Figure 2.1: Most parsimonious cladogram of the Zingiberales (adapted from Kress, 1990).

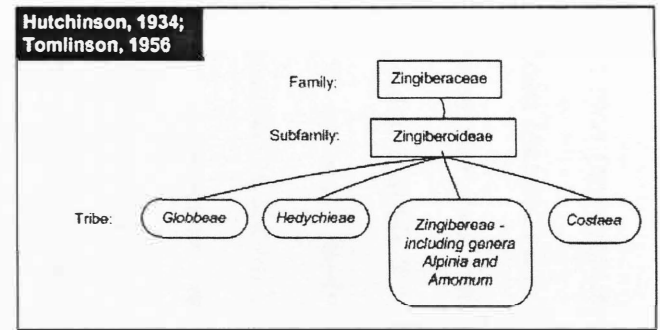
Zingiberaceae was originally divided into two subfamilies, *Zingiberoideae* and *Costoideae* as shown in Figure 2.2. Three tribes were recognized within subfamily *Zingiberoideae*: *Globbeae*, *Hedychieae* and *Zingibereae* (including genus *Alpinia* and *Amomum*). *Hedychieae* has well-developed and free lateral staminodes and *Zingibereae* has reduced, absent or united lateral staminodes to labellum (Jaramilla & Kress, 1997; Kress, 2002).

In 1934, *Costoideae* was reduced as a tribe (*Costaeae*) within the family Zingiberaceae. Finally, Nakai (1941) raised the *Costoideae* as a separate family due to differences of distichous arrangement of the leaves (Kress, 1990; 1995; 2001). Therefore *Costaceae* is considered as a distinct family from the Zingiberaceae (Tomlinson, 1969).

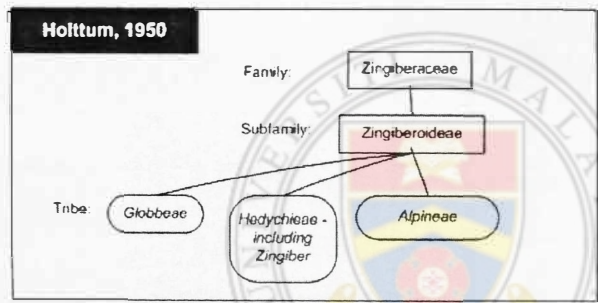
Holttum (1950) removed *Zingiber* from the *Alpinia* group and renamed the tribe as *Alpineae*. *Zingiber* was placed in *Hedychieae*. The removal of *Zingiber* from *Alpineae* based on the plane of distichy of the leaves and cytological support was supported by a few authors, e.g. Burt and Smith in 1964 and Mahanty in 1970. Then, *Zingiber* was separated from *Hedychieae* based on differences on floral characters and vegetative features. Therefore, *Zingibereae* was recognised as a separate tribe, represented by the only genus *Zingiber* (Burt & Olatunji, 1972).



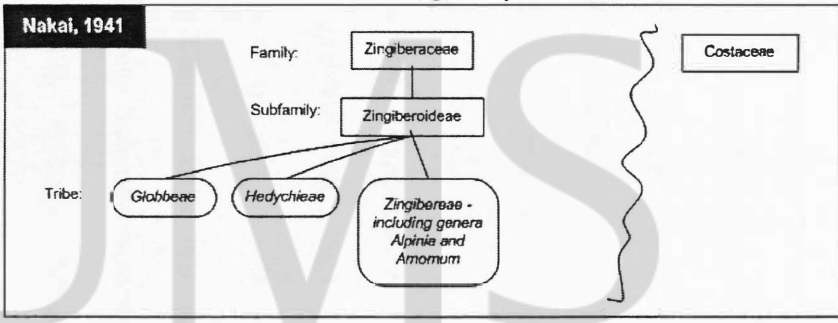
Subfamily Costaceae reduced as a tribe in subfamily Zingiberoideae



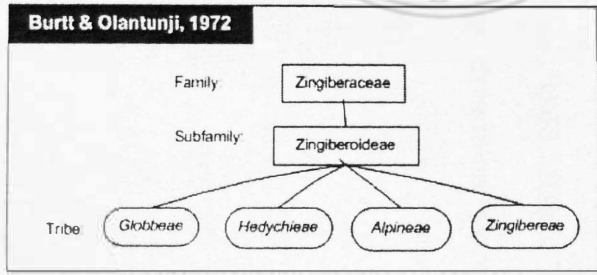
Tribe Costaea elevated as family Costaceae



Zingiber moved into tribe Hedychieae



Zingiber raised as tribe Zingibereae
Current Classification based on morphological data



Proposed classification based on molecular data

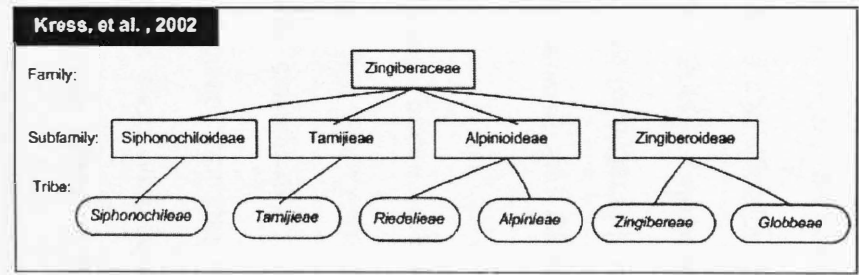


Figure 2.2: History of classification of subfamilies and tribes of Zingiberaceae since 1889.

The currently accepted classification based on morphology of Zingiberaceae is divided into four tribes, namely, Hedychieae (16 genera), Alpinieae (seven genera), so nice (one genus) and Globbeae (one genus) (Mabberley, 1997). 33 genera have been recognized in Southeast Asia (Mood, 1999). Recently, a classification based on molecular data was proposed (Kress, Prince & Williams, 2002), Zingiberaceae is divided into six tribes: Siphonochilae (one genus), Tamijieae (one genus), Alpinieae (16 genera), Riedelieae (four genera), Zingibereae (25 genera) and Globbeae (4 genera) as shown in Table 2.1.

Previous revisions on Zingiberaceae have been based primarily on morphological characters until the 1990s. Not much study has been carried out on the chemical constituents of gingers in relation to their classification. However, studies done on the comparison of a few species showed that the chemical compounds varied distinctively between species and has slight variations within species (Larsen, *et al.*, 1999). A comprehensive review was done on the plant chemistry of large number of plant families provided chemical structures and their systematic importance. Chemical structures of a few species of Zingiberaceae were presented. Comparison was made on the variation of chemical constituents on *Alpinia nutans* (Andr.) Rosc. and five related *Alpinia* species and degradation of gingerol to shogaol in *Zingiber officinale* Rosc. (Hegnauer, 1963, 1986). To date, comparison between chemical and molecular data to determine phylogenetic relationships have been rarely attempted (Harborne, 2000).

Table 2.1: Proposed placement of the genera in the new classification of the family Zingiberaceae.

Subfamily Siphonochiloideae W.J. Kress	Subfamily Tamijioideae W.J. Kress	Subfamily Alpinioideae Link	Subfamily Zingiberoideae Haask.
Tribe Siphonochileae W.J. Kress	Tribe Tamijieae W.J. Kress	Tribe Alpinieae A. Rich.	Tribe Zingibereae Meisn.
<i>Siphonochilus</i>	<i>Tamijia</i>	<i>Anframomum</i> <i>Alpinia</i> <i>Amomum</i> <i>*Aulotandra</i> <i>*Cyphostigma</i> <i>*Elettaria</i> <i>Elettariopsis</i> <i>Etingera</i> <i>*Geocharis</i> <i>*Geostachys</i> <i>Hornstedtia</i> <i>*Leptosolena</i> <i>Paramomum</i> <i>Plagiostachys</i> <i>Renealmia</i> <i>Vanoverberghia</i>	<i>Boesenbergia</i> <i>Camptadra</i> <i>Cautleya</i> <i>Cornukaempferia</i> <i>Curcuma</i> <i>Curcumorpha</i> <i>Distichochlamys</i> <i>*Haplochorema</i> <i>Hedychium</i> <i>Hitchenia</i> <i>Kaempferia</i> <i>*Laocanthus</i> <i>*Nanochilus</i> <i>*Paracautleya</i> <i>*Parakaempferia</i> <i>Pommereschea</i> <i>Pyrgophyllum</i> <i>Rhynchanthus</i> <i>Roscoea</i> <i>Scaphochlamys</i> <i>Smithantris</i> <i>*Stadiochilus</i> <i>Stahlianthus</i> <i>Zingiber</i>
		Tribe Riedelieae W.J. Kress <i>Burbidgea</i> <i>Pleuranthodium</i> <i>Riedelia</i> <i>Siamanthus</i>	Tribe Globbeae Meisn. <i>Gagnepainia</i> <i>Globba</i> <i>Hemiorchis</i> <i>Mantisia</i>
		Incertae Sedis <i>Siliquamomum</i>	Incertae Sedis <i>Caulokaempferia</i>

*Asterisks indicate taxa not sampled in the molecular phylogenetic analyses; these genera are tentatively placed based on morphological features (adapted from Kress, Prince & Williams, 2002).

In 2002, Kress, Prince & Williams proposed a classification based on nuclear internal transcribed spacer (ITS) and plastid *matK* regions of 104 species in 41 genera representing all four subfamilies of the Zingiberaceae. This study suggested that at least some morphological traits were homoplasious and three of the tribes were paraphyletic. It was discovered that the African genus *Siphonochilus* and Bornean genus *Tamijia* were the basal clades. The classification of the Zingiberaceae that recognizes four subfamilies and six tribes: Siphonochiloideae (Siphonochilae), Tamijiodeae (Tamijieae), Alpinoideae (Alpinieae, Riedelieae) and Zingiberoideae (Zingibereae, Globbeae). Morphological features were congruent with this classification and the taxonomic status of various monotypic genera. The tribe Zingibereae includes most of former Hedychieae as well as the genus *Zingiber* (Figure 2.3).

The above study provide a picture on the relationships between genera but not sufficient to illustrate a detailed phylogenetic relationship of the family Zingiberaceae. More studies on the phylogeny of each genus will definitely increase the understanding of the phylogeny of this family. Future investigations will help us better understand the relationship within each genus and also will provide a larger data matrix into the family level phylogenetic compilation.