A STUDY OF THE INFRAGENERIC CLASSIFICATION OF *ALPINIA* ROXB. (ZINGIBERACEAE) USING MOLECULAR DATA

ACHARIYA RANGSIRUJI

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF EDINBURGH

1999

ABSTRACT

Alpinia Roxb. is a complex genus of *c.* 227 species distributed from India and Sri Lanka to China and Japan and South East Asia to Australia and Fiji. The most recent infrageneric classification divides it into two subgenera, 11 sections and 12 subsections (Smith 1990a). Earlier accounts were produced by Schumann (1904) and Holttum (1950). Although similar morphological characters were examined, the classifications differ widely and no consensus classification has yet been found. I have therefore taken a new approach using molecular data to generate putative phylogenies which provide further understanding of the genus in terms of its infrageneric classification and relationships among species.

Fifty seven species of *Alpinia* and its outgroup were sampled and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA was sequenced. Based on my ITS analyses I discovered three important clades with well supported branch lengths of ≥ 20. First, ITS results (BS = 100%, DI > +2) clearly supported Smith's (1991) classification of *Pleuranthodium* as a distinct genus which can be completely removed from *Alpinia*. Second, a clade, consisting of *A. galanga*, *A. nigra* and *A. conchigera* (BS = 100%, DI > +2) indicates a close relationship among these species. However, according to Smith (1990a) *A. galanga* is placed with other species in section *Alpinia* subsection *Alpinia* (with non-tubular bracteoles) whereas *A. nigra* and *A. conchigera* are placed within section *Allughas* (with tubular bracteoles). The position of *A. galanga* in section *Allughas* was confirmed by results of a phylogenetic analysis of the spacer between *trn*L (UAA) 3' exon and *trn*F (GAA) of chloroplast DNA (BS = 65%, DI = +1). Therefore, it appears that *A. galanga* has evolved within section *Allughas* and the absence of tubular bracteoles is a convergence with section *Alpinia*. Third, ITS data revealed a clade (BS = 100%, DI > +2) which includes species from both Smith's (1990a) subgenera *Alpinia* and *Dieramalpinia*. The result was supported by that of the chloroplast spacer (BS = 100%, DI = +1). In addition, it was found that *Alpinia* may be non-monophyletic as the basal group of subgenus *Alpinia* section *Fax* (Smith 1990a) has two African *Renealmia* species as a sister group. This clade however, is only weakly supported (BS < 50%, DI = +1).

The mapping of morphological characters onto the ITS strict consensus tree revealed some congruence between these two types of data. Based on the ITS cladogram the relationships of several species appear to reflect geographical distribution, therefore, general patterns of evolution within *Alpinia* are discussed in relation to biogeography.

ACKNOWLEDGEMENTS

I wish to express my deep gratitude to my main supervisors, Dr Quentin Cronk and Dr Mark Newman, for their guidance, patience and encouragement throughout my study. I also thank my other supervisors, Dr Linda A. Gilmore and Prof Richard Ambler, for their kindness and understanding.

I wish to thank the Regius Keeper and all the staff of the Royal Botanic Garden Edinburgh (E) for the use of the herbarium, library and excellent facilities in the molecular laboratories as well as for the SEM work. I thank the horticulturists for taking care of the ginger collection so that my study of living plants could be accomplished.

I also thank Dr Michael Möller (E) for his expert advice in PCR, constant help in the laboratories and useful suggestions in molecular analyses. I thank Dr Kwiton Jong (E) for his help in bringing some specimens from Malaysia to Edinburgh, and Miss Nicola Preston for performing automated DNA sequencing at the Institute of Cell and Molecular Biology, the University of Edinburgh.

My sincere gratitude goes to the following people/institutions for providing me with plant material as well as pressed voucher specimens required in this study.

1. Prof W.J. Kress at the National Museum of Natural History (Smithsonian Institution, USA).
2. Mr D. Orr and Mr J. Mood at Waimea Arboretum and Botanical Garden (Hawaii, USA).
3. Dr R.T. Hirano and Miss K. Shigematsu at Harold L. Lyon Arboretum (the University of Hawaii at Manoa, Hawaii, USA).
4. The Royal Botanic Gardens Sydney (Australia).
5. Dr H. Ibrahim at the Department of Botany, University of Malaya (Malaysia).
6. Mr A. Weerasooriya at the Royal Botanic Gardens Peradeniya (Sri Lanka).
7. Dr M. Chase, Mr M. Powell and Mrs J. Cowley at the Royal Botanic Gardens, Kew (UK).
8. Mr P. Wilkie and Dr Q. Cronk at E (UK).
9. Dr D. Harris at E (UK).
10. The Director, Dr Weerachai Na Nakorn, and the staff of Queen Sirikit Botanical Garden (Thailand).

I also would like to thank Mr J. Mood and Mr A. Lamb for arranging a trip to Sabah (Malaysia) which allowed me to get acquaintance with some *Alpinia* and other gingers in the wild.

I thank my friends and relatives in Thailand as well as in Edinburgh for their help and encouragement in my study.

Last but not least, I would like to thank my parents and sister for their confidence, continuous love and support throughout my study. My deep gratitude also goes to the Royal Thai Government for their financial support and consideration of the significance of further education.

DECLARATION

I declare that this thesis has been composed by myself and that it contains no material which has been accepted for the award of a degree in any university. All quotations have been distinguished by quotation marks and other sources have been clearly acknowledged.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

1.1 GENERAL TAXONOMIC CONSIDERATIONS

1.1.1 General introduction to the order Zingiberales, the family Zingiberaceae and the tribe Alpineae

In most modern taxonomic treatments, Zingiberales (Scitamineae or Arillatae) is considered to consist of eight families, namely Zingiberaceae, Costaceae, Marantaceae, Cannaceae, Musaceae, Lowiaceae, Heliconiaceae and Strelitziaceae. **Table 1** (modified from Kress 1990) illustrates systems of classification of the Zingiberales which had undergone continual refinement and division. Bentham and Hooker (1883) recognised the Zingiberales as one family (Scitamineae) which included four tribes: Museae, Zingibereae, Maranteae and Canneae. Later Petersen (in Engler and Prantl 1889a-d) elevated the rank of these four tribes to family (Musaceae, Zingiberaceae, Marantaceae, and Cannaceae). Then Schumann (in Engler 1900, 1902, 1904) subdivided the two families Musaceae and Zingiberaceae into five subfamilies. In the Zingiberaceae, the Costoideae were recognised as distinct from the Zingiberoideae on the basis of their spiral phyllotaxy and lack of oil cells. Schumann also further subdivided the Zingiberaceae into four tribes, three in the Zingiberoideae: Zingibereae, Hedychieae, Globbeae and the tribe Costeae in the Costoideae. Later Hutchinson (1934) eliminated the distinction of the two subfamilies, but retained the four tribes, and also raised the Lowioideae and Strelitzioideae to family level and separated them from the Musaceae. In 1941, Nakai suggested that the Costoideae and the Heliconieae be elevated to family rank based on additional morphological differences. After showing that certain outstanding anatomical features of the Costoideae were unique not only to the family Zingiberaceae, but also to the order Zingiberales as a whole, Tomlinson (1956) accepted Nakai's view. The recognition of eight families in the order was

Table 1. Systems of classification of the Zingiberales. Modified from Kress (1990).

Bentham and Hooker (1883)	Petersen (Engler and Prantl 1889a-d)	Schumann (Engler 1900, 1902, 1904)[1]	Hutchinson (1934)	Nakai (1941)[2]	Kress (1990)
Family: Scitamineae	No rank	Order: Scitamineae	Order: Scitamineae	Order: Zingiberales	Order: Zingiberales
Tribes:	Families:	Families:	Families:	Families:	**Suborders**:
Museae	Musaceae	Musaceae	Musaceae	Musaceae	**Musineae**
	Tribes:	Subfamilies:			Family: Musaceae
	Museae	Musoideae			
		Strelitzioideae	Strelitziaceae	Strelitziaceae	**Strelitzineae**
		Tribes:			Family: Strelitziaceae
		Strelitzieae			
		Heliconieae			
		Lowioideae	Lowiaceae	Lowiaceae	**Lowineae**
					Family: Lowiaceae
	Heliconieae			Heliconiaceae	**Heliconineae**
					Family: Heliconiaceae
Zingibereae	Zingiberaceae	Zingiberaceae	Zingiberaceae	Zingiberaceae	**Zingiberineae**
		Subfamilies:	Tribes:		Superfamilies:
		Zingiberoideae	Zingibereae		Zingiberareae
		Tribes:	Hedychieae		Families:
		Zingibereae	Globbeae		Zingiberaceae
		Hedychieae	Costeae		
		Globbeae			
		Costoideae		Costaceae	Costaceae
		Tribe:			
		Costeae			
					Cannariae
					Families:
Maranteae	Marantaceae	Marantaceae	Marantaceae	Marantaceae	Marantaceae
Canneae	Cannaceae	Cannaceae	Cannaceae	Cannaceae	Cannaceae

[1] Also included Loesener (in Engler and Prantl 1930a,b); Winkler (in Engler and Prantl 1930a,b).

[2] Also included Tomlinson (1962); Takhtajan (1980); Cronquist (1981); Dahlgren *et al.* (1985).

followed by modern authors, particulary those interested in systems of classification and inter relationships of families on a broad scale (Takhtajan 1980; Cronquist 1981; Dahlgren and Rasmussen 1983; Dahlgren *et al.* 1985). In 1990, based on the classification of Nakai and Tomlinson, Kress accepted the eight families as working hypotheses of monophyletic groups within the Zingiberales and cladistic analyses of the order were carried out to test hypotheses on family boundaries. On the basis of the morphological characters and his resulting cladogram, Kress (1990) proposed a new phylogenetic classification that recognised eight families, two superfamilies, and five suborders within the Zingiberales. It should be noted that throughout my thesis the description of the family Zingiberaceae will not include the Costoideae, unless otherwise mentioned as a distinct family (Costaceae).

Zingiberaceae, the largest family in the Zingiberales, comprises approximately 48 genera and 1200 species (Willis 1985 plus recently published genera). The family has its greatest concentration in the Indo-Malaysian region (South East Asia) but extends through Tropical Africa to Central and South America (Rendle 1930). The exceptions are for *Roscoea* Sm. which has spread far into temperate regions (the mountains of China, Burma, North India and the Himalayan range) and *Cautleya* (Royle ex Benth.) Hook.f. which goes nearly as far (Cowley 1982).

This is a family of medicinal and economic significance. Several pieces of work dealing with potential uses of the Zingiberaceae, both medicinal and economic, are found in Chopra *et al.* 1956; Mitsui *et al.* 1976; Perry 1980; Itokawa *et al.* 1987; Ahamad and Ahmed 1991 and Jitoe *et al.* 1992. Examples of products from the Zingiberaceae include ginger (rhizomes of *Zingiber officinale* Roscoe), turmeric (an ingredient of curry powder and the source of a yellow dye from rhizomes of *Curcuma longa* L. (*C. domestica* Valeton), Melegueta pepper (seeds of *Aframomum melegueta* (Roscoe) K.Schum. and other species), cardamom (capsules

and seeds of *Elettaria cardamomum* (L.) Maton), and galangal (rhizomes of *Alpinia galanga* (L.) Willd.). In addition many genera such as *Hedychium* Koenig, *Kaempferia* L., *Curcuma* L., and *Alpinia* Roxb. are grown as ornamentals.

A taxonomic history of the Zingiberaceae is available in Holttum (1950, p. 14-18). In addition, Burtt and Smith (1972a) made a thorough chronological study of the key species which are concerned in the typification of the main genera, subgenera and sections. In these notes important contributions to the systematics of the family were laid out, and some useful information of those provided with adequate references are shown in **Table 2**.

Table 2. Some useful information from the history of the systematics of Zingiberaceae. Data tabulated from Holttum (1950), and Burtt and Smith (1972a) where adequate references were available.

Author	Year	Significant contributions to the systematics of Zingiberaceae
Linnaeus, C.	1753	Five genera (*Amomum*, *Alpinia*, *Curcuma*, *Kaempferia*, and *Costus*) were recognised in Monandria Monogynia.
Koenig, J.F.	1783	The first good botanical descriptions were made from living plants.
Retzius, A.J.	1791	Koenig's notes were published with detailed descriptions of plants; each was headed by a name, a phrase or some other identifying words. The use of the anther as the chief organ in the classification was mentioned.
Willdenow, C.L.	1797	Some improvement on Retzius's classification was made.
Roscoe, W.	1807	True Scitamineae (true aromatic plants) were separated from Cannae using characters such as anthers and styles.
Roxburgh, W.	1812	The plants were separated into two divisions: (i) truly herbaceous (*Curcuma*, *Kaempferia*, *Zingiber*, and *Globba*), and (ii) less herbaceous (*Canna*, *Phrynium*, *Amomum*, and *Alpinia*). Characters of the root, leaf, and inflorescence were described.

Table 2 (continued).

Author	Year	Significant contributions to the systematics of Zingiberaceae
Blume, C.L.	1827	The family was subdivided into natural groups which formed the basis of Meisner's tribes.
Baker, J.G.	1890	The Zingiberaceae of India (including those known from the Malay Peninsula) were described.
Ridley, H.N.	1899	Ridley's first account of the Scitamineae of the Malay Peninsula was published.
Schumann, K.	1904	A monograph of the whole family was published.
Valeton, T.H.	1904	Thorough studies of the Zingiberaceae of Java were carried out. The work was continued through 1913, 1914 and 1918.
Gagnepain, F.	1908	Descriptions of new species of Zingiberaceae (mainly from Indo-China) and an account of the family were written.
Loesener, T.H.	1930a	An account of Zingiberaceae was published following the work of Valeton.

Most species of Zingiberaceae occupy shady habitats on the primary forest floor. The family is characterised vegetatively as herbaceous perennials, usually with thick, fleshy, sympodially branched **rhizomes** covered with distichous scale leaves. Erect leaf-bearing **stems**, always unbranched, have a terminal inflorescence, or the inflorescence and leafy stems may be separated, or acaulescent. **Leaves** simple, basal or cauline, usually distichous, with or without a petiole between blade and sheath. **Blades** often asymmetric, linear or broad obovate to elliptic. **Sheaths** overlapping and forming a pseudostem surrounding a thin true stem, open or closed. **Ligule** usually well developed at junction of blade with petiole or sheath. **Inflorescence** often in conelike heads, open racemes, panicles, rarely solitary; **bracts** and **bracteoles** when present subtending the flowers on alternate sides in a cyme, or in a cincinnus. **Flowers** highly diverse in appearance, mostly

zygomorphic, usually lasting one day or less. **Calyx** tubular, usually three-toothed, often split on one side. **Corolla tube** usually slender, divided into three lobes; the dorsal one larger than the lateral ones. **Labellum** large (usually the largest organ of the flower), petaloid or non-petaloid, often two-lobed; and sometimes two petaloid (or tooth-like) **lateral staminodes** free or fused to the labellum. The labellum, two lateral staminodes (if present) and one fertile **stamen** are parts of the androecium (this has been the subject of much discussion, for further detail see * below). **Filament** broad or narrow, joined to the flower tube at the base or to the labellum or the staminodes above the insertion of the corolla lobes. **Anther** variably broadened or appendaged, sometimes apically extended into fleshy or petaloid crest. **Style** slender, filiform, and usually enveloped in a channel of the fertile stamen, bearing the ciliated **stigma** that protrudes beyond the anther. **Ovary** inferior, with two variable **epigynous glands** (nectaries) attached on the upper surface. **Fruit** a three-valved dehiscent capsule, or fleshy, berrylike, and usually crowned by the remains of the perianth. **Seeds** small, numerous and usually arillate.

The established pattern of the Zingiberaceae with the subfamily Zingiberoideae was for long to recognise three tribes: Zingibereae, Hedychieae and Globbeae. Basically, these tribes are different in terms of the form of the lateral staminodes (Zingibereae: small or absent; Hedychieae: petaloid, free from labellum;

* Payer (1857), van Tiegham (1868, 1871), Eichler (1875), Schumann (1904), and Rendle (1930), all believed that the labellum, regarded as derived by a congenital fusion of two antero-lateral staminodes, and the fertile stamen, constitute the inner whorl of the androecium, while the outer whorl are thought to be represented by the two lateral staminodes, which are postero-lateral in postion (the anterior member of this whorl being considered as always absent). Brown (1830), Thompson (1933, 1934, 1936) and Gregory (1936) had different views about the origin of these organs of the androecium. After Holttum (1950, p. 11-12) surveyed the various interpretations of the labellum of Zingiberaceae, Rao and his colleagues (Rao *et al.* 1954; Rao and Pai 1959, 1960; Rao and Gupte 1961) investigated the subject in detail and ultimately arrived at the same conclusion as Payer's (1857). The fact that the labellum is a double structure, formed from the two united antero-lateral staminodes of the inner whorl also suggests the outcome of the obviously bilobed labellum of genera such as *Kaempferia* and *Burbidgea* Hook.f. Schumann (1904) however, believed that the labellum did not have the same origin throughout the family, but this did not seem to affect his classification.

Globbeae: petaloid, free from labellum), the number of locules in the ovary (Zingibereae: 3; Hedychieae: 3; Globbeae: 1), and the nature of the placenta (Zingibereae: axile; Hedychieae: axile; Globbeae: basal-axile or parietal). [However, for this last point about the placentation, a recent discovery by Mangaly and Hamsa (cited in Mangaly and Sabu 1992) showed that placentation in the whole family of Zingiberaceae is parietal. Their explanation seemed to lie in the fact that various degrees of paracarpy occur as a result of the intrusion of the placenta into the ovary chamber during development.] The classification of the Zingiberoideae was then unsatisfactory because the genus *Zingiber* of Schumann's Zingiberaceae differs from the rest of the members of the tribe Zingibereae in having petaloid lateral staminodes. Therefore, Holttum (1950) transferred *Zingiber* to the tribe Hedychieae and consequently named the remainder as Alpineae. This tribe Hedychieae however, should be renamed Zingibereae since now that it contained the type genus *Zingiber*. In 1972 Burtt and Olatunji placed *Zingiber* in a tribe of its own, and since then, four tribes have been recognised: Alpineae Meisn., Zingibereae B.L.Burtt & Olatunji, Hedychieae Petersen and Globbeae Meisn.

In contrast to the other three tribes (Zingibereae, Hedychieae and Globbeae) the tribe Alpineae possesses several distinct features including: (i) transverse distichy - plane of insertion of leaf perpendicular to the direction of rhizome whereas in Zingibereae, Hedychieae and Globbeae it is parallel (Weisse 1932, 1933), (ii) small, linear or tooth-like lateral staminodes at the base of the labellum, or completely absent lateral staminodes (Holttum 1950), and (iii) epidermal stegmata or cells containing a single large silica body (with the exception of a number of species of Alpineae which lack stegmata and a few species of *Globba* (Globbeae) that contain them) (Tomlinson 1956; Olatunji 1970). Alpineae is the largest tribe in Zingiberaceae which consists of 22 genera and 800 species. Except for *Renealmia* L.f. which only occurs in Tropical America and Africa, and *Aframomum* K.Schum.

which is found only in Africa, all other genera are widely distributed in Tropical Asia including Eastern Malesian regions (New Guinea and the surrounding islands) (Chen and Huang 1996).

In 1981 Smith constructed synoptic keys to the tribes Zingibereae, Hedychieae, Globbeae and to members of the tribe Alpineae whose inflorescences are borne separately from the leaves on a leafless scape. Later Smith (1985, 1986) also produced essential keys to the Bornean genera of the tribe Alpineae. She divided this tribe into two main groups based on the position of the inflorescence. The first category includes genera with inflorescence terminal on the leafy shoot: *Burbidgea* Hook.f., *Plagiostachys* Ridl. and *Alpinia*, while the other category includes genera with inflorescence borne separately from the leaf shoot (radical inflorescences): *Hornstedtia* Retz., *Amomum* Roxb., *Etlingera* Giseke (*Achasma* Griff., *Nicolaia* Horan. and *Geanthus* Valeton), *Elettariopsis* Baker, *Elettaria* Maton, *Geocharis* Ridl., and *Geostachys* (Baker) Ridl.

1.1.2 The genus *Alpinia*

Alpinia Roxb., the largest genus of Zingiberaceae, consists of approximately 227 species (Smith 1990a) (see **Figure 46 A-N** for colour plates illustrating the variation within *Alpinia* and some other genera). This is almost 20% of the total number of species of the family. The genus is distributed from India and Sri Lanka to China and Japan and South East Asia to Australia and Fiji (Smith 1990a). Many species of these aromatic rhizomatous herbs are used as culinary spice and as ingredients in traditional Chinese, Indian and South East Asian medicines. Important species include *Alpinia galanga* (L.) Willd., *A. officinarum* Hance, *A. oxyphylla* Miq., *A. nigra* (Gaertn.) B.L.Burtt, *A. zerumbet* (Pers.) B.L.Burtt & R.M.Sm., *A. suishaensis* Hayata, *A. malaccensis* (Burm.f.) Roscoe, *A. calcarata* Roscoe, *A. mutica* Roxb., *A. conchigera* Griff., *A. rafflesiana* Wall. ex Baker, *A. javanica* Blume, and *A. scabra* (Blume) Baker (Watt 1883; Council of Scientific &

Industrial Research India 1948; The Institute of Chinese Materia Medica China Academy of Traditional Chinese Medicine 1989).

Alpinia, in common with other genera in the tribe Alpineae, is generally characterised by fairly tall aerial shoots with distichous leaves whose plane of distichy is transverse to the rhizome. Almost without exception, the plants bear terminal inflorescences on the leafy shoots. The lateral staminodes are absent or are represented by small tooth-like structures at the base of the labellum. The genus is named in honour of Prosper Alpinus, the seventeenth-century Italian botanist. The generic name *Alpinia* was first used by Linnaeus (1753, p. 2) for the Tropical American species *A. racemosa*. Later the younger Linnaeus (1781, p. 79) described a related species, also from Tropical America, as *Renealmia exaltata*. Subsequent authors placed Asiatic species in both genera. However, Roxburgh (1812), Schumann (1904) and some later authors applied the name *Alpinia* mainly to Asiatic species, and used the name *Renealmia* for those in Tropical America and West Africa. This generic nomenclature was not satisfactory because the type species of *Alpinia* was an American species. Eventually the name *Renealmia* was conserved for the American and African species and *Alpinia* L. was rejected so a new name was required for the Asiatic species. To solve this problem the committee appointed at Amsterdam proposed that the Asiatic species previously in *Alpinia* L. be transferred to the conserved *Alpinia* Roxb. 1812, non L. 1753 with *A. galanga* (L.) Willd. as its type.**

** **The lectotypification of *A. galanga* (L.) Willd.** In 1762 (p. 3) Linnaeus described *Maranta galanga* and in the protologue, he referred solely to Rumphius (1747) citing the excellent plate (t. 63). There is no specimen of *Maranta galanga* at LINN (Burtt & Smith 1972a, Smith 1990a) or at BM, UPS or in S. Hence, it appears that Rumphius's (1747) illustration is the sole basis for the lectotypification of *Maranta galanga* L. (= *A. galanga* (L.) Willd.). This accords with the comment of Valeton (1917, p. 153) that "*Galanga major* Rumph. is the whole basis of *Maranta galanga* LINN., which typifies *Alpinia galanga* Sw.; the plate is very generally cited in botanical literature under *Alpinia galanga* (L.) Sw." Following this, Rumphius's (1747) illustration (Herb. Amb. **5**: 143, t. 63) is chosen here explicitly as a lectotype of *Maranta galanga* L., and therefore, of *Alpinia galanga* (L.) Willd.

Swartz (1791, p. 8) treated *Maranta galanga* L., associating it with *Alpinia* and citing Rumphius's (1747) illustration. However, his short account does not constitute an explicit new combination. Willdenow (1797, p. 12) therefore, was the first person to establish the name *Alpinia galanga* for *Maranta galanga*. Throughout my work therefore, I use Willdenow's authority for *A. galanga*.

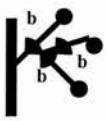
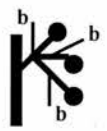
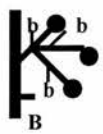
1.2 TWO IMPORTANT INFRAGENERIC CLASSIFICATIONS IN THE HISTORY OF *ALPINIA*

The earliest, relatively comprehensive infrageneric classification of *Alpinia* is Schumann's (1904). On the basis of characters of the bracteole, Schumann divided *Alpinia* into five subgenera and 27 sections. Dealing with species occurring in the Malay Peninsula only, Holttum (1950) proposed that *Alpinia* be divided into four genera, based mainly on the inflorescence-structure and bracteole character. Details of these two important classifications and characters used are summarised in **Table 3**.

Table 3. Two important infrageneric classifications in the history of *Alpinia* and their characters used.

Schumann's (1904) classification and characters used	Illustrations (**B** = bract, **b** = bracteole)
- Schumann divided *Alpinia* into five subgenera and 27 sections based mainly on characters of the bracteole. - Subgenera *Autalpinia* K.Schum., *Probolocalyx* K.Schum. and *Catimbium* Horan. emend. K.Schum. comprised species with or without bracts; bracteoles when present, are open to the base. - Subgenus *Dieramalpinia* K.Schum. covered all species with tubular bracteoles. - Subgenus *Rhizalpinia* K.Schum. contained species with radical inflorescences; most of them have been transferred to other genera.	

Table 3 (continued).

Holttum's (1950) classification and characters used	Illustrations (**B** = bract, **b** = bracteole)
- Holttum divided *Alpinia* into four genera based on the inflorescence-structure and the character of bracteoles. - Genus *Cenolophon* Blume has inflorescences bearing single flowers directly on the main axis; bracts usually small; no bracteoles. The genus also includes Schumann's subgenus *Probolocalyx*. - Genera *Alpinia*, *Catimbium* Juss. and *Languas* Koenig have inflorescences bearing lateral cincinni of two or more flowers; bracts none or small; bracteoles always present. - **(A)** Genus *Alpinia*: bracteoles funnel- or cup-shaped. The genus includes Schumann's subgenus *Dieramalpinia*. - **(B)** Genus *Catimbium*: bracteoles split to the base, usually large; bracts absent. - **(C)** Genus *Languas*: bracteoles split to the base, always small; bracts present and usually small. This genus is analogous to Schumann's subgenus *Autalpinia* section *Hellenia*.	 (A)  (B)  (C) 

Holttum (1950) criticised Schumann's (1904) monograph of Zingiberaceae for the fact that Schumann studied mainly dried material, and had little knowledge of living plants of the family. Holttum concluded that Schumann's work had led to much confusion due to his lack of knowledge of the inflorescence-structure and his failure to realise that certain single floral structures, such as the anther crest, usually

are not sufficiently distinctive and constant to characterise genera. Greater problems also arise because much type material of Schumann was lost during the war in Berlin. Although Holttum believed that Schumann's (1904) account was almost valueless for his Malayan species, the existence of Schumann's classification of *Alpinia* did provide a good reference for later authors (Wu 1981, Smith 1990a).

With Holttum's (1950) classification of *Alpinia* Burtt and Smith (1972a) pointed out that the genera *Catimbium* and *Languas* were illegitimate. Nearly all the species concerned in *Catimbium* have names available in *Alpinia* and it would be irresponsible to re-name these segregate genera without greater confidence in their validity. Similarly the use of *Languas* is not valid for *Alpinia* Roxb. non L. after the latter became a *nomen conservandum*. In 1981, Wu mainly inherited Schumann's classification, but removed the subgenus *Rhizalpinia* and transferred some species among different subgenera. The most recent infrageneric classification of *Alpinia* is that of Smith (1990a).

1.3 SMITH'S (1990A) INFRAGENERIC CLASSIFICATION OF *ALPINIA*

The character of the labellum was used by Smith (1990a) to classify *Alpinia* into two subgenera: *Alpinia* (with concave and petaloid labellum) and *Dieramalpinia* (with labellum always held erect, almost always pressed against the stamen and non-petaloid except for the apical part in some species). **Figure 1** illustrates the distribution of Smith's two subgenera in contrast with **Figure 2** for Schumann's subgenera. It is obvious from both maps that subgenus *Alpinia* occurs throughout the entire geographical range of the genus including India and Sri Lanka, Hainan, Taiwan, Japan, South East Asia, plus New Guinea and the surrounding islands. Smith's subgenus *Alpinia* also extends to the east coast of Australia, but some species present were recognised by Schumann in his subgenus *Dieramalpinia*. Smith has excluded the subgenus *Dieramalpinia* from continental Asia while

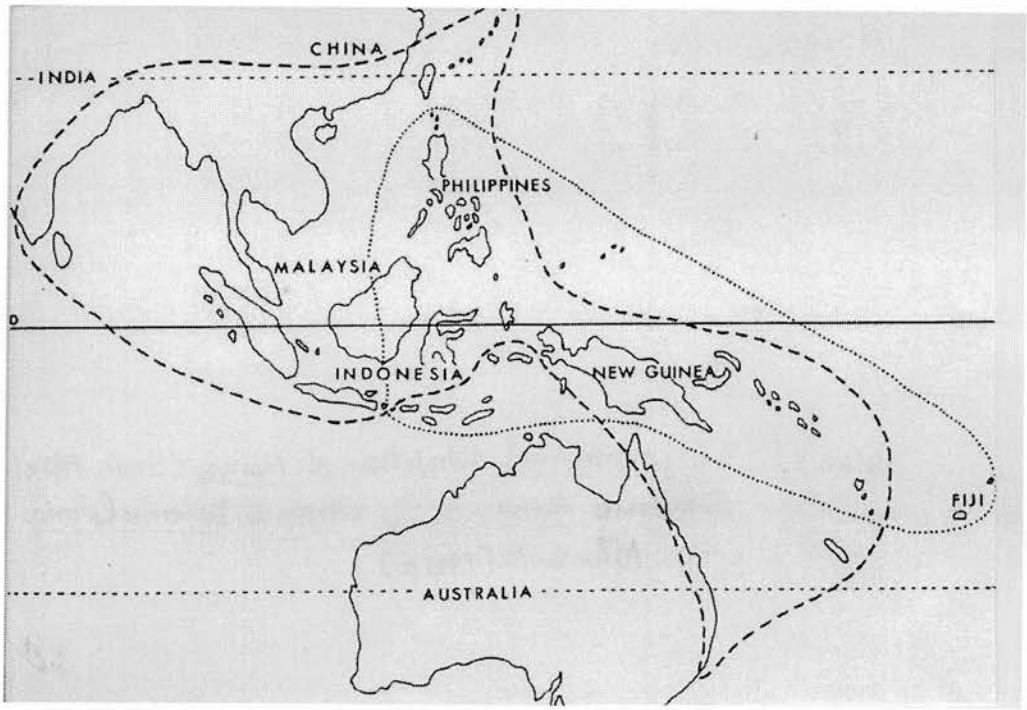


Figure 1. The geographical distribution of *Alpinia* (Smith 1990a). Subgenus *Alpinia* -----; subgenus *Dieramalpinia* ..... After Smith (1990a).

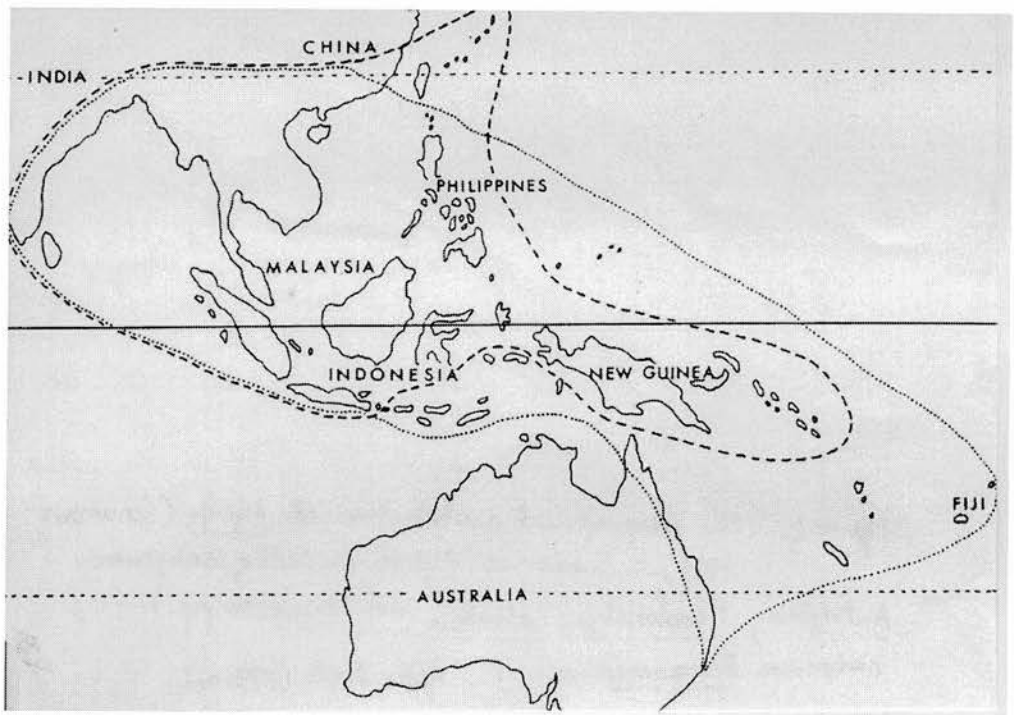


Figure 2. The geographical distribution of *Alpinia* (Schumann 1904). Subgenus *Alpinia* (including subgenera *Autalpinia*, *Probolocalyx*, *Catimbium* and *Rhizalpinia* p.p.) -----; subgenus *Dieramalpinia* ..... After Smith (1990a).

Schumann retained it. **Table 4** gives details of the total number of species and some useful characters of Smith's (1990a) infrageneric classification of *Alpinia*.

Table 4. The total number of species and some useful characters of Smith's (1990a) infrageneric classification of *Alpinia*. Data tabulated from Smith (1990a).

Subgenus *Alpinia* (7 sections, 10 subsections)

Taxon	Total No. of species	Useful characters (B = bracts; b = bracteoles; Infl. = inflorescence; Fls. = flowers; L = labellum; lvs. = leaves)
Section *Alpinia*		b: absent, if present open to the base.
Subsection *Alpinia*	24	B: usually small, soon deciduous; Fls.: small, many; L: small (< 2 cm long).
Subsection *Presleia* (Valeton) R.M.Sm.	18	B: minute, soon deciduous; Fls.: small, in cincinni of 2-3; L: deeply bilobed, lateral lobes bifid forming a quadrate organ.
Subsection *Paniculatae* R.M.Sm.	2	B: calyptrate; Infl.: paniculate; Fls: single.
Subsection *Cenolophon* (Blume) R.M.Sm.	24	B: minute / calyptrate; b: absent; Fls: single; Infl.: never branched.
Subsection *Catimbium* (Horan.) R.M.Sm.	35	B: absent; b: always enclosing the flower buds, falling soon; Fls.: in cinnini; L: > 3 cm long (commonly yellow-orange heavily marked crimson red).

Table 4 (continued).

Taxon	Total No. of species	Useful characters (B = bracts; b = bracteoles; Infl. = inflorescence; Fls. = flowers; L = labellum; lvs. = leaves)
Subsection *Probolocalyx* (K.Schum.) R.M.Sm.	1	B: membranous, soon deciduous; b: calyptrate; Fls: in cincinni of 2-3; L: convolute.
Section *Didymanthus* K.Schum.	2	Short stem; lvs.: 1-4; B: oblong; b: absent; L: entire / bilobed at the apex.
Section *Kolowratia* (Presl) Loes.	4	B: large, persistent / small, caducous; b: tubular / non-tubular; Calyx: coriaceous; L: obovate / rectangular; Stigma: large, funnel-shaped.
Section *Fax* R.M.Sm.	3	B: conspicuous involucre-like, sterile; b: tubular / non-tubular; Infl.: terminal / radical.
Section *Guillainia* (Vieill.) K.Schum.	3	B: large, persistent; b: tubular; Fls.: in cincinni; Corolla tube: long, well exserted from the calyx.
Section *Arctiflorae* R.M.Sm.	1	B: conspicuous, persistent; b: tubular, narrowly cylindric; Fls.: in cincinni; Anther: sessile.
Section *Allughas* K.Schum.		B: small, soon deciduous (occasionally large and persistent); b: tubular; Fls.: always in cincinni.

Table 4 (continued).

Taxon	Total No. of species	Useful characters (B = bracts; b = bracteoles; Infl. = inflorescence; Fls. = flowers; L = labellum; lvs. = leaves)
Subsection *Allughas* (K.Schum.) R.M.Sm.	9	B: small, soon deciduous; Dorsal petal: with a conical cucullate apex; L: up to 4 cm long.
Subsection *Odontychium* (K.Schum.) R.M.Sm.	1	Dorsal petal: cucullate; L: up to 4 mm wide in lower two-thirds.
Subsection *Strobidia* (Miq.) R.M.Sm.	4	Dorsal petal: rounded, hood not pointed; L: strongly concave, with a wart-like callus at the base.
Subsection *Caeruleae* R.M.Sm.	4	Dorsal petal: rounded; L: small, without a callus; Mature fruit: blue.
Stamen **Non-petaloid labellum**  **Subgenus *Dieramalpinia*** (4 sections, 2 subsections)		
Taxon	Total No. of species	Useful characters (B = bracts; b = bracteoles; Infl. = inflorescence; Fls. = flowers; L = labellum; lvs. = leaves)
Section *Pycnanthus* K.Schum.		B: small / absent, rarely involucre-like; b: tubular; L: linear / narrowly oblong; Filament always with subapical teeth.
Subsection *Pycnanthus* R.M.Sm.	25	B: small / absent; Fls.: borne singly / in cincinni; L: linear / narrowly oblong; Filament: subapically dentate.

Table 4 (continued).

Taxon	Total No. of species	Useful characters (B = bracts; b = bracteoles; Infl. = inflorescence; Fls. = flowers; L = labellum; lvs. = leaves)
Subsection *Amomiceps* (K.Schum.) R.M.Sm.	1	B: large, involucre-like; L: linear, the apex expanded into a small petaloid area; Infl.: large, congested.
Section *Eubractea* K.Schum.	4	B: conspicuous, not always persistent; L: connate to the base of the filament into a distinct tube above the petals; Fls.: in the upper half of cincinni sometimes functionally male.
Section *Myriocrater* K.Schum.	18	B: absent / minute; b: tubular; Infl.: often pendulous; Fls.: all but the first or rarely the second flower functionally male; L: oblong.
Section *Dieramalpinia* K.Schum.	44	B: usually large, often brightly coloured, always persistent; Infl.: pendulous; L: narrowly oblong to triangular.

1.4 AIMS

As can be seen from the previous section of the infrageneric classifications of *Alpinia*, Schumann, Holttum and Smith reach radically different conclusions using similar morphological data, and no consensus classification has yet been found. Therefore, my present study of *Alpinia* was carried out with three main aims:

- To investigate whether a new and alternative approach using molecular data to produce phylogenetic trees (cladograms) could provide a better understanding of the infrageneric classification of *Alpinia*. The trees will test Smith's (1990a) classification.
- To examine whether the trees, when combined with morphological and biogeographical data, can accurately reflect the evolutionary relationships of *Alpinia*.
- To confirm Smith's (1991) work that *Pleuranthodium* is a distinct genus, and can be completely removed from *Alpinia*.

CHAPTER 2: MOLECULAR APPROACHES TO THE STUDY OF THE INFRAGENERIC CLASSIFICATION OF *ALPINIA*

2.1 INTRODUCTION

2.1.1 Molecular techniques for plant phylogenetic studies

Molecular phylogenetics involves the study of evolutionary relationships among organisms or genes using molecular biology and statistical techniques. In situations where morphological variation is limited or the homology of morphological features is unclear, molecular approaches may provide new insights and solutions for some of the organism systematics. These approaches can be divided into two main categories which involve analyses based on proteins or nucleic acids (in this case, only DNA (not RNA) will be discussed).

(1). Protein assays

(i). Protein immunology

The method employs immunological techniques where the reaction of antibodies produced in a particular host (usually a rabbit) against antigens of one species is measured and used to compare genetic distance with other species by examining their differences in antigen-antibody reactivities. Examples for the use of this method in the study of plant relationships are given by Olsen-Stojkovich *et al.* (1986), Fischer and Jensen (1990), and Kolberg *et al.* (1994). However, this method is now rarely used because of problems with non-specific interactions and the availability of sequence data.

(ii). Protein sequencing

Following the pioneer amino acid sequencing work of Fitch and Margoliash (1967), Boulter *et al.* (1970) began to investigate the use of protein sequences in plant phylogeny. A number of sequences of cytochrome *c* (Boulter *et al.* 1972) and

plastocyanin (Boulter *et al.* 1977, 1979) were published, and later reviewed by Scogin (1981) from the taxonomic point of view. Studies by Martin and collaborators (e.g. Martin *et al.* 1983; Martin and Dowd 1984a-c; Martin and Dowd 1986) demonstrate that the protein sequencing approach does indeed have phylogenetic merit. Methods of sequencing involve the use of an automatic sequencer along with other techniques such as High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) for amino acid identification (reviewed in Martin and Jennings 1983).

(iii). Protein electrophoresis

This technique involves the migration of proteins at different rates under the influence of an electric field. The number of amino acids with positive side chains (lysine, arginine and histidine) and those with negative side chains (aspartic acid and glutamic acid) present in a protein determines its net charge. Migrational properties of each protein depends on this charge, the shape and size of the protein. Two types of protein data can be obtained using electrophoretic methods. One is from isozymes, which are functionally similar forms of enzymes; and the other is from allozymes, which are variants of polypeptides representing different allelic alternatives of the same gene locus. Many studies (e.g. McIntyre 1988; Jarvie and Barkworth 1990; Kazan and Muehlbauer 1991; Potter and Doyle 1992) have used enzyme electrophoresis to study relationships among related taxa or to estimate divergence of particular genes in related species.

(2). DNA assays

(i). DNA-DNA hybridisation

The double-stranded nature of DNA, and the weak hydrogen bonds between paired nucleotides on the two complementary strands provide the main basic principles for this method. By boiling DNA solution at 100°C the hydrogen bonds

are broken and the two DNA strands are separated. Upon cooling and under conditions where duplex formation can occur, the solution is mixed with DNA strands from a single sample or species (in which case homoduplexes are produced) or strands from two species (forming heteroduplexes). The thermal stabilities of the homoduplexes and heteroduplexes are measured and the difference in the stability provides a quantitative estimate of the genetic divergence between two species. This method has been applied to areas of plant phylogenetics as shown by Stam *et al.* (1988) and Bot *et al.* (1989a, b).

(ii). Restriction site analyses

The methods rely on the ability of restriction endonucleases which cleave the DNA at specific sites and generate fragments of reproducible size. These fragments are then electrically separated through agarose gels, hybridised to radioactively labelled DNA probes, and autoradiographed. The observed band fragments are then compared among individuals or species. With the advent of polymerase chain reaction (PCR) technology (Mullis *et al.* 1986; Saiki *et al.* 1988), several potential molecular techniques which involve the use of restriction enzymes and PCR are commonly employed to infer plant phylogenies. These techniques are such as: (i) restriction fragment length polymorphisms (RFLPs, e.g. Havey and Muehlbauer 1989; Debener *et al.* 1990; Miller and Tanksley 1990; Gentzbittel *et al.* 1992; Monte *et al.* 1993), (ii) restriction site mapping (e.g. Jansen *et al.* 1991; Downie and Palmer 1992a; Doyle and Doyle 1993), (iii) amplification fragment length polymorphisms (AFLPs, e.g. Kardolus *et al.* 1998), and (iv) minisatellite DNA (e.g. Zentgraf *et al.* 1992).

(iii). DNA sequencing

This method is used to determine all or part of the nucleotide sequence of a specific DNA molecule. In 1977, two alternative techniques for DNA sequencing were developed; they are Maxam-Gilbert (chemical) sequencing (Maxam and

Gilbert 1977), and Sanger dideoxy sequencing (Sanger *et al.* 1977). Both techniques yield a range of different sized fragments which have the same 5' ends but whose 3' ends vary according to the position of specific bases within the target sequence. These fragments are then electrophoretically size-separated to generate a sequence ladder of the original target (and its complementary strand) which can be read following autoradiography. In addition to these techniques, there are two modern techniques; cycle sequencing and automated sequencing, the former involves the use of PCR in the preparation of sequencing reactions. As modifications of Sanger dideoxy sequencing are used in cycle sequencing and in automated sequencers, Sanger's method is preferred in most laboratories to Maxam and Gilbert's method. For this study of *Alpinia*, results were initially produced manually by Sanger's method which was designed as a preliminary study of DNA sequencing. Later, combined methods of cycle sequencing and automated sequencing were employed.

In most phylogenetic studies, DNA sequencing is the method of choice (compared to all the above methods) because (i) DNA sequences encode all heritable variation of phenotypic characters in the uniform language of a linear sequence of four different nucleotides (A, C, G, T) which form discrete characters that provide an enormous amount of information valuable for phylogenetic analyses. (ii) DNA sequences allow researchers to examine the pattern of mutation in terms of nucleotide substitutions and insertions or deletions (indels) which are useful sources of characters. In addition, transitions and transversions, which are the two kinds of nucleotide substitution, may be useful in character weighting. (iii) Due to the fact that different parts of the nuclear and organelle genomes evolve at different rates, DNA sequence data of these parts can provide a phylogenetic record from very recent (using fast evolving regions or molecules) to ancient (using slow evolving regions or molecules) times. (iv) Many DNA sequence data have been compiled in

the GenBank data base and are accessible to all researchers. (v) DNA sequences can be obtained from sources such as herbarium specimens and even fossils, with the use of PCR which requires only a minute amount of DNA template which may be badly degraded and have relatively low molecular weight.

2.1.2 The use of nuclear ribosomal DNA and chloroplast DNA in plant phylogenetic studies

(1). The internal transcribed spacer (ITS) region of nuclear ribosomal DNA

Ribosomal DNA (rDNA) is the set of DNA sequences that code for the synthesis of ribosomal RNA (rRNA). In higher plants the genes are present in each nuclear haploid cell as multiple copies ranging from 200 (*Linum usitatissimum*) to 22000 (*Vicia faba*) (Rogers and Bendich 1987). Copies of rDNA exist in long tandem arrays, at one or a few chromosomal loci, forming the nucleolar organising region (Long and Dawid 1980). Although there is variability among copies of rDNA within individuals, the rDNA repeat units of a single plant are highly homogeneous as a result of concerted evolution (Arnheim *et al.* 1980). Processes such as unequal crossing over (Smith 1976), gene conversion (Fogel and Mortimer 1969; Ohta 1984; Walsh 1986; Hillis *et al.* 1991), transposition, and slippage replication or RNA-mediated exchanges (Drouin and Dover 1990) have been described as "molecular drive" (Dover 1982, 1986) whose mechanisms can fix mutations that spread through a multigene family (in a population or species) in a relatively short time resulting in the overall sequence homogeneity of the rDNA repeats.

The structure of higher plant nuclear ribosomal DNA (nrDNA) (**Figure 3**) is similar to that in other eukaryotes (Long and Dawid 1980 for review). Each repeat unit consists of genes coding for 18S small subunit (SSU), 5.8S and 25S large subunit (LSU) rRNAs, separated by two internal transcribed spacers (ITS1 and

ITS2). The whole region is transcribed as a single large precursor rRNA which is processed subsequently by cleavages of ITS1 and ITS2 resulting in the mature rRNAs (reviewed in Brown and Shaw 1998). The region separating the transcription unit is called the intergenic spacer (IGS) which ranges in length from one to eight kilobases in most plants (Jorgensen and Cluster 1988). The IGS region contains tandem subrepeat sequences which vary interspecifically in length (usually from 100 to 200 base pairs (bp); see Appels and Dvorak 1982; Jorgensen *et al.* 1982; Yakura *et al.* 1984; Saghai-Maroof *et al.* 1984; McMullen *et al.* 1986) while within species subrepeat length varies only slightly. It has been suggested that the length variable region of the IGS has a role in the recombination and evolution of the rDNA gene family (Federoff 1979). The subrepeats within this region may serve as enhancers of transcription (Flavell and O'Dell 1979; Kohorn and Rae 1982, 1983; Reeder *et al.* 1983; Reeder 1984).

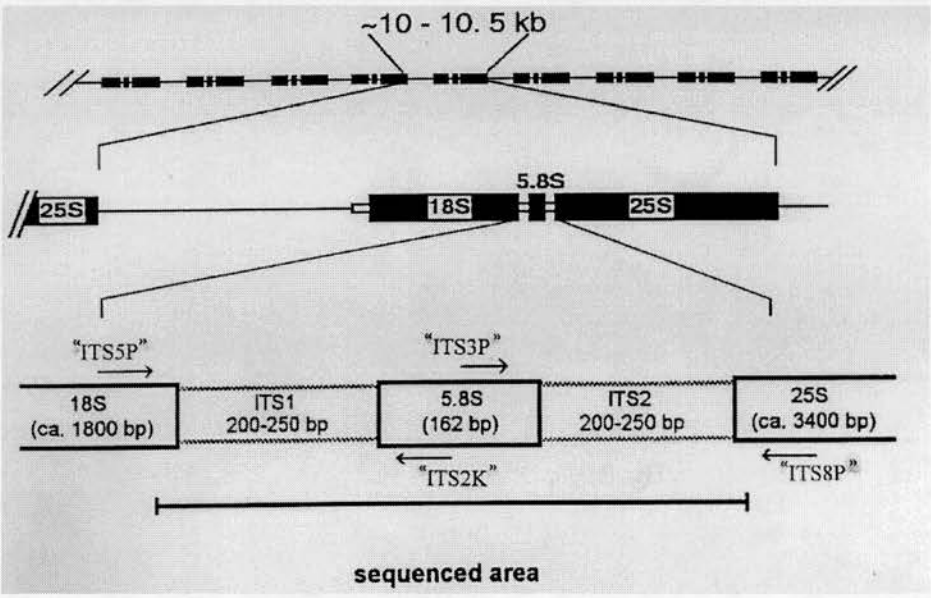


Figure 3. Repeat units of the nuclear ribosomal DNA and the organisation of the ITS region. Arrows indicate orientation and approximate position of primer sites. Primer names (in quotation marks) and sequences are modified from White *et al.* (1990). Primer "ITS2K" was designed during this course of study.

In contrast to the IGS, the region which contains 5.8S, ITS1 and ITS2 (or ITS region; **Figure 3**) in angiosperms is relatively short and evolutionarily conservative in length (565-700 bp; see Baldwin *et al.* 1995), although in some non-flowering seed plants the length of this region varies substantially (975-3125 bp; see Liston *et al.* 1996). The small size of the ITS region and the conserved nature of its flanking coding regions (18S, 5.8S and 25S) make this marker particularly appropriate to direct sequencing of amplified DNA from PCR with universal primers which can be used for a wide diversity of organisms from fungi to flowering plants (White *et al.* 1990; Table 2). Several ITS sequences were reported from various crops such as rice (Takaiwa *et al.* 1985), melon (Kavanagh and Timmis 1988), tomato (Kiss *et al.* 1988), mustard (Rathegeber and Capesius 1989), mung bean (Schiebel and Hemleben 1989), tobacco (Venkateswarlu and Nazar 1991), wheat (Chatterton *et al.* 1992a, b), rye (Chatterton *et al.* 1992c), oats (Chatterton *et al.* 1992d), and barley (Chatterton *et al.* 1992e). While the 18S and 25S coding regions have been used to address phylogenetic questions at the family level or higher taxonomic levels in plants (Zimmer *et al.* 1989; Hamby and Zimmer 1992), ITS sequences appear to be useful for assessing relationships at lower taxonomic levels such as among genera or species. This is because the sequences of the spacer regions evolve more rapidly than coding regions in general (Brown *et al.* 1972; Appels *et al.* 1986), and are more variable as a result of mutagenic processes such as single-base substitutions, and indels (Venkateswarlu and Nazar 1991). Following the use of ITS sequences in phylogenetic studies of animals (Gonzalez *et al.* 1990) and fungi (Lee and Taylor 1992; O'Donnell 1992), Baldwin (1992, 1993) demonstrated that ITS region is phylogenetically informative for infra- and intergeneric relationships in subtribe Madiinae of Compositae. Other examples are given by Suh *et al.* 1993; Wojciechowski *et al.* 1993; Baum 1994; Hsiao *et al.* 1994; Kim and Jansen 1994; Nickrent *et al.* 1994; Sun *et al.* 1994; Campbell *et al.* 1995;

Hsiao *et al.* 1995; Oxelman and Lidén 1995; Soltis and Kuzoff 1995; Susana *et al.* 1995; Buckler and Holtsford 1996a; Downie and Katz-Downie 1996; Soltis *et al.* 1996; Choi and Kim 1997; Möller and Cronk 1997a; Pridgeon *et al.* 1997; Eldenäs *et al.* 1998.

It has been suggested that both ITS1 and ITS2 regions play an important role in processing mature rRNAs from primary transcripts in yeasts (Musters *et al.* 1990; van der Sande *et al.* 1992) as well as in higher plants (Venkateswarlu and Nazar 1991; Liu and Schardl 1994). According to Venkateswarlu and Nazar (1991) substantial sequence conservation is observed in the ITS1 and ITS2 regions of higher plants under study and secondary "crucifix or tRNA-like" structures assumed from these conserved motifs may be critical to rRNA maturation in bringing the termini of the 18S, 5.8S, and 25S rRNAs into relative proximity for processing. In divergent organisms such as yeast, toad and mouse, these "core-like" structures appear to be expanded to different degrees through mutations and duplications or deletions. Although the secondary structure and its proposed biological function may have evolutionary constraints on the ITS region, the effect of these is not great. Schlötterer *et al.* (1994) demonstrated that at least in the case of *Drosophila* only a few evolutionary constraints were observed on ITS sequences and thus they might be expected to evolve at or near the neutral rate. Poor alignability of the Compositae ITS sequences reported by Baldwin (1992) with species from other plant families also suggests lack of long-term evolutionary constraints on much of the ITS region.

Therefore, based on the characteristics of the ITS region (small size, highly conserved flanks, high copy numbers, rapid concerted evolution and length conservation within many angiosperms), it appears that the ITS is an ideal gene to examine the infrageneric structure of *Alpinia*. There are however, a number of disadvantages associated with the use of this region. First, the ITS region provides a relatively small amount of sequence data. In angiosperms, this region contains less

than 700 bp. (Baldwin *et al.* 1995) and only approximately 400-500 bp of ITS1 and ITS2 sequence are included in a phylogenetic analysis because the 5.8S rRNA (*c.* 160 bp) exhibits low levels of sequence variation. Huelsenbeck and Hillis (1993) suggested that sequences of such short length are less effective for accurate tree reconstruction than longer sequences under most conditions and types of analysis. Secondly, the presence of some indels can cause ambiguity in sequence alignment of the ITS region and different alignments can result in different tree topologies as shown by Soltis *et al.* (1996). Thirdly, the occurrence of multiple copies of the ITS region at different loci or as a multigene family presents problems of homology assessment in determining whether the sequences being compared are paralogous or orthologous (Fitch 1970). Fourthly, homogenisation of nrDNA repeats is not instantaneous; partial and uneven homogenisation may give rise to individual plants which contain a mixture of older and more-derived alleles. This brings about the potential for polymorphism at ITS nucleotide sites (Buckler and Holtsford 1996). If concerted evolution is slower than speciation, then a single genome will contain divergent paralogues (descendants of a duplicated ancestral gene) (Baldwin *et al.* 1995) whose relationships can remain unidentified and, where infrequent recombination between paralogues takes place, can result in erroneous species phylogenies (Sanderson and Doyle 1992 cited in Buckler *et al.* 1997). Fifthly, when ancient hybridisation followed by rapid concerted evolution and lineage sorting (Neigel and Avise 1986; reviewed by Doyle 1992) occurred, it can be difficult to detect hybrid derivatives from ITS sequence data and this could sometimes lead to discordance between phylogenies based on ITS and morphology (e.g. in Wojciechowski *et al.* 1993). Next, ITS sequences may exhibit high levels of homoplasy and high sequence divergence which could generate conflicting results among different data sets (Baldwin 1992). Finally, with the use of PCR and ITS universal primers, low levels of contamination may be obtained. This has happened

in a study of *Mimulus* (Scrophulariaceae) (Ritland *et al.* 1993), where the amplified sequences were more similar to green algae than to higher plants (Hershkovitz and Lewis 1996). In order for the ITS region to provide a more meaningful interpretation of plant phylogenies, data from other sources such as chloroplast DNA and morphology may be required.

(2). The noncoding regions between *trn*T (UGU) and *trn*F (GAA) of chloroplast DNA

Chloroplasts contain their own autonomously replicating DNA. Although the majority of proteins present in the chloroplasts are encoded by nuclear DNA, the rest are encoded by chloroplast DNA (cpDNA) and synthesised by the chloroplast transcription-translation machinery. In addition to their important role in photosynthesis these proteins perform many metabolic functions, including the reduction of nitrate and sulphate, starch metabolism and steps in the pathways for the biosynthesis of fatty acids, amino acids and chlorophyll (Whitfeld and Bottomley 1983; Palmer 1987). Generally, the inheritance of cpDNA is from one parent, either maternally as in most flowering plants or paternally as in some gymnosperms (Palmer 1987). **Figure 4** illustrates the rice (*Oryza sativa*, Poaceae) chloroplast genome which may serve as a useful model for other monocots including Zingiberaceae. Among land plants, the typical chloroplast genome consists of a single circular DNA molecule, ranging in size from 134 to 160 kilobases (kb) and is characterised by two inverted repeats (IRs) of *c.* 25 kb which divide the remainder of the genome into one large single copy (LSC) and one small single copy (SSC) region (Palmer 1985a; Sugiura 1989). The size of this genome may however, vary due to some internal rearrangements, for example, the IRs which can be highly altered as observed in a group of legumes (Palmer and Thompson 1982) and all

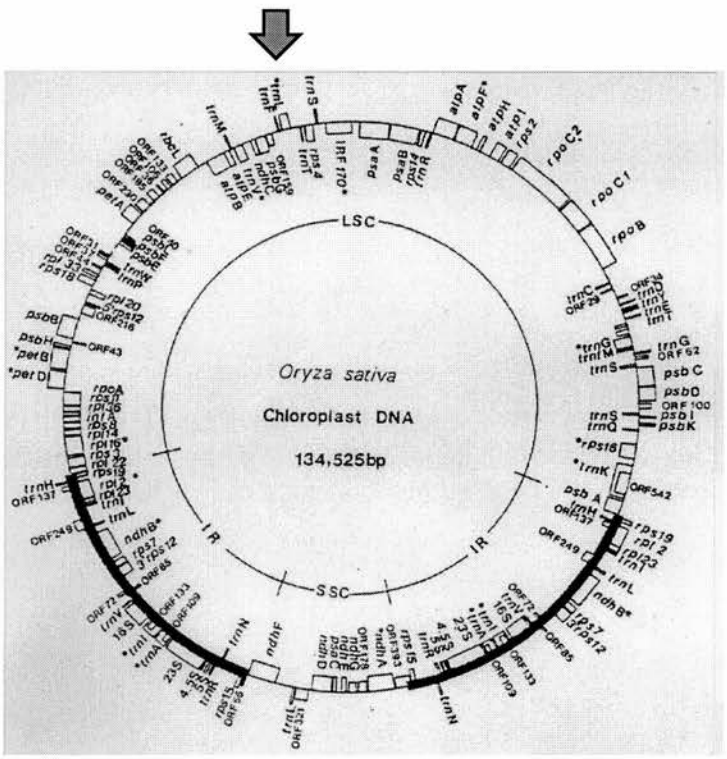


Figure 4. The *Oryza sativa* chloroplast genome with three distinct regions: LSC: large single copy region; IR: inverted repeat; and SSC: small single copy region. The arrow indicates the region between *trn*T (UGU) and *trn*F (GAA). After Hiratsuka *et al.* (1989).

conifers (Raubeson and Jansen 1992) where one copy of the IR is lacking; and in geranium where the size of the IR is greatly increased (Palmer *et al.* 1987). Other types of rearrangements include inversions and the insertion or deletion of genes and introns in the genome (Downie and Palmer 1992b).

The chloroplast genome is useful for the study of plant systematics and it has several advantages relative to the plant nuclear and mitochondrial genomes (reviewed in Palmer 1985a, b, 1987; Birky 1988; Palmer *et al.* 1988; Clegg 1989; Crawford 1990). First, it is relatively small and abundant in leaf cells, thus facilitating DNA extraction and analysis. Second, with the determination of the complete DNA sequences of several taxa (a bryophyte, *Marchantia* (Ohyama *et al.* 1986); a dicot, tobacco (Shinosaki *et al.* 1986); a monocot, rice (Hiratsuka *et al.*

1989); a parasitic dicot, *Epifagus* (Wolfe *et al.* 1992); a black pine, *Pinus thunbergii* (Tsudzuki *et al.* 1992), and an alga, *Euglena* (Hallick *et al.* 1993)) which represent virtually the full range of plant diversity, useful comparative studies of the structure, gene content, nucleotide sequences and mode and tempo of cpDNA have been carried out (reviewed in Clegg *et al.* 1994). The molecular information obtained is used to support branches of evolutionary research. Third, rates of nucleotide substitution of cpDNA are relatively slow and therefore, the genome is appropriate for the resolution of plant phylogenetic relationships at deep levels of evolution (i.e. at the interspecific level and above). Two molecular approaches including restriction site mapping of the entire chloroplast genome (e.g. Palmer *et al.* 1988, Jansen *et al.* 1991, Olmstead and Palmer 1992), and sequencing of the cpDNA gene such as *rbc*L which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase (RuBisCo) (e.g. Doebley *et al* 1990; Soltis, *et al.* 1990; Giannasi *et al.* 1992; Olmstead *et al.* 1992; Chase *et al.* 1993), have been widely used. Other genes suitable for phylogenetic inference are listed in Table 1 (p. 1213) of Olmstead and Palmer (1994).

For the evolutionary study of closely related taxa (e.g. at the infrageneric level), regions of cpDNA selected should evolve more rapidly than do coding regions. Noncoding regions do not code for tRNA, rRNA or protein, and therefore, display the highest frequency of mutations (Palmer *et al.* 1988; Clegg *et al.* 1991). These regions appear to diverge through nucleotide substitutions and indel events (Clegg and Zurawski 1991; Palmer 1991). Several studies based on the molecular evolution of variable noncoding regions in cpDNA of closely related taxa (Zurawski *et al.* 1984; Doebley *et al.* 1987; Aldrich *et al.* 1988; Ogihara *et al.* 1988; Stein and Hachtel 1988; Wolfson *et al.* 1991; Nimzyk *et al.* 1993) have shown that many indel events are associated with short direct repeats especially for small length mutations of 1-10 bp which occur frequently and are probably caused by slipped-strand

mispairing during replication and repair (Takaiwa and Sugiura 1982; Zurawski *et al.* 1984). Larger length mutations (10-1000 bp) are less common and may arise from recombination (Palmer 1991).

In 1991, Taberlet *et al.* investigated the region between the *trn*T (UGU) and *trn*F (GAA) genes located in the LSC unit of cpDNA (indicated by the arrow in **Figure 4**). Within this region three noncoding regions are found (**Figure 5**): (i) an intergenic spacer between *trn*T (UGU) and the *trn*L (UAA) 5' exon, (ii) the *trn*L (UAA) intron, and (iii) another intergenic spacer between the *trn*L (UAA) 3' exon and *trn*F (GAA). PCR primer sets which were constructed based on conserved sequences of the tRNA genes that flank the noncoding regions appeared to work well for different plant groups including bryophytes, pteridophytes, gymnosperms and angiosperms (Taberlet *et al.* 1991, Table 1). The three noncoding regions appear to have a roughly threefold higher rate of nucleotide substitution than the *rbc*L sequences (Gielly and Taberlet personal communication, cited in Böhle *et al.* 1994), and are therefore, expected to be useful for plant molecular systematics, at least at the infrageneric level. Based on these regions a study has been suggested for *Acer* (at the interspecific level, Taberlet *et al.* 1991), and various analyses aiming primarily at phylogenetic reconstruction have been carried out at the infrageneric level (e.g. Böhle *et al.* 1994; Gielly and Taberlet 1994; Gielly and Taberlet 1996; Gielly *et al.* 1996), and at the generic level (van Ham *et al.* 1994). These three noncoding regions are composed of single-copy sequences and therefore the problems of homology assessment should not be found. In addition, these regions are located distantly from the two inverted repeats, hence short inversions will not occur. Such events can yield homoplasious information as shown by Sang *et al.* (1997) in the *psb*A-*trn*H intergenic spacer. As a complementary data set to that produced by the work on ITS, the two chloroplast noncoding regions (ii) and (iii) were chosen for this present study of the infrageneric relationships of *Alpinia*.

Although the use of cpDNA molecule may be prone to significant error from hybridisation and introgression events due to the high potential for interspecific gene flow (reviewed in Rieseberg and Soltis 1991), comparison of cpDNA and nrDNA phylogenies should solve the problems (Smith and Sytsma 1990; Rieseberg 1991; Wendel *et al.* 1991) and confirm species relationships (Sytsma and Schaal 1985; Rieseberg *et al.* 1988; Wallace and Jansen 1990).

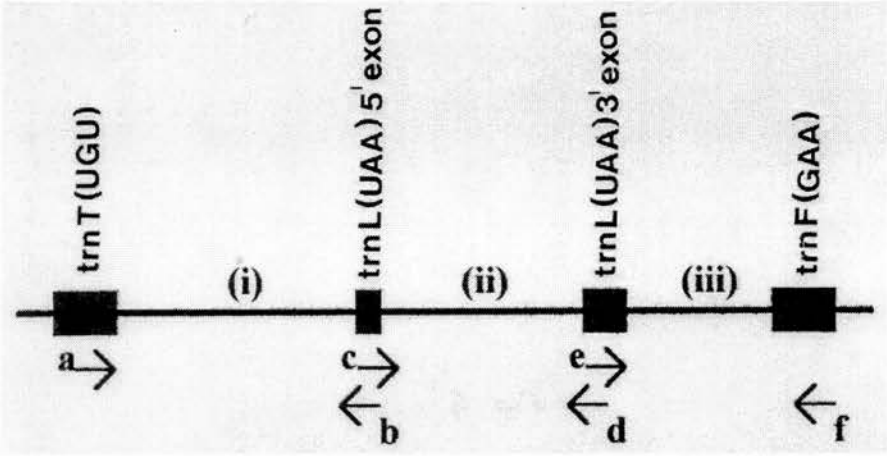


Figure 5. The chloroplast DNA region between the *trn*T (UGU) and the *trn*F (GAA) genes. Arrows indicate positions and directions of universal primers (Taberlet *et al.* 1991) used to amplify three noncoding regions: (i) an intergenic spacer between *trn*T (UGU) and the *trn*L (UAA) 5' exon, (ii) the *trn*L (UAA) intron, and (iii) another intergenic spacer between the *trn*L (UAA) 3' exon and *trn*F (GAA). After Taberlet *et al.* (1991).

2.2 MATERIALS

2.2.1 Origin of plant material

The present study of the infrageneric classification of *Alpinia* was based on DNA sequences of the ITS region of 57 taxa of Zingiberaceae. Almost all plant material was taken from living plants at the Royal Botanic Garden Edinburgh (E, UK), the Royal Botanic Gardens, Kew (UK), the National Museum of Natural History (Smithsonian Institution, USA), Waimea Arboretum and Botanical Garden (Hawaii, USA), Harold L. Lyon Arboretum (the University of Hawaii at Manoa, Hawaii, USA), the Royal Botanic Gardens Sydney (Australia), the Department of Botany, University of Malaya (Malaysia) and the Royal Botanic Gardens Peradeniya (Sri Lanka) - see acknowledgements. There was also a small number of taxa (six) where leaf material was taken from herbarium specimens at E. The majority of the voucher specimens were freshly prepared at E. The rest were either prepared elsewhere and loaned to E, or sent dried and unmounted to E with further flowers sent moist in cotton wool soaked with alcohol. Herbarium specimens were then prepared at E. Flowers were preserved in Copenhagen mixture (10:1:8 mixture of methylated spirit, glycerol and water) in a spirit collection (E). However, for certain species of *Alpinia* and its outgroup no voucher specimens are yet available. This is mainly because some of these species flower infrequently. Nonetheless, they were used in this study following identification by an authority on Zingiberaceae, Prof W.J. Kress at the National Museum of Natural History (Smithsonian Institution, USA). Other plant specimens with accession numbers could be checked from their sources. For the 57 taxa of Zingiberaceae under study the origin of the specimens and additional useful information are given in **Table 5**.

2.2.2 Outgroup taxa

To investigate the phylogenetic relationships of *Alpinia*, appropriate outgroup selection was essential. The outgroup should be systematically close enough to *Alpinia* to allow sequence alignment and yet distant enough to enable unequivocal rooting of the tree. Examination of the outgroup helps to ensure that the ingroup cladograms obtained are parsimonious, not only within the ingroup, but also with the related taxa. Nine taxa of four outgroup genera (*Burbidgea*, *Elettariopsis*, *Pleuranthodium* and *Renealmia*) were selected for this study (see **Table 5**) based on the following criteria. (i) As with *Alpinia* these four genera belong to the tribe Alpineae. (ii) In terms of floral morphology, *Pleuranthodium* and *Renealmia* are very close to *Alpinia*. In contrast, *Burbidgea* and *Elettariopsis* are more distantly related to *Alpinia* and their floral structures can be easily distinguished from one another.

2.2.3 Ingroup taxa

The forty eight taxa of *Alpinia* under study are good representatives of almost all sections and subsections of *Alpinia* based on Smith's (1990a) infrageneric classification (see **Table 5**). Sampling did not appear to be a problem for subgenus *Alpinia*, but for subgenus *Dieramalpinia* the material was inadequate. This problem is mainly due to the difficulty of access to plant material from Eastern Malesia, where the species of subgenus *Dieramalpinia* are found.

Table 5. Sources, and voucher specimens for the 57 species (58 accessions) of Zingiberaceae from which ITS sequences were obtained for this study. Twenty-two species marked by * were included in the analyses of the intergenic spacer between *trn*L (UAA) 3' exon and *trn*F (GAA). Taxonomic treatment of *Alpinia* follows Smith's (1990a) system. Abbreviations used in the table are E: The Royal Botanic Garden Edinburgh; herb.: DNA extraction from a herbarium specimen; HLA: Harold L. Lyon Arboretum; K: The Royal Botanic Gardens, Kew; NMNH (SI): The National Museum of Natural History (Smithsonian Institution); NSW: The Royal Botanic Gardens Sydney; PDA: The Royal Botanic Gardens Peradeniya; UM: University of Malaya; USNH: United States National Herbarium; WAI: Waimea Arboretum & Botanical Garden; - denotes no voucher. Accession numbers follow the abbreviations (when available).

Taxon	Source and Accession No.	Voucher
Outgroup		
Burbidgea schizocheila Hackett	E, 19851903	A. Rangsiruji 6 (E)
Elettariopsis unifolia (Gagnep.) M.F.Newman	E, 19901449	M.F. Newman 747 (E)
Pleuranthodium floccosum (Valeton) R.M.Sm.	HLA, L-78.0460	KMN 3010 (HLA, E)
Pleuranthodium floribundum (K.Schum.) R.M.Sm.	WAI, 75s1701	-
Pleuranthodium papilionaceum (K.Schum.) R.M.Sm.	E, 19751791	A. Rangsiruji 31 (E)
Pleuranthodium racemigerum (F.Muell.) R.M.Sm.	WAI, 87p300	-
Pleuranthodium schlechteri (K.Schum.) R.M.Sm.*	WAI, 75p168	-
Renealmia aff. *africana* Benth. ex Hook.f.	K	D. Harris 4927 (K)
Renealmia battenbergiana Cummins ex Baker*	E, 19740104	A. Rangsiruji 27 (E), C 8482 (E)

Table 5 (continued).

Taxon	Source and Accession No.	Voucher
Ingroup		
Gen. *Alpinia*		
Subg. *Alpinia*		
Section *Alpinia*		
Subsections:		
Alpinia		
A. coriacea T.L.Wu & S.J.Chen	NMNH (SI), AC 95-5539	3308886 (USNH)
A. galanga (L.) Willd.*	E, 19771077	A. Rangsiruji 3 (E)
A. galanga (L.) Willd.	KMN 3543 (E) (herb.)	KMN 3543 (E)
A. intermedia Gagnep.*	NMNH (SI), AC 94-5330	-
A. japonica Miq. var. *kiushiana* Kitam.*	HLA, L-84.0177	-
A. maclurei Merr.	NMNH (SI), AC 95-5540	-
A. polyantha D.Fang	NMNH (SI), AC 94-3744 JK	-
A. suishaensis Hayata	E, 19791028	A. Rangsiruji 37 (E), C 8468 (E)
Presleia		
A. aquatica (Retz.) Roscoe*	J. Mood, M 669	Slide M 669 (A. Rangsiruji)
A. brevilabris C. Presl*	NMNH (SI), AC 94-5335	-
A. flabellata Ridl.	J. Mood, M 504	A. Rangsiruji 40 (E)
A. foxworthyi Ridl.	NMNH (SI), AC 94-5339	-
Paniculatae		
A. ligulata K.Schum.*	J. Mood, M 336	-
A. nieuwenhuizii Valeton*	J. Mood, M 557	Slide M 577 (A. Rangsiruji)
Cenolophon		
A. officinarum Hance*	NMNH (SI), AC 95-5553	-
A. oxymitra K.Schum.	E (herb.)	(E)
A. oxyphylla Miq.	E, 19902064	A. Rangsiruji 34 (E)
A. aff. *shimadai* Hayata*	E, 19934175	A. Rangsiruji 15 (E)

Table 5 (continued).

Taxon	Source and Accession No.	Voucher
Subsections:		
Catimbium		
A. blepharocalyx K.Schum. var. *blepharocalyx*	NMNH (SI), AC 95-5521	3308901 (USNH)
A. blepharocalyx K.Schum. var. *glabrior* (Hand.-Mazz.) T.L.Wu*	E, 19901453	A. Rangsiruji 21 (E)
A. calcarata Roscoe	E, 19750164	A. Rangsiruji 25 (E)
A. formosana K.Schum.	NMNH (SI), AC 94-5336	-
A. kwangsiensis T.L.Wu & S.J.Chen	E, 19901472	-
A. latilabris Ridl.	J. Mood, M 552	Slide M 552 (A. Rangsiruji)
A. malaccensis (Burm.f.) Roscoe*	E, 19751793	A. Rangsiruji 14 (E)
A. mutica Roxb.	E, 19763131	A. Rangsiruji 1 (E)
A. zerumbet (Pers.) B.L.Burtt & R.M.Sm.	E, 19751777	A. Rangsiruji 18 (E)
Probolocalyx		
A. glabra Ridl.	E (herb.)	C 8316 (E)
Sections:		
Didymanthus		
A. pumila Hook.f.*	NMNH (SI), AC 96-308	-
Kolowratia		
A. elegans (Presl) K.Schum.*	NMNH (SI), AC 94-5290	3304282 (USNH)
Fax		
A. abundiflora B.L.Burtt & R.M.Sm.	A. Weerasooriya (PDA)	Unmounted (K)
A. fax B.L.Burtt & R.M.Sm.	A. Weerasooriya (PDA)	Unmounted (K)
Guillainia		
A. purpurata (Vieill.) K.Schum.	WAI, 80p18	A. Rangsiruji 9 (E)
Arctiflorae		
A. arctiflora F.Muell.	NSW, 904264	A. Rangsiruji 48 (E)

Table 5 (continued).

Taxon	Source and Accession No.	Voucher
Sections:		
Allughas		
Subsections:		
Allughas		
A. javanica Blume*	UM	A. Rangsiruji 53 (E)
A. nigra (Gaertn.) B.L.Burtt*	WAI, 80p172	A. Rangsiruji 55 (E)
A. rafflesiana Wall. ex Baker*	UM	A. Rangsiruji 52 (E)
Strobidia		
A. conchigera Griff.*	NMNH (SI), AC 95-5512	3308910 (USNH)
Caeruleae		
A. arundelliana (F.M.Bailey) K.Schum.	NSW, 842143	260007 (NSW), A. Rangsiruji 49 (E)
A. caerulea Benth.	NSW 862220	200238 (NSW), A. Rangsiruji 50 (E)
A. modesta (F.Muell.) K.Schum.*	NSW 904273	234226 (NSW), A. Rangsiruji 51 (E)
Odontychium	None	None
Subg. *Dieramalpinia*		
Section *Pycnanthus*		
Subsections:		
Pycnanthus		
A. boia Seem.	E (herb.)	2782 (E)
Amomiceps	None	None
Sections:		
Eubractea		
A. eubractea K.Schum.	E (herb.)	529 (E, Leiden)
Myriocrater		
A. coeruleoviridis K.Schum.	E (herb.)	240 (E)
A. vulcanica Elmer	P. Wilkie, 29129	A. Rangsiruji 23 (E)
Dieramalpinia		
A. carolinensis Koidz.*	HLA, L-81.0366	KMN 3569 (HLA, E)
A. luteocarpa Elmer (*scorpoidea*)	WAI, 87s571	A. Rangsiruji 2 (E)
A. oceanica Burkill*	E, 19691132	A. Rangsiruji 5 (E)
A. vittata W.Bull	HLA, L-72.0535	KMN 1455 (HLA)

Note: *A. luteocarpa* Elmer (*scorpoidea*) is a problematic species. The specimen I have used here did not match with the type specimen (Elmer 17926) at K which only contains vegetative characters. The specimen used, although possessing inflorescences, lacked a labellum from each flower (or, if the labellum was present, it was very reduced). For her specimens at E, Smith encountered the same problem. She called these specimens "*A. scorpoidea*" but this species has not been validly published. Therefore, throughout my thesis I shall refer to it as *A. luteocarpa* (*scorpoidea*).

Table 6. PCR and sequencing primers for the ITS region (see **Figure 3**) and for the region between *trn*L (UAA) 5' exon and *trn*F (GAA) (see **Figure 5**). Primer "ITS2K" was designed during this course of study while primers "ITS3P", "ITS5P" and "ITS8P" were modified from White *et al.* (1990). Primers c, d, e, and f were obtained from Taberlet *et al.* (1991).

Name	Location	Direction	Sequence
"ITS2K"	5.8S nrDNA	Reverse	5'-GGCACAACTTGCGTTCAAAG-3'
"ITS3P"	5.8S nrDNA	Forward	5'-GCATCGATGAAGAACGTAGC-3'
"ITS5P"	18S nrDNA	Forward	5'-GGAAGGAGAAGTCGTAACAAGG-3'
"ITS8P"	25S nrDNA	Reverse	5'-CACGCTTCTCCAGACTACA-3
c	*trn*L (UAA) 5' exon	Forward	5'-CGAAATCGGTAGACGCTACG-3'
d	*trn*L (UAA) 3' exon	Reverse	5'-GGGGATAGAGGGACTTGAAC-3'
e	*trn*L (UAA) 3' exon	Forward	5'-GGTTCAAGTCCCTCTATCCC-3'
f	*trn*F (GAA)	Reverse	5'-ATTTGAACTGGTGACACGAG-5'

2.3 METHODS

2.3.1 Total genomic DNA extraction

For each taxon, fresh leaf material of no larger than 2 cm^2 was collected. For studies involving PCR it is crucial that any material used for DNA extraction is healthy (i.e. free from insect, fungal and viral damage). The material was placed into a snap-top plastic bag, dried and preserved with approximately one teaspoonful of self-indicating silica gel. Prior to extraction of the DNA, the leaves were usually stored at 4°C overnight to be destarched. Total genomic DNA was isolated from the silica gel dried leaf material using a modification of the CTAB method of Doyle and Doyle (1987), with no further purification. In the case of the herbarium specimens, two alternative methods to isolate DNA were conducted using either 2x "hot" CTAB or the DNeasy™ Plant Mini Kit (Qiagen Ltd., UK).

(1). A modification of the CTAB method of Doyle and Doyle (1987) for silica gel dried leaf material

Reagents:

- 2x CTAB extraction buffer: 2% w/v CTAB (cetyltrimethylammonium bromide), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1% PVP-40T, and 0.2% (v/v) beta-mercaptoethanol (added immediately prior to use).

Properties of 2x CTAB:

The components of the extraction buffer have particular functions to protect DNA from degradation by native enzymes and secondary plant metabolites. (i) CTAB is a cationic detergent which aids in the lysis of cell membranes and will form complexes with nuclic acids. (ii) NaCl aids in the formation of nucleic acid-CTAB complexes. (iii) EDTA chelates divalent ions, particularly Ca^{2+} and Mg^{2+}, and prevents the activity of metal-dependent nucleases. (iv) PVP-40T forms complexes with secondary plant products such as complex polyphenols, tannins and

quinones. The substance will inhibit browning of the DNA extract. (v) Beta-mercaptoethanol which can be added prior to use, is a reducing agent which protects DNA against quinones, disulphides, peroxidases and polyphenol oxidases.

- "Wet" chloroform: chloroform/isoamyl alcohol (24:1)
- Isopropanol
- Wash buffer: 76% ethanol, 10 mM ammonium acetate

Procedure:

1. A small amount of leaf material (a single paper-punch size) was placed into an Eppendorf tube where sand and 200 µl of 2x CTAB extraction buffer were added. The material was then ground with a plastic pestle. A further 800 µl of the extraction buffer was added, the contents were mixed gently, and incubated at 65°C for 30 min with occasional inversion.

2. The tube was allowed to cool to room temperature before 200 µl "wet" chloroform was added. Invert the tube gently 3 or 4 times (each time to obtain a momentary single phase), followed by 2 min of centrifugation at 13000 rpm to separate the layers.

3. The aqueous (upper) phase was removed to a clean Eppendorf tube where it was re-extracted with 200 µl "wet" chloroform and step 2 was repeated.

4. The aqueous (upper) phase was removed to a clean Eppendorf tube and 600 µl of cold (-20°C) isopropanol was added. The contents were mixed gently and allowed to stand for 15 min. Then the tube was centrifuged for 2 min at 13000 rpm.

5. The supernatant was removed and 1 ml of wash buffer was added. The tube was vigorously agitated to release the pellet from the bottom, and then left standing for at least 30 min.

6. The tube was centrifuged for 2 min at 13000 rpm and the supernatant was removed. To dry the pellet, the tube was inverted and placed in an oven at 45°C for 5-10 min. The pellet was then resolved in 50 µl sterile distilled water and stored at

-20°C until required.

(2). "Hot" CTAB method for herbarium material

The method followed the procedure in (1) except: (i) 2x CTAB buffer was heated to 65°C prior to use. (ii) Liquid nitrogen was used instead of sand to ease the grinding of leaf material. (iii) 50 µl RNase A (1 mg/ml) was added to a mixture of the CTAB and ground leaf material, and the contents were kept at 65°C for 1 hr (instead of 30 min) before proceeding to the next step of "wet" chloroform extraction. (iv) In the presence of the wash buffer DNA was left at 4°C overnight.

(3). The DNeasy™ Plant Mini Kit (Qiagen Ltd., UK) for herbarium material and difficult plant species

The DNeasy™ Plant Mini Kit (Qiagen Ltd., UK) was designed for rapid and reproducible DNA purification from a wide range of plant species. The kit uses spin column technology to isolate highly pure DNA free from inhibitory contaminants. According to Qiagen (Qiagen News (issue 2) 1998) DNeasy technology has been used to obtain DNA from more than 40 different plant species and also from difficult plant and fungal species. Thus, the kit was chosen for DNA extractions from herbarium specimens as well as from difficult species of Zingiberaceae under study.

Procedure (modified from Qiagen):

1. A small amount of leaf material was placed into an Eppendorf tube and ground under liquid nitrogen to a fine powder.

2. 400 µl of Buffer AP1 and 20 µl of RNase A stock solution (20 mg/ml) were added and the tube was agitated vigorously using a vortex.

3. The mixture was incubated at 65°C for 30 min and mixed by inverting the tube 2-3 times during incubation.

4. Then 130 µl of Buffer AP2 was added and the contents were mixed and incubated for 10 min on ice.

5. On the QIAshredder spin column sitting in a 2-ml collection tube, the contents were applied and centrifuged for 2 min at 13000 rpm.

6. Flow-through fraction from step 5 was transferred to a new tube without disturbing the pellet.

7. 0.5 volume of Buffer AP3 and 1 volume of ethanol (96-100%) were added and the contents were mixed by pipetting.

8. 650 µl of the mixture from step 7 was applied onto the DNeasy mini spin column sitting in a 2-ml collection tube, and centrifuged for 1 min at 8000 rpm. The flow-through was discarded. This step was repeated with the remaining sample.

9. The DNeasy column is placed in a new 2-ml collection tube with 500 µl Buffer AW added and they were centrifuged for 1 min at 8000 rpm.

10. The flow-through was discarded and again 500 µl Buffer AW was added and centrifuged, this time for 2 min at 13000 rpm.

11. The DNeasy column is transferred to an Eppendorf tube and 100 µl of preheated (65°C) Buffer AE was applied directly onto the DNeasy column membrane, followed by a centrifugation for 1 min at 8000 rpm.

2.3.2 Agarose gel electrophoresis

This method relies on the fact that DNA molecules carry a negative charge effect. On an agarose gel, when an electric field is applied, the DNA molecules will travel through a complex network of pores to reach the positive electrode. The smaller the molecule, the faster it can migrate through the gel. Gel electrophoresis will therefore separate DNA molecules according to their relative sizes. In this study the method was used basically, to check the presence of the DNA, its quality and approximate quantity.

Reagents:

- 10x TBE buffer stock (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA (pH 8.0))

- Loading solution (Promega, USA: 0.25 M disodium-EDTA, 50% glycerol, 0.1% SDS, 0.01% bromophenol blue, 0.01% xylene cyanol)

- DNA size marker (Lambda-*Hin*dIII; Promega, USA)

Procedure (for a small gel (6 cm × 7.5 cm)):

1. A gel mould was prepared by sealing the open ends with tape, and an appropriate gel comb was aligned vertically in the mould.

2. 1% agarose (Promega, USA) was then prepared by adding 0.3 g of agarose in a 50-ml flask with 30 ml 1x TBE buffer (diluted from 10x TBE stock). The ingredients were mixed thoroughly, and heated in a microwave until all of the particles had gone into solution.

3. The slightly cooled agarose was poured into the level mould. The gel was then left to set as it turned opaque.

4. The comb was carefully removed to prevent tearing of the wells or the teeth separating them. The tape was also removed from the mould and the gel was placed in the rig submerged in 1x TBE buffer.

5. 1/5 volume of loading solution was added to DNA samples and each sample was then loaded into the gel well using a Gilson Pipetteman (usually P10). A DNA size marker (Lambda-*Hin*dIII) was also included on the gel.

6. The gel apparatus was connected to the electrical supplies with the appropriate current (60 mA to 80 mA) and the gel was run for about 1 to 1½ hr.

7. Then the gel mould was removed from the apparatus and the gel was stained in an ethidium bromide bath (500 ml distilled water with 0.5 μg/ml ethidium bromide) for 10-20 min with gentle shaking.

8. The DNA fragments on the gel were visualised under UV light and then photographed using a Polaroid camera.

2.3.3 PCR amplifications and conditions for the ITS region and the region between *trn*L (UAA) 5' exon and *trn*F (GAA)

The PCR is an *in vitro* technique which allows the amplification of a specific DNA region that lies between two regions of known DNA sequence. Three basic steps including denaturation, annealing, and extension are involved in this technique to produce millions of identical DNA copies within a short time even when the starting sample contains only one original target sequence.

(1). Primer design

In this present study of *Alpinia* one primer ("ITS2K") was designed to match specifically with the region in 5.8S rDNA of *Alpinia* and its outgroup. Following the alignment of homologous sequences in the 5.8S rDNA region of seven taxa of *Alpinia*, four taxa of the outgroup and a carrot (Yokota *et al.* 1989) the design of "ITS2K" primer was achieved with the aid of software, OLIGO (National BioScience). The primer was constructed by taking into consideration the following important points:

1. A highly conserved region of 5.8S rDNA with the length of 18-25 bp was selected.

2. The first three bases of the 3' end of the primer are complementary to the target sequence, therefore, both DNA strands were firmly annealed and this is essential for efficient polymerase binding.

3. Primer pairs for ITS1 region ("ITS2K" and "ITS5P") do not have complementary 3' ends. This reduces the chance that the primers will pair with each other and amplify to form primer artifacts or primer dimers.

4. The base composition of "ITS2K" primer was almost equivalent for all four bases, and no palindromic base sequences were present, thus, it is unlikely that the primer will fold and pair with itself.

(2). Basic reactions for PCR (double stranded DNA amplifications)

Procedure (for 50 µl reactions):

1. For each reaction the following ingredients were mixed in a microcentrifuge tube (0.2 ml PCR tube): 31.5 µl sterile distilled water (33.5 µl for the negative control), 5 µl of 10x Dynazyme™ reaction buffer (1x: 10 mM Tris HCl, pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100; Finnzymes Oy, Finland), 1 µl of 10 mM deoxyribonucleoside triphosphate (dNTP) mix (Sigma Chemicals, UK), 5 µl of each primer (final concentration 1 µM; Oswel DNA Service, UK), 2 µl of total genomic DNA (none for the negative control) and 0.5 µl (1U) of Dynazyme™ II thermostable DNA polymerase (Finnzymes Oy, Finland).

Note: Different primer pairs were used to amplify different regions of the genes (see **Figure 3** and **Figure 5** as well as **Table 6**).

- The complete ITS region: primers "ITS 5P" and "ITS 8P".
- The ITS1 region: primers "ITS5P" and "ITS2K".
- The ITS2 region: primers "ITS3P" and "ITS8P".
- The region between *trn*L (UAA) 5' exon and *trn*F (GAA): primers c and f.
- The intergenic spacer between *trn*L (UAA) 5' exon and *trn*L (UAA) 3' exon: primers c and d.
- The intergenic spacer between *trn*L (UAA) 3' exon and *trn*F (GAA): primers e and f.

2. All the ingredients were well mixed and centrifuged briefly to ensure that there was no froth in the tube.

3. The tube was then placed in the thermal cycler (GeneAmp PCR System 9600, Perkin Elmer, USA) and the PCR cycle was achieved by completing three major steps after an initial denaturation step at 94°C for 3 min.

(i). Denaturation (94°C for 1 min). In this step, heat is used to denature the DNA from double to single strands.

(ii). Annealing (for ITS primers: 55°C for 2 min; and for *trn*L primers: 50°C for 1 min). In this phase, the temperature is lowered close to the primer's melting temperature so that binding of the primers to the appropriate sites in the template DNA can occur with high specificity.

(iii). Extension (for ITS primers: 72°C for 1.5 min; and for *trn*L primers: 72°C for 2 min). In this step, the addition of new bases to the free 3' ends of the primers is performed by the heat-stable DNA polymerase whose optimum temperature is about 72°C.

The PCR cycle was repeated 30 times for the ITS region and 35 times for the region between *trn*L (UAA) 5' exon and *trn*F (GAA), followed by a 5-min final extension step at 72°C. In the case of the herbarium specimens used for the ITS study, the cycle started at 94°C for 10 min as an initial incubation, with 40 cycles. Gel electrophoresis was then carried out to check each PCR product. The procedure followed section **2.3.2**, except for the use of 1.5% gel and the 123 bp ladder (Sigma Chemicals, UK) as a size marker.

Under certain circumstances where the ITS region or the region between *trn*L (UAA) 5' exon and *trn*F (GAA) experienced difficulties in PCR amplification such as no product was obtained, or the amplification contained more than one band, several attempts were made to adjust the PCR conditions. However, in the case of the region between *trn*L (UAA) 5' exon and *trn*F (GAA) PCR products always contained multiple bands (**Figure 11**). As a result, each cpDNA noncoding region i.e. the *trn*L (UAA) intron, and the intergenic spacer between the *trn*L (UAA) 3'

exon and *trn*F (GAA) had to be amplified individually with primer pairs c and d, and e and f, respectively. The results obtained were as follows: for each taxon, the combination of primer c and primer d produced multiple bands while that of the primer e and primer f produced only one clear and distinctive band (**Figure 12**). Methods used to increase the stringency of the PCR conditions were carried out, but failed to improve the amplification with primer c and primer d. Therefore, only the PCR products between the *trn*L (UAA) 3' exon and *trn*F (GAA) generated from the pair of primer e and primer f were used as potential templates for cpDNA sequencing.

2.3.4 Purification of PCR products

Prior to sequencing it is important that amplified DNA fragments are purified in order to remove contaminants such as residual PCR primers and other nucleotides. Contamination of sequencing reactions with PCR components can reduce the quality of sequence data, affecting accuracy, signal intensity, and read length (Sauer *et al.* 1998). In the molecular study of *Alpinia* QIAquick system (Qiagen Ltd., UK) was employed to clean up contaminating DNA fragments following PCR by using a unique silica gel membrane technology.

(1). QIAquick™ PCR Purification Kit (Qiagen Ltd., UK)

Procedure:

1. 5 volumes of Buffer PB were added to 1 volume of the PCR reaction and mixed.

2. A QIAquick spin column was placed in a 2-ml collection tube and the sample was applied onto the column, and centrifuged for 1 min at 13000 rpm.

3. After discarding the flow-through the column was placed back into the same tube and 750 µl Buffer PE was added and centrifuged for 1 min at 13000 rpm.

4. The flow-through was again discarded and the column was placed back into the same tube and centrifuged for an additional 1 min at 13000 rpm.

5. Then the column was transferred to a clean Eppendorf tube, and 40 µl of sterile distilled water was added to the centre of the column. This was left standing for 1 min before centrifugation for 1 min at 13000 rpm.

Gel electrophoresis was once again carried out to determine the approximate concentration of the purified PCR products (1 µl of each sample loaded) by comparing the intensity of each product against the intensity of the 123 bp size marker (the concentration of the first 123 bp ladder band is roughly equal to 30 ng/µl, and 10 ng/µl in the second band).

(2). QIAquick™ Gel Extraction Kit (Qiagen Ltd., UK)

For the PCR reactions which yielded products of more than one band, the method of gel extraction was employed in order to purify and obtain a single band product from the gel.

Reagents (not supplied in the kit):

- Isopropanol
- 3 M sodium acetate (pH 5.0)

Procedure:

1. A gel comb with wide teeth was used in this case to prepare a 1.5% gel, and for each PCR product that contained multiple bands, all of its available volume (~ 40 µl) was loaded onto the gel. The gel was allowed to run for as long as 2 hrs so that the bands were well separated from one another.

2. The required DNA fragment was then excised from the gel and weighed in a tube.

3. 3 volumes of Buffer QG were added to 1 volume of the gel, and the contents were incubated at 50°C for 10 min. The gel was allowed to dissolve completely as the tube was flicked and inverted every 2-3 min during the incubation.

4. 1 gel volume of isopropanol was added with 10 µl of 3 M sodium acetate (pH 5.0), and the contents were well mixed.

5. A QIAquick spin column was placed in a 2-ml collection tube, the sample was loaded and centrifuged for 1 min at 13000 rpm.

6. The flow-through was discarded and the column was placed back into the same tube.

7. 500 µl of Buffer QG was added to the column and then centrifuged for 1 min at 13000 rpm.

8. 750 µl of Buffer PE was then added to the column which was left standing for 5 min before centrifugation for 1 min at 13000 rpm.

9. The flow-through was discarded and the column was centrifuged for an additional 1 min at 13000 rpm.

10. The column was placed in a clean Eppendorf tube and the DNA was eluted by adding 30 µl of sterile distilled water to the centre of the column. This was left standing for 1 min and then centrifuged for 1 min at 13000 rpm.

The presence of the required fragment from the PCR product was checked by running a 1.5% gel for 1 hr with the 123 bp ladder to confirm the correct size of the product.

2.3.5 DNA Sequencing protocols

(1). Manual sequencing by Sanger's method performed for the ITS region

(i). Preparation of sequencing gel

Reagents:

- 20x Glycerol Tolerant Buffer (20x GTB (1 litre): 216 g Tris Base, 72 g Taurine, and 4 g Na_2 EDTA.$2H_2O$)
- Ammonium persulphate
- TEMED
- Running buffer (0.8x GTB)

Procedure:

1. Acrylamide sequencing gel stock solution contains urea, 20x GTB, and acrylamide 40% ready mix. To prepare 500 ml of 6% acrylamide sequencing gel stock, 20 ml of 20x GTB was added to a flask containing 250 g of urea. Then 75 ml of acrylamide 40% ready mix was added and followed by 100 ml of distilled water. The ingredients were dissolved on a magnetic stirrer with hot plate and the solution was finally topped up with distilled water to 500 ml. This 6% working solution was kept at 4°C until required.

2. Gel plates were cleaned thouroughly with soapy water and 70% ethanol. The inner surfaces of the plates were occasionally treated with silane to smoothen the surfaces and prevent gel sticking to the plates.

3. The plates were clamped together with spacers between them and all sides of the plates except the top were tightly sealed.

4. Prior to pouring the gel, 600 µl of ammonium persulphate and 6 µl of TEMED were added to 75 ml of 6% acrylamide stock solution. The mixture was swirled carefully avoiding the formation of air bubbles.

5. From a beaker the gel solution was poured slowly and constantly between the plates and no air bubbles should be formed.

6. A sharkstooth comb was inserted (with the teeth pointing upwards) between the plates at the top and the gel was allowed to set for at least 1 hr.

7. The running buffer (0.8x GTB) was poured on the comb with a Pasteur pipette. The comb was removed and cleaned in distilled water before inserted back into the gel with the teeth pointing downwards.

8. The gel was clamped onto the gel running apparatus whose reservoirs were filled with the running buffer.

9. Each well formed by the sharkstooth comb was cleaned by using a micropipette. The gel was pre-run with the power of 50 W.

(ii). DNA sequencing reactions for double stranded PCR products (using Sequenase PCR Product Sequencing Kit; Amersham Life Science Inc., USA)

(a). Enzymatic pre-treatment of the PCR product

1. To 5 µl of the purified PCR product (0.2-0.5 pmol): 1 µl of Exonuclease I (10 units/µl) and 1 µl of Shrimp Alkaline Phosphatase (2 units/µl) were added.

2. The contents were mixed, centrifuged and incubated at 37°C for 15 min. During this stage, the Exonuclease I removed residual single stranded primers and other single stranded DNA produced by PCR, and the Shrimp Alkaline Phosphatase removed the remaining dNTPs from the PCR mixture which would otherwise interfere with the labelling step of the sequencing process.

3. The two enzymes were then inactivated by heating to 80°C for 15 min.

(b). Preparation of the annealing mixture

1. 1 µl of an ITS primer (0.8 µM) and 2 µl of sterile distilled water were added to the enzyme-treated PCR mix. The mixture was incubated at 100°C for 3 min, and cooled immediately on ice for 5 min.

2. The mixture was centrifuged briefly and kept on ice.

(c). The preparation of termination and labelling reactions

1. Eppendorf tubes were labelled with A, C, G and T for each set of a primer used and the PCR product, and 2.5 µl of each Termination Mixture (A, C, G, T) was added.

2. 4-dNTP labelling was used by diluting the stock labelling mix 1:5 in sterile distilled water. Each sequence required 2 µl of this diluted mixture.

3. Four termination tubes from step 1 (A, C, G, T) were pre-warmed for 1 min at 37°C.

4. For each labelling reaction, the following components were added to ice-cold annealed DNA mixture from step **(b)**: 2 µl of reaction buffer, 1 µl of DTT (0.1 M), 2 µl of diluted labelling mix, 0.5 µl of [α-^{35}S] dATP (5 µCi), and 2 µl of Sequenase DNA Polymerase. The reaction was mixed thouroughly, centrifuged briefly and kept at room temperture for 5 min.

5. For each termination reaction (pre-warmed in step 3), 3.5 µl of labelling reaction was added to each termination tube (A, C, G, T). The contents were mixed and the incubation of the termination reactions at 37°C was continued for 5-10 min.

6. The reactions were stopped by adding 4 µl of Stop Solution.

(d). Gel loading

1. The samples were heated to 75°C for 2 min immediately before loading onto sequencing gel (2-3 µl/lane).

2. For the length between 200-300 bp of ITS1 or ITS2, the gel was run for approximately 3-5 hrs to obtain well separated DNA fragments.

(iii). Autoradiograph of the sequencing gel

Reagents:

- Fixing solution: 10% methanol and 10% acetic acid

Procedure:

1. At the end of the electrophoresis run, the sequencing apparatus was disconnected from the power pack and the running buffer was disposed of safely.

2. The gel plates were then removed from the apparatus and laid flat on a bench. The plates were slowly separated using the end of a metal spatula so that the gel remained attached to the lower plate.

3. The right hand corner of the gel (away from the side of the gel that was loaded first) was cut off to mark the orientation of the gel for subsequent manipulations. The gel (together with its supporting plate) was transferred to a shallow bath containing 10% methanol and 10% acetic acid in water (fixing solution) and fixed for 15 min. The urea and sucrose were removed, thus enabling the gel to dry completely afterwards.

4. The gel and its supporting plate were lifted out of the fixing solution. A piece of Whatman 3 MM paper was placed on top of the gel and when gentle pressure was applied, the gel became firmly attached to the paper.

5. A piece of Saran Wrap was carefully laid on top of the gel, avoiding any bubbles forming.

6. The gel was dried 30-40 min under vacuum on a commercial gel dryer set at 80°C.

7. When the gel was dried the Saran Wrap was removed. The gel was then exposed to an X-ray film (Kodak XAR-5) for 24-48 hrs at room temperature. The edge of the film was also cut off and used for the gel orientation.

8. The autoradiograph was developed and the sequence of the gel was read on a light box.

(2). Cycle sequencing and automated DNA sequencing

Procedures involve the use of Sanger sequencing with fluorescently labelled (rather than radioactively labelled) DNA fragments. Using dye labelled dideoxynucleotides as chain terminators (as in this present study), the reactions are performed in a single tube (instead of 4 tubes as in the manual DNA sequencing), and can be run on a single gel lane. Therefore, the sequence of up to 64 samples can be analysed in one gel within a short time with a high level of accuracy.

(i). Preparation of sequencing reactions

Procedure:

1. For each sequencing reaction (10 µl) the components supplied in the sequencing kit (a Dye Terminator Cycle-Sequencing Ready-Reaction Kit with AmpliTaq® DNA Polymerase; Perkin Elmer, USA) were added in sequence to a 0.2 ml PCR tube as follows.

- 3 µl of the sterile distilled water.
- 4 µl of the reaction mix (supplied in the kit).
- 0.5 µl of the primer. The primers were identical to those used for PCR, but at a lower concentration (3.2 pmol/µl). Both forward and reverse primers were employed in each region to allow confirmation of the accuracy of the sequences. For the control reaction, 4 µl of the primer (supplied in the kit) was added.
- 2.5 µl of the DNA template (purified PCR product). For the control reaction, 2 µl of the DNA (supplied in the kit) was required.

2. The final contents were mixed thouroughly and then centrifuged briefly.

3. The sample was placed in the thermal cycler and run for 25 cycles with the following conditions: 96°C for 10 sec (denaturation step), 50°C for 5 sec (annealing step), and 60°C for 4 min (extension step).

(ii). Purification of sequencing reactions

Procedure:

1. Each sequencing reaction (10 µl) was transferred onto a 0.6 ml PCR tube containing 50 µl of ethanol (96-100%) and 2 µl of 3 M sodium acetate. The contents were mixed gently and kept on ice for 10 min.
2. The tube was then centrifuged for 30 min at 13000 rpm.
3. The ethanol was carefully aspirated using a pipette and 250 µl of 70% ethanol was added to wash the pellet. The contents were then centrifuged for 2 min at 13000 rpm.
4. The tube, with its lid opened, was vacuum dried for 3 min at medium rate and finally it should be completely dried.
5. The tube was stored at -20°C and this was ready for the automated DNA sequencing.

(iii). Gel preparation and loading

For *Alpinia* study, gel preparation and loading for the automated DNA sequencing was performed by Miss N. Preston (at the Institute of Cell and Molecular Biology, the University of Edinburgh) using the Applied Biosystems Model 377 DNA sequencer. During electrophoresis, these fragments are detected individually by a scanning laser. Results are recorded directly into computer and interpreted by computer software into a DNA sequence.

2.3.6 Sequence analyses

For each taxon two sequences of the same DNA region obtained by forward and reverse primers were interpreted simultaneously using Factura™ version 2.0 (feature identification software). Therefore, the accuracy of each base in the sequences was confirmed. Following the published sequences of the 5.8S rDNA

gene and the ITS region in carrot and broad bean ribosomal DNA (Yokota *et al.* 1989), I determined both ITS1 and ITS2 regions for the 57 taxa of Zingiberaceae under study. Sequence Navigator™ version 1.0.1 software was used with the CLUSTAL option for the initial alignments of both ITS regions. These alignments were subsequently adjusted by eye. The number and size of indel events were examined and the G+C content was analysed. In PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0d64 (Swofford 1998), sequence characteristics such as sequence divergence, number of constant sites, variable sites, informative sites and autapomorphic sites were calculated. In addition, the number of transitions, transversions and their respective ratios were obtained using MacClade version 3.01 (Maddison and Maddison 1992).

To determine the sequence boundaries of the intergenic spacer between *trn*L (UAA) 3' exon and *trn*F (GAA), sequences of the first five taxa of Zingiberaceae (both *Alpinia* and its outgroup) under study were compared with published data of the complete sequence of the tobacco chloroplast genome (Shinozaki *et al.* 1986). Later more sequences (17) were added to the same data matrix producing 22 taxa in total. The remaining procedures for sequence analysis of this cpDNA followed those for the ITS.

2.3.7 Phylogenetic analyses

Sequence data were analysed using PAUP*. Since the ITS analyses contained a large number of taxa (57), the use of exact algorithms (exhaustive and branch-and-bound methods) became limited by the excessive amount of computing time needed. Therefore, heuristic approaches, which are considered to be approximate methods that do not guarantee optimality but are relatively fast and efficient, were employed. For *Alpinia* and its outgroup under study aligned sequences of the combined ITS1 and ITS2 regions with coded gaps (0 was coded for

an insertion and 1 was coded for a deletion) contained 472 characters. However, in the phylogenetic analyses of these taxa only parsimony-informative characters (188; 39.8%) which were unordered and equally weighted were included. Accelerated transformation (ACCTRAN) was selected for character-state optimisation. This procedure can be thought of as preferring reversals over parallelisms. It accelerates changes toward the root and maximises early gains, thus forces subsequent reversals (Swofford and Maddison 1987). Gaps were treated as missing values and multistate taxa were interpreted as uncertain. The general search strategy using heuristic approaches (Möller, personal communication) were then carried out to obtain the most parsimonious tree(s).

1. With option MULPARS "off" a search run of 500000 replicates without swapping was conducted using random-addition sequence. The overall and shortest tree length obtained in this analysis was noted (n).

2. Following a setting of options: MULPARS "on" and with no more than three trees of length greater than or equal to the tree length obtained in step 1 (n) with one step shorter (n-1) was saved in each replicate, a search run of 100000 replicates with nearest-neighbour-interchanges (NNI; a fast swapping algorithm) was conducted using random-addition sequence. All shortest trees obtained in this analysis were then saved to a tree file.

(The use of the random-addition sequence has been suggested as a means to detect any multiple islands of most parsimonious trees (Maddison 1991)).

3. With three options: MULPARS, STEEPEST DESCENT and COLLAPSE "on" a complete search was carried out using tree-bisection-reconnection (TBR) swapping for all trees which were previously saved in the memory. The most parsimonious tree(s) obtained in this step was saved and used for further investigation in phylogenetic studies of *Alpinia*.

[Based on PAUP 3.1 User's Manual (Swofford 1993):

MULPARS option: when "on" will save all equally parsimonious trees.

STEEPEST DESCENT option: when "on" will not abandon a round of swapping until all input trees from the previous round have been examined by the swapping algorithm.

COLLAPSE option: when "on" will collapse any zero-length branches.]

To demonstrate the robustness of the phylogenetic trees a method of bootstrapping (Felsenstein 1985) is commonly employed. This method estimates confidence intervals of internal branches in phylogenetic analyses by repeated resampling from the original data with replacement to produce a series of new matrices of the same size as the original, each of which can be analysed to find the best-fit tree. In this phylogenetic study of *Alpinia* which involved a large data set of ITS sequences, normal bootstrapping performed under a heuristic search for 1000 replicates would require a long period of time to reach a completion. Therefore, fast and efficient calculation of bootstrap values (BS, 1000 replicates with only branch support of > 50% was retained) was conducted in PAUP*. In addition, decay index analyses (DI: Bremer 1988; Donoghue *et al.* 1992) were performed by using simple-addition sequence with TBR swapping and comparing strict consensus trees based on saving trees of progressively longer lengths (up to +2). This type of analysis provides an indication of the robustness of the data by determining which clades persist in a consensus tree as parsimony is relaxed. Fit measures of the phylogenetic trees including the consistency index (CI = m/s (Kluge and Farris 1969) where m is the minimum possible length of a tree based on the number of variant characters and s is the actual tree length), retention index (RI = M-s/M-m (Farris 1989) where M is the maximum possible tree length) and rescaled consistency index (RC = CI×RI (Farris 1989)) were also calculated. These indices range from zero to one with higher values (close to one) indicating that characters in the data set are more

congruent with each other and with the tree (less amount of homoplasy is expected) (Maddison and Maddison 1992). Finally, the g_1 statistic (Huelsenbeck 1991; Hillis and Huelsenbeck 1992), a measure of the phylogenetic signal in the data matrix based on skewness of a tree length distribution, was obtained in PAUP* by setting to RANDOM TREES search and evaluating 10000 random trees. To improve parsimony tree estimates by reducing the effect of homoplasious characters in ITS sequence data, a successive weighting approach (Farris 1969) was carried out. Under heuristic search with TBR swapping, the characters were re-weighted based on the CI values. This process was repeated until no change in tree topology was observed.

In the case of the *trn*L (UAA) 3' exon and *trn*F (GAA) spacer, sequences of the 22 taxa of Zingiberaceae were analysed with coded gaps (length of the matrix = 325 bp) using a branch-and-bound search with simple-addition sequence and COLLAPSE and MULPARS options in effect. *Pleuranthodium schlechteri* was used to root the tree instead of *Elettariopsis unifolia* (as in ITS analyses) due to limited number of outgroup taxa available for the analysis. The bootstrap values (1000 replicates) were calculated using a normal heuristic search, the g_1 value and all the essential indices (DI, CI, RI, RC) were obtained following the methods applied for the ITS.

CHAPTER 3: RESULTS OF MOLECULAR APPROACHES TO THE STUDY OF THE INFRAGENERIC CLASSIFICATION OF *ALPINIA*

3.1 MOLECULAR TECHNIQUES

3.1.1 Total genomic DNA extraction

(1). A modification of the CTAB method of Doyle and Doyle (1987) for silica gel dried leaf material

Although extracting DNA from fresh leaves is still the preferred method, silica gel dried samples have proven themselves a nearly equivalent source (Chase and Hills 1991). In this present study of *Alpinia* most of the leaf material was collected when fresh from various places (see section **2.2**), placed in bags with silica gel and returned by surface mail. The material still arrived in a suitable condition for DNA extraction. The use of silica gel is simple, inexpensive and therefore, represents an ideal method for preserving plant samples collected from nearby and easily accessible localities as well as those from remote areas.

Figure 6 shows that the extraction of DNA using 2x CTAB buffer with silica gel dried leaf material often yielded high molecular weight DNA. However, the DNA obtained was sheared because the leaves of Zingiberaceae under study were thick, waxy and fibrous, and a great deal of mechanical grinding was required with the use of sand to obtain a homogeneous mixture of the leaf material and buffer. In this experiment, no RNase was added to the mixture, so a large amount of RNA still remained in the extract.

(2). "Hot" CTAB method for herbarium material

In this study of *Alpinia* one of the major difficulties encountered was the collection of living plant samples, particularly in subgenus *Dieramalpinia*, from geographically restricted areas such as New Guinea and the surrounding islands.

Thus, herbarium specimens potentially represented an invaluable source of material for molecular analyses. Thirteen herbarium specimens which had enough leaves and were collected from seven to 42 years ago were selected. For each specimen a small amount of tissues was taken from the greenest and most healthy leaves. In some occasions however, brownish material of certain species was used due to the limited number of specimens.

During extraction the material was ground in the presence of liquid nitrogen which increased the efficiency of grinding and RNase was added to remove RNA. Although the resulting DNA pellet was left in the wash buffer at 4°C overnight, a large amount of blackish material that co-precipitated with DNA at the precipitation step (with isopropanol) still remained. Results of the DNA obtained are shown in **Figure 7**. The DNA extracts were highly degraded and for some specimens (lanes 3 and 5) no DNA or only a small amount of DNA was recovered.

(3). The DNeasy™ Plant Mini Kit (Qiagen Ltd., UK) for herbarium material and difficult plant species

A relatively high molecular weight DNA was obtained from the 42-year-old herbarium specimen of *Alpinia boia* (**Figure 8**, lane 2) although it was degraded. The use of the DNeasy™ Plant Mini Kit also yielded pure and intact DNA extracts from three difficult species (using silica gel dried leaf material) where the CTAB method produced poor results.

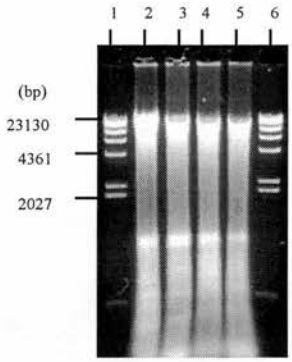


Figure 6. Genomic DNA extracts from silica gel dried leaf samples using a modification of the CTAB method of Doyle and Doyle (1987). Lane 2: *Alpinia galanga*; lane 3: *A. officinarum*; lane 4: *A. ligulata*; lane 5: *A. nieuwenhuizii*. Lanes 1 and 6: Lambda-*Hin*dIII marker.

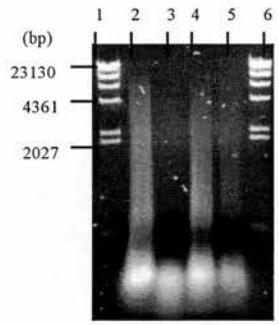


Figure 7. Genomic DNA extracts from herbarium leaf material using "hot" CTAB method. Lane 2: *Alpinia glabra* (25 years); lane 3: no DNA was recovered from *A. biakensis* (18 years); lane 4: *A. eubractea* (17 years); lane 5: *A. pyramidata* (7 years). Lanes 1 and 6: Lambda-*Hin*dIII marker.

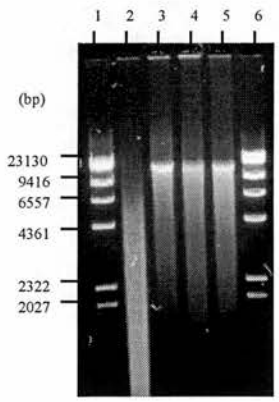


Figure 8. Genomic DNA extracts from herbarium material and difficult plant species using DNeasy™ Plant Mini Kit (Qiagen Ltd., UK). Herbarium specimen: lane 2: *Alpinia boia* (42 years); silica gel dried specimens: lane 3: *A. carolinensis*; lane 4: *A. abundiflora*; lane 5: *A. fax*. Lanes 1 and 6: Lambda-*Hin*dIII marker.

3.1.2 PCR amplifications of the ITS region and the region between *trn*L (UAA) 5' exon and *trn*F (GAA)

All DNA extracts obtained from both silica gel dried and herbarium specimens were used for PCR amplifications of the complete ITS region with primers "ITS5P" and "ITS8P". Results are shown in **Figure 9**. It was observed that each amplification product had a length of approximately 800 bp. In certain cases where no PCR product was obtained (e.g. lane 4 in **Figure 9**), different primer combinations were used for the amplifications of individual ITS1 (primers "ITS2K" and "ITS5P") and ITS2 (primers "ITS3P" and "ITS8P") regions (**Figure 10**). However, when the amplifications were still unsuccessful, DNA extracts were usually diluted in sterile distilled water in a series of 1/10, 1/100 and 1/1000 to decrease the amount of PCR inhibitors. This method worked well with three *Alpinia* species namely *A. abundiflora*, *A. fax* and *A. vulcanica*.

In the case of the herbarium specimens it was observed that six out of 13 had their DNA extracts amplifiable. Results showed that there seemed to be no apparent correlation between the age of a herbarium specimen and the success of DNA amplification. For the herbarium material, important factors which appear to influence DNA degradation as well as amplification ability of the DNA are the developmental stage of the collected tissues, the method of drying (duration and conditions of preparation), and chemical components (PCR inhibitors) of the species (reviewed in Savolainen *et al.* 1995). It was observed that poor DNA results were obtained from brownish material of herbarium specimens of Zingiberaceae. This may be due to the fact that taxonomists in the past used alcohol (70% ethanol) to preserve their plant material prior to drying. According to Doyle and Dickson (1987) treating leaf material in this way would accelerate the rate of DNA breakdown.

In addition to the ITS region, PCR amplifications were initially carried out in the chloroplast DNA region between *trn*L (UAA) 5' exon and *trn*F (GAA) using primers c and f in five taxa of Zingiberaceae. Results in **Figure 11** showed that each amplification product contained multiple bands. Therefore, an alternative approach using different sets of primer was carried out to obtain two separate regions of *trn*L (UAA) 5' exon and *trn*L (UAA) 3' exon (primers c and d), and *trn*L (UAA) 3' exon and *trn*F (GAA) (primers e and f). **Figure 12** shows that in all three taxa (not including one without products in lanes 6 and 7) successful results were obtained with primers e and f for single and distinctive bands while primers c and d resulted in several bands. Therefore, for additional taxa (17) the amplifications were based on the use of the former primer pair which produced smaller fragments of the region between *trn*L (UAA) 3' exon and *trn*F (GAA).

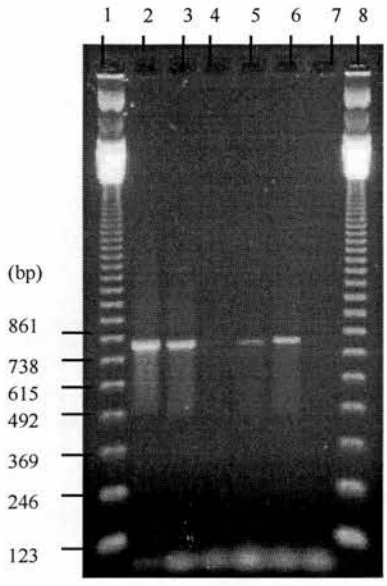


Figure 9. PCR amplification products of the complete ITS region. Lane 2: *Alpinia nigra*; lane 3: *A. suishaensis*; lane 4: no PCR product was obtained for *A. maclurei*; lane 5: *Renealmia battenbergiana*; lane 6: *R.* aff. *africana*. Lane 7: PCR negative control; lanes 1 and 8: 123 bp ladder.

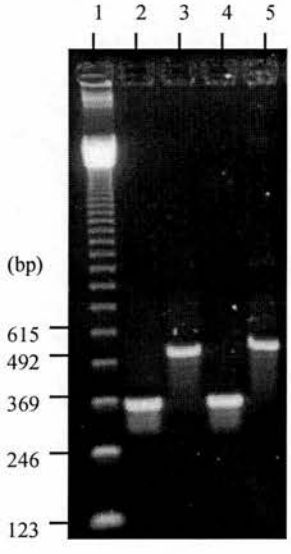


Figure 10. PCR amplification products of individual ITS1 and ITS2 regions. Lane 2: *Alpinia maclurei* (ITS1); lane 3: *A. maclurei* (ITS2); lane 4: *A. purpurata* (ITS1); lane 5: *A. purpurata* (ITS2). Lane 1: 123 bp ladder

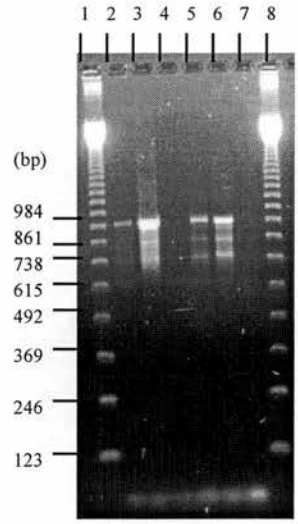


Figure 11. PCR amplification products of the chloroplast DNA region between *trn*L (UAA) 5' exon and *trn*F (GAA) using primers c and f. Lane 2: *Alpinia conchigera*; lane 3: *A. galanga*; lane 4: no product of *A. polyantha*; lane 5: *A. intermedia*; lane 6: *Pleuranthodium schlechteri*. Lane 7: PCR negative control; lanes 1 and 8: 123 bp ladder.

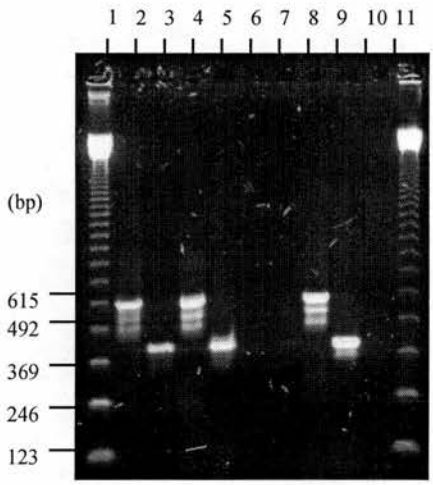


Figure 12. PCR amplification products of two chloroplast DNA regions between (i) *trn*L (UAA) 5' exon and *trn*L (UAA) 3' exon (using primers c and d), and (ii) *trn*L (UAA) 3' exon and *trn*F (GAA) (using primers e and f). Lane 2: *Alpinia conchigera* (region (i)); lane 3: *Alpinia conchigera* (region (ii)); lane 4: *A. intermedia* (region (i)); lane 5: *A. intermedia* (region (ii)); lane 6: no product of *A. polyantha* (region (i)); lane 7: no product of *A. polyantha* (region (ii)); lane 8: *Pleuranthodium schlechteri* (region (i)); lane 9: *P. schlechteri* (region (ii)). Lane 10: PCR negative control; lanes 1 and 11: 123 bp ladder.

3.1.3 Purification of PCR products

(1). QIAquick™ PCR Purification Kit (Qiagen Ltd., UK)

It was observed in **Figure 13** that amplified DNA fragments obtained after the purification step were clean. The kit was supplied with the optimised binding buffer (Buffer PB) which prevented oligonucleotides of ≤ 40 bp from binding to the silica gel membrane, ensuring complete removal of residual PCR primers (most PCR primers used had lengths between 19-22 bp) and other nucleotides, as well as salts and DNA polymerase. The amplified DNA fragments (concentrations:10-30 ng/μl) were then suitable for sequencing.

(2). QIAquick™ Gel Extraction Kit (Qiagen Ltd., UK)

The same principle of bind-wash-elute procedure was used in both QIAquick™ PCR Purification and QIAquick™ Gel Extraction Kits for fast and efficient clean up of amplified PCR fragments. In cases such as that shown in **Figure 14**, when the PCR product contained more than one band, only the bands with specific amplification (the upper ones) were extracted and purified from the gel. Results (data not shown) yielded smaller amounts of DNA but it was pure and good enough as a template for sequencing reactions.

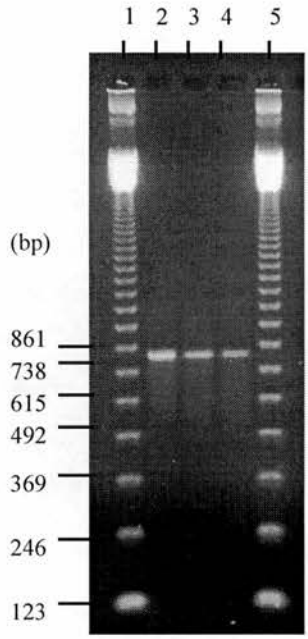


Figure 13. PCR purification products of the complete ITS region using QIAquick™ PCR Purification Kit (Qiagen Ltd., UK). Lane 2: *Alpinia nigra*, lane 3: *A. suishaensis*, lane 4: *Renealmia* aff. *africana*. Lanes 1 and 5: 123 bp ladder.

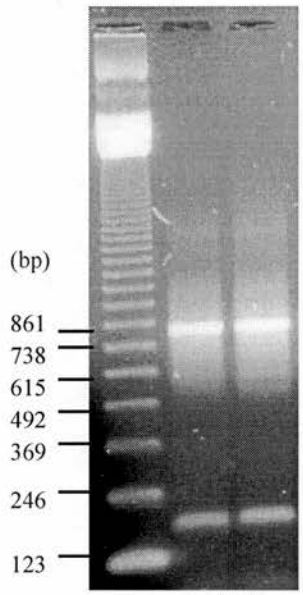


Figure 14. PCR amplification product of the ITS region of *Alpinia oceanica*. The upper bands contained the required product with an approximate length of 800 bp (compared with 123 bp ladder on the first lane). These bands were removed from the gel and purified using QIAquick™ Gel Extraction Kit (Qiagen Ltd., UK).

3.1.4 DNA Sequencing

(1). Manual sequencing by Sanger's method performed for the ITS region

In order to study and understand techniques of DNA sequencing, four DNA samples of *Alpinia galanga*, *A. zerumbet*, *Burbidgea schizocheila* and *Elettariopsis unifolia* were used in manual sequencing by Sanger's method to obtain ITS1 and ITS2 regions.

When sequencing double stranded PCR products, sufficient DNA template must be present to provide enough terminations close to the primer to visualise small products and to obtain strong bands on autoradiograms. In this study 160-200 bp were read per sequencing reaction. **Figure 15** shows parts of the sequences of the 5.8S rDNA and ITS2 region of *A. galanga* and *B. schizocheila*. The sequences were read from 5' to 3' as follows (base differences between the two sequences are highlighted):

A. galanga: TTGGGCGTCAT**G**GCATCGTCGC**C**TTTGCTCCTTGCTTTGCTG**C**TGG

B. schizocheila: TTGGGCGTCAT**T**GCATCGTCGC**T**TTTGCTCCTTGCTTTGCTG**G**TGG

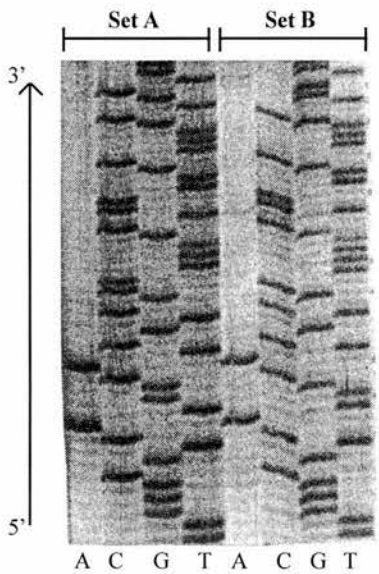


Figure 15. Autoradiograms derived from sequencing of double stranded DNA show parts of the sequences of the 5.8S rDNA and ITS2 region of *A. galanga* (set A) and *B. schizocheila* (set B).

(2). Cycle sequencing and automated DNA sequencing

Compared to the manual sequencing above, a method which combined cycle sequencing and automated DNA sequencing was less time consuming. This was mainly because in the manual sequencing a single radioisotopic reporter ([α-^{35}S] dATP) was used to analyse each of the four bases (A, C, G, T) which required the preparation of four separate reactions that consumed four gel lanes while for the other two types of sequencing a fluorescent reporter (dye labelled dideoxynucleotides) was used and the reactions were performed in a single tube and run on a single gel lane. Manual sequencing also required an ample amount of time for the exposure and development of the resulting autoradiographic patterns. In addition, on each autoradiogram only one base was read and recorded at a time and data obtained were not in a form ready to be used with any computer applications. With the results of cycle sequencing and automated DNA sequencing a high level of accuracy was observed although there were some possible errors such as misidentifying a detected base, inserting a base, or failing to detect a base at all. Skilled interpretation was required to analyse the sequence data. **Figure 16** illustrates parts of the ITS1 region of *Alpinia galanga* obtained by cycle sequencing and automated DNA sequencing.

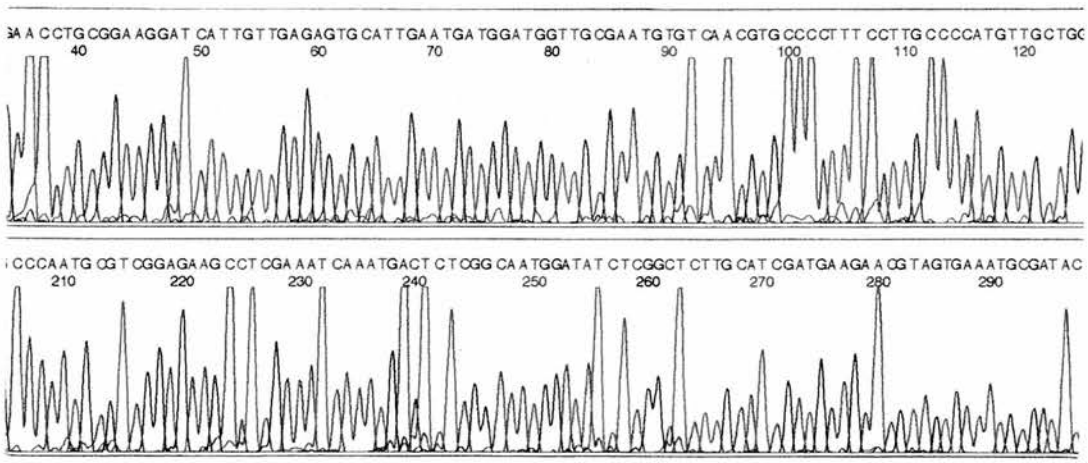


Figure 16. An electropherogram of parts of the ITS1 region of *Alpinia galanga* obtained by cycle sequencing and automated DNA sequencing. The height of each of the four coloured lines indicates the relative intensity of fluorescence that corresponds to each of the four labelled dideoxynucleotides. Hence, the peaks may be read directly as DNA sequences (bases indicated above the electropherogram).

3.2 SEQUENCE AND PHYLOGENETIC ANALYSES

3.2.1 Sequence and phylogenetic analyses of the ITS region for 57 taxa of Zingiberaceae

(1). Sequence alignment

Alignment of ITS sequences of 57 taxa of Zingiberaceae resulted in a 460 bp long data matrix (without coded gaps) as shown in **Figure 17**. The alignment did not include 5.8S rDNA (162 bp) because this coding region is highly conserved and therefore almost completely uninformative for the phylogenetic analysis of *Alpinia*. For each taxon the actual spacer length of the combined ITS1 and ITS2 regions was given in square brackets at the end of the sequence. Although the ITS sequence alignment of these 57 taxa of *Alpinia* and its outgroup was straightforward and easily accomplished by eye (following the initial alignments using the Sequence Navigator™ version 1.0.1 software), alignment ambiguities were encountered due to the presence of several indel events (positions: 181-192, 305-308, 336-340, 394-404). These ambiguous sites (identified by * in **Figure 17**) were excluded from the calculations of the number and size of indels, the number of constant sites, variable sites, informative sites and autapomorphic sites.

(2). Sequence characteristics and analyses

Sequence characteristics and analyses of the ITS region for *Alpinia* and its outgroup are summarised in **Table 7**.

(i). ITS length variation

The combined ITS1 and ITS2 regions of the 57 taxa under study varied in length from 387 to 429 bp. The length of ITS1 ranged from 186 to 191 bp while that of ITS2 was greater and ranged from 197 to 238 bp. The unambiguous alignment (see **Figure 17**) of all taxa required 7 (**a-g**, size: 1-2 bp) and 11 (**h-r**, size: 1-6 bp) independent indel events in ITS1 and ITS2 respectively, which accounted

Table 7. Sequence characteristics of ITS1 and ITS2 regions for 57 taxa of Zingiberaceae.

Sequence characteristics	ITS1	ITS2	ITS1 and ITS2
Length range (total) (bp)	186-191	197-238	387-429
Length mean (total) (bp)	188.1	227.6	415.8
Length range (ingroup) (bp)	186-191	197-238	387-429
Length mean (ingroup) (bp)	188.1	228.3	416.4
Length range (outgroup) (bp)	187-189	216-226	405-415
Length mean (outgroup) (bp)	188.3	223.8	412.1
Aligned length (without coded gaps) (bp)	200	260	460
Length of ambiguous sites (bp)*	12	20	32
Aligned length after exclusion of ambiguous sites (bp)*	188	240	428
G+C content range (%)	50.0-62.2	54.3-65.5	53.1-63.9
G+C content mean (%)	53.9	59.6	56.8
Sequence divergence range (ingroup) (%)	0-20.9	0-19.7	0.2-19.1
Sequence divergence range (total) (%)	7.0-25.3	6.5-20.5	7.7-20.6
Number of indels (total)*	7	11	18
Size of indels (total) (bp)*	1-2	1-6	1-6
Number of constant sites (%)*	80 (42.6)	109 (45.4)	189 (44.2)
Number of variable sites (%)*	108 (57.4)	131 (54.6)	239 (55.8)
Number of informative sites (%)*	88 (46.8)	88 (36.7)	176 (41.1)
Number of autapomorphic sites (%)*	20 (10.6)	43 (17.9)	63 (14.7)
Number of transitions (minimum)	128	156	284
Number of transversions (minimum)	55	43	98
Transition/transversion ratio (ts/tv ratio) (average across all maximally parsimonious trees)	2.3	3.6	2.9
Skewness of tree length distribution (g_1 value for 10000 random trees)	-0.5	-0.7	-0.6

(* based on alignment excluding ambiguous sequence sites).

for the length variation of the ITS region. In ITS2 there were two indels (**i** and **k**) of three and six bp which are critical and apparent synapomorphies uniting *Alpinia conchigera*, *A. galanga* and *A. nigra*. In addition, there were three other indels (**m**, **n** and **q**) of three, one and six bp which occurred among a group of *Alpinia* consisting of *A. arundelliana*, *A. modesta*, *A. caerulea*, *A. oceanica*, *A. vittata*, *A. purpurata*, *A. arctiflora*, *A. elegans*, *A. eubractea*, and *A. luteocarpa* (*scorpoidea*). It is unlikely that all five identical indels could have occurred twice independently.

(ii). ITS sequence divergence and informative/uninformative sites

Among *Alpinia* and its outgroup accessions, pairwise sequence comparisons indicated ITS1 sequence divergence ranging from 7.0 to 25.3%. Within *Alpinia* ITS1 sequence divergence ranged from 0 to 20.9%. ITS2 was slightly less variable and sequence divergence values were observed from 6.5 to 20.5% between the ingroup and outgroup taxa, and 0 to 19.7% within the ingroup. Pairwise comparisons of individual taxa across both spacer regions revealed 7.7 to 20.6% sequence divergence between *Alpinia* and its outgroup, while only 0.2 to 19.1% divergence occurred among *Alpinia*.

Of 428 unambiguously aligned sites of the ITS region, 189 (44.2%) were constant, 176 (41.1%) were potentially informative phylogenetically, and 63 (14.7%) were autapomorphies, unique to individual taxa. Despite the fact that ITS2 contained several indels which were potentially informative, this region had a lower percentage of informative sites (36.7%) than that of ITS1 (46.8%) due to a high number of autapomorphic characters (43; 17.9%).

(iii). ITS base composition and transition/transversion ratio

The G+C content in the ITS region of the 57 taxa of Zingiberaceae under study was in the range of 53.1 to 63.9%. A similar amount of variation was observed in both ITS1 (50.0-62.2%) and ITS2 (54.3-65.5%). The total number of transitions in the ITS region was 284, with 128 in ITS1 and 156 in ITS2. In

contrast, the total number of transversions (98) was much smaller than that of transitions, with only 55 in ITS1 and 43 in ITS2. The average ratio of transition/transversion (ts/tv) across all maximally parsimonious trees was slightly higher in ITS2 (3.6) than in ITS1 (2.3) and the combined ITS1 and ITS2 regions (2.9).

(3). Phylogenetic analyses

Searching for multiple islands of most parsimonious trees (see section **2.3.7**) based on the complete data matrix of the ITS region, which included coded gaps and all ambiguous sequence sites but excluded uninformative characters, yielded the following results from the heuristic search. First, 73 trees were obtained with an overall tree length of n = 555 steps (under settings: MULPARS "off" with 500000 replicates using random-addition sequence without swapping). Next, 180 shortest trees with 555 steps were obtained (under settings: MULPARS "on" with no more than three trees of length greater than or equal to n-1 = 554 saved in each replicate, with 100000 replicates using random-addition sequence with NNI swapping). Finally with MULPARS, STEEPEST DESCENT and COLLAPSE options "on", the complete search using TBR swapping yielded a similar result of 180 equally parsimonious trees with 555 steps. The overall outcome of these procedures showed that no shorter tree was found in other islands of equally parsimonious trees.

Figure 18 illustrates the strict consensus tree derived from all 180 parsimonious trees (tree length = 555 steps) with accompanying bootstrap values above the branches and decay indices below. The bootstrap values (BS) ranged between 52 and 100%. Results of the decay index (DI) analyses revealed 8662 trees at ≤ 556 steps (DI = +1) and 38338 trees at ≤ 557 steps (DI = +2). While performing DI = +3 the computer ran out of memory and the search was terminated. Each of these 180 parsimonious trees had the following fit measures: CI = 0.4847, RI =

0.8129 and RC = 0.3940. In the presence of the uninformative characters the analysis yielded 180 equally parsimonious trees of 624 steps. The tree topology remained unaltered even though there were some changes in the branch lengths. In general, the values of the CI and RC increased (0.5417 and 0.4403) while that of the RI remained the same.

From the ITS analysis where uninformative characters were ignored, one of the 180 trees (tree length = 555 steps) was arbitrarily chosen and is presented in **Figure 19** to illustrate the number of character changes on each branch (branch length), as optimised by ACCTRAN in PAUP*. Significant branch lengths (20, 27 and 33) which led to three important clades containing *Pleuranthodium* (*Pleuranthodium* clade), *Alpinia galanga* (*A. galanga* clade) and *A. eubractea* (*A. eubractea* clade) are indicated in red.

When all informative characters of the combined ITS1 and ITS2 data matrix were re-weighted according to the CI value (0.4847) three equally parsimonious trees of length 272947 steps were produced with CI = 0.618, RI = 0.869 and RC = 0.537. The strict consensus tree obtained from this analysis (**Figure 20**), although it did not change dramatically in topology as compared to **Figure 18**, showed a better resolution of the relationships of *Alpinia* as indicated in green boxes. Following the successive weighting of the characters (using CI = 0.618), an identical tree topology of the strict consensus tree was obtained.

Pleuranthodium, a genus which occurs mainly in New Guinea and Australia, was recognised as a section in *Alpinia* by Schumann (1904). In 1991 Smith adopted this section at generic level. Based on the ITS analyses (**Figures 18** and **19**) results clearly supported the distinctiveness of the genus (BS = 100%, DI > +2, branch length = 20). In addition, within the *Pleuranthodium* clade there are two separate lineages of (i) *P. floccosum* and *P. racemigerum* (BS = 98%, DI > +2) and (ii) *P. schlechteri*, *P. papilionaceum* and *P. floribundum* (BS = 97%, DI > +2) which

appear to support Smith's classification of the genus into two sections, *Pleuranthodium* and *Psychanthus*. It is noteworthy that the result shown in **Figure 20** also confirmed the position of *Pleuranthodium* as a distinct genus. However, when the informative characters of the ITS region (excluding the coded gaps) were weighted with transversions over transitions by a factor of three, corresponding to the value of the average ts/tv ratio in the ITS region (2.9), the resulting consensus tree (**Figure 21**) computed from 144 equally parsimonious trees (tree length = 776 steps, CI = 0.543, RI = 0.837, RC = 0.454) showed that *Pleuranthodium* is nested within *Alpinia* with *A. rafflesiana*, *A. javanica* and *Burbidgea schizocheila* as sister groups (see the red box).

The next and most important clade in the study of the infrageneric classification of *Alpinia* and the relationships among species includes *Alpinia conchigera*, *A. galanga* and *A. nigra*. *Alpinia galanga*, the type species of the genus and hence a member of section *Alpinia* subsection *Alpinia* (with non-tubular bracteoles) is shown here closely related to *A. nigra* and *A. conchigera* of section *Allughas* (with tubular bracteoles). The *A. galanga* clade is strongly supported by a BS value of 100% and a DI value of > +2 with a branch length equal to 27 (**Figures 18** and **19**). The same feature of *A. galanga* being placed within section *Allughas* is shown in both **Figures 20** and **21** (after the informative characters of the ITS region were re-weighted based on the CI value and weighted 3x to accommodate the ts/tv ratio, respectively). The analyses of the ITS region involved the use of only one sequence of *A. galanga* because the two sequences obtained from silica gel dried and herbarium specimens were identical and thus provided a confirmation that both sequences were correct.

The *A. eubractea* clade which has 33 character changes on the branch (**Figure 19**) comprises species from both Smith's (1990a) subgenera *Alpinia* and *Dieramalpinia*. The clade (**Figure 18**) is well supported by a BS value of 100% and

a DI value of > +2 and contains the following species: *Alpinia eubractea*, *A. elegans*, *A. luteocarpa* (*scorpoidea*), *A. arctiflora*, *A. arundelliana*, *A. modesta*, *A. caerulea*, *A. purpurata*, *A. oceanica* and *A. vittata*. Results based on re-weighted characters using the CI values (**Figure 20**) and weighted characters using ts/tv ratio of the ITS region (**Figure 21**) supported the grouping of these ten species with more resolved branches.

The *A. rafflesiana* clade which contains two species of section *Allughas* from Malaysia, *A. rafflesiana* and *A. javanica* (**Figures 18** and **19**; BS = 100%, DI = > +2, branch length = 15) forms a natural group. It is obvious that other taxa in this section (*A. conchigera*, *A. nigra*, *A. arundelliana*, *A. modesta*, and *A. caerulea*) were not derived from the same common ancestor and thus the section appears to be polyphyletic.

Figures 18 - 20 show that a monophyletic clade which consists of Smith's (1990a) subgenus *Dieramalpinia* only is present. The *A. carolinensis* clade, comprising *A. carolinensis*, *A. boia* and *A. coeruleoviridis*, is strongly supported with a BS value of 99%, a DI value of > +2, and a branch length equal to 18.

Results of the ITS analyses also revealed other less well defined clades in terms of the branch lengths. (i) *A. aquatica* clade (**Figures 18** and **19**; BS = 95%, DI = > +2, branch length = 5) contains four species of Smith's (1990a) section *Alpinia* subsection *Presleia*. The clade however, includes an unresolved branch of *Alpinia vulcanica*, a species from the Philippines that belongs to subgenus *Dieramalpinia* section *Myriocrater*. (ii) *A. glabra* clade (BS = 95%, DI = > +2, branch length = 8) contains *A. glabra* (a monotypic species of section *Alpinia* subsection *Probolocalyx*), *A. nieuwenhuizii* and *A. ligulata* (section *Alpinia* subsection *Paniculatae*). It is interesting to note that all three taxa have their origin in Borneo. (iii) *A. polyantha* clade, although it does not exist in the strict consensus tree (**Figure 18**), may contain Smith's section *Alpinia* subsection *Alpinia* with *A. pumila*,

a species from section *Didymanthus*. It appears that within section *Alpinia* subsection *Alpinia* there may be more than one separate lineage; a clade which contains *A. intermedia*, *A. suishaensis* and *A. maclurei* is present. (iv) *A. zerumbet* clade (BS = 82%, DI = +2, branch length = 5) has Smith's section *Alpinia* subsection *Catimbium* with a few species (*A.* aff. *shimadai* and *A. officinarum*) of subsection *Cenolophon* nested within it. There are however, other species (*A. oxymitra* and *A. oxyphylla*) of subsection *Cenolophon* which occur elsewhere on the trees and do not seem to be closely related.

Based on the ITS cladograms (**Figure 18 - Figure 20**) *Alpinia* appears to be non-monophyletic. The basal group of subgenus *Alpinia* section *Fax* (*A. fax* clade) has representatives of African *Renealmia* as sister to it. However, the clade is only weakly supported with a DI value of +1 and a branch length equal to 4 (BS is less than 50% and therefore not shown in **Figure 18**).

The analysis of skewness (g_1 statistic) of the tree length distribution of 10000 random trees for 57 taxa of *Alpinia* and its outgroup in the ITS region resulted in a negative value of -0.6. This figure which indicates a left-skew tree length distribution suggests that the ITS sequence data set is potentially informative of phylogenetic history (Hillis and Huelsenbeck 1992), although Källersjö *et al.* (1992) pointed out that this type of evaluation can be misleading.

3.2.2 Sequence and phylogenetic analyses of the region between *trn*L (UAA) 3' exon and *trn*F (GAA) for 22 taxa of Zingiberaceae

(1). Sequence alignment

Figure 22 illustrates a sequence alignment of the chloroplast spacer between *trn*L (UAA) 3' exon and *trn*F (GAA) of *Alpinia* and its outgroup. The alignment had a total length of 320 bp (without coded gaps) and required eight independent

indels (**s-z** ; size: 1-8 bp). Square brackets at the end of each sequence contained a number which indicated the actual spacer length of a taxon.

(2). Sequence characteristics and analyses

Sequence characteristics and analyses of this region for the 22 taxa of Zingiberaceae are summarised in **Table 8**.

The total length of the spacer ranged from 295 to 310 bp. Pairwise comparisons between *Alpinia* and its outgroup ranged from 1.0 to 8.3% sequence divergence. This short spacer contained a relatively high number of constant sites (273; 85.3%) and low number of phylogenetically informative sites (14; 4.4%). The mean G+C content across the 22 taxa was 33.1% and the ts/tv ratio was 1.3.

(3). Phylogenetic analyses

The strict consensus tree (**Figure 23**) was derived from 11 equally parsimonious trees, each with a length of 56 steps. The tree had the following fit measures: CI = 0.946, RI = 0.953, RC = 0.902. Although the tree did not provide clear phylogenetic resolution of the taxa examined due to the presence of many invariable sites and the lack of potentially informative sites, this region of the chloroplast DNA contained at least three informative indels (**u**, **w** and **y** in **Figure 22**) which are significant for the grouping of certain taxa of *Alpinia* that supported ITS results. The lack of indel **u** (eight bp) indicated that species from section *Alpinia* subsection *Cenolophon* (*A.* aff. *shimadai* and *A. officinarum*) would be grouped with those from subsection *Catimbium* (*A. blepharocalyx* var. *glabrior* and *A. malaccensis*) (BS = 70%). Similarly, the grouping of *A. rafflesiana* and *A. javanica* (BS = 95%) was clear due to the absence of indel **w** (eight bp). The presence of indel **y** (six bp) certainly confirmed the grouping of *A. galanga* with *A. conchigera* and *A. nigra* (BS = 65%). The same category which contained members

of both Smith's (1990a) subgenera *Alpinia* and *Dieramalpinia* was observed. The group included *A. oceanica*, *A. elegans* and *A. modesta* and was strongly supported with a BS value of 100%. In general, the results of molecular phylogenetic studies based on the spacer between *trn*L (UAA) 3' exon and *trn*F (GAA) are congruent with those of the ITS region.

Table 8. Sequence characteristics of the chloroplast spacer between *trn*L (UAA) 3' exon and *trn*F (GAA) for 22 taxa of Zingiberaceae.

Sequence characteristics	The spacer between *trn*L (UAA) 3' exon and *trn*F (GAA)
Length range (total) (bp)	295-310
Length mean (total) (bp)	302.3
Length range (ingroup) (bp)	295-310
Length mean (ingroup) (bp)	302.1
Length (outgroup) (bp)	304
Aligned length without coded gaps (bp)	320
G+C content range (%)	31.4-35.4
G+C content mean (%)	33.1
Sequence divergence range (ingroup) (%)	0-5.0
Sequence divergence range (total) (%)	1.0-8.3
Number of indels (total)	8
Size of indels (total) (bp)	1-8
Number of constant sites (%)	273 (85.3)
Number of variable sites (%)	47 (14.7)
Number of informative sites (%)	14 (4.4)
Number of autapomorphic sites (%)	33 (10.3)
Number of transitions (minimum)	5
Number of transversions (minimum)	4
Transition/transversion ratio (ts/tv ratio) (average across all maximally parsimonious trees)	1.3
Skewness of tree length distribution (g_1 value for 10000 random trees)	-0.5

Figure 17. Sequence data matrix of aligned ITS1 and ITS2 regions of nuclear ribosomal DNA for 57 taxa of Zingiberaceae. ITS1 ranges from site 1 to 200 and ITS2 ranges from site 201 to 460. Uncertain nucleotide states are coded as follows: N = A/C/G/T, K = G/T, R = A/G, S = C/G, W = A/T, Y = C/T; hyphens denote alignment gaps; * shows ambiguous sites; **a**-**r** above the nucleotide matrix indicate the position of alignment gaps; nucleotides in bold denote phylogenetically informative insertions; numbers in square brackets at the end of sequences indicate the actual length of the combined ITS1 and ITS2 regions. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

Taxon	ITS1
	10 20 30 40 50 60 70 80 90
	. . . .
	a b c d e
Elettariopsis unifolia	TTGTTGAG--AGAGCATTGAATGATGGATGGTTGTGAATATGTCAGCTTGCCCCTT-CTTTG-CCTC-ACGTC--AGTGGGCAATTGACT
Burbidgea schizocheila	TTGTTGAG--AGAGCATAGAATGATGGATGGCTGTGAATGTGTCAACGTGTCCCTT-TCTCG-TCCC-ATATC--AGTGGGCGTTTGACC
Pleuranthodium schlechteri	TTGTTGAG--AGAGCACAGAATGATGGATGGTTGTGAATGTGTCAACGTGTCCCTTTTCTCG-TCCC-ATGTC--AGTGGGCGTTTGACC
Pleuranthodium papilionaceum	TTGTTGAG--AGAGCACAGAATGATGGATGGTTGTGAATGTGTCAACGTGTCCCTTTTCTCG-TCCC-ATGTC--AGTGGGCGTTTGACC
Pleuranthodium floribundum	TTGTTGAG--AGAGCACAGAATGATGGATGGTTGTGAATGTGTCAACGTGTCCCTTTTCTCG-TCCC-ATGTC--AGTGGGCGTTTGACC
Pleuranthodium floccosum	TTGTTGAG--AGAGCATAGAATGATGGATGATTGTGAATGCGTCAACGTGTCCCCTTTCTCG-TCCC-ATGTC--AGTGGGCGTTTGACC
Pleuranthodium racemigerum	TTGTTGAG--AGAGCATAGAATGATGGATGATTGTGAATGCGTCAACGTGTCCCTTTTCTCG-TCCC-ACGTC--AGTGGGCGTTCGACC
Renealmia battenbergiana	TTGTTGAGGGAGAGCATTGAATGATGGATGGTTGTGAATGTGTTAACGTGCCCCTT-CCTTG-CCCC-ATGTT--GGTGTGCAATTGACC
Renealmia aff. africana	TTGTTGAGGGAGAGCATTGAATGATGGATGGTTGTGAATGTGTTAACGTGCCCCTT-CCTTG-CCCC-ATGTT--GGTGTGCAATTGACC
Alpinia abundiflora	TTGTTGAG--AGAGCATGGAATGATGGATGGTTGTGAATGTGTCAATGTGCCCCTT-CCTTG-CCCC-ATGTT--GGCGGGCAATCGACC
Alpinia fax	TTGTTGAG--AGAGCATTGAATGATGGATGGCTGTGAACGTGTCAACGCGCCCCTT-CCTTG-CCCC-ATGTT--GGTGGGCAATTGATC
Alpinia arundelliana	TTGTTGAG--AGAGCATCGACCAACGGATGGTTGTGAACGTGTCAACGTGCCCCTTTCCTTGGCCCCCATGTC--GGCGGCCAACTGGAC
Alpinia modesta	TTGTTGAG--AGAGCATCGACCAACGGATGGTTGTGAATGTGTCAACGTGCCCCTTTCCTTGGCCCCCATGTC--GGCGGCCAACCGGAC
Alpinia caerulea	TTGTTGAG--AGAGCATCGACCAACGGATGGTTGTGAATGTGTCAACGTGCCCCTTTCCTTGGCCCCCATGTC--GGCGGCCAACTGGAC
Alpinia oceanica	TTGTCGAG--AGAGCATCGAACAACGGACGATCGCGAATGTGTCAACGCGCCCCTTTCGTTGGCCCC-ATGTC--GGCGGCCAACCGACC
Alpinia vittata	TTGTCGAG--AGAGCATCGAACAACGGACGATCGCGAATGTGTCAACGCGCCCCTTTCGTCGGCCCC-ATGTC--GGCGGCCAACCGACC
Alpinia purpurata	TTGCTGAG--AGAGCATCGAACAACGGATGGTCGTGAATGTGTCAACATGCCCGTTTCCTTGGCCCC-ATGTC--GGCGGCCAACTGACC
Alpinia arctiflora	TTGCTGAG--AGAGCATAGACCAACGGATGGTTGCGAATGTGTCAACGTGCCCCTTTCGTTGGCCCC-GTGCG--GGCGGCCAACTGGTC
Alpinia elegans	TTGTTGAG--AGAGCATCGAACAACGGATGGCTGCGAATGTGTCAACGTGCCCCTTTCCTCGGCCCC-ACGTC--GGCGGCCAATTGACC
Alpinia eubractea	TTGTTGAG--AGAGCATTGAACAACGGATGGTTGTGAACGTGTCAACGTGCCCGTCTCCTAGGCCCC-ATGTT--GGTGGCCAACCGACC
Alpinia luteocarpa (*scorpoidea*)	TTGTTGAG--AGAGCATTGAACAACGGATGGCTGCGAATGTGTCAACGTGCCCCTTTCTTTGGCCCC-ATGTC--GGCGGCCAATTGACC
Alpinia carolinensis	TTGTTGAG--AGAGCATCGAATGACGGATGGCCGTGAACGTGTCAATGTGCCCCTTTAATCG-CCCC-ATGTT--GGTGGCCGACCGACC
Alpinia coeruleoviridis	TTGTTGAG--AGAGCATTGAATTACGGATGGTTGTGAATGTGTCAATGTGCCCCTTTAATTG-CCCC-ATGTT--GGTGGCCGACCGATC
Alpinia boia	TTGTTGAG--AGAGCATTGAATGACGGATGGCTGTGAATGTGTCAATGTGCCCCTTTAATTG-CCCC-ATGTT--GGTGGCCGACCGACC
Alpinia rafflesiana	TTGTTGAG--AGAGCATTGAATGACGGATGATTGTGAATGTGTCAACGTGCCCCATTCCTTG-CCCC-ATGTT--GGTGGGCGACTGACC
Alpinia javanica	TTGTTGAG--AGAGCATTGAATGACGGATGATTGTGAATGTGTCAACGTGCCCCATTCGTTG-CCCC-ATGTT--GGTGGGCGACTGACC
Alpinia conchigera	TTGTTGAG--AGTGCATTGAATGATGGATGGTTGCGAATGTGTCAACGTGCCCCTTTCCTTG-CCCC-ATGTT--GTTGGGCAATTGATC

Figure 17 (continued).

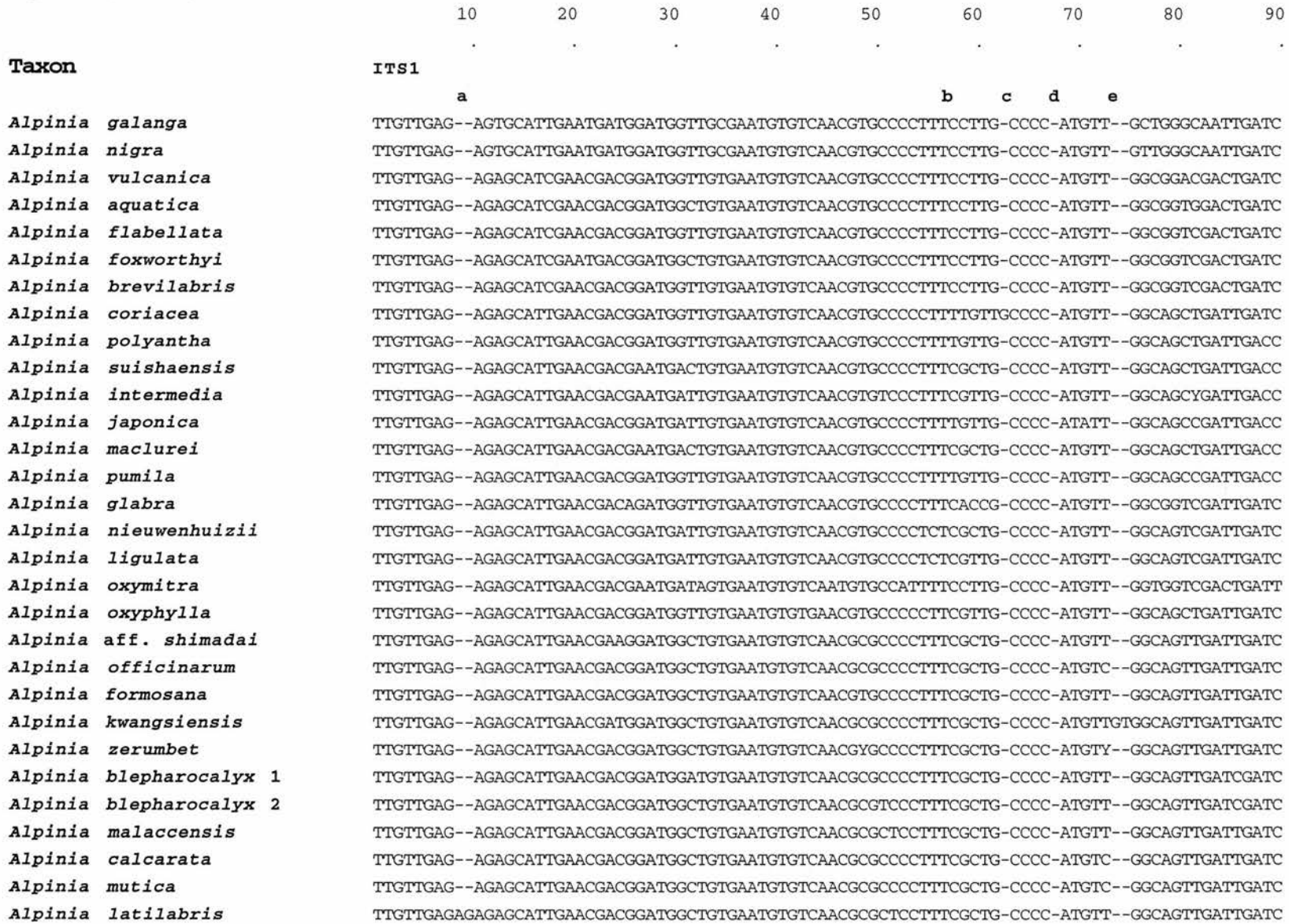


Figure 17 (continued).

```
                                  100        110        120        130        140        150        160        170        180
                                   .          .          .          .          .          .          .          .          .
Taxon                                                                                    f                    g
Elettariopsis unifolia            GTAACTCGGTGCGAGCAGCACCAAGGAACAATGAACTTAGAAGCAGAGGGCCC-TCGGCGTGCGCGGGG-AGCCTTATGCGTCGGAGATG
Burbidgea schizocheila            CTACTTCGGTGCGATCGGCACCAAGGAACAATGAACTCAGAAGGAGAGGGCCC-TCGGCGTGCATGGGG-AGCCCAATGCTTCGGAGATG
Pleuranthodium schlechteri        CTACTTCGGTGCGATCGGCACCAAGGAATAATGAACTCAGAAGGAGAGGGCCC-TCGGCGTGCGTGGGG-AGCCCAATGCTTCGGAGACA
Pleuranthodium papilionaceum      CTACTTCGGTGCGATCGGCACCAAGGAATAATGAACTCAGAAGGAGAGGGCCC-TCGGCGTGCGTGGGG-AGCCCAATGCTTCGGAGACA
Pleuranthodium floribundum        CTACTTCGGTGCGATCGGCACCAAGGAATAATGAACTCAGAAGGAGAGGGCCC-TCGGCGTGCGTGGGG-AGCCCAATGCTTCGGAGACA
Pleuranthodium floccosum          CTACTTCGGTGCGATCGGCACCAAGGAATAATGAACTCAGAAGGAGAGGGCCC-TCGGCGTGCGTGGGG-AGCCCAATGCTTCGGAGACA
Pleuranthodium racemigerum        CTACTTCGGTGCGATCGGCACCAAGGAATAATGAACTCAGAAGGAGAGGGCCC-TCGGCGTGCGTGGGG-AGCCCAATGCTTCGGAGACA
Renealmia battenbergiana          GTAGCTCGGTGCGATCGGCACCAAGGAACAATGAACTTAGAAGCAGAGGGCCC-TCGATGTGCGCGGGG-AGCCCAATGCGGAGGAGATG
Renealmia aff. africana           GTAGCTCGGTGCGATCGGCACCAAGGAACAATGAACTTAGAAGCAGAGGGCCC-TCGATGTGCGCGGGG-AGCCCAATGCGGAGGAGATG
Alpinia abundiflora               GTAGCTCGGTGCGATCGGCACCAAGGAACAATGAACTTAGAAGCAGAGGGCCC-TCGATGTGCGCGGGG-AGCTCAATGCGTCTGAGATG
Alpinia fax                       GTAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTTAGACGCAGAGGGCCC-TCGATGTGCGCGGGG-AGCTCAATGCGTCGGAGATG
Alpinia arundelliana              ATAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGTGGGCCC-TCGGCGTGCACGGGGGAGTCCACTGCATAAGAGATG
Alpinia modesta                   ATAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGTGGGCCC-TCGGCGTGCACGGGGGAGTCCACTGCATAAGAGATG
Alpinia caerulea                  ATAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGTGGGCCC-TCGGCGTGCACGGGGGAGTCCACTGCATAAGAGATA
Alpinia oceanica                  GTAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGCGGGCCC-TCGGCGTGCACGGGG-AGTCCACCGCACAAGAGATG
Alpinia vittata                   GTAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGCGGGCCC-TCGGCGTGCACGGGG-AGTCCACCGCACAAGAGATG
Alpinia purpurata                 GTAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGCGGGCCC-TCGGCGTGCACGGGG-AGTCCACTGCATAAGAGATG
Alpinia arctiflora                GTAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGTGGGCCC-TCGGTGTGCACGGGG-AGTCCACTGCAGAAGAGATG
Alpinia elegans                   GTAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGTGGGCCC-TCGACGTGCACGGGG-AGTCGATTGCATAAGAGATG
Alpinia eubractea                 GTAGCTCGGTGCGATCGGCACCAAGGAACAACGAATTCTGAAGCAGTGGGCCC-TCGGTGTGCACGGGG-AGTCCATTGCATAAGAGATG
Alpinia luteocarpa (scorpoidea)   GTAGCTCGGTGCGATCGGCACCAAGGAACAACGAACTCAGAAGCAGCTGGGCCCTCGATGTGCACGGGG-AGTCCATTGCATAAGAGATG
Alpinia carolinensis              GTAGCTCGGTGCGATCCGCACCAAGGAAAAATGAACTCAGAAGCAGAGGGCCC-TCGGCGTGCGCGGGG-AGCCCAATGCGATGTAGATG
Alpinia coeruleoviridis           GTAGCTCGGTGCGATCAGCACCAAGGAAAAATGAACTCAGAAGCAGAGGGCCC-TCGGCGTGCGCGGGG-AGCCCAATGCGTTGGAGATG
Alpinia boia                      GTAGCTCGGTGCGATCAGCACCAAGGAAAAATGAACTCAGAAGCAGAGGGCCC-TCGGCGTGCGCGGGG-AGCCCAATGCGTTGGAGATG
Alpinia rafflesiana               GGAGCTCGGTGCGATCGGCACCAAGGAACAATGAACTCAGAAGCAAAGGGCTC-TCGGTGTGCGTGCGA-GGCCCAATGCTTCGGAGATG
Alpinia javanica                  GGAGCTCGGTGCGATCGGCACCAAGGAACAATGAACTCAGAAGCAAAGGGCTC-TCGGTGTACGTCCAA-GGCCCAATGCTTCGGAGATG
Alpinia conchigera                GTAGCTCGGTGCGATCGGCACCAAGGAATAATAAACTGAGAAGCAGAGGGCCC-TCGGTGTGTGCGGGG-AGCCCAATGCGTCGGAGAAG
Alpinia galanga                   GTAGCTCGGTGCGATCGGCACCAAGGAATAATAAACTGAGAAGCAAAGGGCCC-TCGGTGTGTGCGGGG-AGCCCAATGCGTCGGAGAAG
Alpinia nigra                     GTAGCTCGGTGCGATCGGCACCAAGGAATAATAAACTGAGAAGCAGAGGGCCC-TCGGTGTGTGCGGGG-AGCCCAATGCGTCGGAGAAG
```

Figure 17 (continued).

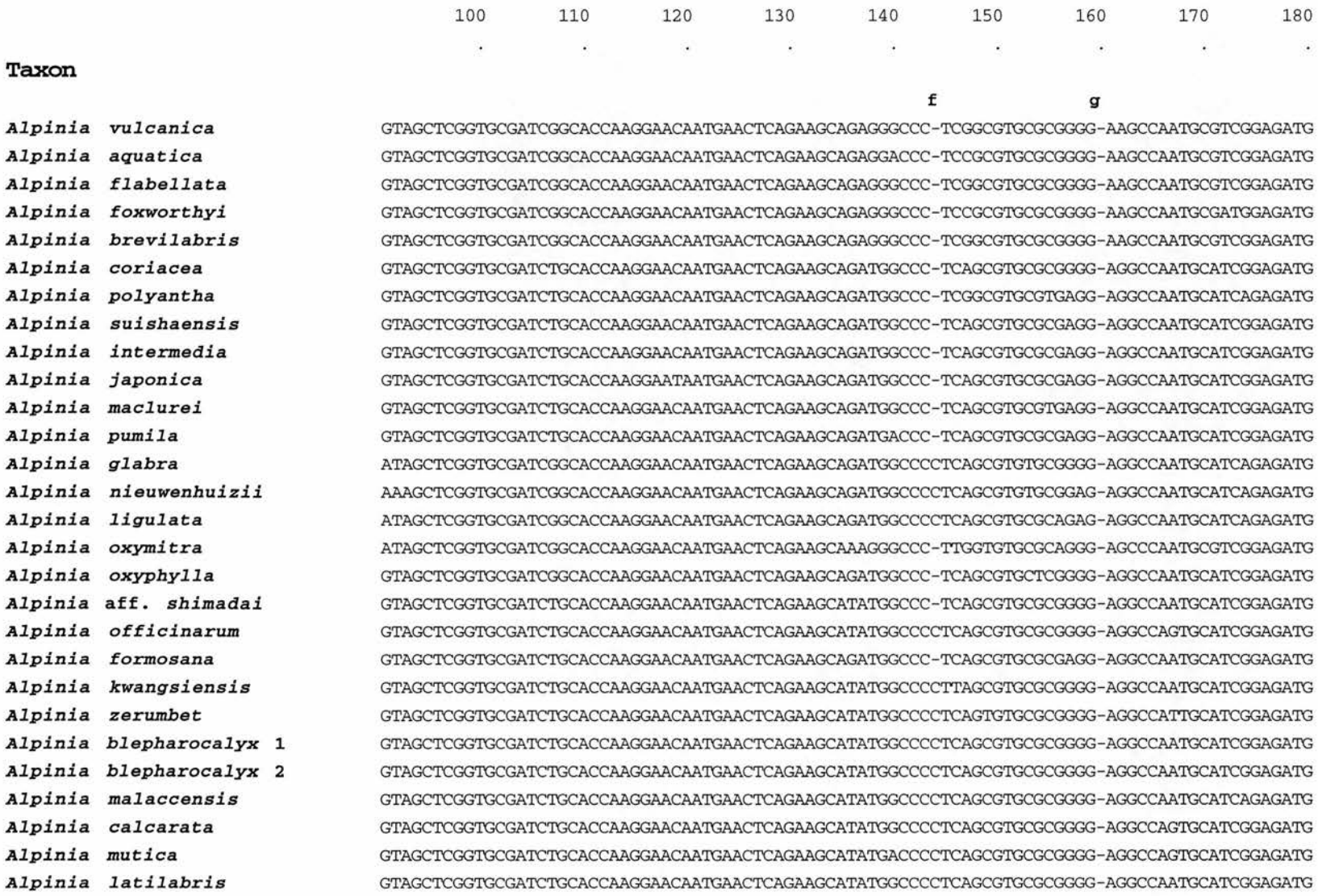


Figure 17 (continued).

```
                                190       200       210       220       230       240       250       260       270
                                 .         .         .         .         .         .         .         .         .
Taxon                                      ITS2
                                ************                                h     i                                 j
Elettariopsis unifolia          ---CCTCGGAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTT--GTTG---GTGCCAAGCGCGGAAATTGACCTCGTGTGCC--CTCG
Burbidgea schizocheila          G---CTCGAAA-TAAAATGAATCGTCGCTTTTGCTCCTTGCTTT--GCTG---GTGGCAAGYGCGGAAATTGACCTCGTGTGCC--CTCG
Pleuranthodium schlechteri      G---CTCGAAAATCAAATGAATCGTCGCTTTTGCTCCCTGCCTT--GCTG---GCGGCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Pleuranthodium papilionaceum    G---CTCGAAAATCAAATGAATCGTCGCTTTTGCTCCCTGCCTA--GCTG---GCGGCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Pleuranthodium floribundum      G---CTCGAAAATCAAATGAATCGTCGCTTTTGCTCCCTGCCTA--GCTG---GCGGCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Pleuranthodium floccosum        GA---TCGAAA-TCAAATGAATCGTCGCTTTTGCTCCTTGCCTT--GCTG---GCGGGAAGCGCGGATATTGACCTCGTGTGCC--CTCG
Pleuranthodium racemigerum      GA---TCGAAA-TCAAATGAATCGTCGCTTTTGCTCCTTGCCTT--GCTG---GCGGGAAGCGCGGATATTGACCTCGTGTGCC--CTCG
Renealmia battenbergiana        ---CCACGGAA-TCGAATGAATCGTCGCCTTTGCTCCTCTCTTT--GTTG---GTGTCAAGTGCGAAAATTGGCCTCGTGTGCC--CTCG
Renealmia aff. africana         ---CCACGGAA-TCGAATGAATCGTCGCCTTTGCTCCTCTCTTT--GTTG---GCGTCGAGTGCGAAAATTGGCCTCGTGTGCC--CTCG
Alpinia abundiflora             ---CCTCAGAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTTT-GCTG---GTGCCAAGCGCGGAAATTGGCCTCTTGTGCC--CTCG
Alpinia fax                     ---CCTCATAA-TCTAATGAATCGTCGCCTTCGCTCCTTGCTTTT-GCTG---GTGCCAAGCGCGGAAATTGGCCTCTTGTGCC--CTCG
Alpinia arundelliana            ----CTTGGAA-TCGAATGAATCATCGCCTTTGCTCCTTGCTCT--GCTG---GTGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia modesta                 ----CTTGGAA-TCGAATGAATCATCGCCTTTGCTCCTTGCTCT--GCTG---GTGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia caerulea                ----CTTGGAA-TCGAATGAATCATCGCCTTTGCTCCTTGCTCT--GCTG---GTGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia oceanica                ----CTCGGAA-TCGAACGAATCATCGCCTTTGCTCCTTGCTCT--GCTG---GTGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia vittata                 ----CTCGGAA-TCGAACGAATCATCGCCTTTGCTCCTTGCTCT--GCTG---GTGCCAAGCGCGGAAATTGGCCCCGTGTGCC--CTCG
Alpinia purpurata               ----CTCGGAA-TCGAATGAATCATCGCCTTTGCTCCTTGCTCTTTGCTG---GTGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia arctiflora              ----CTTGGAA-TCGAATGAATCATCGCCTTTGCTCCTTGCTCT--GCTG---GTGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia elegans                 ----CTCGGAA-TCGAATGAATCATCGCCTTTGCTCCTAGCTTT--GCTG---GTGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia eubractea               ATG-CTTGGAA-TCTAATGAATCATCGCCTTTGCTCCTTGCTTT--GCTG---GCGCTAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia luteocarpa (scorpoidea) ----CTTGGAA-TCGAATGAATCATCGCCTTTGCTCCTTGCTTT--GCTG---GTGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia carolinensis            ---CCTCGGAA-TCATATGAATCGGCGCCTTTGCTCCTCTGCTTT-GTCG---GGGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia coeruleoviridis         ---CCTCGGAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTT--GTCG--GGGGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia boia                    AA---TCGGAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTT--GTCG--GGGGCCAAGCGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia rafflesiana             ---CCTCGGAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTT--GCTG---GTGCCAAGCGCGGATATTGGCCTCGTGTGCC--CTCG
Alpinia javanica                ---CCTCGTAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTT--GCTG---GTGCCAAGCGCGGATATTGGCCTCGTGTGCC--CTCG
Alpinia conchigera              ---CCTCGAAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTT--GCTGCTAGTGCTAAGTGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia galanga                 ---CCTCGAAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTT--GCTGCTGGTGCTAAGTGCGGAAATTGGCCTCGTGTGCC--CTCG
Alpinia nigra                   ---CCTCGAAA-TCAAATGAATCGTCGCCTTTGCTCCTTGCTTT--GCTGCTGGTGCTAAGTGCGGAAATTGGCCTCGTGTGCC--CTCG
```

Figure 17 (continued).

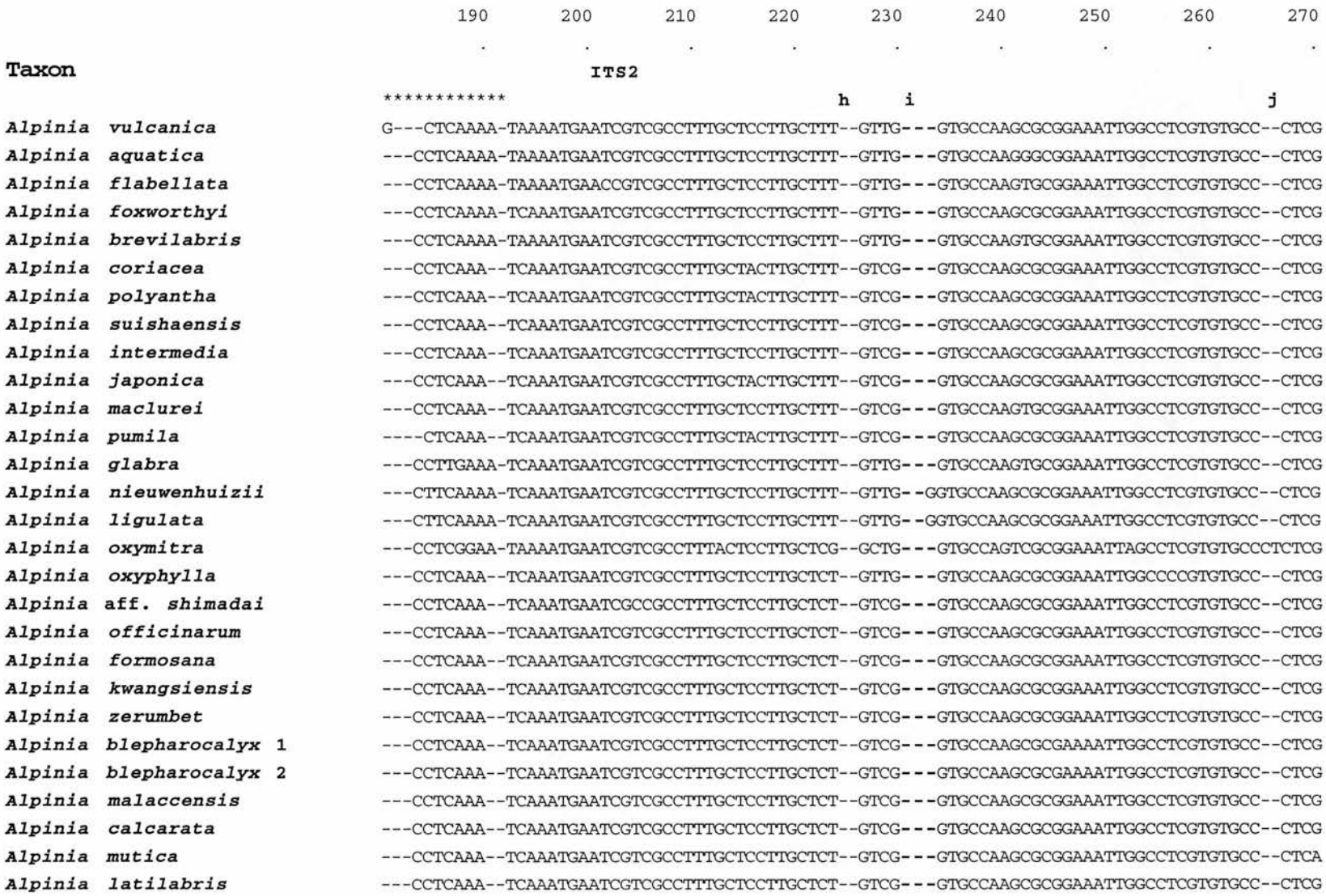


Figure 17 (continued).

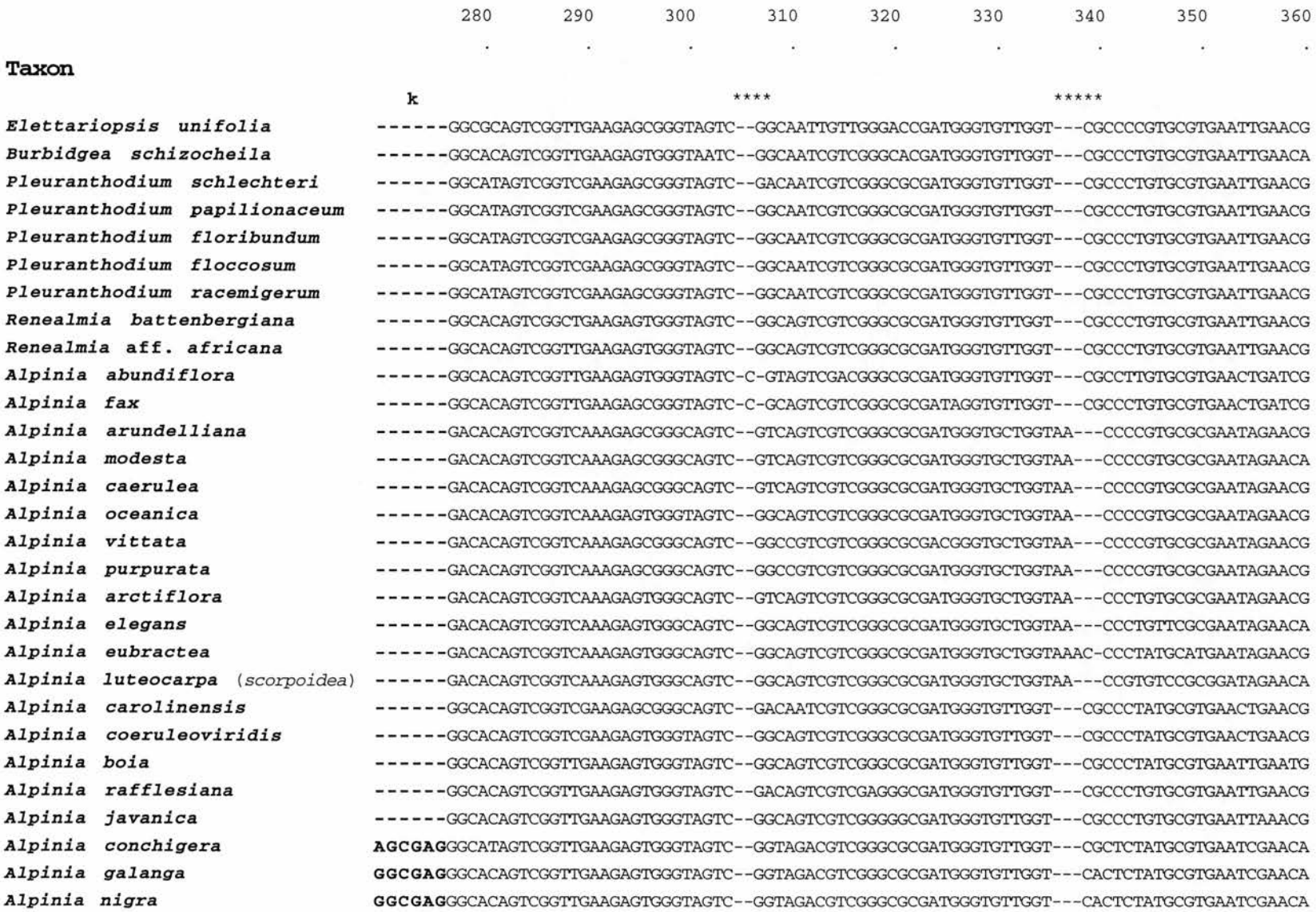


Figure 17 (continued).

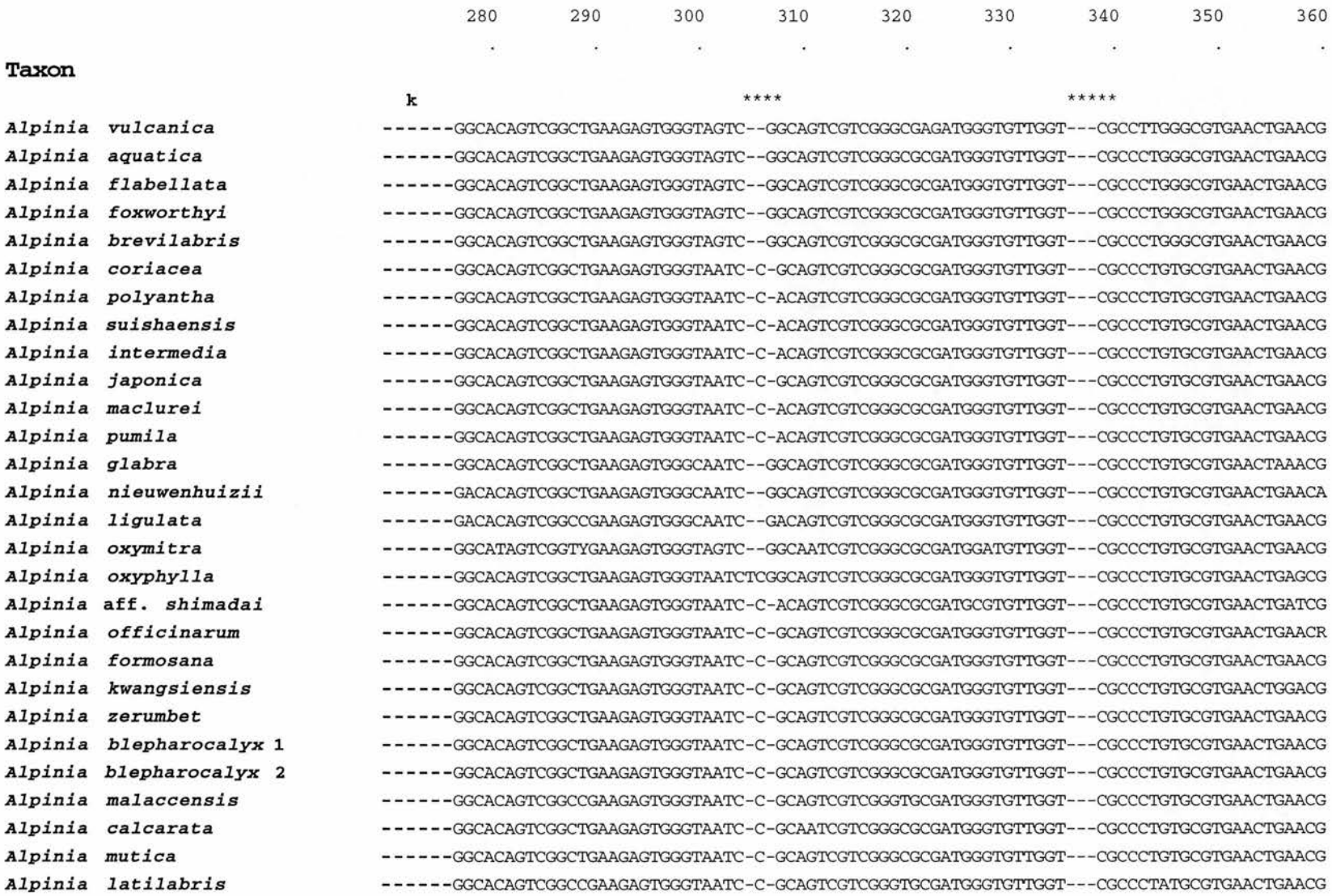


Figure 17 (continued).

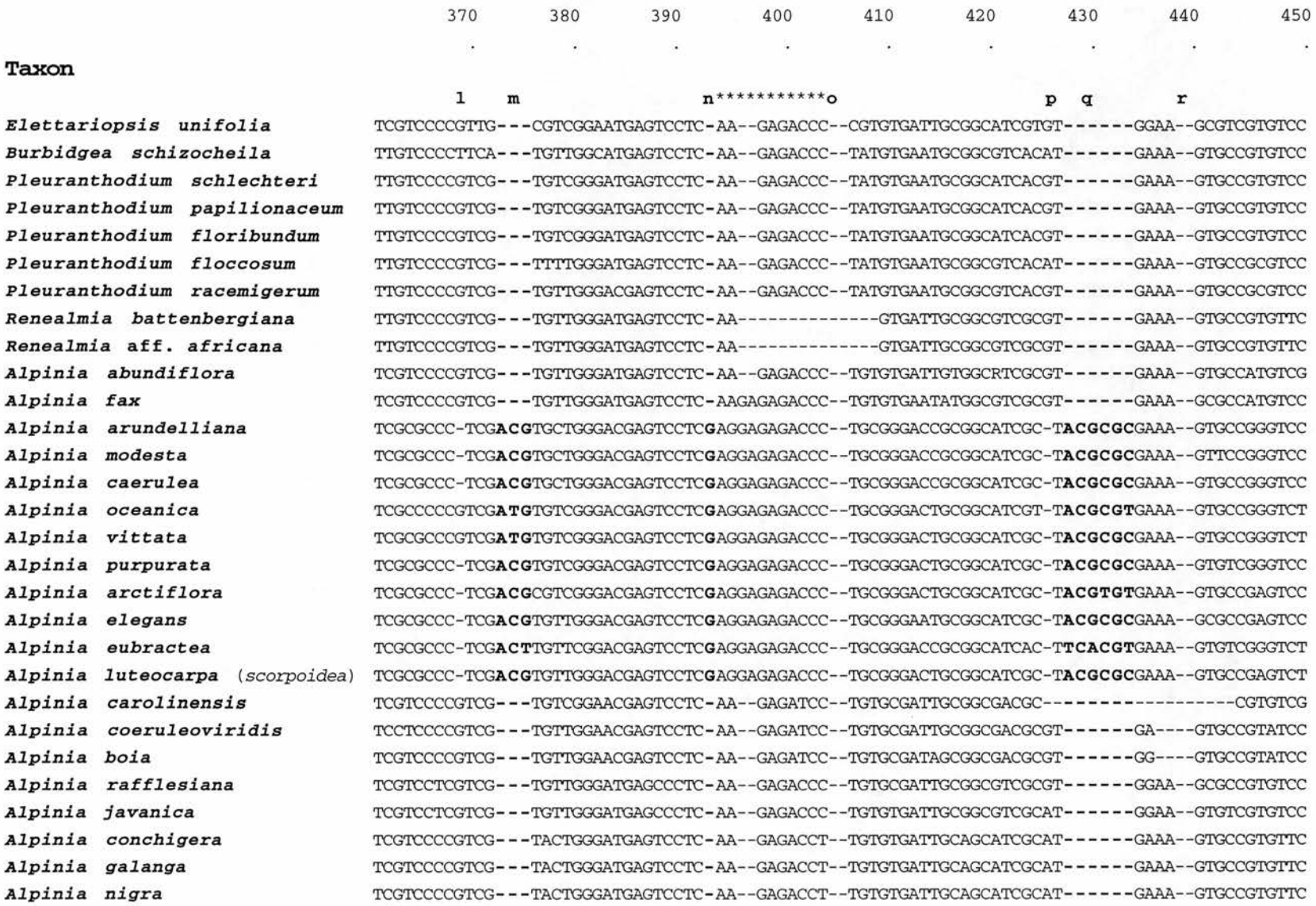


Figure 17 (continued).

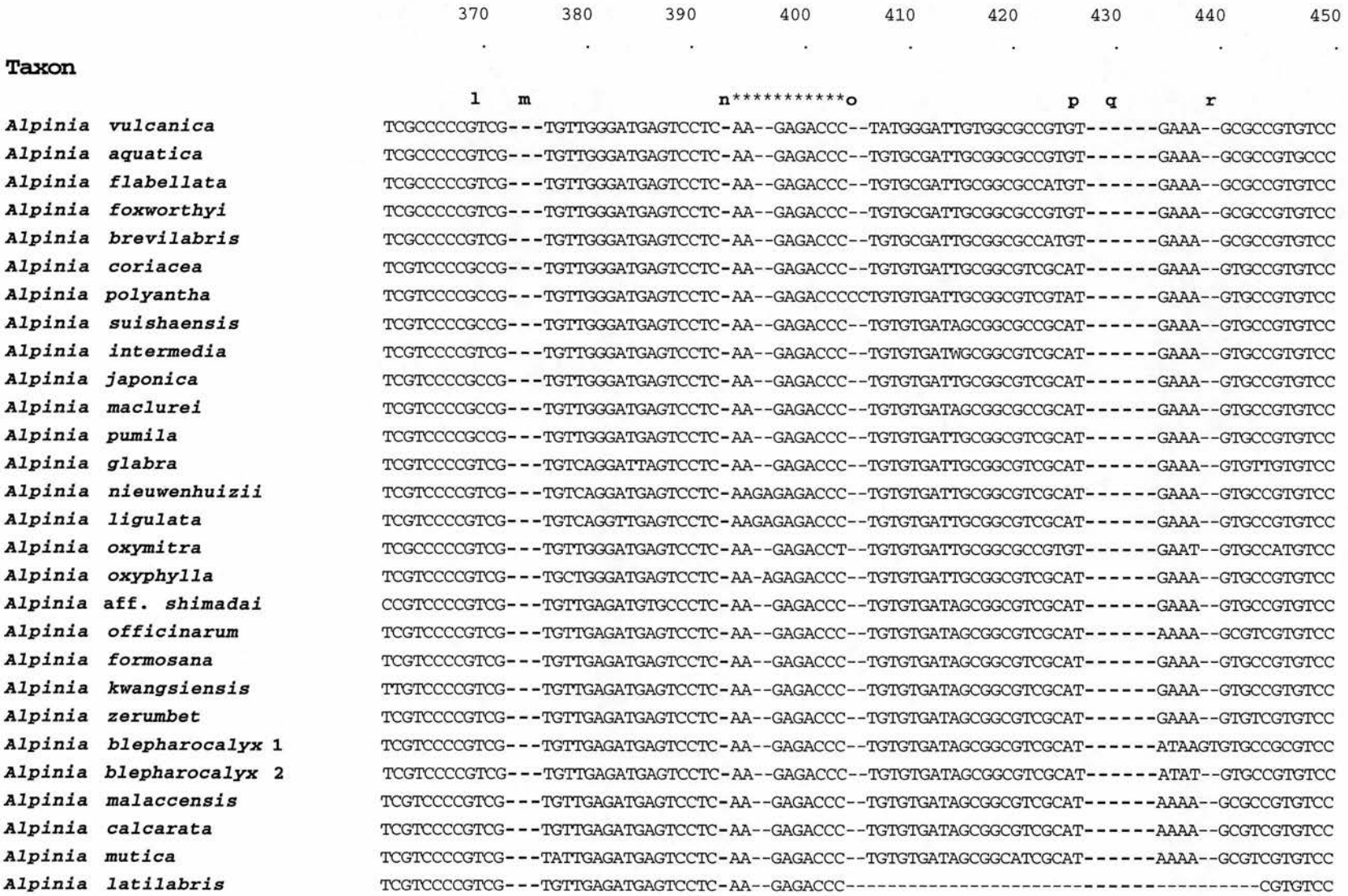


Figure 17 (continued).

Taxon	460	
Elettariopsis unifolia	ATCATATTGT	**[413]**
Burbidgea schizocheila	ATCTGATTGT	**[413]**
Pleuranthodium schlechteri	ATCTGATTGT	**[415]**
Pleuranthodium papilionaceum	ATCTGATTGT	**[415]**
Pleuranthodium floribundum	ATCTGATTGT	**[415]**
Pleuranthodium floccosum	ATCTGATTGT	**[414]**
Pleuranthodium racemigerum	ATCTGATTGT	**[414]**
Renealmia battenbergiana	GTCATATTGT	**[405]**
Renealmia aff. africana	GTCATATTGT	**[405]**
Alpinia abundiflora	GTCATATTGT	**[414]**
Alpinia fax	GTCATATTGT	**[416]**
Alpinia arundelliana	GTCGGTTTGT	**[426]**
Alpinia modesta	GTCGGTTTGT	**[426]**
Alpinia caerulea	GTCGGTTTGT	**[426]**
Alpinia oceanica	GTCGGTTTGT	**[425]**
Alpinia vittata	GTCGGTTTGT	**[425]**
Alpinia purpurata	GTCGGTTTGT	**[426]**
Alpinia arctiflora	GTCGGTTTGT	**[424]**
Alpinia elegans	GTCGATTTGT	**[424]**
Alpinia eubractea	GTCGGTTTGT	**[429]**
Alpinia luteocarpa (*scorpoidea*)	GTCGATTTGT	**[425]**
Alpinia carolinensis	ATCAAATTGT	**[405]**
Alpinia coeruleoviridis	GTCAAATTGT	**[413]**
Alpinia boia	GTCAAATTGT	**[413]**
Alpinia rafflesiana	ATCAGATTGT	**[414]**
Alpinia javanica	ATCAGATTGT	**[414]**
Alpinia conchigera	ATTAGATTGT	**[423]**
Alpinia galanga	ATCATATTGT	**[423]**
Alpinia nigra	ATCATATTGT	**[423]**

Figure 17 (continued).

Taxon		460	
Alpinia	*vulcanica*	ATCAGATTGT	[414]
Alpinia	*aquatica*	ATCAGATTGT	[414]
Alpinia	*flabellata*	ATCAGATTGT	[414]
Alpinia	*foxworthyi*	ATCAGATTGT	[414]
Alpinia	*brevilabris*	ATCAGATTGT	[414]
Alpinia	*coriacea*	ATCAAATTGT	[414]
Alpinia	*polyantha*	ATTAAATTGT	[415]
Alpinia	*suishaensis*	ATCAAATTGT	[413]
Alpinia	*intermedia*	ATCAAATTGT	[413]
Alpinia	*japonica*	ATCAAATTGT	[413]
Alpinia	*maclurei*	ATCAAATTGT	[413]
Alpinia	*pumila*	ATCAAATTGT	[412]
Alpinia	*glabra*	ATCAAATTGT	[415]
Alpinia	*nieuwenhuizii*	ATCAAATTGT	[418]
Alpinia	*ligulata*	ATCAAATTGT	[418]
Alpinia	*oxymitra*	ATCAGATTGT	[416]
Alpinia	*oxyphylla*	ATCAGATTGT	[416]
Alpinia	aff. *shimadai*	ATCAAATTGT	[413]
Alpinia	*officinarum*	ATCAAATTGT	[414]
Alpinia	*formosana*	ATCAAATTGT	[413]
Alpinia	*kwangsiensis*	ATCAAATTGT	[416]
Alpinia	*zerumbet*	ATCAAATTGT	[414]
Alpinia	*blepharocalyx* 1	ATCAAATTGT	[416]
Alpinia	*blepharocalyx* 2	ATCAAATTGT	[414]
Alpinia	*malaccensis*	ATCAAATTGT	[414]
Alpinia	*calcarata*	ATCAAATTGT	[414]
Alpinia	*mutica*	ATCAAATTGT	[414]
Alpinia	*latilabris*	ATCAAATTGT	[387]

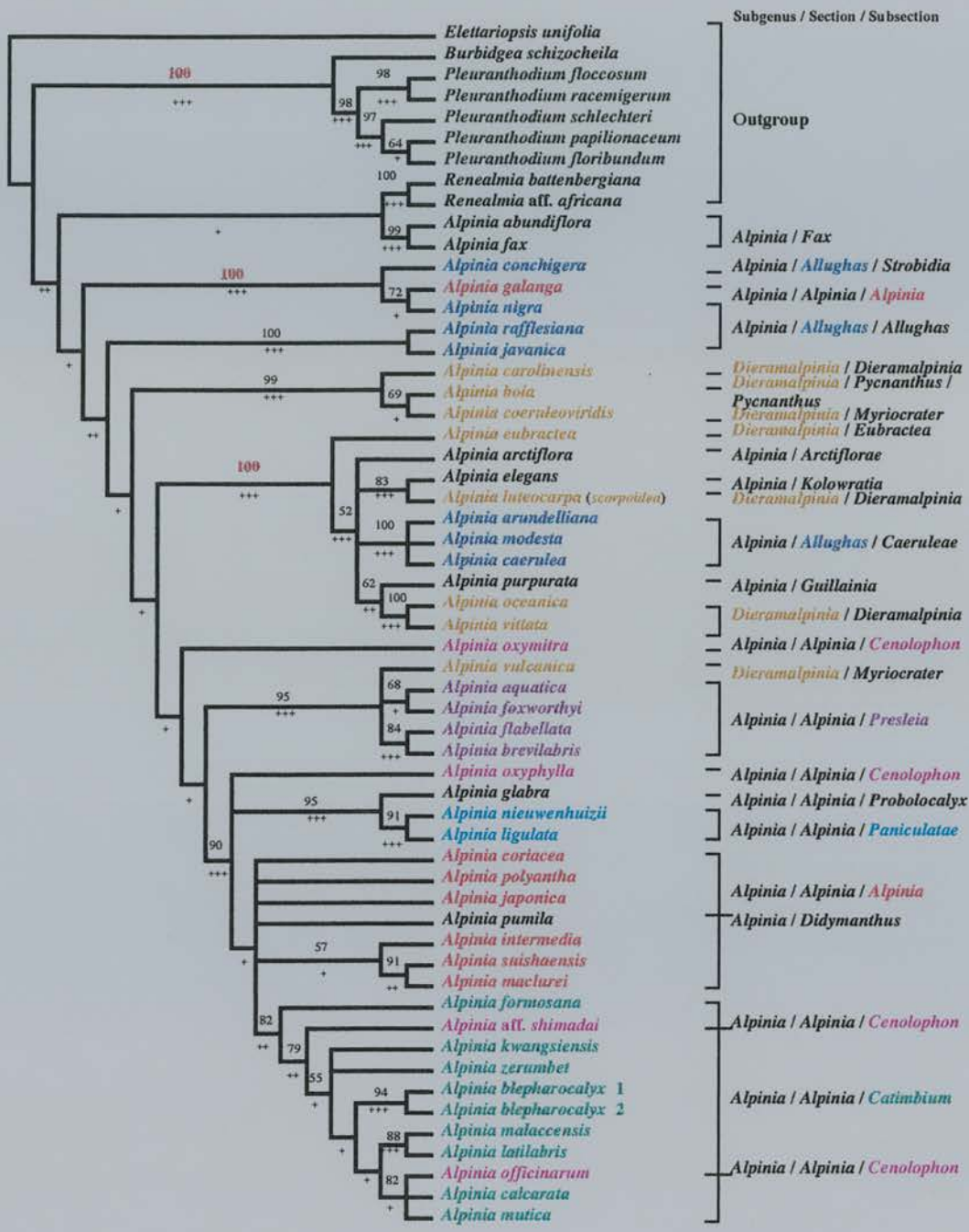


Figure 18. The strict consensus tree derived from 180 equally parsimonious trees of length 555 steps based on equally weighted parsimony analysis of the informative characters of the combined ITS1 and ITS2 regions plus coded gaps. Fit measures of the tree: CI = 0.4847, RI = 0.8129, RC = 0.3940. Numbers above the branches are bootstrap values (%) of 1000 replicates. Plus signs below the branches indicate decay indices (+: DI = +1, ++: DI = +2, +++: DI > +2). Coloured taxa are based on Smith's (1990a) classification. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

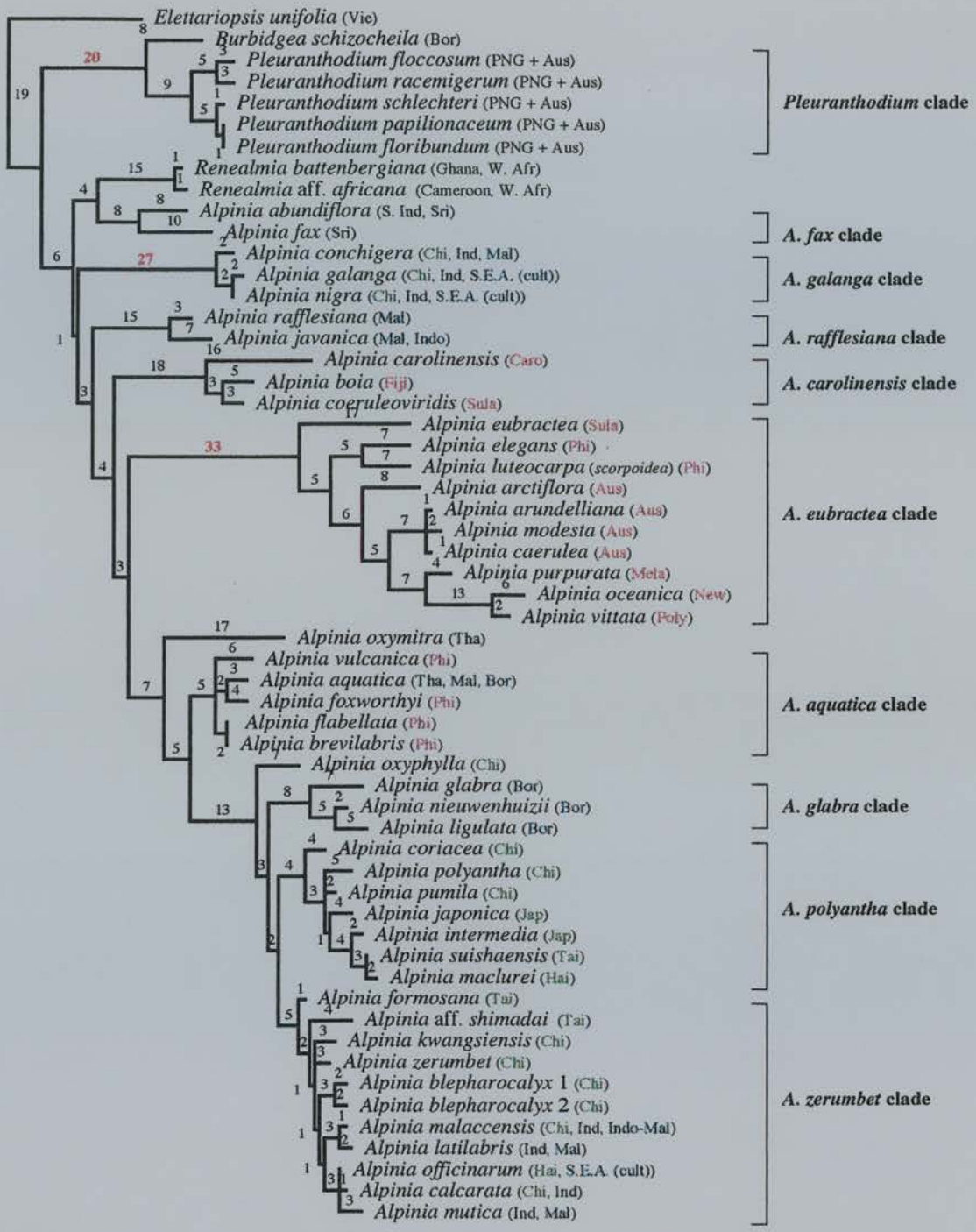


Figure 19. One of 180 equally parsimonious trees of 555 steps based on equally weighted parsimony analysis of the informative characters of the combined ITS1 and ITS2 regions plus coded gaps. Fit measures of the tree: CI = 0.4847, RI = 0.8129, RC = 0.3940. Numbers above or below the branches indicate branch lengths (character changes on the branches). Several clades which were mentioned in the text are shown here: *Pleuranthodium* clade, *A. fax* clade, *A. galanga* clade, *A. rafflesiana* clade, *A. carolinensis* clade, *A. eubractea* clade, *A. aquatica* clade, *A. glabra* clade, *A. polyantha* clade and *A. zerumbet* clade. The following abbreviations are used to represent areas (countries) for the geographical distribution of the 57 taxa of Zingiberaceae under study. Aus: Australia; Bor: Borneo; Caro: Caroline Island; Chi: China; Hai: Hainan; Ind: india; Indo: Indonesia; Indo-Mal: Indo-Malaysian region; Jap: Japan; Mal: Malaysia; Mela: Melanesia; New: New Britain; Phi: the Philippines; PNG: Papua New Guinea; Poly: Polynesia; S.E.A.: South East Asia (cult: cultivated); S. Ind: South India; Sri: Sri Lanka; Sula: Sulawesi; Tai: Taiwan; Tha: Thailand; Vie: Vietnam; W. Afr: West Africa. The distributions are based on: Hepper (1968; for *Renealmia*), Smith (1985; for *Burbidgea*), Smith (1990a; for *Alpinia*), Smith (1991; for *Pleuranthodium*), and Newman (1997; for *Elettariopsis*). *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

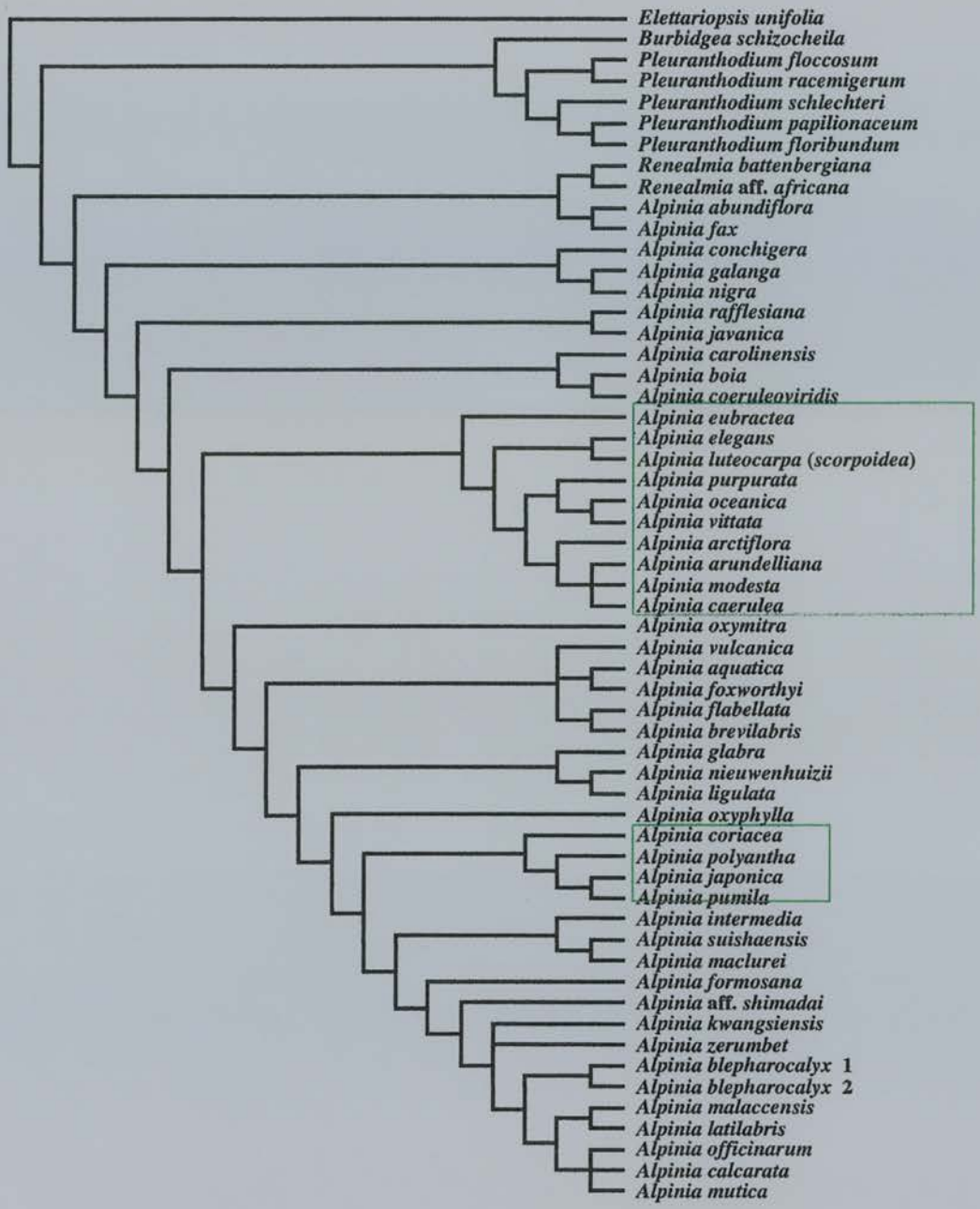


Figure 20. The strict consensus tree obtained from three equally parsimonious trees of length 272947 steps based on parsimony analysis of the informative characters of the combined ITS1 and ITS2 regions plus coded gaps; the characters were re-weighted according to the CI value (0.4847). Fit measures of the tree: CI = 0.618, RI = 0.869, RC = 0.537. Green boxes contain groups of *Alpinia* whose relationships are more resolved than those shown in **Figure 18**. Successive weighting of the characters (using CI = 0.618) yielded an identical tree topology of the strict consensus tree. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

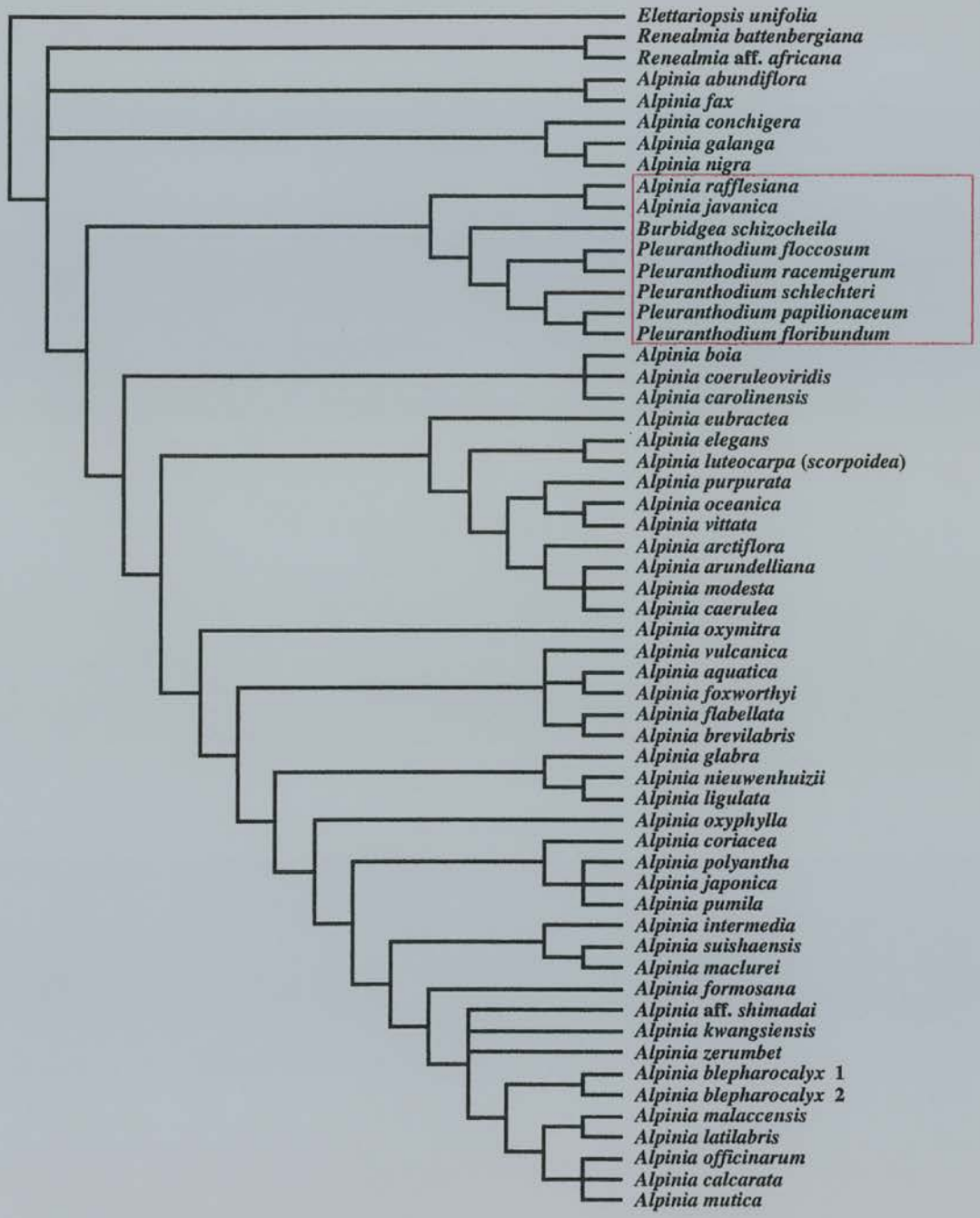


Figure 21. The strict consensus tree obtained from 144 equally parsimonious trees of length 776 steps based on parsimony analysis of the informative characters of the combined ITS1 and ITS2 regions excluding coded gaps with transversions being weighted over transitions by a factor of three. Fit measures of the tree: CI = 0.543, RI = 0.837, RC = 0.454. The red box indicates the fact that *Pleuranthodium* is found nested within *Alpinia*. The clade shows *A. rafflesiana*, *A. javanica* and *Burbidgea schizocheila* as sister groups of *Pleuranthodium*. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

Figure 22. Sequence data matrix of aligned spacer region between *trn*L (UAA) 3' exon and *trn*F (GAA) of chloroplast DNA for 22 taxa of Zingiberaceae. Hyphens denote alignment gaps; **s-z** above nucleotide matrix indicate the position of alignment gaps; nucleotides in bold denote phylogenetically informative insertions; numbers in square brackets at the end of sequences indicate the actual length of the spacer region. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior*.

```
                                10        20        30        40        50        60        70        80        90
                                .         .         .         .         .         .         .         .         .

Taxon
                                                                                                                s
Pleuranthodium schlechteri      CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGG
Renealmia battenbergiana        CATCAGCAGCACAGATCTAAGAACATTCACTAATTAACTCACTCACTCCAATTTTTCACAACACAAATGTATCCGAGCTAAAATCCTTGG
Alpinia modesta                 CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGG
Alpinia oceanica                CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGG
Alpinia elegans                 CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGG
Alpinia carolinensis            CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGG
Alpinia rafflesiana             CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAGATGTATCCGAACTAAAATCCTTGG
Alpinia javanica                CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAGATGTATCCGAACTAAAATCCTTGG
Alpinia conchigera              CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGG
Alpinia galanga                 CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGG
Alpinia nigra                   CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGG
Alpinia aquatica                CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAGTGTATCCGAACTCAAATC-TTGG
Alpinia brevilabris             CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAGTGTATCCGAACTCAAATC-TTGG
Alpinia intermedia              CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTCAAATC-TTGG
Alpinia japonica                CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTCAAATC-TTGG
Alpinia pumila                  CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTCAAATC-TTGG
Alpinia nieuwenhuizii           CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAATACAAATGTATCCGAACTCAAATC-TTGG
Alpinia ligulata                CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAATACAAATGTATCCGAACTCAAATC-TTGG
Alpinia aff. shimadai           CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTCAAATC-TTGG
Alpinia officinarum             CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTCAAATC-TTGG
Alpinia blepharocalyx 1         CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTCAAATC-TTGG
Alpinia malaccensis             CATCAGCGATTCAGTTCAAACAAAATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTCAAATC-TTGG
```

Figure 22 (continued).

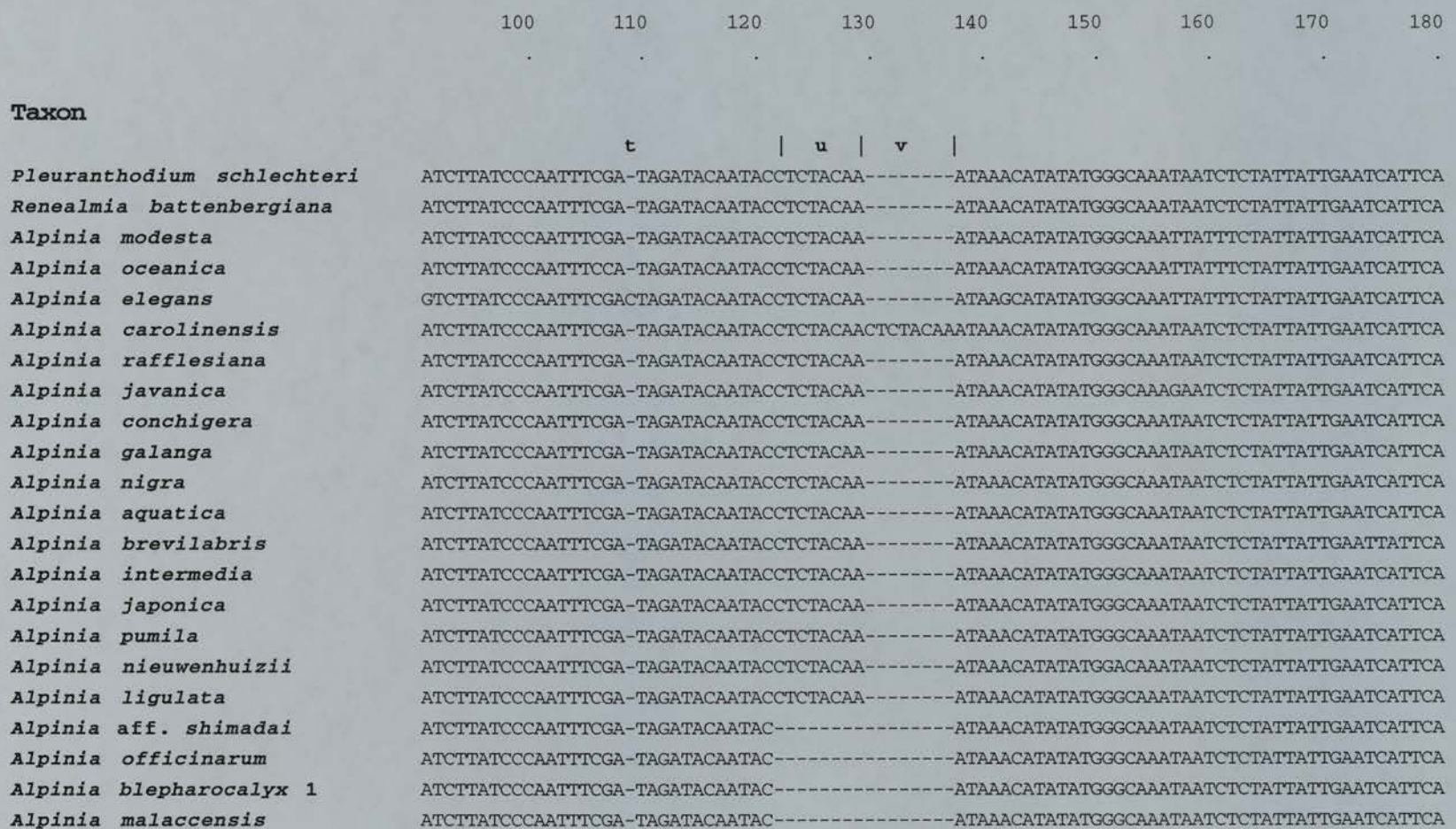


Figure 22 (continued).

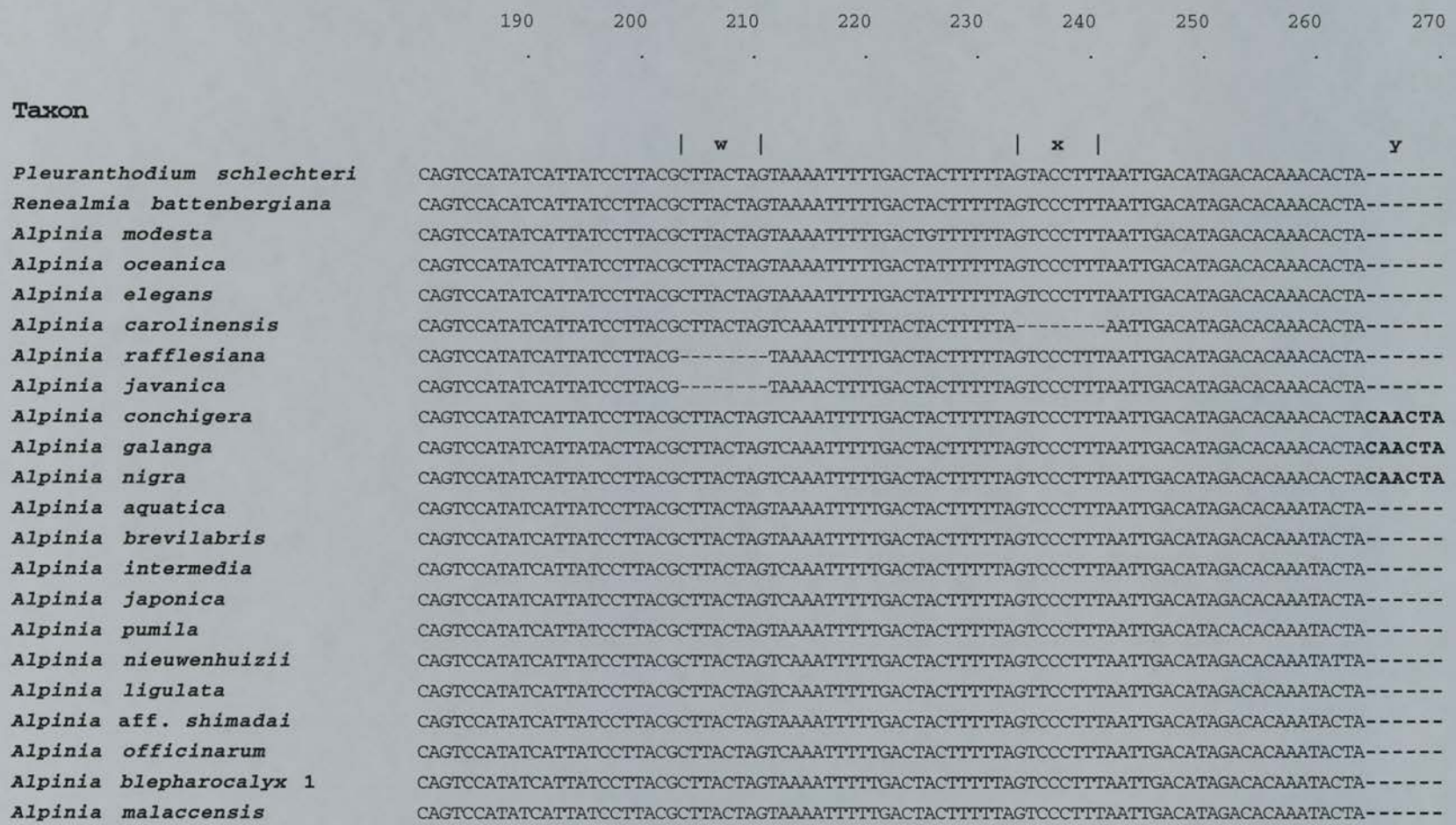


Figure 22 (continued).

```
                                  280        290        300        310        320
                                   .          .          .          .          .

Taxon
                                                                     z
Pleuranthodium schlechteri        CATCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [304]
Renealmia battenbergiana          CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [304]
Alpinia modesta                   TACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CATTTGGTAGAGC  [304]
Alpinia oceanica                  TACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CATTTGGTAGAGC  [304]
Alpinia elegans                   TACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CATTTGGTAGAGC  [305]
Alpinia carolinensis              CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [304]
Alpinia rafflesiana               CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [296]
Alpinia javanica                  CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [296]
Alpinia conchigera                CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [310]
Alpinia galanga                   CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [310]
Alpinia nigra                     CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [310]
Alpinia aquatica                  CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [303]
Alpinia brevilabris               CACCAGTATGATGGATGGGAAATGGTCGGGATACGC-CAGTTGGTAGAGC  [303]
Alpinia intermedia                CACCAGTATGATGCATGGGAAATGGTCGGGATAGCCGCAGTTGGTAGAGC  [304]
Alpinia japonica                  CACCAGTATGATGCATGGGAAATGGTCGGGATAGCCGCAGTTGGTAGAGC  [304]
Alpinia pumila                    CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [303]
Alpinia nieuwenhuizii             CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [303]
Alpinia ligulata                  CACCAGTATGATGCATGGGAAATGGTCGGGATAGCT-CAGTTGGTAGAGC  [303]
Alpinia aff. shimadai             CACCAGTATGATGCATGGGAAATAGTCGGGATAGCT-CAGTTGGTAGAGC  [295]
Alpinia officinarum               CACCAGTATGATGCATGGGAAATAGTCGGGATAGCT-CAGTTGGTAGAGC  [295]
Alpinia blepharocalyx 1           CACCAGTATGATGCATGGGAAATAGTCGGGATAGCT-CAGTTGGTAGAGC  [295]
Alpinia malaccensis               CACCAGTATGATGCATGGGAAATAGTCGGGATAGCT-CAGTTGGTAGAGC  [295]
```

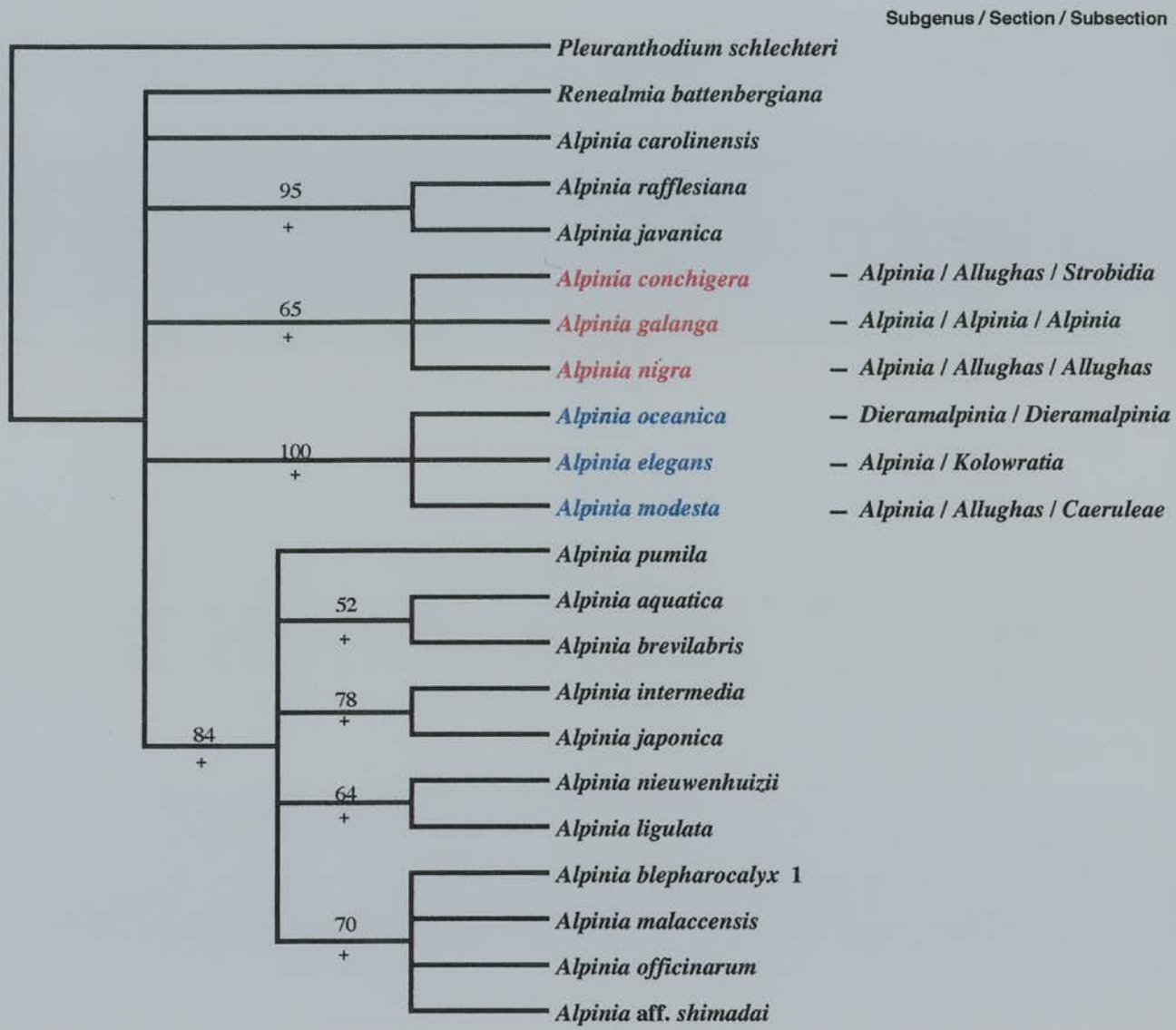


Figure 23. The strict consensus tree derived from 11 equally parsimonious trees of length 56 steps based on equally weighted parsimony analysis of the chloroplast DNA between *trn*L (UAA) 3' exon and *trn*F (GAA) plus coded gaps. Fit measures of the tree: CI = 0.946, RI = 0.953, RC = 0.902. Numbers above the branches are bootstrap values (%) of 1000 replicates. Plus signs below the branches indicate a decay index of +1. Taxa in red indicate the fact that *Alpinia galanga* is included with species of section *Allughas*. Taxa in blue show the grouping of *Alpinia* that contains both Smith's (1990a) subgenera *Alpinia* and *Dieramalpinia*. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior*.

CHAPTER 4: MORPHOLOGICAL APPROACHES TO THE STUDY OF THE INFRAGENERIC CLASSIFICATION OF *ALPINIA*

4.1 INTRODUCTION

According to Burtt and Smith (1976), Valeton (1918) and Holttum (1950) used living plant specimens extensively. Therefore using of their studies requires good knowledge of living plants. Dried herbarium specimens of Zingiberaceae are often difficult to examine because certain characters may be lost or cannot be easily elucidated. Therefore, ideally, material should be collected by preserving some flowers, or better whole inflorescences, in spirit (70% ethanol or Formalin Acetic acid Alcohol (1:1:18 mixture of 40% formaldehyde, glacial acetic acid and 70% ethanol)) and by taking colour photographs of the inflorescence-structure as well as the floral parts. Burtt and Smith (1976) published notes on the collection and preservation of the inflorescences, bracts, bracteoles, fruit, and leaf shoots of Zingiberaceae. They suggested that detailed field observations should include characters such as the presence of non-persistent bracts and bracteoles, the occurrence of monoecism in the plant population, the variation in ligule, petiole and lamina size throughout the entire shoot, and the relationship of the plane of distichy of the leaf shoot to the rhizome.

4.1.1 Useful morphological and anatomical characters for the infrageneric classification of *Alpinia*

(1). Bract and bracteole characters

Schumann (1904), Holttum (1950) and Smith (1990a) all used the presence or absence of the bract and bracteole, and the size (large or small) and type (persistent or soon deciduous) of these two characters in their classifications of *Alpinia*. The distinction between tubular (cup-shaped) and non-tubular (open)

bracteoles appeared to be important and diagnostic to characterise taxa (see sections **1.2** and **1.3** for details of the infrageneric classifications of *Alpinia* by Schumann (1904), Holttum (1950) and Smith (1990a)). However, it should be noted that in Smith's (1990a) classification, section *Kolowratia* contains species with both tubular and non-tubular bracteoles. In addition, both types of bracteoles have been found within a single inflorescence of *Alpinia abundiflora* (section *Fax*).

(2). The structure and habit of the inflorescence

Holttum (1950) and Smith (1990a) laid much importance on the inflorescence-structure of *Alpinia*. In their classifications most of the taxa have inflorescences bearing flowers in cincinni, while *Cenolophon* has inflorescences bearing single flowers directly on the main axis. For the habit of the inflorescence, Schumann (1904) classified subgenus *Rhizalpinia* on the basis of their radical inflorescences. However, most of the species in this subgenus have been transferred to other genera such as *Amomum*. Therefore, most *Alpinia* have inflorescences terminal on the leafy shoot, except Smith's (1990a) section *Fax* which contains two species namely, *A. abundiflora* whose inflorescence may be terminal but is more often radical, and *A. fax* whose inflorescence is always radical.

(3). The form of the labellum

Smith's (1990a) subdivision of *Alpinia* into subgenera is mainly based on the form of the labellum. Although petaloid and non-petaloid characters of the labellum are fairly constant and distinct throughout the genus, certain species of *Alpinia* with a non-petaloid labellum have the apex occasionally expanding into a small petaloid area.

(4). The type of the stigma

The morphology of the stigma in *Alpinia* was observed in a small number of species (8 species in subgenus *Alpinia* and 7 species in subgenus *Dieramalpinia*) by Smith (1990a). In most sections of subgenus *Alpinia* the stigma is usually well expanded with a terminal, rounded to elliptic, frequently ciliate orifice. In sections *Fax* (*A. abundiflora*) and *Arctiflorae* (*A. arctiflora*) however, the orifices are probably ventrally placed. In subgenus *Dieramalpinia* the stigma is commonly erect with a usually rounded orifice placed subapically, at the tip or, more often, on the ventral face.

(5). The type of fruit and seed in *Alpinia* based on anatomical characters

Studies of fruit and seed anatomy of 21 Chinese *Alpinia* species were carried out by Liao and Wu (1996a, b). While fruit characters were obtained from the exocarp, mesocarp and endocarp, seed characters were obtained from the aril, perisperm, endosperm, embryo, symmetry of the seed, structures of seed coat, micropylar region, and chalazal region (see **Table 9** for details). Both anatomical studies yielded four types of fruit and seed which are "Polyantha", "Conchigera", "Oxyphylla", and "Zerumbet". Wu's (1981) system of *Alpinia* classification was supported by these four types of fruit and seed as follows: subgenus *Alpinia* by "Polyantha" type, subgenus *Dieramalpinia* by "Conchigera" type, subgenus *Probolocalyx* by "Oxyphylla" type and subgenus *Catimbium* by "Zerumbet" type. However, it should be noted that no voucher specimens were stated in these studies of fruit and seed anatomy, therefore, species identification may be uncertain.

Table 9. The anatomical characters of fruit and seed types of *Alpinia* (adapted from Liao and Wu 1996a, b). Ticks in bold-face type (✓) denote distinctive characters for different fruit and seed types.

Fruit and seed type	"Polyantha"	"Zerumbet"	"Conchigera"	"Oxyphylla"
Fruit character				
1	✓	✓	✓	
2				✓
3			✓	
4	✓	✓	✓	
5		✓		
6		✓		
7		✓		
8	✓		✓	✓
9			✓	
10	✓	✓		✓
Seed character				
11			✓	
12	✓	✓		✓
13	✓		✓	✓
14		✓		
15			✓	
16	✓	✓		✓
17			✓	✓
18		✓		
19	✓		✓	✓
20		✓		
21			✓	

Table 9 (continued).

Fruit and seed type	"Polyantha"	"Zerumbet"	"Conchigera"	"Oxyphylla"
Fruit character				
22		✓		✓
23	✓		✓	✓
24		✓		
25				✓
26			✓	
27	✓			
28		✓		
29				✓
30			✓	
31	✓			
32		✓		

Fruit characters:

1: Cells of exocarp arranged regularly and nearly with the same size.
2: Cells of exocarp arranged irregularly and varied in size.
3: Hypodermis present in mesocarp.
4: Sclerotic cell layer present in mesocarp.
5: Dehiscent zone present in mesocarp.
6: Fibrous cap present in mesocarp.
7: Parenchymatous cells in inner part of mesocarp are of volume larger than that in middle part, linear-oblong or irregular in shape, with well developed intercellular space and arranged tangentially or obliquely.
8: Parenchymatous cells in inner part of mesocarp are of volume smaller than that in middle part, roundish or oblong in shape, with small intercellular space and arranged only tangentially.
9: The innermost cell layer of mesocarp is composed of a row of roundish parenchymatous cells with extremely large volume.
10: The innermost cell layer of mesocarp is composed of oblong occasionally roundish parenchymatous cells with volume similar to the neighbouring cells.

Seed characters:

11: Number of cell layers of seed coat is less than eight.

12: Number of cell layers of seed coat is eight or more than eight.

13: Hypodermal cells contain pigment bodies.

14: Hypodermal cells contain small amount of pigment bodies or not; cells with small volume and compressed cell walls.

15: Parenchymatous cells of pigment cell layers contain pigment cells only.

16: Parenchymatous cells of pigment cell layers contain some translucent cells scattered among the pigment cells.

17: Cells of outermost layer of pigment cell layers contain pigment bodies.

18: Cells of outermost layer of pigment cell layers contain very few pigment bodies or none.

19: Seeds are without postchalazal bundle.

20: Seeds have postchalazal bundle.

21: Mesophyll contains some pigment cells and translucent cells with large volume.

22: Mesophyll contains some pigment cells and translucent cells with small volume.

23: Seeds are without diaphragm.

24: Seeds have diaphragm.

25: Positions of gap of endotesta and chalazal pigment cell group (CPG) are near the top.

26: Positions of gap of endotesta and CPG are at dorsal side and near the top.

27: Positions of gap of endotesta and CPG are at dorsal side and far from the top.

28: Positions of gap of endotesta and CPG are near the centre.

29: Seeds are symmetrical (with gap of endotesta and CPG at the top).

30: Seeds are slightly asymmetrical.

31: Seeds are highly asymmetrical.

32: Seeds are symmetrical (with gap of endotesta and CPG near the centre).

4.1.2 The hunt for additional characters in *Alpinia* systematics

Detailed studies of other morphological characters which were not used by Schumann, Holttum or Smith in their classifications were carried out in this thesis. These included the types of stomata, rhizomes, and pollen of both *Alpinia* and its outgroup. The form of the stigma was also examined following Smith's (1990a) observations that differing stigma types occur and they may strengthen the case for the classification of *Alpinia*.

(1). The stomata

The structure of the stomata in Zingiberaceae was previously described by Tomlinson (1956, 1969), Stebbins and Khush (1961) and Olatunji (1970, 1980). Stebbins and Khush (1961) grouped Zingiberaceae with other families of Zingiberales including Musaceae, Strelitziaceae and Cannaceae based on their type of stomatal complexes with two guard cells and four to six subsidiary cells around the guard cells in all four directions. Within Zingiberaceae Tomlinson (1956) reported that tetracytic stomata (stomata with two lateral and two terminal subsidiary cells associated with guard cells and are **distinct in size** from the other epidermal cells) were dominant but in 1969 he grouped the family with those whose stomata were paracytic (stomata with two visibly modified lateral subsidiary cells placed parallel to the guard cells but with terminal neighbouring cells which are **not different** from other epidermal cells). Based on detailed studies of the development of stomata Olatunji (1970) observed that tetracytic stomata were prevalent in Zingiberaceae.

(2). The rhizomes

The rhizomes of Zingiberaceae are always sympodial. Typically, each new stem-forming bud first extends horizontally underground, then the end of the stem

turns upwards producing an erect shoot which bears leaves or flowers, or both. Subsequently new growth comes by the development of one or more buds from nodes near the base of the erect shoot.

The structure of rhizomes is varied. According to Bell and Bryan (1991) two basic types of rhizome occur; those rhizomes which possess long and slender sympodial units are described as leptocaul, and those which have relatively short and bulky sympodial units are described as pachycaul. Studies of rhizomatous growth patterns have been recorded for several plants such as bamboos (McClure 1966); *Carex* and *Aegopodium* (Smirnova 1967); *Croomia* and *Thalassia* (Tomlinson 1970); *Lycopodium* (Primack 1973); *Ripogonum* (Tomlinson and Esler 1973); *Medeola* (Bell 1974); *Eleocharis* (Routledge 1986); and *Iris* (Kron and Stewart 1994). In Zingiberaceae and Costaceae the work was extensively carried out by two investigators namely, Hallé (1967, 1979) for *Aframomum, Costus*, *Hornstedtia*, *Amomum*, *Alpinia*, *Phaeomeria* (= *Etlingera*) and *Tapeinochilus*; and Bell (1979) for *Alpinia*. Bell (1979) observed that the branching system of the rhizome elements of *Alpinia speciosa* L. (*A. zerumbet* (Pers.) B.L.Burtt & R.M.Sm.) conformed to Tomlinson's model (**Figure 24**) as described for tree architecture by Hallé and Oldeman (1970, English translation by Stone 1975). It is possible that this model was derived from Holttum's drawing (**Figure 25**) based on his studies of the growth-habits of monocotyledons (Holttum 1955). In addition, the rhizomes of *A. speciosa* (*A. zerumbet*) formed repeated Y-shaped branching which produced near-perfect hexagonal grids (**Figure 26**). Such an organised structure of the hexagons was believed to provide the plants with economical exploration and exploitation of the substrate (Bell 1979). However, according to Bell and Tomlinson (1980) certain species in genera such as *Hedychium*, *Zingiber*, *Aframomum*, *Costus* and *Heliconia* developed near-linear branching systems instead of the Y-shaped rhizome patterns.

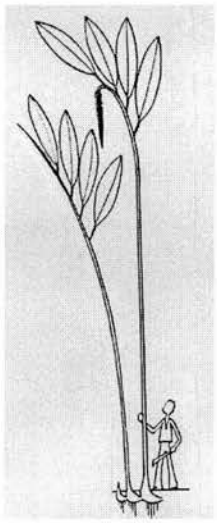


Figure 24. Tomlinson's model illustrates *Alpinia boia*, one of the largest gingers with the leafy shoots up to 10 m high arising from a massive underground rhizome. After Hallé *et al*. (1978).

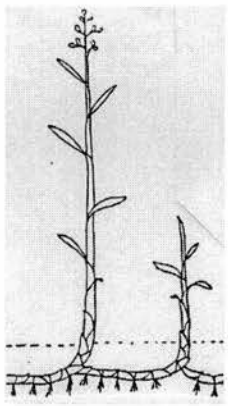


Figure 25. Holttum's drawing shows a regular rhizomatous growth with sympodial units and each erect stem ending in an inflorescence. After Holttum (1955).

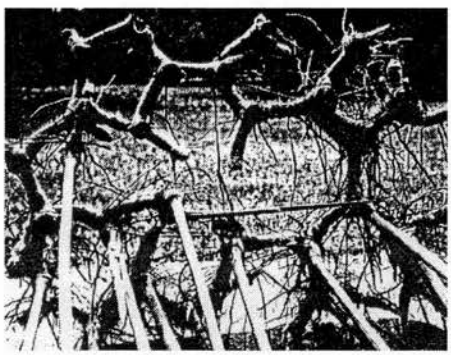


Figure 26. Bell's photograph of the rhizomes of *Alpinia zerumbet* with repeated Y-shaped branching which produced near-perfect hexagonal grids. After Bell (1976, 1979).

(3). The stigmas

The study of stigma morphology in Zingiberaceae was first carried out, to a small extent, by Valeton (1913, 1914). The topic was then much neglected as no one really paid attention to the form of the stigma. Smith (1990a) examined some fresh and spirit material of *Alpinia* stigmas (see section **4.1.1**), and discovered different types which, she believed, may be useful for classification if there was enough material for study.

(4). The pollen

Pollen morphology is important in the understanding of the taxonomy and phylogeny of the order Zingiberales (Dahlgren 1977; Dahlgren and Clifford 1982; Kress and Stone 1983; Dahlgren *et al.* 1985). Previously, it was believed that pollen grains of this order were exineless and inaperturate (Dahlgren and Clifford 1982). However, recent studies indicate that both an exine layer and aperturate pollen do exist within members of the order (Hesse and Waha 1982; Kress and Stone 1982; Skvarla and Rowley 1988; Chen 1989; Mangaly and Nayar 1990).

According to a study of the palynology of South Indian Zingiberaceae by Mangaly and Nayar (1990; Table 1, p. 355-357), pollen of the majority of taxa (*Alpinia*, *Amomum*, *Boesenbergia*, *Globba*, *Elettaria*, *Hedychium* and *Kaempferia*) was spheroidal or subspheroidal, and mostly inaperturate. In contrast, pollen of *Zingiber* and *Curcuma* which were ovoid to elliptic possessed a sulcate aperture (monosulcate). [However, in the case of *Zingiber*, Liang (1988) and Chen (1989) regarded the pollen grains as inaperturate. Their studies were supported by Theilade *et al.* (1993) who believed that aperture-like structures observed in a few grains were most probably artifacts caused by the SEM preparation procedures.] Mangaly and Nayar (1990) found that a very thin (reduced) layer of the exine occurred in all taxa, but it was totally lacking in *Kaempferia galanga* L. The possession of this much

reduced exine layer in Zingiberaceae causes problems using the standard palynological technique involving acetolysis. Mangaly and Nayar (1990) showed that a simple preparation which involved mounting the pollen directly in glycerine jelly or in water was useful. Based on their study, Mangaly and Nayar reported that in *Alpinia*, surfaces of the exine were either smooth (psilate pollen) or spiny (spinose pollen).

4.2 MATERIALS

For the study of the stomata (both abaxial and adaxial surfaces) mature leaves from living specimens (at E) or from voucher specimens were used. In the case of the rhizome study only fresh living specimens available at E were examined. While the material of the stigma was mainly obtained from the spirit collection, pollen material was obtained from the spirit collection as well as dried voucher specimens at E. Although there were some limits to the source of the material for the present morphological studies, it was hoped that results obtained may provide useful information for the classification of *Alpinia*. **Table 10** illustrates morphological characters examined for *Alpinia* and its outgroup.

Table 10. Morphological characters examined (✓) for *Alpinia* and its outgroup.

Taxon	Morphological characters examined			
	Stomata	Rhizomes	Stigmas	Pollen
Outgroup genera:				
Burbidgea	-	✓	-	✓
Elettariopsis	✓	✓	✓	✓
Pleuranthodium	✓	✓	✓	✓
Renealmia	✓	-	-	✓
Ingroup:				
Subgenus *Alpinia*				
Section *Alpinia*				
Subsection *Alpinia*	✓	✓	✓	✓
Subsection *Presleia*	✓	-	-	✓
Subsection *Paniculatae*	-	-	✓	✓
Subsection *Cenolophon*	✓	✓	✓	✓
Subsection *Catimbium*	✓	✓	✓	✓
Subsection *Probolocalyx*	-	-	✓	✓

Table 10 (continued).

Taxon	Morphological characters examined			
	Stomata	Rhizomes	Stigmas	Pollen
Section *Didymanthus*	-	-	-	-
Section *Kolowratia*	✓	-	✓	✓
Section *Fax*	✓	-	-	✓
Section *Guillainia*	✓	-	✓	✓
Section *Arctiflorae*	-	-	-	✓
Section *Allughas*				
Subsection *Allughas*	✓	-	✓	✓
Subsection *Odontychium*	-	-	-	-
Subsection *Strobidia*	✓	-	-	✓
Subsection *Caeruleae*	✓	-	✓	✓
Subgenus *Dieramalpinia*				
Section *Pycnanthus*				
Subsection *Pycnanthus*	-	-	-	-
Subsection *Amomiceps*	-	-	-	-
Section *Eubractea*	-	-	-	-
Section *Myriocrater*	-	-	-	✓
Section *Dieramalpinia*	✓	✓	✓	✓
Number of species studied	15	13	17	37

4.3 METHODS

4.3.1 The stomata (light microscopy; the method partly followed Olatunji (1980))

1. A portion of the mature leaf was boiled in 2% sodium hydroxide solution for about 5-10 min. This treatment allows the epidermis to be separated easily in most material.

2. The material was held down, with a pair of blunt forceps, on a piece of tile, irrigated with water. The tissues on top of the epidermis were scraped off carefully with a sharp scalpel until the epidermis underneath was reached.

3. The epidermis was mounted in distilled water on a slide to make a temporary preparation.

4. Shapes and sizes of the stomata were recorded and drawings and photographs were made using an axioskop microscope fitted with drawing and photographic attachments.

4.3.2 The rhizomes (external morphology)

1. The rhizomes were dug up and soil particles were removed using clean water.

2. Branching systems, sizes, colours and odours were noted and photographs were taken.

4.3.3 The stigmas (scanning electron microscopy)

The specimens obtained from the spirit collection were critical point dried, mounted, sputter-coated and then placed in the scanning electron microscope (SEM) for examination and photography.

(1). Critical point drying (CPD; using K 850 critical point dryer)

1. The specimens which were well fixed in the spirit were cut to an appropriate size that was small enough to fit into the CPD carrying basket.

2. The specimens were then taken through a series of increasing concentrations of ethanol (50%, 70%, 90%) and acetone (100%) for dehydration.

3. The basket was transferred swiftly into the pre-cooled CPD (5°C) chamber taking care not to let the specimens dry out.

4. The chamber was filled with liquid CO_2 while the pressure built to approximately 5515.8 kN/m^2 (800 psi).

5. The specimens were allowed to soak in the liquid CO_2 for about 5 min before the pressure was released (the exhaust valve was slowly opened) and the liquid CO_2 level dropped. The specimens were however, still covered at all times with the liquid CO_2.

6. The fluid exchange (steps 4 and 5) was repeated 10 times so that the acetone was completely removed from the specimens.

7. The chamber was again filled with the liquid CO_2 to the level marked with a red line and the temperature was increased to 35-40°C with the pressure of no more than 8963.2 kN/m^2 (1300 psi). At this stage all liquid CO_2 was converted into gaseous state (the Critical Point: critical temperature = 31.1°C, critical pressure = 7391.2 kN/m^2 (1072 psi)) without surface tension.

8. The CPD system was then slowly depressurised while maintaining the temperature.

9. Once the pressure dropped to zero the specimens were transferred to desiccant as soon as possible. At this point the specimens became highly hydroscopic and therefore, mounting and sputter-coating were done without delay to reduce the risk of damage.

(2). Mounting of the specimens

After critical point drying the specimens became brittle. They were handled carefully while mounting onto SEM aluminium stubs usually with carbon discs or

dye (silver in methyl isobutyl ketone) as a support and to reduce the background effect.

(3). Sputter-coating

1. The mounted specimens were transferred into the sputter coater chamber and argon was flushed through for 10 sec.

2. The chamber was evacuated to 9.3-13.3 N/m^2 (0.07-0.1 Torr) after which the purge light came on for 1 min.

3. Coating of the specimens in metal took place at a preset deposition rate (25 mA) and for a preset time (2.5 min). The coating produces a conductive surface preventing the build-up of negative charge which would otherwise cause image distortion.

(4). Scanning electron microscopy (Zeiss 962 SEM)

A scanning electron microscope produces a three dimensional image from secondary electrons reflected from the surface of a specimen. The image leads to a better understanding of the spatial relations of features and can reveal unsuspected details (Heywood 1971). Heywood (1971, 1984) had foreseen the impact of SEM in the study of taxonomic characters of pollen grains and spores, leaf surfaces (especially stomatal architecture), and seed and fruit surfaces which is important in plant systematics and evolution. In this present study of *Alpinia* the SEM was employed to examine the form of the stigma and the type of hair cells. The method of the SEM is as follows.

Procedure:

1. With the supply of nitrogen the system was ventilated before mounted specimens were placed in the SEM chamber.

2. The system was then evacuated for several minutes before the high voltage and filament buttons were switched on. The high voltage (5 kV) together

with the working distance (12 mm) were adjusted to attain the optimum resolution of the image.

3. In the scanning mode the magnification, focus, and contrast/brightness were adjusted.

4. The image was stored in memory and the output was obtained through a video graphic printer.

4.3.4 **The pollen** (light microscopy)

1. The pollen grains from the spirit collection and dried voucher specimens were mounted directly in distilled water on a slide to make a temporary preparation.

2. The specimen was observed under the axioskop microscope. Shapes, sizes and external structures of the pollen were recorded and photographs were taken.

4.3.5 The use of MacClade (version 3.01; Maddison and Maddison 1992) to analyse phylogeny and character evolution of *Alpinia*

MacClade (Maddison and Maddison 1992) and PAUP (Swofford 1991) programmes were designed to achieve compatibility and complementarity. Following a search strategy for parsimonious trees in PAUP, MacClade is generally used to explore the trees further and analyse character evolution upon them. The programme can be used to combine different sets of data, for instance to use a molecular tree in order to determine the dynamics of evolution of morphological data. In this present study of *Alpinia* a strict consensus tree of the ITS region of 57 taxa of Zingiberaceae obtained from PAUP* was imported into MacClade. Distinctive characters of the bract, bracteole, labellum, pollen, fruit and seed were coded as unordered, and binary- or multi-state characters with equal weight, in MacClade. These characters were mapped onto the ITS cladogram (with no

optimisation) to trace character evolution along the branches and to confirm or deny the classification of *Alpinia* based on the ITS region.

CHAPTER 5: RESULTS OF MORPHOLOGICAL APPROACHES TO THE STUDY OF THE INFRAGENERIC CLASSIFICATION OF *ALPINIA*

5.1 ADDITIONAL CHARACTERS OF *ALPINIA* AND ITS OUTGROUP

5.1.1 The stomata

For 15 species examined across 14 taxa of *Alpinia* and its outgroup (see **Table 10**), it was observed that the leaves were amphistomatic, although the stomata were usually much more abundant on the abaxial than the adaxial epidermis. The SEM result (**Figure 27**) showed that the stomata were regularly orientated with the guard cells placed more or less parallel to the long axes of the veins. From my observations of the stomata of these 15 species (e.g. in **Figure 28 A-H**), there seemed to be no other recognisable cells which were clearly distinct from epidermal cells except for the two visibly modified lateral subsidiary cells (L.S.C.). Thus, it appeared that these species have mainly paracytic stomata. However, according to Olatunji (1970) tetracytic stomata were dominant in Zingiberaceae. His study of the developmental processes yielded evidence to support this. In his unpublished Ph.D. thesis Olatunji (1970) pointed out the fact that the true relationships of terminal subsidiary cells (T.S.C.) especially of tribe Alpineae, were concealed in mature stomata, therefore, any investigator examining the mature epidermis alone might take the stomatal complex as paracytic.

In general, the nature, the size and shape of the stomata were constant throughout the species examined. Their sizes, including the guard cells, ranged approximately from 20 to 30 μm. All the guard cells were bean-shaped, and the epidermal cells were more or less hexagonal with straight walls. However, in *Alpinia rafflesiana* and *A. javanica* (**Figure 29 A** and **B**) there were some interesting features which are maybe partly accounted for by the close relationship of the two species. Results from the present study showed that both species had (i) no significant difference in the stomatal densities on the two surfaces of the epidermis

(i.e. in one mm^2 there were 30-43 stomata on the abaxial surface and 28-39 stomata on the adaxial surface), (ii) cuticular striations radiating out over the lateral subsidiary cells, and (iii) numerous unbranched hairs on the upper and lower surfaces of the epidermis.

Figure 27. A photograph from the SEM shows regularly orientated stomata with guard cells parallel to the long axes of the veins on abaxial epidermis of *Alpinia suishaensis* (× 200).

Figure 28. The appearance of mature stomata from the leaf (× 40) under the axioskop microscope. **A**: *Renealmia battenbergiana*; **B**: *Alpinia galanga*; **C**: *A. nigra*; **D**: *A. conchigera*; **E**: *A. oxyphylla*; **F**: *A. zerumbet*; **G**: *A. elegans*; **H**: *A. oceanica*. G.C. = guard cells; L.S.C. = lateral subsidiary cells; T.S.C. = terminal subsidiary cells.

Figure 29. The appearance of mature stomata from the leaf (× 20) under the axioskop microscope of **A**: *Alpinia rafflesiana* and **B**: *A. javanica*. C.S. = cuticular striations (radiating out over the lateral subsidiary cells); H. = unbranched hairs.

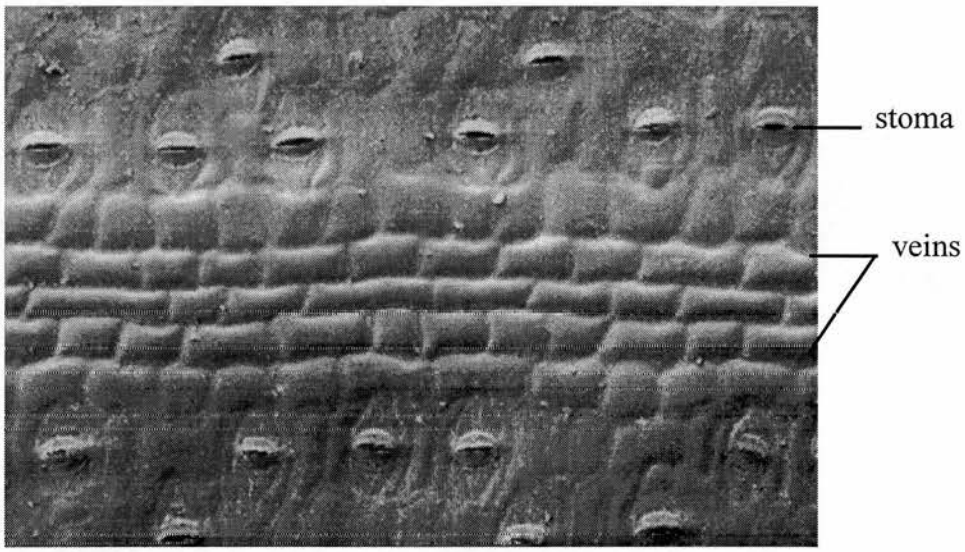


Figure 27.

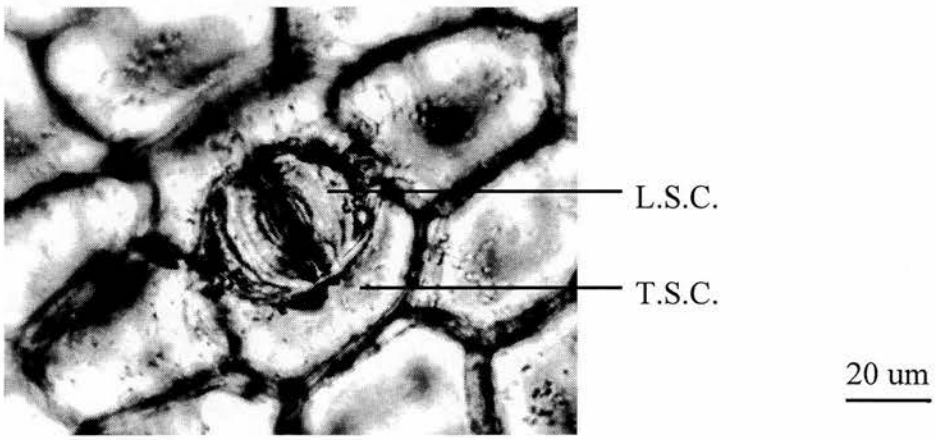


Figure 28 A.

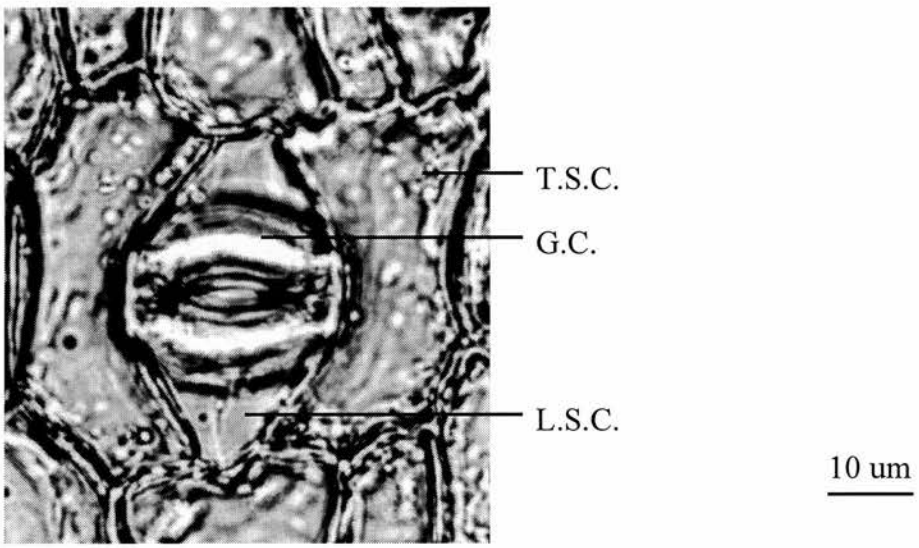


Figure 28 B.

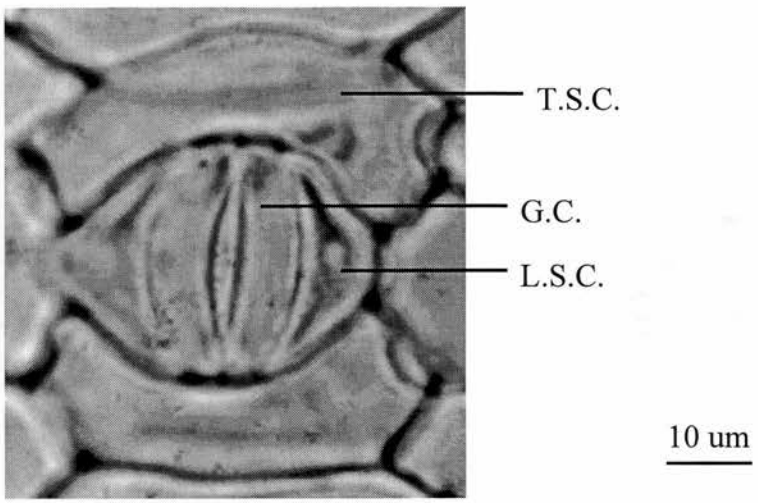


Figure 28 C.

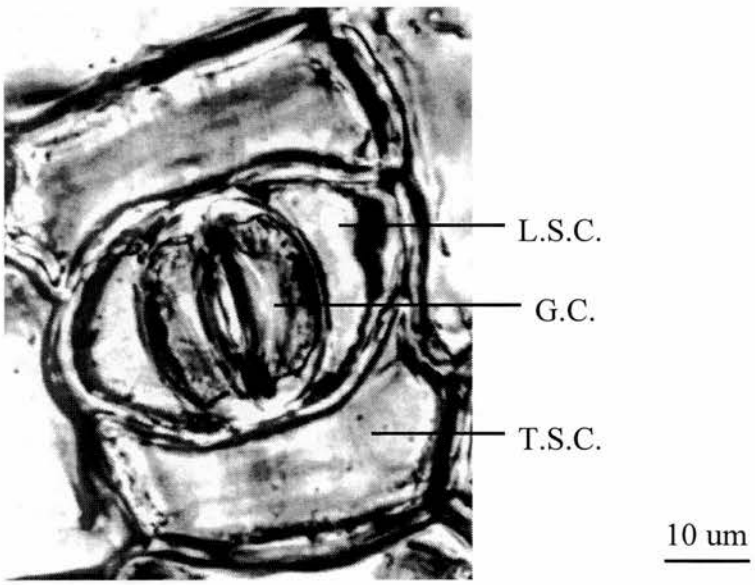


Figure 28 D.

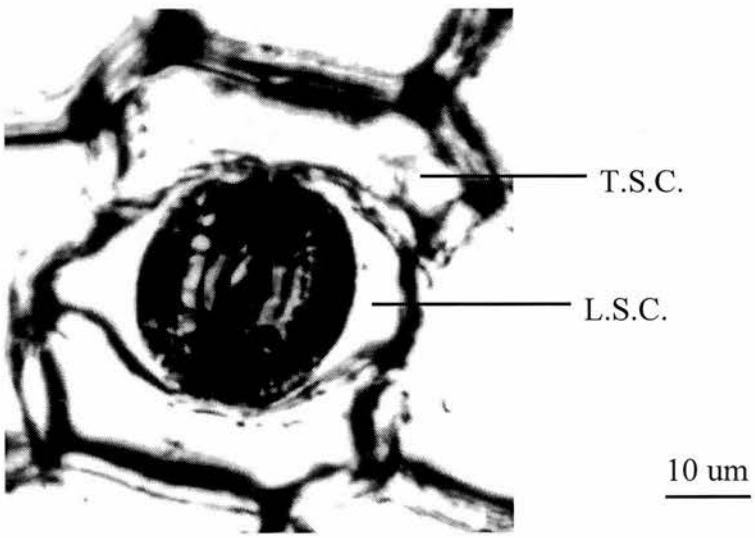


Figure 28 E.

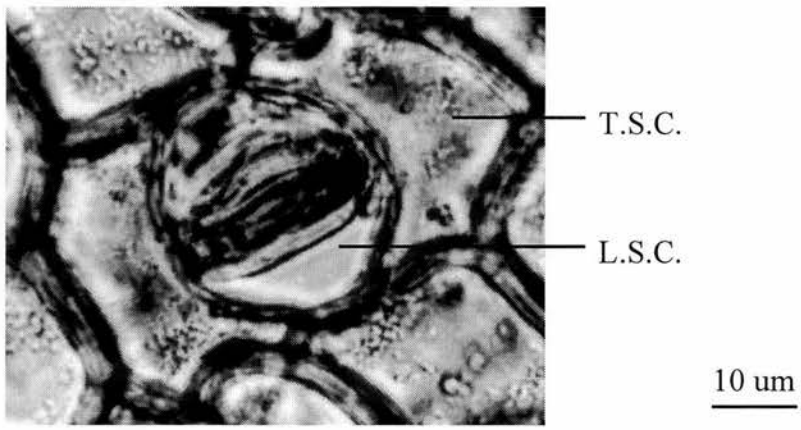


Figure 28 F.

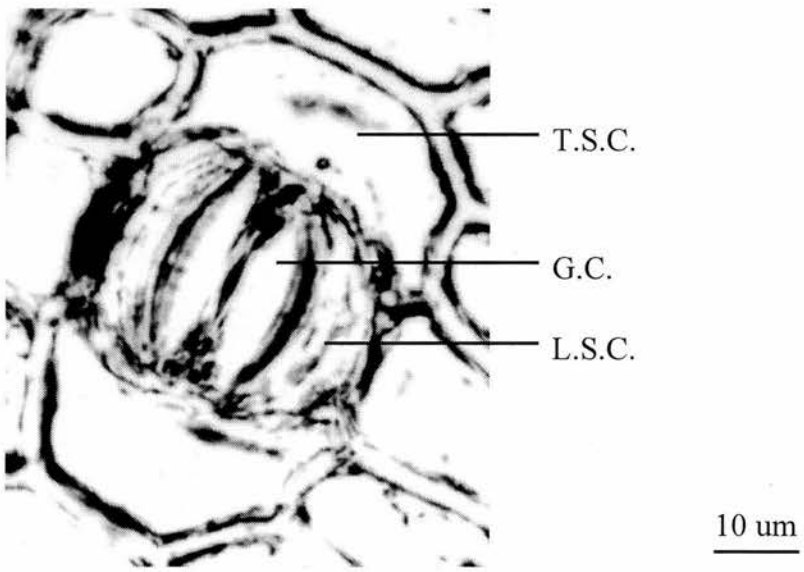


Figure 28 G.

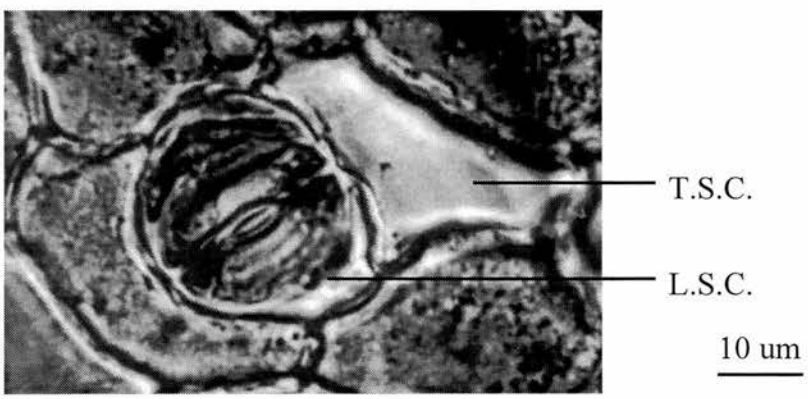


Figure 28 H.

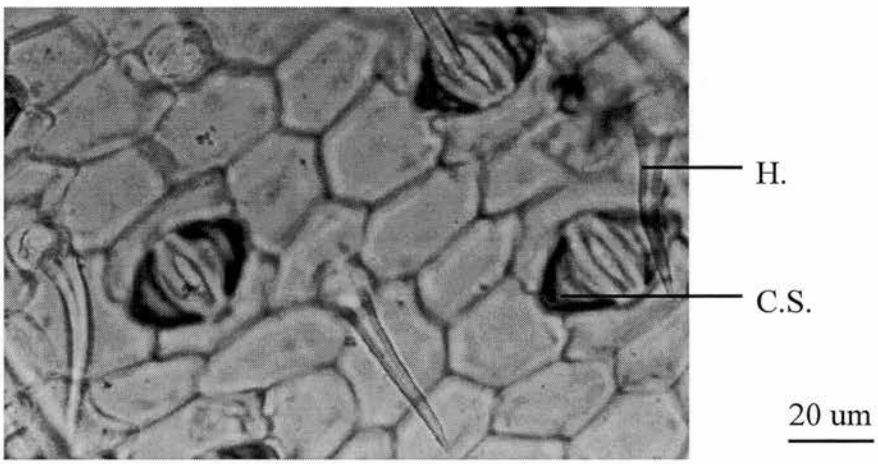


Figure 29 A.

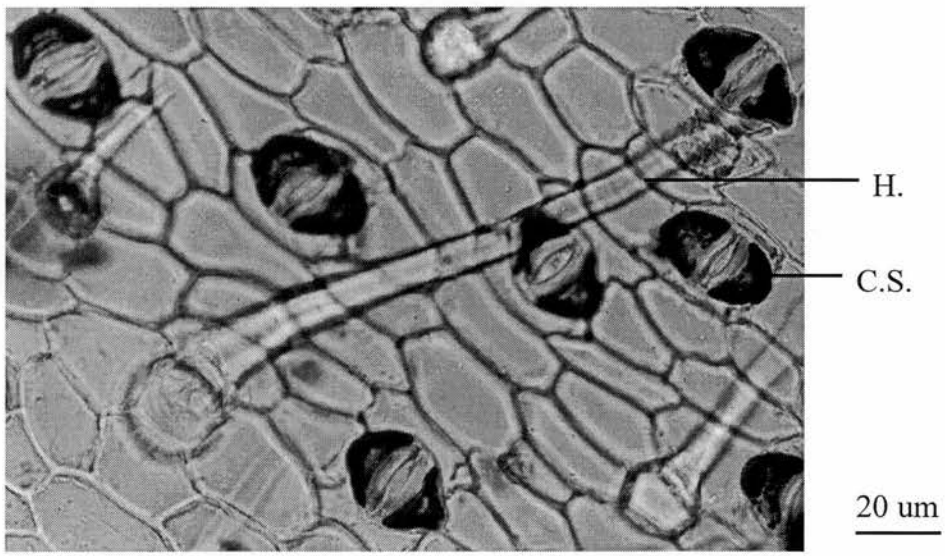


Figure 29 B.

5.1.2 The rhizomes

All rhizomes of the 13 species from 7 taxa of Zingiberaceae under study (see **Table 10**) possessed distichous scale leaves at the nodes. Most rhizomes contained dense root systems which held the soil particles tightly. The length of rhizome produced by each new growth appeared to be characteristic of each species. However, among different species of *Alpinia* as well as different genera (*Burbidgea*, *Elettariopsis* and *Pleuranthodium*) the rhizomes varied considerably in size (also depending on their age). Older rhizomes usually had yellowish brown colour while younger ones were pinkish white. All rhizomes under study had an aromatic smell, except those of *Burbidgea schizocheila* which were odourless. The direction of the growth of rhizomes in all taxa examined was transverse to the plane of distichy of the leaves. Aerial, leafy shoots arising from underground rhizomes of *Alpinia*, *Burbidgea* and *Pleuranthodium* had terminal inflorescences, while the inflorescences of *Elettariopsis* developed at the base of the leafy shoots at ground level.

The study of rhizome architecture is commonly ignored merely because the excavation of plants in a large area in the plot is time consuming and usually requires manpower. In this study, rhizomes of the 13 species of *Alpinia* and its outgroup were obtained from plants grown in pots (some excavations were carried out from very large pots and one example is shown in **Figure 30)**. Since rhizome growth patterns can be the result of genetically determined developmental instructions interacting with environmental factors (reviewed in Cain and Cook 1988; Stoll *et al.* 1998), the rhizomes of plants grown in the pots (under artificial conditions) may have structures which deviate from those found in nature. Nonetheless, the material used in this study revealed three basic types of rhizome.

(1). Linear and leptocaulous rhizomes

The sympodial units did not branch and they were slender and wide-creeping, so that the plants were rather spread out. Rhizomes of this type were observed in *Elettariopsis unifolia* (**Figure 31**) and *Pleuranthodium papilionaceum*.

(2). Linear and pachycaulous rhizomes

The sympodial units did not branch and they were relatively thick and short, so the leafy shoots which they produced were clumped. This type of rhizome was observed in *Burbidgea schizocheila* (**Figure 32 A**), *Alpinia galanga* (**Figure 32 B**) and *A. oceanica*.

(3). Y-shaped rhizomes

Two daughter branches developed in the horizontal plane at either side of the distal end of the plagiotropic portion of the parent branch. It was observed that the majority of the Zingiberaceae under study (8 species of *Alpinia*; e.g. in **Figure 33 A-D**) possessed this type of rhizome. However, these Y-shaped patterns did not always lead to the formation of hexagonal grids as shown in Bell's example (see **Figure 26)**.

Figure 30. The excavation of rhizomes of *Alpinia* sp. (subsection *Catimbium*) from a large pot. Scale: marker measuring 14 cm.

Figure 31. Linear and leptocaulous rhizomes of *Elettariopsis unifolia* (scale: centimetres).

Figure 32. Linear and pachycaulous rhizomes. **A**: *Burbidgea schizocheila* (scale: centimetres); **B**: *Alpinia galanga* (scale: marker measuring 14 cm).

Figure 33. Y-shaped rhizomes. **A**: *Alpinia suishaensis* (lateral view); **B**: *A. suishaensis* (top view) (scale: marker measuring 14 cm); **C**: *A. malaccensis*; **D**: *Alpinia* sp. (subsection *Catimbium*).

Figure 30.

Figure 31.

Figure 32 A.

Figure 32 B.

Figure 33A.

Figure 33B.

Figure 33C.

Figure 33D.

5.1.3 The stigmas

Across 12 different taxa of *Alpinia* and its outgroup (see **Table 10**), 17 species were examined for the form of the stigma and the type of hair cells using the SEM. Ideal material (14) taken from spirit collection produced good results with well preserved structures of the stigma (e.g. in **Figure 34 A-L**). However, due to the lack of this type of material in most species of *Alpinia*, the use of herbarium specimens (3) was attempted. These specimens were revived in water which was brought to the boil, then they underwent similar steps for the spirit material used in the SEM preparation procedures (see section **4.3.3**). Results showed some distortions of the stigmas, and damage of the hair cells was clearly observed (e.g. in **Figure 35**). Therefore, to avoid any misleading results caused by the distortions, only the results obtained from the spirit material will be interpreted and discussed.

All the stigmas examined were non-papillate, and their sizes measured from the longest axis of the orifice ranged approximately from 0.2 mm (for *Pleuranthodium papilionaceum,* **Figure 34 A**) to 3.0 mm (for *Alpinia elegans,* **Figure 34 I**). Except for *Pleuranthodium papilionaceum* stigma which was non-ciliated, all the other stigmas had smooth and unbranched hairs (e.g. in **Figure 36**) mostly along the edge of the orifice and styles (but in certain species, for example, *Alpinia zerumbet* (**Figure 34 H**) dense hairs covered the whole body of the stigma).

The shape of the stigmas was very diverse throughout the species examined. *Pleuranthodium papilionaceum* (including all other *Pleuranthodium* species - reviewed in Smith 1991) had a clavate, geniculate stigma with a very small orifice (**Figure 34 A**). *Elettariopsis triloba* (and all other species of this genus - reviewed in Kam 1982) on the other hand, had distinct characteristics of the stigma with broad triangular ciliate mouth (**Figure 34 B**). Within *Alpinia*, the stigmas were more or less obconical or funnel-shaped. Based on the structure and position of the orifice alone, two basic types of stigma were recognised.

(1). Stigmas with relatively wide open orifices placed apically or subapically

The stigmas of this type were observed in the majority of the specimens studied. All of them belonged to Smith's (1990a) subgenus *Alpinia*. Examples are given in **Figure 34 C-J**.

(2). Stigmas with long and narrow orifices placed on the flattened surface

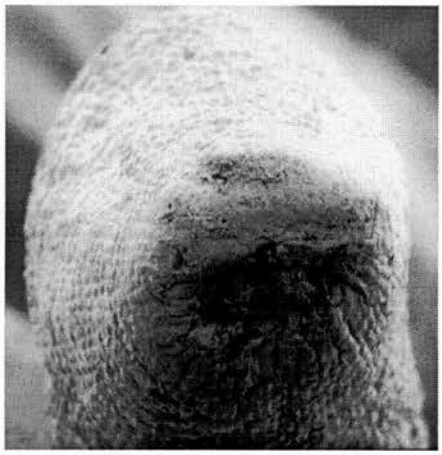
From this study it was observed that both *Alpinia oceanica* and *A. vittata*, the only two well preserved specimens available from Smith's (1990a) subgenus *Dieramalpinia*, possessed this type of stigma (**Figure 34 K-L**).

The results obtained indicated some support for Smith's (1990a) observations at subgeneric level. However, as with Smith, I encountered the same problem of lack of good material for the study. The specimens used did not represent the whole range of Smith's infrageneric classification of *Alpinia*. Therefore, it is not yet certain if the character of the stigma is in fact useful for the classification.

Figure 34. The appearance of stigmas in the SEM (numbers in brackets at the end of species names indicate approximate sizes of the stigma measured from the longest axis of the orifice). **A**: *Pleuranthodium papilionaceum* (0.2 mm); **B**: *Elettariopsis triloba* (2.1 mm); **C**: *Alpinia suishaensis* (0.5 mm); **D**: *A. galanga* (1.0 mm); **E**: *A. nigra* (0.7 mm); **F**: *A. caerulea* (0.5 mm); **G**: *A. oxyphylla* (0.9 mm); **H**: *A. zerumbet* (1.6 mm); **I**: *A. elegans* (3.0 mm); **J**: *A. purpurata* (0.6 mm); **K**: *A. oceanica* (1.7 mm); **L**: *A. vittata* (2.2 mm).

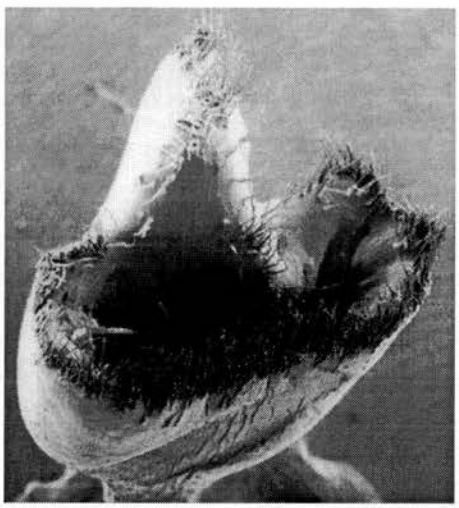
Figure 35. A photograph from the SEM of the stigma from a herbarium specimen of *Alpinia ligulata*. The result shows some distortions of the stigma and damage of the hair cells.

Figure 36. A photograph from the SEM shows smooth and unbranched hairs (approximate length: 74-103 μm) on the margin of the orifice of *Alpinia elegans*.



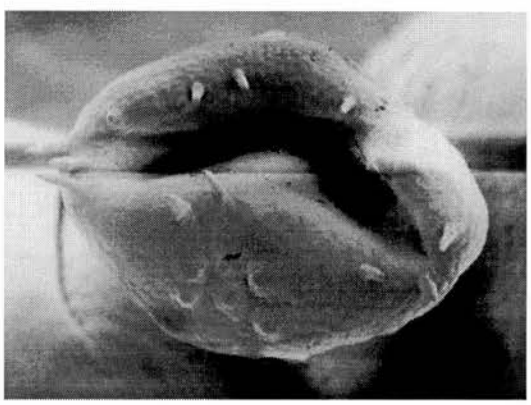
0.1 mm

Figure 34 A.



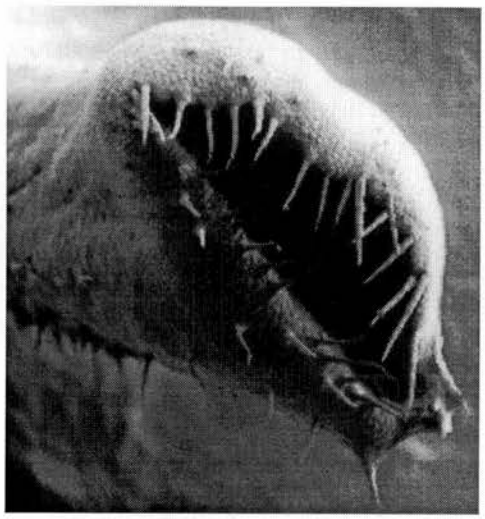
0.42 mm

Figure 34 B.



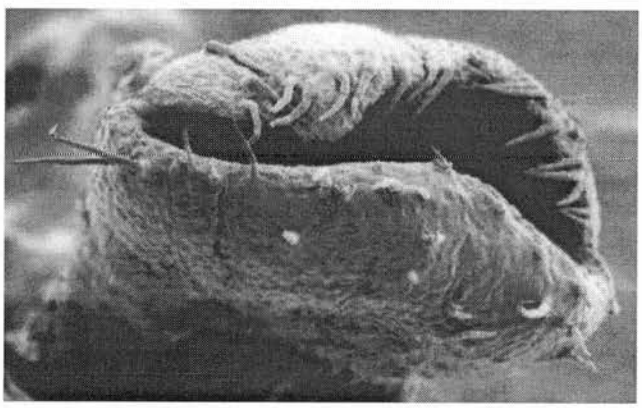
0.25 mm

Figure 34 C.



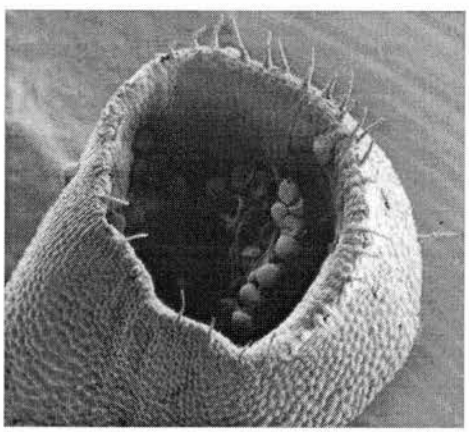
0.2 mm

Figure 34 D.



0.17 mm

Figure 34 E.

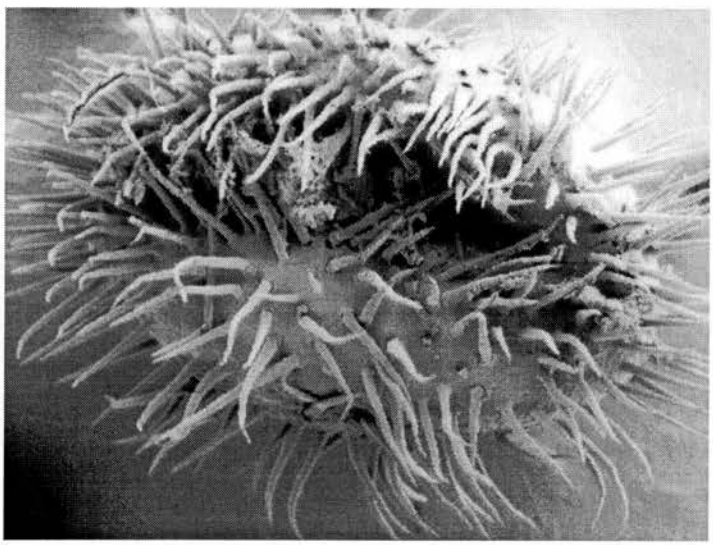


0.25 mm

Figure 34 F.

0.2 mm

Figure 34 G.

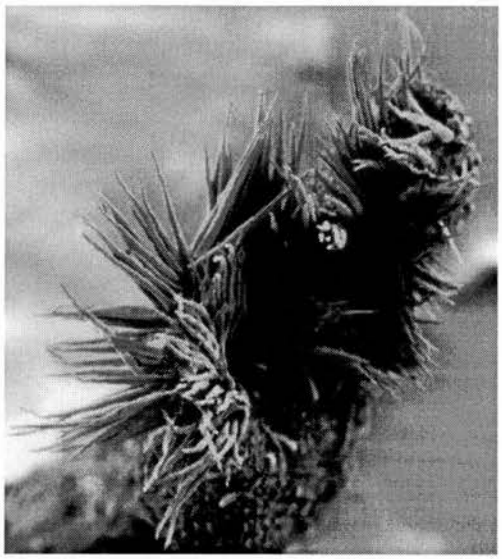


0.27 mm

Figure 34 H.

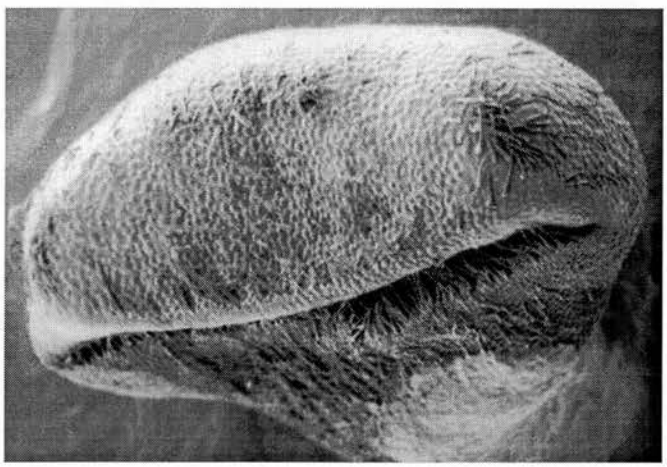
0.35 mm

Figure 34 I.



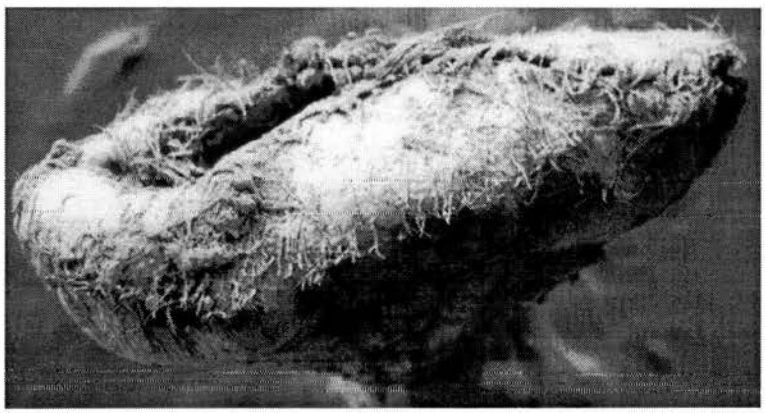
0.12 mm

Figure 34 J.



0.24 mm

Figure 34 K.



0.3 mm

Figure 34 L.

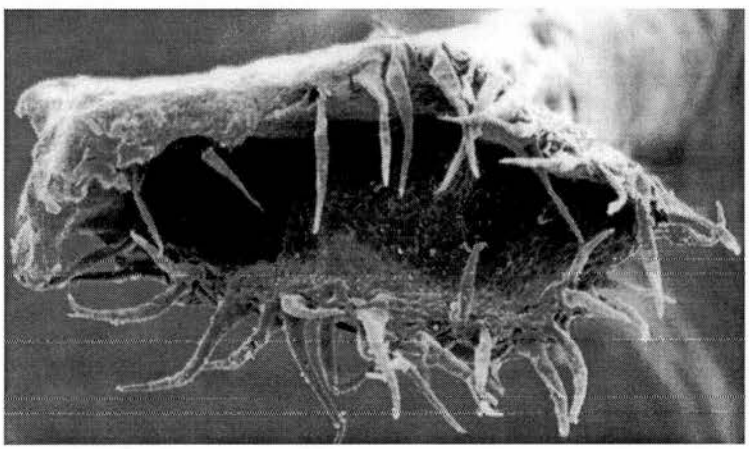


Figure 35.

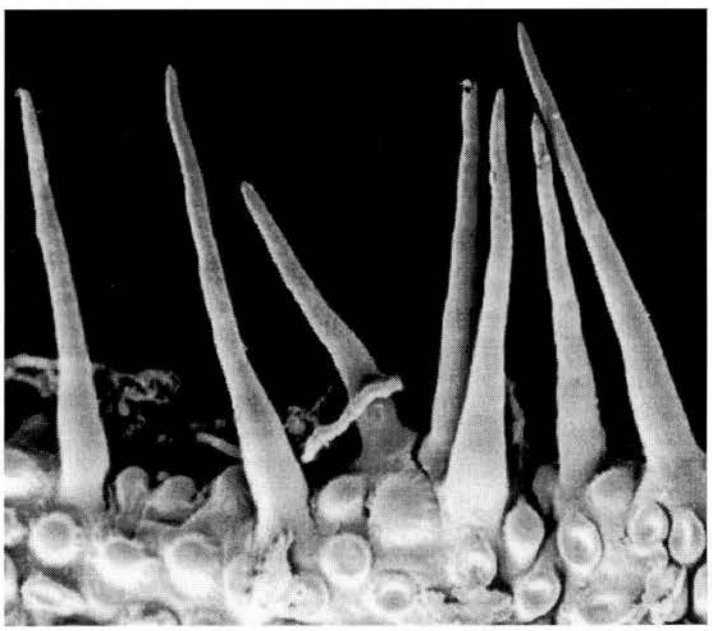


Figure 36.

5.1.4 The pollen

The pollen morphology was observed from 37 species encompassing 19 taxa of Zingiberaceae (see **Table 10**). Most of the pollen were spheroidal, but some were subspheroidal to ovoid (e.g. in **Figure 37 E** of *Alpinia carolinensis*). The average size (measured from the equatorial diameter) taken from 10 readings of pollen grains selected at random for each species ranged approximately from 60 to 120 μm. The pollen was inaperturate, and based on the external structure of the exine, it was divided into two types: smooth and spiny. Results obtained from this study are in accordance with those reported by Mangaly and Nayar (1990).

(1). Smooth (psilate) pollen

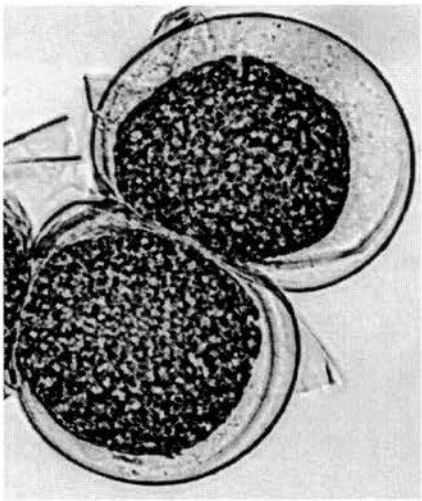
It was observed that outgroup species including *Elettariopsis unifolia*, *Burbidgea schizocheila*, *Pleuranthodium papilionaceum* and *Renealmia battenbergiana* possessed this type of pollen. Within *Alpinia*, smooth pollen grains occurred in both subgenera *Alpinia* and *Dieramalpinia* of Smith (1990a). Of 37 species studied, 17 had smooth pollen grains (see **Table 12** and **Figure 44**). Examples are given in **Figure 37** (**A-F**).

(2). Spiny (spinose) pollen

From the study it was found that most species in section *Alpinia* subsections *Catimbium*, *Paniculatae*, *Presleia*, *Alpinia*, *Cenolophon* and *Probolocalyx* and a few species in section *Allughas* had spiny pollen. Of 37 species studied, 20 possessed this type of pollen (see **Table 12** and **Figure 44**). Examples are shown in **Figure 38** (**A-F**).

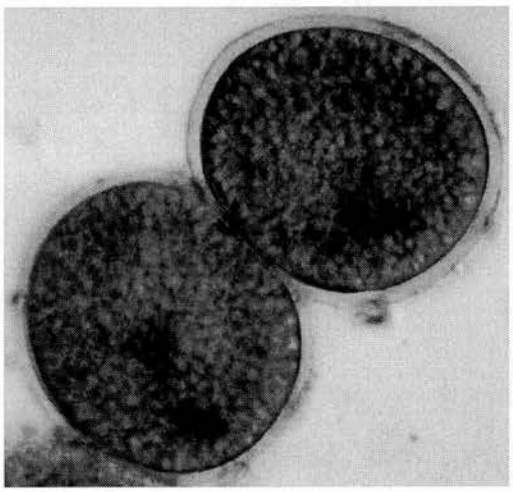
Figure 37. Smooth (psilate) pollen grains (× 40) under the axioskop microscope. **A**: *Alpinia modesta*; **B**: *A. caerulea*; **C**: *A. purpurata*; **D**: *A. elegans*; **E**: *A. carolinensis*; **F**: *A. vittata*.

Figure 38. Spiny (spinose) pollen grains (× 40) under the axioskop microscope. **A**: *Alpinia suishaensis*; **B**: *A. galanga*; **C**: *A. nigra*; **D**: *A. ligulata*; **E**: *A. blepharocalyx* var. *glabrior*; **F**: *A. calcarata*.



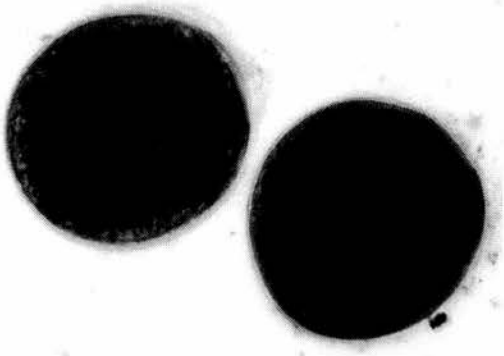
20 um

Figure 37 A.



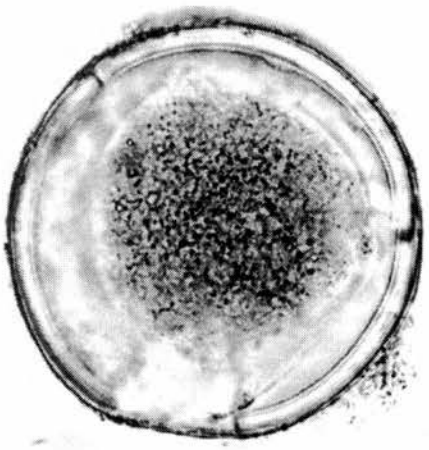
20 um

Figure 37 B.



23.4 um

Figure 37 C.

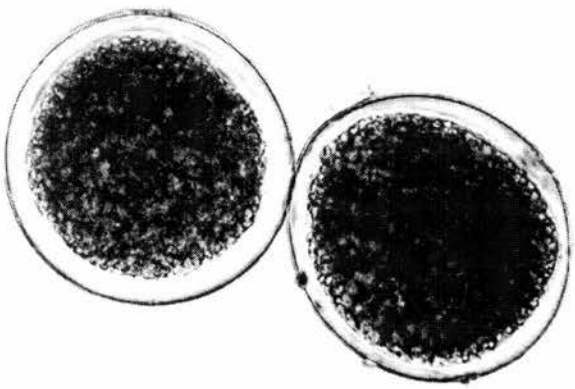


15 um

Figure 37 D.

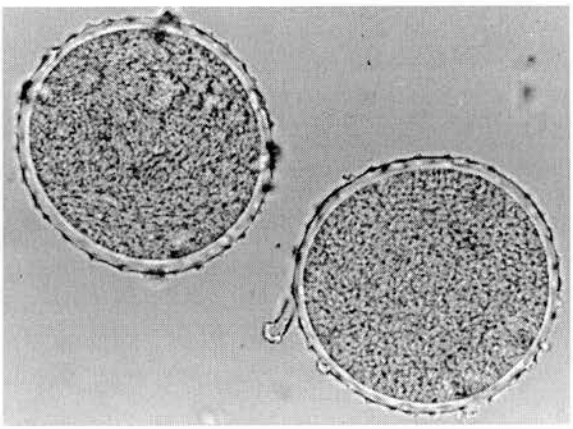
14.6 um

Figure 37 E.



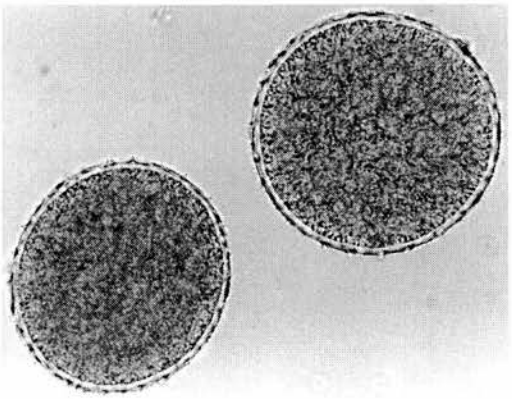
23.7 um

Figure 37 F.



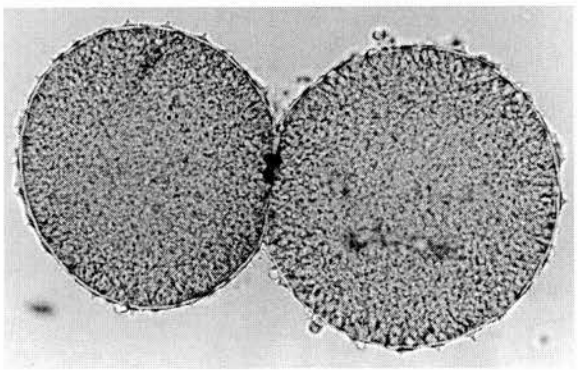
23.5 um

Figure 38 A.



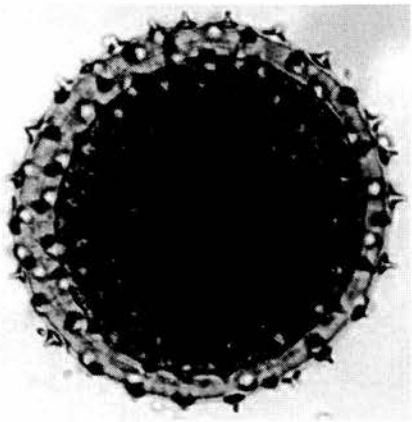
62.5 um

Figure 38 B.



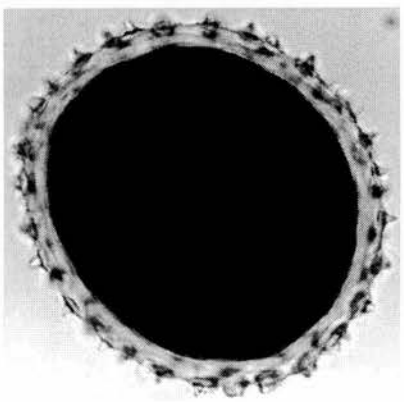
32.4 um

Figure 38 C.



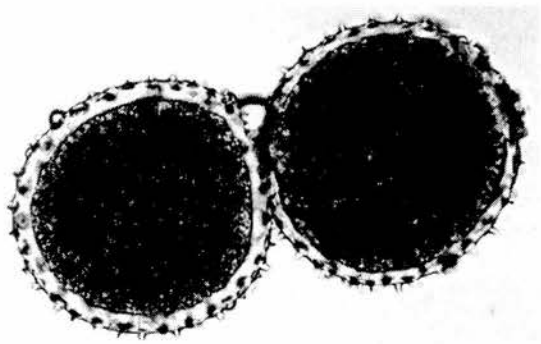
16 um

Figure 38 D.



17.8 um

Figure 38 E.



24.2 um

Figure 38 F.

5.2 THE USE OF MACCLADE TO ANALYSE PHYLOGENY AND CHARACTER EVOLUTION OF *ALPINIA*

5.2.1 Characters and character states

Binary- or multi-state characters were defined for the distinctive characteristics of the bract, bracteole, labellum, pollen, fruit and seed of *Alpinia* and its outgroup (see **Table 11**). Coded data (**Table 12**) were obtained and employed in MacClade to map different character states onto the ITS strict consensus tree (**Figure 18**). Results of the mapping are shown in **Figures 39-45**.

Table 11. Character states for *Alpinia* and its outgroup in the study of character evolution using MacClade.

1. Presence of bract
   0: absent; 1: present
2. Type of bract
   0: soon deciduous (or caducous after anthesis); 1: persistent
3. Presence of bracteole
   0: absent; 1: present
4. Type of bracteole
   0: tubular (cup-shaped); 1: non-tubular (open to the base); 2: tubular and non-tubular (in the same inflorescence)
5. Type of labellum
   0: petaloid; 1: non-petaloid; 2: non-petaloid (except apex expanding into a small petaloid area); 3: petaloid or non-petaloid
6. Type of pollen
   0: smooth; 1: spiny
7. Type of fruit and seed (see **Table 9** for details)
   0: "Polyantha" type; 1: "Zerumbet" type; 2: "Conchigera" type;
   3: "Oxyphylla" type
   (Note: The fruit and seed data are entirely based on Liao and Wu 1996a, b)

Table 12. Coded data using the character states (from **Table 11**) for morphological characters of *Alpinia* and its outgroup. (Data based on Hepper 1968; Kam 1982; Smith 1975, 1985, 1990a, 1991; Liao and Wu 1996a, b; Newman 1997; various original papers appeared in Smith 1990a; and my own observations of the specimens). Question marks indicate data insufficient and hyphens indicate characters inapplicable.

Character	Bract		Bracteole		Labellum	Pollen	Fruit and Seed
Taxon	Presence	Type	Presence	Type	Type	Type	Type
Burbidgea schizocheila	0	-	0	-	1	0	?
Elettariopsis unifolia	1	1	1	1	0	0	?
Pleuranthodium floccosum	?	?	?	?	0	?	?
Pleuranthodium floribundum	?	?	?	?	0	?	?
Pleuranthodium papilionaceum	1	0	1	1	0	0	?
Pleuranthodium racemigerum	1	0	1	1	0	?	?
Pleuranthodium schlechteri	?	?	?	?	0	?	?
Renealmia aff. *africana*	1	1	1	0	0	?	?
Renealmia battenbergiana	1	1	1	0	0	0	?
Alpinia abundiflora	1	1	1	2	0	0	?
A. aquatica	1	0	?	?	0	?	?

Table 12 (continued).

Character	Bract		Bracteole		Labellum	Pollen	Fruit and Seed
Taxon	Presence	Type	Presence	Type	Type	Type	Type
A. arctiflora	1	1	1	0	0	0	?
A. arundelliana	1	?	1	0	0	?	?
A. blepharocalyx var. *blepharocalyx*	0	-	1	1	0	?	?
A. blepharocalyx var. *glabrior*	0	-	1	1	0	1	?
A. boia	1	1	1	0	1	?	?
A. brevilabris	1	0	1	1	0	?	?
A. caerulea	1	1	1	0	0	0	?
A. calcarata	0	-	1	1	0	1	1
A. carolinensis	1	1	1	0	1	0	?
A. coeruleoviridis	?	?	1	0	1	?	?
A. conchigera	1	0	1	0	0	1	2
A. coriacea	1	1	1	1	0	?	?

Table 12 (continued).

Character	Bract		Bracteole		Labellum	Pollen	Fruit and Seed
Taxon	Presence	Type	Presence	Type	Type	Type	Type
A. elegans	1	1	1	0	3	0	?
A. eubractea	1	1	1	0	2	?	?
A. fax	1	1	1	0	0	0	?
A. flabellata	1	0	?	?	0	1	?
A. formosana	0	-	1	1	0	1	?
A. foxworthyi	1	0	?	?	0	1	?
A. galanga	1	0	1	1	0	1	2
A. glabra	1	0	1	1	0	1	?
A. intermedia	1	0	1	1	0	1	?
A. japonica	1	0	1	1	0	?	0
A. javanica	1	0	1	0	0	0	?
A. kwangsiensis	0	-	1	1	0	?	1
A. latilabris	0	-	1	1	0	1	?
A. ligulata	1	0	0	-	0	1	?

Table 12 (continued).

Character	Bract		Bracteole		Labellum	Pollen	Fruit and Seed
Taxon	Presence	Type	Presence	Type	Type	Type	Type
A. luteocarpa (*scorpoidea*)	1	1	1	0	?/-	0	?
A. maclurei	1	0	1	1	0	?	0
A. malaccensis	0	-	1	1	0	1	1
A. modesta	1	1	1	0	0	0	?
A. mutica	0	-	1	1	0	1	?
A. nieuwenhuizii	1	0	0	-	0	1	?
A. nigra	1	0	1	0	0	1	2
A. oceanica	1	1	1	0	1	0	?
A. officinarum	1	0	0	-	0	?	3
A. oxymitra	1	0	0	-	0	1	?
A. oxyphylla	1	0	0	-	0	1	3
A. polyantha	1	0	1	1	0	?	0
A. pumila	1	1	0	-	0	?	0
A. purpurata	1	1	1	0	0	0	?

Table 12 (continued).

Character	Bract		Bracteole		Labellum	Pollen	Fruit and Seed
Taxon	Presence	Type	Presence	Type	Type	Type	Type
A. rafflesiana	1	0	1	0	0	?	?
A. aff. *shimadai*	?	?	0	-	0	1	?
A. suishaensis	1	0	1	1	0	1	0
A. vittata	1	1	1	0	1	0	?
A. vulcanica	1	1	1	0	1	0	?
A. zerumbet	0	-	1	1	0	1	1

5.2.2 Inferences of *Alpinia* character evolution based on ITS data and nonmolecular features (morphology) with reference to Smith's (1990a) classification

(1). The presence or absence of the bract

The mapping of the presence or absence of the bract onto the ITS strict consensus tree required three evolutionary steps. The absence of the bract distinguished the *A. zerumbet* clade from all other *Alpinia* clades in **Figure 39**. This clade comprises Smith's (1990a) section *Alpinia* subsection *Catimbium* (*A. formosana*, *A. kwangsiensis*, *A. zerumbet*, *A. blepharocalyx* var. *glabrior*, *A. blepharocalyx* var. *blepharocalyx*, *A. malaccensis*, *A. latilabris*, *A. calcarata* and *A. mutica*) except *A.* aff. *shimadai* (data insufficient) and *A. officinarum* which belong to the same section but in subsection *Cenolophon*. Based on this result it appeared that the common ancestor of the species under study possessed bracts. During the course of evolution the bracts were completely lost in subsection *Catimbium*. This subsection could form a natural group with its remarkable characteristic of large flowers with showy labella.

(2). The type of the bract (soon deciduous, persistent)

Persistent bracts appeared to have evolved several times independently within the species of Zingiberaceae studied. With a few exceptions, this character appears to be congruent with ITS data. **Figure 40** shows that the *A. eubractea* clade which consists of the species from both Smith's (1990a) subgenera *Alpinia* (*A. arctiflora*, *A. elegans*, *A. arundelliana*, *A. modesta*, *A. caerulea* and *A. purpurata*) and *Dieramalpinia* (*A. eubractea*, *A. luteocarpa* (*scorpoidea*), *A. oceanica* and *A. vittata*) forms a natural group on the basis of the presence of the persistent bract (although data were insufficient for *A. arundelliana*). In addition, three species in the *A. carolinensis* clade (*A. carolinensis*, *A. boia* and *A. coeruleoviridis* (data

insufficient)) from subgenus *Dieramalpinia* were united based on the same character. Two closely related taxa in the *A. fax* clade (*A. abundiflora* and *A. fax*) and two African *Renealmia* species also had this type of bract.

(3). The presence or absence of the bracteole

The majority of the species examined in this study possessed bracteoles. Therefore, mapping of the bracteole character (presence or absence) onto the ITS strict consensus tree (**Figure 41**) did not show a significant value in understanding phylogeny and classification of *Alpinia* except that the absence of the bracteole united two closely related species, *A. nieuwenhuizii* and *A. ligulata* (Smith's (1990a) section *Alpinia* subsection *Paniculatae*) together. Although similar character (the absence of the bracteole) is essential in Smith's classification of section *Alpinia* subsection *Cenolophon*, the grouping of the species in this subsection does not exist in the ITS cladogram. In the *A. aquatica* clade no bracteoles were seen in any material studied of the species from Smith's (1990a) section *Alpinia* subsection *Presleia*, with the exception of *A. brevilabris* whose bracteoles are very small.

(4). The type of the bracteole (tubular, non-tubular, tubular and non-tubular)

The type of the bracteole shows some degrees of congruence with ITS data. The mapping of this character required five evolutionary steps. **Figure 42** illustrates that basal clades which include species such as *Renealmia battenbergiana*, *R.* aff. *africana*, *Alpinia fax*, *A. rafflesiana*, *A. javanica* and other species from the *A. carolinensis* and the *A. eubractea* clades all had tubular bracteoles. In contrast, more advanced clades which include other *Alpinia* species such as *A. brevilabris*, *A. glabra*, *A. coriacea*, *A. polyantha*, *A. japonica*, *A. intermedia*, *A. suishaensis*, *A. maclurei* and species in the *A. zerumbet* clade all possessed non-tubular bracteoles. Despite the fact that some outgroup taxa (*Elettariopsis unifolia*, *Pleuranthodium*

racemigerum and *P. papilionaceum*) had non-tubular bracteoles, it appeared that Holttum's (1950) assumption of tubular bracteoles being regarded as primitive was correct.

Smith (1990a) used the type of the bracteole as a main character to distinguish section *Alpinia* (with non-tubular bracteoles) from section *Allughas* (with tubular bracteoles). However, based on ITS data *A. galanga*, the type species of the genus and a member of section *Alpinia* subsection *Alpinia* was found closely related to *A. nigra* and *A. conchigera* in section *Allughas*. Therefore, it appeared that *A. galanga* has evolved within section *Allughas* and the absence of tubular bracteoles is a convergence with section *Alpinia*. It is significant that both these forms of bracteoles have been found within a single inflorescence in *A. abundiflora* of Smith's section *Fax*. Moreover, in Smith's treatment of section *Kolowratia* species with non-tubular and those with tubular bracteoles were placed together. Therefore, the feature of bracteoles by itself, appears not to be useful at sectional level.

(5). The type of the labellum (petaloid, non-petaloid, non-petaloid (except apex), petaloid or non-petaloid)

The type of the labellum shows some conflicts with the ITS data. Smith (1990a) used this character (petaloid versus non-petaloid) to subdivide *Alpinia* into two subgenera, *Alpinia* (with petaloid labellum) and *Dieramalpinia* (with non-petaloid labellum). Smith also recognised the labellum type which was non-petaloid but with the apex occasionally expanding into a small petaloid area and she included it with the non-petaloid labellum. For certain species the form of the labellum was unclear and I specified it as petaloid or non-petaloid. **Figure 43** shows that the *A. eubractea* clade contains species with all types of the labellum. The petaloid labellum was found in *A. arctiflora*, *A. arundelliana*, *A. modesta*, *A. caerulea* and *A.*

purpurata whereas the non-petaloid labellum was found in *A. oceanica* and *A. vittata*. Species with the unclear type of the labellum (*A. elegans*) and the non-petaloid labellum with a small petaloid area at the apex (*A. eubractea*) were also present. In addition, *A. luteocarpa* (*scorpoidea*), a species without a labellum or if the labellum was present it was very reduced, was found in this clade. It is important to note that Smith included *A. denticulata* (Ridl.) Holttum in subgenus *Alpinia* (section *Allughas*) although the species possesses the non-petaloid labellum type. Therefore, it appears that the type of the labellum is inconsistent, and by using this sole character to classify *Alpinia*, results can be misleading.

(6). The type of the pollen (smooth, spiny)

Mapping of the type of the pollen onto the ITS strict consensus tree required three steps (**Figure 44**) and results obtained were more or less in agreement. For the Zingiberaceae under study it appeared that the common ancestor had smooth pollen grains. This character was found in the outgroup (*Elettariopsis unifolia*, *Burbidgea schizocheila*, *Pleuranthodium papilionaceum* and *Renealmia bettenbergiana*) as well as in many species of *Alpinia* such as *A. abundiflora*, *A. fax*, *A. javanica*, *A. carolinensis*, *A. vulcanica*, and those in the *A. eubractea* clade (*A. arctiflora*, *A. elegans*, *A. luteocarpa* (*scorpoidea*), *A. modesta*, *A. caerulea*, *A. purpurata*, *A. oceanica* and *A. vittata*). **Figure 44** shows that there are two separate lineages for the evolution of the spiny pollen. The first and more primitive lineage includes all three species in the *A. galanga* clade (*A. galanga*, *A. nigra* and *A. conchigera*). In the second lineage this character was observed in various clades including the *A. aquatica* clade (*A. foxworthyi* and *A. flabellata*), the *A. zerumbet* clade (*A. formosana*, *A. zerumbet*, *A. blepharocalyx* var. *glabrior*, *A. malaccensis*, *A. latilabris*, *A. calcarata* and *A. mutica*), the *A. glabra* clade (*A. glabra*, *A.*

nieuwenhuizii and *A. ligulata*), and in other species such as *A. oxymitra*, *A. oxyphylla*, *A.* aff. *shimadai*, *A. intermedia* and *A. suishaensis*.

(7). The type of the fruit and seed ("Polyantha" type, "Zerumbet" type, "Conchigera" type, "Oxyphylla" type)

Although the anatomical characters of fruit and seed were obtained only from a small number of Chinese *Alpinia* (see **Table 9** for details of these characters), the mapping of these characters revealed three important groups where morphology was complement with ITS data (**Figures 45**). The "Polyantha" type of fruit and seed supported the grouping of *A. pumila* along with other species of Smith's (1990a) section *Alpinia* subsection *Alpinia* (*A. polyantha*, *A. japonica*, *A. suishaensis* and *A. maclurei*) although Smith placed *A. pumila* in section *Didymanthus* based on its unique characteristics of very short stem with few-leaved shoots which are not frond-like. The "Zerumbet" type of fruit and seed supported the *A. zerumbet* clade (*A. kwangsiensis*, *A. zerumbet*, *A. malaccensis* and *A. calcarata*). Finally, it is significant that the "Conchigera" type of fruit and seed was found only in the closely related species *A. galanga*, *A. nigra* and *A. conchigera*.

Figure 39. The ITS strict consensus tree with **the presence or absence of the bract** mapped. The mapping required three evolutionary steps and all character states were unordered. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

Figure 40. The ITS strict consensus tree with **the type of the bract** mapped. The mapping required six evolutionary steps and all character states were unordered. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

Figure 41. The ITS strict consensus tree with **the presence or absence of the bracteole** mapped. The mapping required seven evolutionary steps and all character states were unordered. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

Figure 42. The ITS strict consensus tree with **the type of the bracteole** mapped. The mapping required five evolutionary steps and all character states were unordered. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

Figure 43. The ITS strict consensus tree with **the type of the labellum** mapped. The mapping required six evolutionary steps and all character states were unordered. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

Figure 44. The ITS strict consensus tree with **the type of the pollen** mapped. The mapping required three evolutionary steps and all character states were unordered. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

Figure 45. The ITS strict consensus tree with **the type of the fruit and seed mapped**. The mapping required four evolutionary steps and all character states

were unordered. *Alpinia blepharocalyx* 1 is *A. blepharocalyx* var. *glabrior* and *Alpinia blepharocalyx* 2 is *A. blepharocalyx* var. *blepharocalyx*.

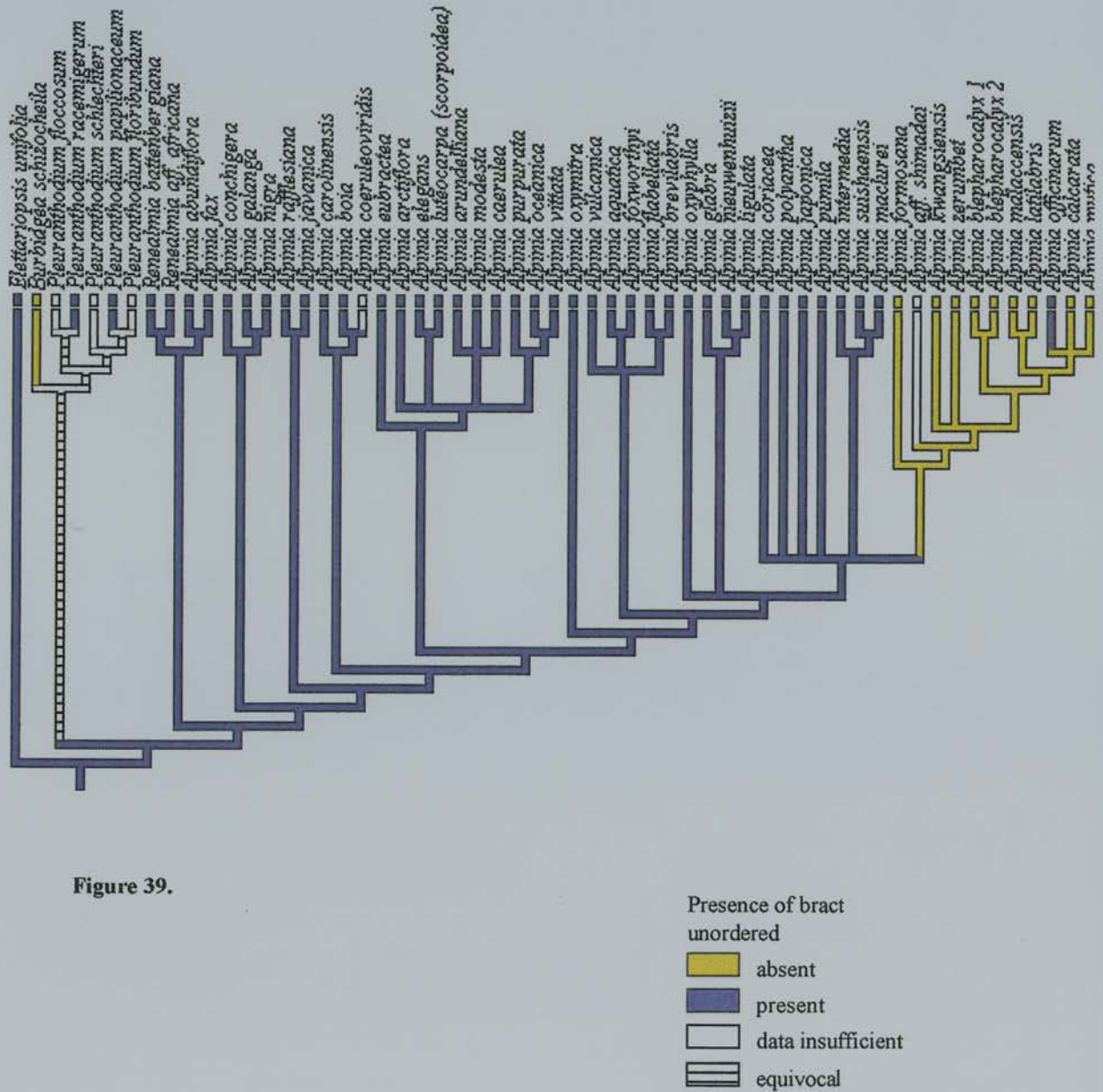

Elettariopsis uniflora
Burbidgea schizochelia
Pleuranthodium floccosum
Pleuranthodium racemigerum
Pleuranthodium schlechteri
Pleuranthodium papilionaceum
Pleuranthodium floribundum
Renealmia bakeriana
Renealmia aff. africana
Alpinia arundiflora
Alpinia fax
Alpinia conchigera
Alpinia galanga
Alpinia nigra
Alpinia raffiesiana
Alpinia javanica
Alpinia carolinensis
Alpinia boia
Alpinia coerulea/viridis
Alpinia subracteata
Alpinia arctiflora
Alpinia elegans
Alpinia lutocarpa (scorpioidea)
Alpinia arundelliana
Alpinia modesta
Alpinia caerulea
Alpinia purpurata
Alpinia oceanica
Alpinia vittata
Alpinia oxymitra
Alpinia vulcanica
Alpinia aquatica
Alpinia foxworthyi
Alpinia flabellata
Alpinia brevilabris
Alpinia oxyphylla
Alpinia glabra
Alpinia nieuwenhuizii
Alpinia ligulata
Alpinia coriacea
Alpinia polyantha
Alpinia japonica
Alpinia pumila
Alpinia intermedia
Alpinia suishaensis
Alpinia macrurei
Alpinia formosana
Alpinia aff. shimadai
Alpinia kwangsiensis
Alpinia zerumbet
Alpinia blepharocalyx 1
Alpinia blepharocalyx 2
Alpinia malaccensis
Alpinia latilabris
Alpinia officinarum
Alpinia calcarata
Alpinia mutica
Presence of bract
unordered
absent
present
data insufficient
equivocal

Figure 39.

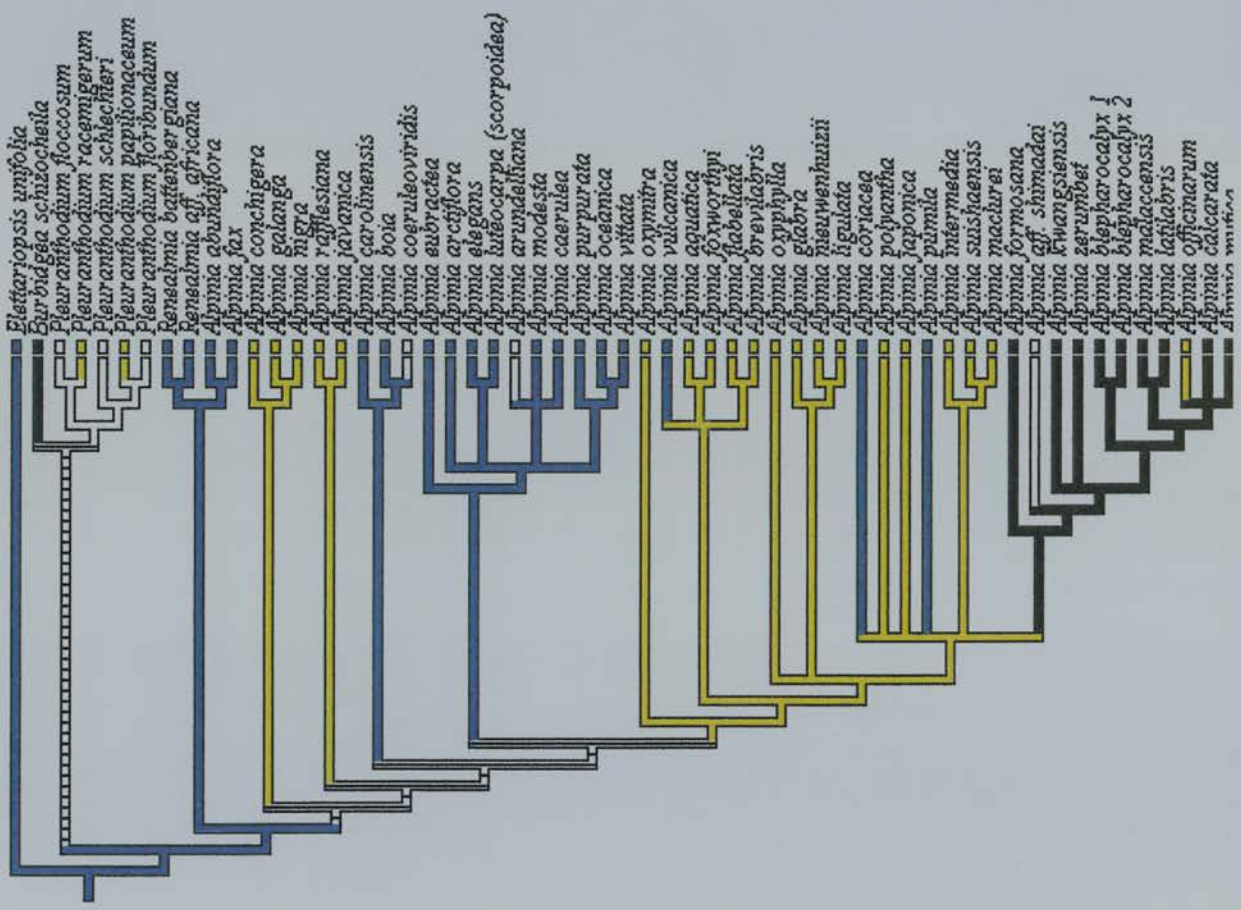


Figure 40.

Type of bract
unordered

- soon deciduous
- persistent
- data insufficient
- data inapplicable
- equivocal

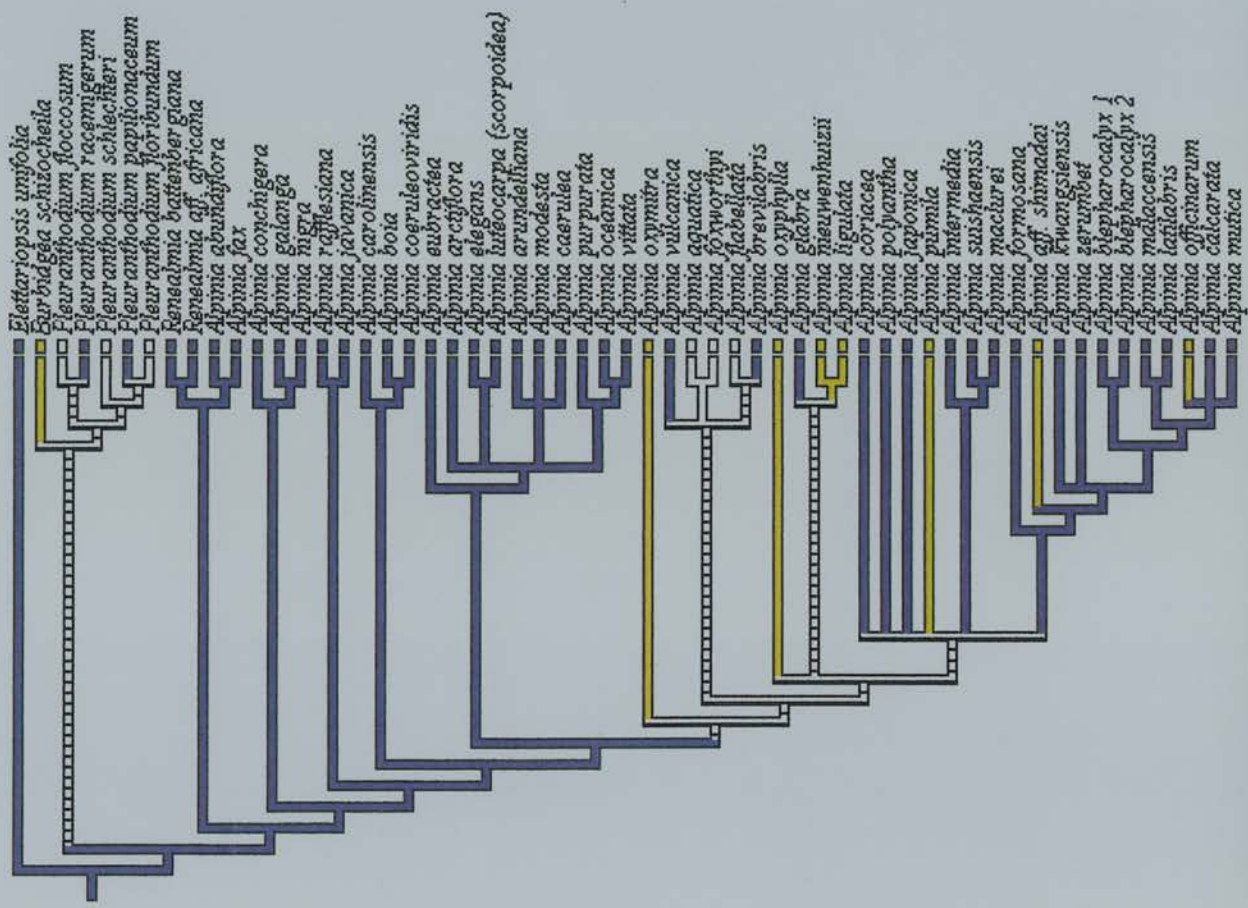


Figure 41.

Presence of bracteole
unordered

- absent
- present
- data insufficient
- equivocal

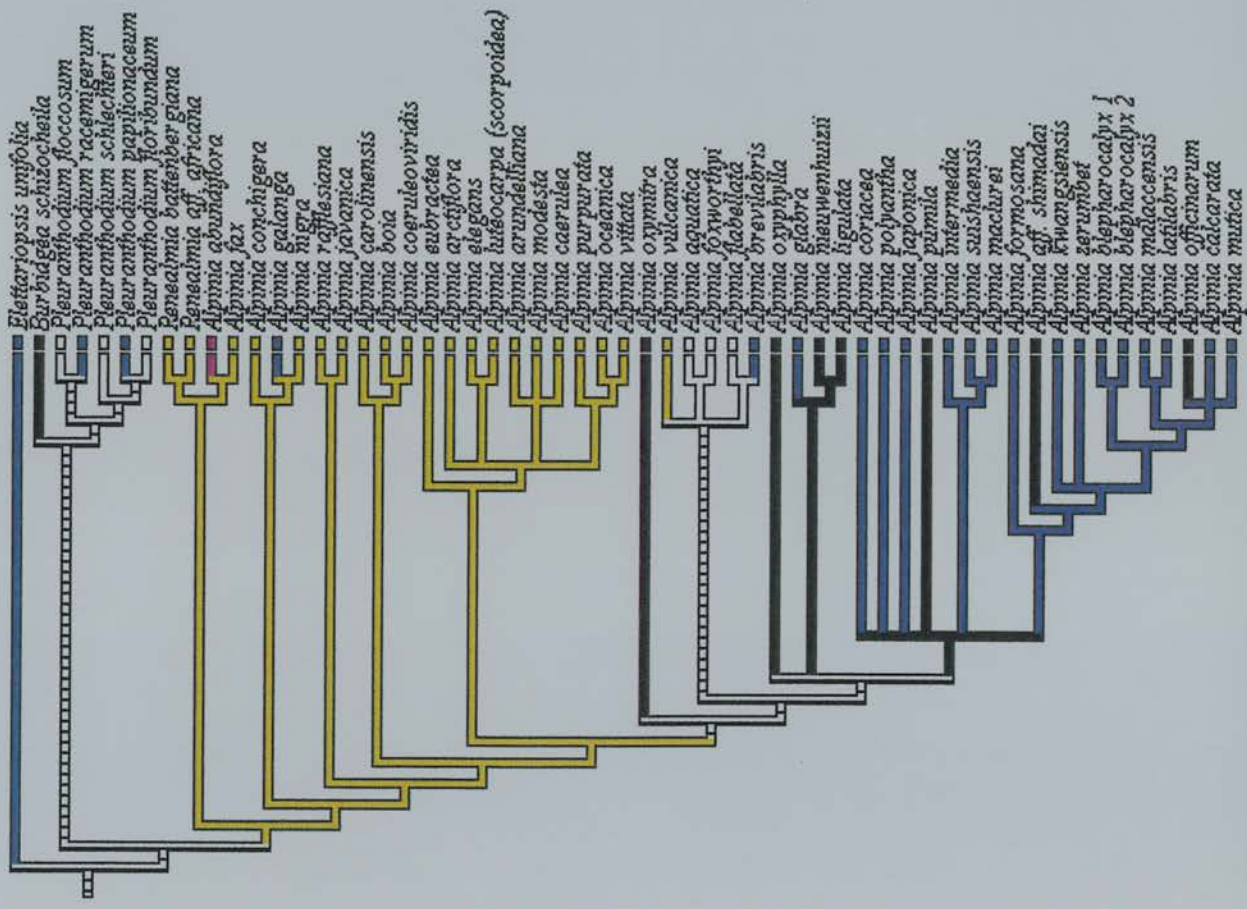


Figure 42.

Type of bracteole
unordered

- tubular
- non-tubular
- tubular and non-tubular
- data insufficient
- data inapplicable
- equivocal

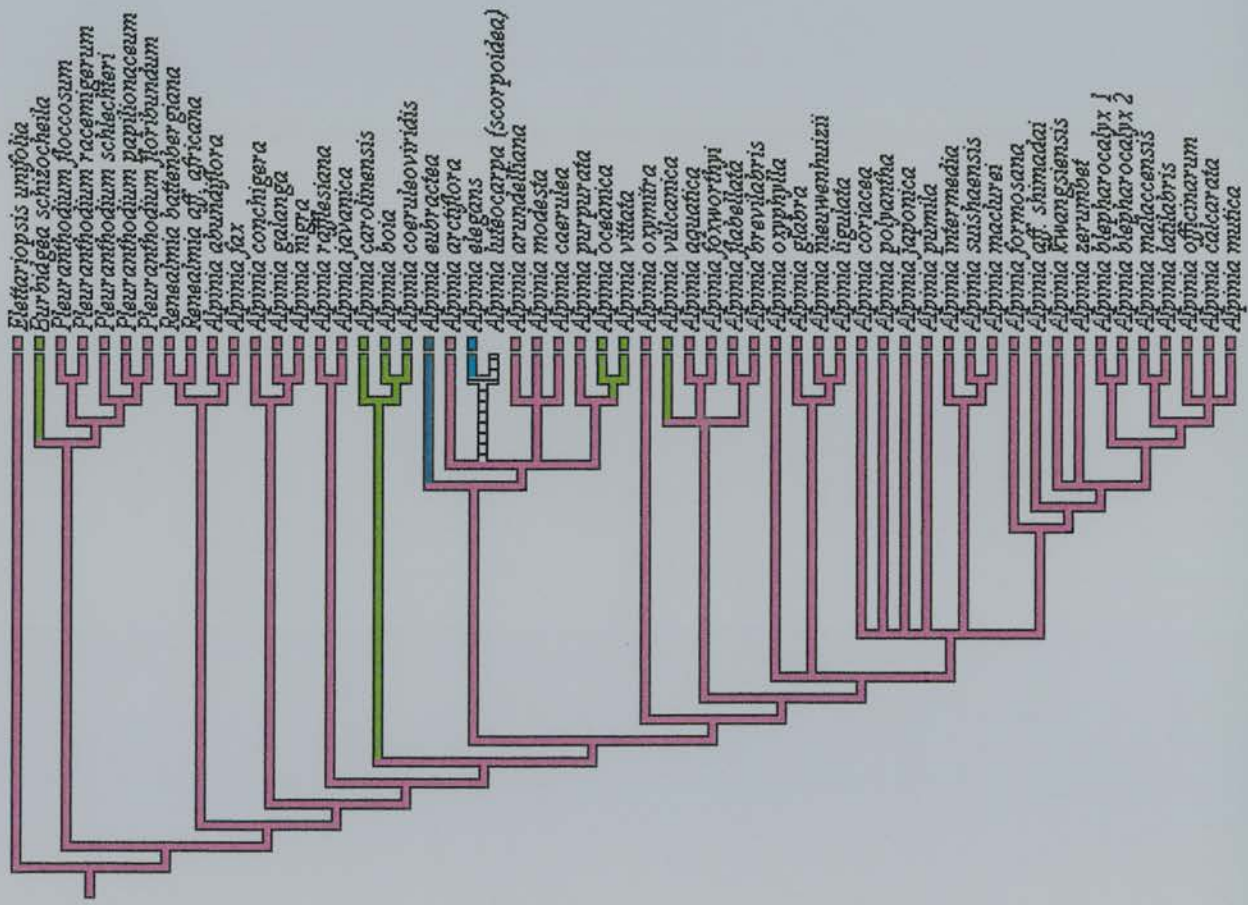


Figure 43.

Type of labellum
unordered

- petaloid
- non-petaloid
- non-petaloid (except apex)
- petaloid or non-petaloid
- equivocal

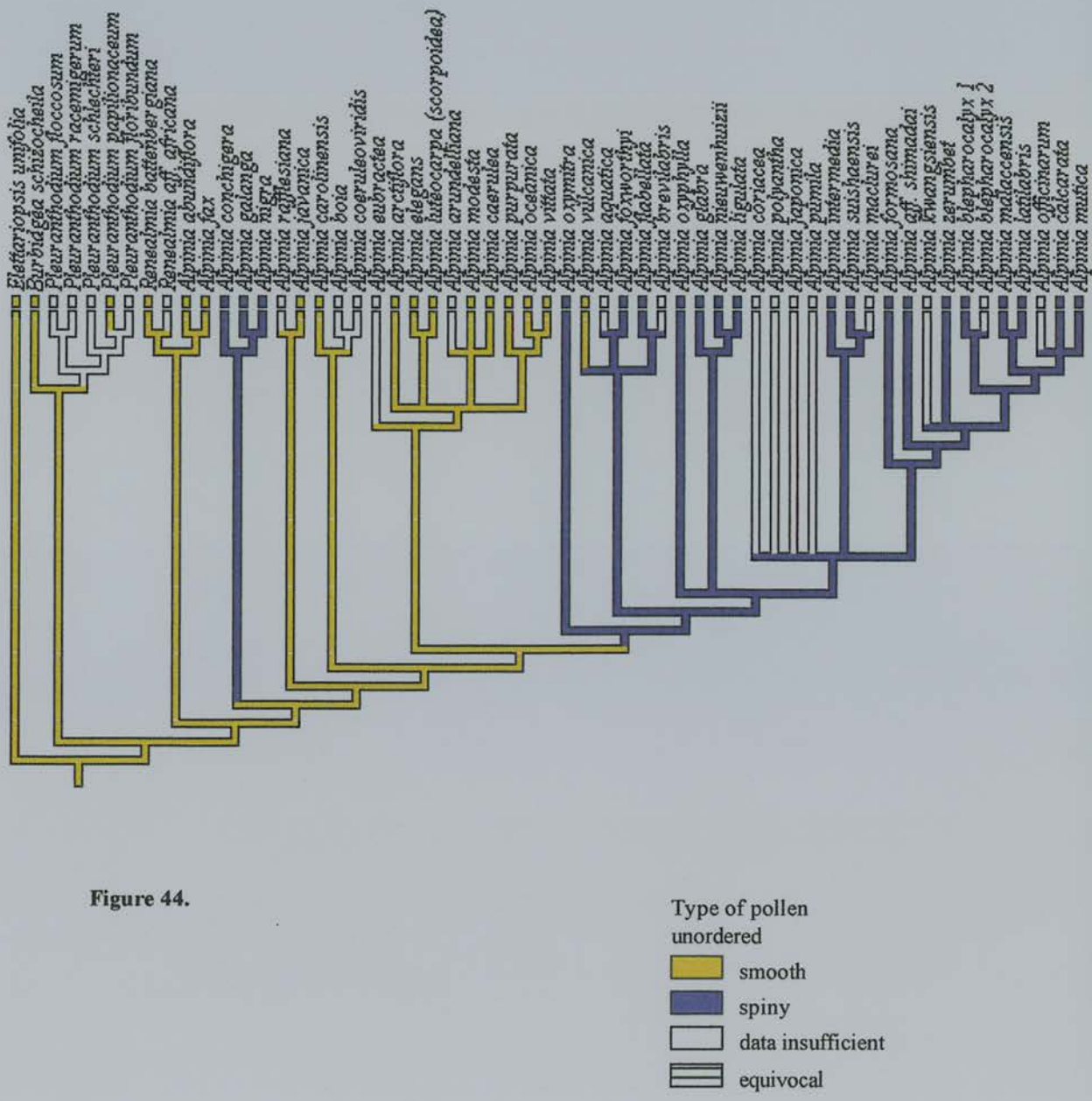


Figure 44.

Type of pollen
unordered
- smooth
- spiny
- data insufficient
- equivocal

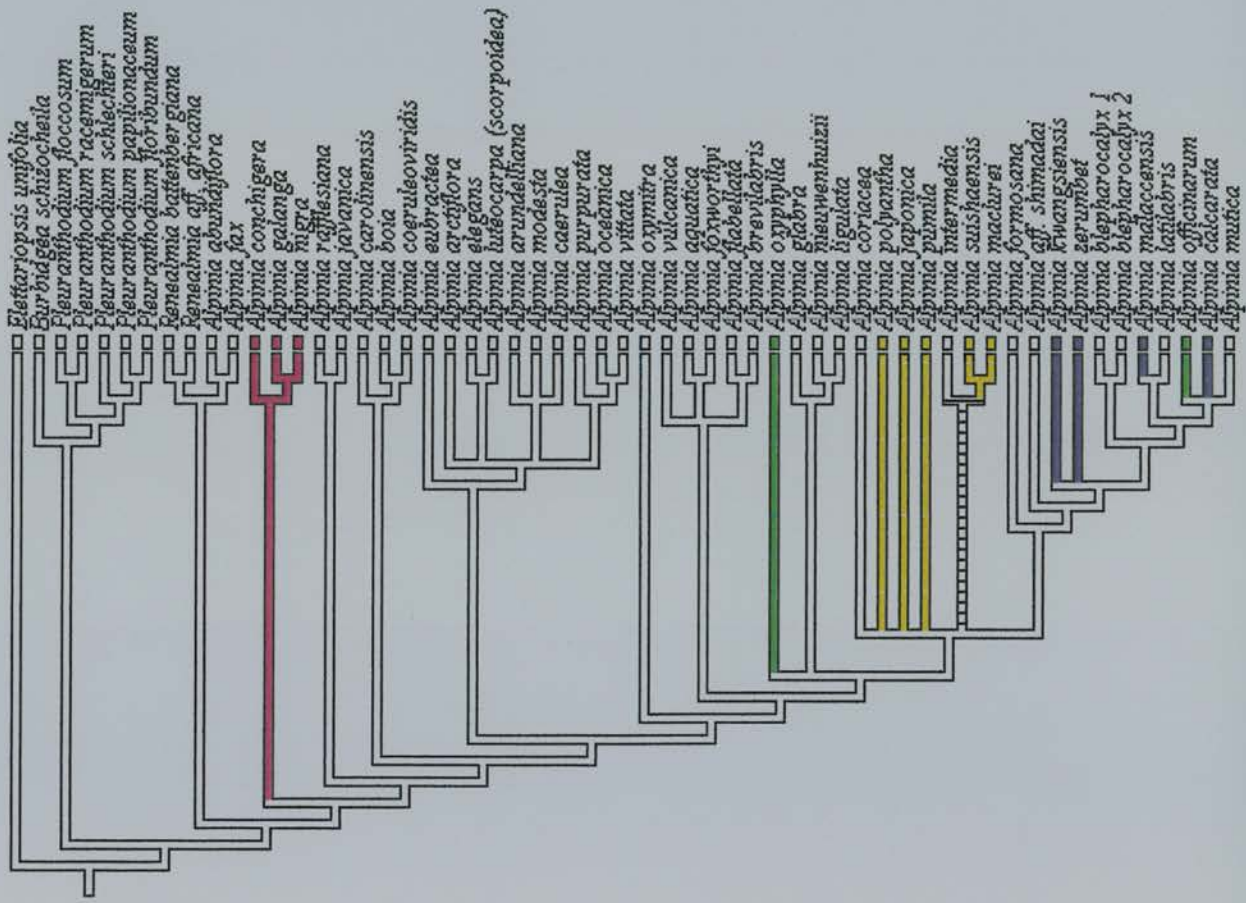


Figure 45.

Type of fruit and seed unordered

- "Polyantha" type
- "Zerumbet" type
- "Conchigera" type
- "Oxyphylla" type
- data insufficient
- equivocal

CHAPTER 6: DISCUSSION BASED ON THE RESULTS OF THE MOLECULAR AND MORPHOLOGICAL APPROACHES TO THE STUDY OF THE INFRAGENERIC CLASSIFICATION OF *ALPINIA*

6.1 MOLECULAR EVOLUTION OF THE ITS REGION

In the Zingiberaceae under study ITS1 is shorter than ITS2. This type of length variation has been reported from other angiosperms such as Betulaceae (Savard *et al.* 1993), Cucurbitaceae (Kavanagh and Timmis 1988; Torres *et al.* 1990) and Viscaceae (Nickrent *et al.* 1994). For these 57 taxa of Zingiberaceae, the length variation of both spacers is due to the occurrence of numerous indel events. In comparison with other substitutions these indels might be less likely to be homoplasious because indels are likely to result in a series of ITS structural changes and hence are probably more constrained (Baldwin *et al.* 1995; Buckler and Holtsford 1996a, b).

Within *Alpinia*, the ranges of sequence divergence values are similar for both ITS1 (0-20.9%) and ITS2 (0-19.7%), indicating a similar substitution rate in both spacers. This similarity of the pairwise sequence divergence is also a feature of other groups of angiosperms such as *Astragalus* (ITS1: 0-10.2%, ITS2: 0-8.8%; Wojciechowski *et al.* 1993), *Viburnum* (ITS1: 0-13.6%, ITS2: 0-11.9%; Donoghue and Baldwin 1993) and *Gilia* section *Giliandra* (ITS1: 0-13.9%, ITS2: 0-12.5%; Porter 1993a, b). In addition, these 57 taxa of Zingiberaceae have a more or less uniform G+C content in ITS1 and ITS2, which conforms with other studies in angiosperms (reviewed in Baldwin *et al.* 1995). This may reflect some degree of coevolution of the two spacers. The average ratio of ts/tv in the combined ITS1 and ITS2 regions of *Alpinia* and its outgroup (2.9) is higher than the expected value (approximately 2.0) for relatively recently diverged sequences (Holmquist 1983) of other angiosperms such as Poaceae (Hsiao *et al.* 1994) because of an excess of

transitions among nucleotide substitutions (reviewed in Wakeley 1996). Transitions may be caused by pyrimidine dimerisation, ionisation, and 5-methylcytosine deamination - phenomena which are related to kinetic processes and not to cell replications (Vairapandi and Duker 1994; von Borstel 1994). In contrast, transversions are more tightly linked to cell replication cycles and generation time (Buckler and Holtsford 1996a), therefore, the occurrence of transversions is less prevalent in nature. In general, however, the features of ITS evolution in *Alpinia* and its outgroup show similarity between the two spacers and with other angiosperm ITS regions.

The chloroplast spacer between *trn*L (UAA) 3' exon and *trn*F (GAA) evolves more slowly than the ITS region. However, despite the fact that it provides very limited phylogenetic information for the present study of *Alpinia*, this chloroplast spacer serves as a useful confirmation for the results based on the ITS region.

6.2 PHYLOGENETIC RELATIONSHIPS WITHIN *ALPINIA* AND BETWEEN *ALPINIA* AND ITS OUTGROUP

6.2.1 The *Pleuranthodium* clade

Schumann (1904) recognised *Pleuranthodium* as a section in subgenus *Autalpinia* K.Schum. (of the genus *Alpinia*) based on its unbranched, racemose inflorescences which sometimes pushed out laterally from the uppermost leaf sheaths, and small bracts and bracteoles. Three species, namely *A. pterocarpa* K.Schum., *A. tephrochlamys* Lauterb. & K.Schum. and *A. pelecystyla* K.Schum. were placed in the section. In 1991, Smith raised *Pleuranthodium* to genus level (with two sections and 23 species) on the basis of its distinct petaloid labellum which is held erect and is strongly cup-shaped. In addition, with the exception of the Australian *P. racemigerum*, *Pleuranthodium* is restricted to New Guinea and the islands of the Bismarck Archipelago (Smith 1990b, note that Smith (1991) should be

consulted as *Pleuranthodium* (K.Schum.) R.M.Sm. is used to replace the illegitimate *Psychanthus* (K.Schum.) Ridl.). Moreover, Valeton (1914, p. 45) mentioned some distinctive characters of the fruit (fleshy and splitting almost to the base with age) which are not present in *Alpinia*.

In this study, results of the ITS analysis revealed that *Pleuranthodium* is monophyletic confirming that it is distinct and thus can be completely removed from *Alpinia*. My results therefore completely vindicate Smith's (1991) treatment of *Pleuranthodium* at generic level.

6.2.2 The *A. galanga* clade

It has already been mentioned in **CHAPTER 3** (p. 78, 81) and **CHAPTER 5** (p. 156) that molecular data (both ITS and the spacer between *trn*L (UAA) 3' exon and *trn*F (GAA)) show *Alpinia galanga* (Smith's (1990a) section *Alpinia*) to be a close relative of *A. nigra* and *A. conchigera* (Smith's (1990a) section *Allughas*). *Alpinia galanga* appears to have evolved within section *Allughas*, despite the fact that it has non-tubular bracteoles and all species in this section have tubular bracteoles. The absence of tubular bracteoles in *A. galanga* appears to be a convergence with section *Alpinia*. Both types of bracteoles are present in the same inflorescence in *A. abundiflora* and Smith (1975) remarked that, although this character is obvious in the fully grown bracteole, it depends on a very minute shift of cell division in the course of development.

It is interesting to note that some authors placed *A. galanga* with either *A. nigra* or *A. conchigera*. In 1832, Roxburgh placed *A. galanga* alongside *A. allughas* Roscoe (*A. nigra* (Gaertn.) B.L.Burtt), together with all species having terminal inflorescences. Ridley (1899) on the other hand, grouped *A. galanga* in section *Hellenia*, along with *A. conchigera* Griff., *A. melanocarpa* (Teijsm. & Binn.) Ridl. (*A. aquatica* (Retz.) Roscoe) and *A. scabra* (Blume) Baker, based on the

characteristics of their small flowers, narrow lips, crested anthers and small globose fruits which contain few seeds. Other lines of evidence which seem to support the close relationship of *A. galanga*, *A. nigra* and *A. conchigera* have come from studies of fruit and seed anatomy of Chinese *Alpinia* by Liao and Wu (1996a, b). All three species possess the "Conchigera" type of fruit and seed. They also share common characters such as non-persistent bracts, petaloid labella and spiny pollen grains. In addition, rhizomes of both *A. galanga* and *A. nigra* (which are often cultivated and occur in abandoned cultivations throughout Thailand and Malaysia) have been used for cooking without local people drawing a distinction between them. This implies that *A. galanga* and *A. nigra* possess similar secondary compounds, a fact that also suggests a close relationship between them.

6.2.3 The *A. eubractea* clade

Analysis of the ITS region showed strong support for the *A. eubractea* clade which contains members of Smith's (1990a) subgenera *Alpinia* and *Dieramalpinia*. In the clade, four sections in subgenus *Alpinia* are represented, these are section *Arctiflorae* (*A. arctiflora*), section *Kolowratia* (*A. elegans*), section *Allughas* subsection *Caeruleae* (*A. arundelliana*, *A. modesta* and *A. caerulea*) and section *Guillainia* (*A. purpurata*). The rest of the species are from section *Eubractea* (*A. eubractea*) and section *Dieramalpinia* (*A. luteocarpa* (*scorpoidea*), *A. oceanica* and *A. vittata*) of subgenus *Dieramalpinia*. It appears from this study that the form of the labellum (petaloid versus non-petaloid) by itself, is inadequate for the division of *Alpinia* into two subgenera as proposed by Smith (1990a). Furthermore, the use of the labellum character by Smith (1990a) is not always consistent for all species (see **CHAPTER 5**, p. 156-157). The fact that some species in *Alpinia* have a petaloid labellum and others have a non-petaloid labellum could be due to two main factors. First, a simple genetic switch which can result in structural or architectural changes

in plant morphogenesis (Gottlieb 1984). Gottlieb (1984) pointed out that in plants many discrete characters such as presence versus absence of structures, or changes in structure, shape or position, divergence may be initiated by changes in a small number of genes and additional genes may act to modify the expression of such traits (see examples in Gottlieb 1984, Tables 1 and 2). Second, a consequence of plant adaptations to specific groups of pollinators (pollination syndromes) (Gottlieb 1984). The labellum is an essential part of the floral structures which are involved in pollinator attraction. It also acts as a platform for some pollinators to enter the corolla to collect nectar. As a result, pollen grains can be transported to different plants and outcrossing is achieved. Examples of pollination syndromes are given by various authors such as Gottlieb (1984, Tables 1 and 2), Kress (1985), Carr (1987), Classen (1987), Ippolito and Armstrong (1993) and Hopkins *et al.* (1998). Thus, certain morphological differences may be misleading in the absence of genetic information and knowledge of developmental interactions. In addition, Crisp (1994) suggested that characters associated with functional syndromes may not be independent and should be used with caution in cladistic analyses.

In his classification of *Alpinia* Schumann (1904) placed *A. elegans*, *A. eubractea* and *A. oceanica* together in section *Eubractea* K.Schum. of subgenus *Dieramalpinia* K.Schum. on the basis of their tubular bracteoles and large persistent bracts. This classification is well supported by the current molecular data. *Alpinia purpurata* (*Guillainia novo-ebudica* F.Muell.) was assigned in *Guillainia* F.Muell. but Schumann (1904) reduced the genus to sectional level (*Guillainia* (Vieill.) K.Schum.) within subgenus *Autalpinia* K.Schum. Later authors then added *A. oceanica* (*Guillainia rechingeri* Gagnep.) and *A. vittata* (*Guillainia vittata* Ridl.) to *Guillainia* (see Smith 1975). Smith (1975) stated that Schumann's section *Guillainia* should be treated as a section of subgenus *Dieramlpinia* if Schumann was aware of the presence of tubular bracteoles. However, based on the broad petaloid

character of the labellum, Smith (1990a) placed *A. purpurata* along with two other species (*A. fusiformis* R.M.Sm. and *A. ludwigiana* R.M.Sm.) in section *Guillainia* subgenus *Alpinia*. Smith's (1990a) section *Allughas* subsection *Caeruleae* includes four species from Australia, they are *A. caerulea*, *A. arundelliana*, *A. modesta* and *A. hylandii* R.M.Sm. This section was placed in subgenus *Alpinia* because each member has a petaloid labellum. Schumann (1904) on the other hand, recognised *A. caerulea* in his section *Allughas* (subgenus *Dieramalpinia* K.Schum.), but *A. arundelliana* and *A. modesta* in section *Hellenia* (Willd.) K.Schum. (subgenus *Autalpinia* K.Schum.). Results of the ITS analyses strongly supported a close relationship between these three species. It appears that these species form a natural group and should be correctly classified in subgenus *Dieramalpinia* with a similar geographical distribution with other members of the same subgenus (distribution of subgenus *Dieramalpinia*: Borneo, Sulawesi, the Philippines, Carolines, the Moluccas, New Guinea and the Bismarck Archipelago, the Solomons to Fiji and Samoa). These species also possess the types of bract (persistent; although data were insufficient for *A. arundelliana*) and bracteole (tubular) which are common among other species in subgenus *Dieramalpinia*. Another species in the *A. eubractea* clade which was problematic is an Australian species, *A. arctiflora*. Schumann (1904) placed it near to *A. caerulea* and other species in his section *Allughas* of subgenus *Dieramalpinia*. In contrast, Smith (1990a) treated it as a monotypic species in section *Arctiflorae* of subgenus *Alpinia*. The ITS strict consensus tree in this case did not provide a solution to the correct placement of this species within *Alpinia*. However, following the successive weighting of the characters based on the CI values (**Figure 20**) results showed a close relationship of *A. arctiflora* to the three species of section *Allughas* (*A. arundelliana*, *A. modesta* and *A. caerulea*). The last species in the *A. eubractea* clade is *A. luteocarpa* (*scorpoidea*). Smith (1990a) was uncertain about both the species identification and

the sectional placement of the species in her subgenus *Dieramalpinia* because the type specimen only contains vegetative characters (see **CHAPTER 2**, p. 39) and in all other specimens which had flowers, they were without labella. Based on ITS analyses this species appeared to be closely allied to *A. elegans*. Therefore, it is probable that *A. luteocarpa* (*scorpoidea*) belongs to Smith's (1990a) section *Kolowratia* of subgenus *Alpinia* (which may be in turn associated with Schumann's (1904) section *Eubractea* of subgenus *Dieramalpinia*) but further material is needed to verify this. The species examined in the *A. eubractea* clade share similar characters including persistent bracts, tubular bracteoles and smooth pollen grains.

6.2.4 The *A. carolinensis* clade

The existence of the *A. carolinensis* clade in ITS analyses revealed a natural grouping of Smith's (1990a) subgenus *Dieramalpinia*. The clade is characterised by the presence of the persistent bract (data were insufficient for *A. coeruleoviridis*), tubular bracteole and non-petaloid labellum type. Schumann (1904) recognised *A. boia* and *A. coeruleoviridis* in his subgenus *Dieramalpinia* but placed them in different sections - *Pycnanthus* K.Schum. and *Oligocincinnus* K.Schum. In her classification, Smith (1990a) also placed *A. boia* in section *Pycnanthus* but *A. coeruleoviridis* and *A. carolinensis* are found in sections *Myriocrater* and *Dieramalpinia* respectively.

6.2.5 The polyphyly of Smith's (1990a) section *Allughas*

In the ITS strict consensus tree (**Figure 18**) the *A. rafflesiana* clade, consisting of *A. rafflesiana* and *A. javanica* (section *Allughas* subsection *Allughas*), forms a natural group which is located between the *A. galanga* clade and the *A. carolinensis* clade. Within the *A. galanga* clade two species belong in section *Allughas* but in different subsections (*A. nigra* in subsection *Allughas* and *A.*

conchigera in subsection *Strobidia*). According to Smith (1990a) *A. arundelliana*, *A. caerulea* and *A. modesta* are also members of section *Allughas* but in subsection *Caeruleae* (these species are found in my *A. eubractea* clade). Therefore, based on ITS analyses Smith's (1990a) section *Allughas* appeared to be polyphyletic. Interestingly, Schumann's (1904) section *Allughas* (subgenus *Dieramalpinia*) only included *A. nigra* and *A. caerulea* whereas *A. arundelliana* and *A. modesta* were assigned in section *Hellenia* (subgenus *Autalpinia*). *Alpinia conchigera*, *A. rafflesiana* and *A. javanica* were classified into three different sections (in subgenus *Dieramalpinia*), namely *Strobidia* (Miq.) K.Schum., *Brachybotrys* K.Schum. and *Javana* K.Schum., respectively. In contrast to Schumann and Smith, Holttum (1950) dealt with species from Malay Peninsula only and grouped *A. conchigera*, *A. rafflesiana* and *A. javanica* in the same genus *Alpinia* based on the presence of tubular bracteoles.

6.2.6 The *A. aquatica* clade

This clade (**Figure 19**) reflects some degree of correlation between *Alpinia* phylogeny and biogeography. All but one species in the clade come from the Philippines (*A. foxworthyi*, *A. flabellata*, *A. brevilabris* and *A. vulcanica*). *Alpinia aquatica* is found from Thailand to Borneo. *Alpinia aquatica, A. foxworthyi*, *A. flabellata* and *A. brevilabris* were classified in Smith's (1990a) section *Alpinia* subsection *Presleia* (subgenus *Alpinia*) on the basis of the very small flowers and petaloid four-lobed labellum. *Alpinia vulcanica* however, was placed in section *Myriocrater* of subgenus *Dieramalpinia*, with non-petaloid labellum. Unique characteristics which are found in *A. vulcanica*, and are shared with other members of section *Myriocrater* are inflorescence unilateral (with cincinni borne on only one side of the main axis), and the exhibition of monoecism - all flowers in a cincinnus are functionally male except the lowermost (first and/or second) flowers which are

female and produce fruits (Elmer 1919; Burtt and Smith 1972b; Smith 1977). Monoecism has not been observed in other sections of subgenus *Dieramalpinia* except section *Eubractea* where only the flowers in the lower half of a cincinnus produce fruit. There are no records of monoecism in subgenus *Alpinia* (Smith 1990a). *Alpinia vulcanica* however, possesses ecristate anthers; a feature not observed in any other members of the same section (Smith 1977). Plant genetics as well as pollination systems seem to play important roles here in producing such diverse forms and functions. At present there are no morphological data which support the grouping of *A. vulcanica* with the four members of section *Alpinia* subsection *Presleia* based on ITS analyses, thus these species form a cryptic clade. However, the presence of *A. vulcanica* in this clade instead of a clade including *A. coeruleoviridis* (another species from section *Myriocrater*) suggests that Smith's (1990a) section *Myriocrater* may not be monophyletic. The inclusion of more samples in the analysis should help to verify this.

6.2.7 The *A. glabra* clade

This is a clade which consists of three species endemic to Borneo. Two species (*A. nieuwenhuizii* and *A. ligulata*) belong to Smith's (1990a) subsection *Paniculatae* and the third species (*A. glabra*) is monotypic in subsection *Probolocalyx*. All three species are however, classified in the same section (*Alpinia*) under subgenus *Alpinia*. According to Smith (1990a) *A. nieuwenhuizii* and *A. ligulata* share a distinctive characteristic of branched inflorescences throughout. *A. glabra* on the other hand, has inflorescences sometimes with short lateral branches at the base. Schumann (1904) recognised *A. glabra* and *A. ligulata* together with 17 other species in his subgenus *Probolocalyx* based on minute (or absent) bracts and bracteoles. Smith (1990a) placed these species in various subsections including *Alpinia*, *Cenolophon*, *Catimbium* and *Paniculatae*. Smith also believed that, as

more material had become available, there would be more than one species involved in her subsection *Probolocalyx*. At present however, ITS analyses revealed that *A. glabra* is closely related to *A. nieuwenhuizii* and *A. ligulata*.

6.2.8 The *A. polyantha* clade

This clade does not exist in the ITS strict consensus tree (but does exist in most of the 180 equally parsimonious trees) due to unresolved branches of species in Smith's (1990a) section *Alpinia* subsection *Alpinia* (*A. coriacea*, *A. polyantha*, *A. japonica*) and section *Didymanthus* (*A. pumila*, a species native to China). The tree however, indicates that this subsection may be paraphyletic since a natural group, containing *A. intermedia*, *A. suishaensis* and *A. maclurei*, is present. In addition, based on the successive weighting of the characters of the ITS region using the CI values (**Figure 20**), results showed the existence of a clade comprising *A. coriacea*, *A. polyantha*, *A. japonica* and *A. pumila* (with the last two species being closely related). Although Schumann (1904) placed *A. japonica* and *A. pumila* in different subgenera (*Probolocalyx* and *Rhizalpinia* respectively), he recognised the fact that these two species share a characteristic of flowers in pairs. Recent studies of the fruit and seed anatomy of the Chinese *Alpinia* by Liao and Wu (1996a, b) also confirmed the grouping of *A. pumila* with Smith's subsection *Alpinia* (*A. polyantha*, *A. suishaensis*, *A. maclurei* and *A. japonica*). In terms of biogeography *A. pumila* and all the other species mentioned share similar distributions in Eastern Asia including China, Hainan, Taiwan and Japan (**Figure 19**). Thus, it appears that *A. pumila* has evolved within Smith's (1990a) section *Alpinia* subsection *Alpinia*. A distinctive feature of *A. pumila* is that the leaf sheaths are very loosely clasping, forming a tuft or, at most, a short leafy shoot, a condition which could be due to plant adaptation to the environment.

6.2.9 The *A. zerumbet* clade

The clade (**Figure 18**), strongly supported by molecular analyses of both the ITS region and the spacer between *trn*L (UAA) 3' exon and *trn*F (GAA), contains nine species of Smith's (1990a) section *Alpinia* subsection *Catimbium* (*A. formosana*, *A. kwangsiensis*, *A. zerumbet*, *A. blepharocalyx* var *glabrior*, *A. blepharocalyx* var *blepharocalyx*, *A. malaccensis*, *A. latilabris*, *A. calcarata* and *A. mutica*), and two species of the same section but in subsection *Cenolophon* (*A.* aff. *shimadai* and *A. officinarum*). All the species present in this clade possess large flowers with showy labella, a remarkable characteristic of subsection *Catimbium*. According to Smith (1990a) the unique characters which distinguish subsection *Cenolophon* from subsection *Catimbium* are that they are strictly single-flowered with crested anthers, very small bracts and no bracteoles. However, the dividing line between the two subsections has become less obvious as subsection *Cenolphon* also includes species with ecristate anthers (e.g. *A. havilandii*), and partially single-flowered inflorescences are known in subsection *Catimbium* (e.g. *A. latilabris* and *A. malaccensis*). The other two species of subsection *Cenolophon* (*A. oxymitra* and *A. oxyphylla*) were scattered on the ITS cladograms and no natural grouping exists in this subsection.

According to ITS results, the species in the *A. zerumbet* clade are found nested within a non-monophyletic subsection *Alpinia*. The results suggest that the characteristic of large flowers with showy labella of subsection *Catimbium* has evolved from smaller flowers, typical of subsection *Alpinia*.

6.2.10 The genus *Alpinia* is possibly not monophyletic

As can be seen from the base of the ITS strict consensus tree (although with insignificant values of DI (+1) and BS (< 50%), **Figure 18**) Smith's (1990a) subgenus *Alpinia* section *Fax* (*A. abundiflora* and *A. fax*) is sister to two African

Renealmia species (*R. battenbergiana* and *R.* aff. *africana*) and appears to be more closely related to them than to the rest of *Alpinia*. These species have persistent bracts, tubular bracteoles (*A. abundiflora* may have both tubular and non-tubular bracteoles in the same inflorescence) and the inflorescence may be terminal and/or radical (terminal inflorescences: *R. battenbergiana*; radical inflorescences: *A. fax* and *R.* aff. *africana*; terminal and radical inflorescences: *A. abundiflora*). Since section *Fax* is endemic to the southernmost part of India and Sri Lanka, it is possible that the species in this section and African *Renealmia* once co-occurred in Gondwanaland and became separated as a result of plate tectonics.

The connection of *Alpinia* and *Renealmia* can be observed from the history of the two genera since the time of Linnaeus (1753) and the younger Linnaeus (1781) (see **INTRODUCTION**, p. 9). According to Holttum (1950), Schumann (1904) described close similarities between the inflorescence of *Renealmia* and that of *Alpinia* subgenus *Dieramalpinia*. Burtt and Smith (1972a) also mentioned the fact that these taxa resemble each other in the presence of tubular bracteoles but due to the lack of good material they were uncertain if *Renealmia* from Tropical America and Africa was distinct from its Asiatic counterpart (*Alpinia*). A study by Olatunji (1970) revealed some anatomical features in common between *Alpinia* species (which belong not only to subgenus *Dieramalpinia*) and *Renealmia*. These included the presence of hypodermal fibres in the lamina, a characteristic of *Renealmia*, which was also found in many *Alpinia* (e.g. *A. vittata*, *A. nigra* and *A. oxymitra*).

According to Olatunji (1970) the presence of scattered stellate hairs on the leaf lamina of *Renealmia* (both Tropical American and African species) is diagnostic. Although this type of hair is also found in other genera such as *Aframomum* K.Schum. (Tomlinson 1956), *Riedelia* Oliv. and *Rhynchanthus* Hook.f., it is absent from *Alpinia* (Olatunji 1970). Another line of evidence which seems to

distinguish the two genera is the chromosome number. While the majority of the species in both *Alpinia* and *Renealmia* are tetraploid, *Alpinia* appears to have a basic chromosome number of x = 12 (2n = 48) and *Renealmia* has x = 11 (2n = 44) (Beltran and Kiew 1984), where x is the number of chromosomes in a set and n is the number of chromosomes in a gamete. Since 12 is the most frequent basic chromosome number in Zingiberaceae, Mahanty (1970) suggested that it is the original basic number for the family and presumably derived from 11 which is the basic number for the Zingiberales.

6.3 BIOGEOGRAPHICAL IMPLICATIONS OF *ALPINIA* PHYLOGENY

Plant biogeography involves an understanding of the history and ancestral events of diversification and dispersal of the plants (with fossil data) in relation to plate tectonics and palaeoclimatology. Recently, molecular data and molecular phylogenies have been successfully used for biogeographical interpretations of several plant groups such as Magnoliaceae (Clifford and Wendel 1990), Paeoniaceae (Sang *et al.* 1995), Leguminosae (Bruneau 1996; Schnabel and Wendel 1998), Sarraceniaceae (Randall *et al.* 1996), Araliaceae (Wen and Zimmer 1996), Oleaceae (Jeandroz *et al.* 1997) Nothofagaceae (Manos 1997) and Gesneriaceae (Möller and Cronk 1997b).

The analysis of the ITS region (**Figure 19**) revealed a pattern of a strong correlation between *Alpinia* and its geographical distribution. The origin of the genus is unknown but *Alpinia* appears to have its main centre of diversity in the Malesian region based on the presence of a remarkably high number of species (*c.* 160 species; Larsen 1996).

The oldest known leaf fossils of Zingiberaceae (large leaves with parallel vein sets showing reduction to a nearly uniform width as found in *Alpinia*, *Renealmia*, *Curcuma*, *Zingiber* and *Etlingera*) were reported from the Late

Cretaceous to the Early Eocene sediments of Western Interior North America. These fossils were assigned to an extinct genus, *Zingiberopsis* (*Z. magnifolia*, *Z. attenuata* and *Z. isonervosa*) due to the apparent lack of ligules in the fossils and the extreme reduction of parallel veins to a single width subset that occurred in *Z. isonervosa*. The nearest extant relative of this genus appeared to be *Alpinia* based on similarities in the leaf architecture. For instance, (i) *Alpinia chinensis* (Retz.) Roscoe (*A. suishaensis*) has the marginal transverse veins oblique to the costa rather than perpendicular to it as in most other genera, and (ii) *A. formosana* and *Z. magnifolia* have a common parallel vein pattern (Hickey and Peterson 1978).

It is probable that *Zingiberopsis* of Western North America became extinct as a result of climatic fluctuations (reviewed in Kubitzki and Krutzsch 1998). From the Cretaceous to the Paleocene the Northern Hemisphere had cool-paratropical conditions. In the Early and Middle Eocene the temperature became warmer and this supported tropical vegetation (Hickey 1981). From the Middle to Late Eocene however, climates gradually cooled, leading to a sharp decline at the Eocene-Oligocene boundary (Wolfe 1978; Collinson *et al.* 1981).

Other records of Zingiberaceae were from the Eocene of Southern England (Chandler 1964) and India (Prakash 1972). Raven and Axelrod (1974) believed the family probably was common in Laurasia and West Gondwanaland.

The phylogenetic analysis of the ITS region (**Figure 19**) illustrated a possible scenario underlying assumptions for the geographical origin of the ancestor of *Alpinia*. For this scenario I assume that *Alpinia abundiflora* and *A. fax* have evolved from a common ancestor with African *Renealmia* and are therefore not closely related to other true *Alpinia* species. Based on the current knowledge of *Zingiberopsis*, *Alpinia* and other genera in Zingiberaceae may have originated in Laurasia. By the Middle to Late Eocene, the plants which survived the climatic fluctuations could have migrated from North America to Asia via Europe. By that

time there were no barriers such as ocean or high mountains in these regions, and the Bering land bridge between North America and Asia was not available until the Miocene (Hsü 1983). [However, some authors, including Takhtajan (1969) and Tiffney (1985) believed that North America was connected to Eurasia in as early as the Late Cretaceous or the Eocene via the North Atlantic land bridge as well as the North Pacific route through the Bering land bridge. Thus this allowed direct and extensive migrations of organisms between the two areas.] This route of migration may account for the presence of the other fossils of Zingiberaceae in Southern England (Chandler 1964). Following the ITS analysis, at the base of the tree, the species in the *A. galanga* clade (*A. conchigera*, *A. galanga* and *A. nigra*) which occur in China, India and are widely cultivated in South East Asia became established. Due to an extensive domestication over a long period of time, the true origin and direction of movement of these species are unclear. Their occurrence in India could have happened after the collision of the Indian plate with Eurasia in the Early Eocene or around 50 million years ago (Patriat and Achache 1984). After the migration of *Alpinia* to South East Asia, *A. rafflesiana* and *A. javanica*, two closely related species native to Malaysia and Indonesia may have arisen. The subsequent eastward and southeastward dispersions could account for the presence of *Alpinia* (*A. carolinensis*, *A. boia*, *A. coeruleoviridis*, *A. eubractea*, *A. elegans*, *A. luteocarpa* (*scorpoidea*), *A. arctiflora*, *A. arundelliana*, *A. modesta*, *A. caerulea*, *A. purpurata*, *A. oceanica* and *A. vittata*) in Central and Eastern Malesia (see * below) and the Australian east coast; the migrations occurred probably via the intervening islands. These migration events could have led to the diversification of the species with tubular bracteoles (except *A. galanga*). In general, it was observed for both plants

* The Malesian region (Johns 1995) includes the following regions:
A. Western Malesia (Sunda shelf): the Malay Peninsula, Sumatra, Java, Borneo and Palawan.
B. Central Malesia: the Philippines (excluding Palawan), Sulawesi, the Lesser Sunda Islands and the Moluccas.
C. Eastern Malesia (Sahul shelf): New Guinea (Irian Jaya and Papua New Guinea).

and animals that the eastward movement across Wallace's Line appears to have been greater than in the opposite direction due to the superior competitive ability of species from a more diverse ecosystem (Briggs 1987). The next event of migration could have involved a northward direction to the Philippines, leading to the presence of *A. vulcanica*, *A. foxworthyi*, *A. flabellata* and *A. brevilabris*. The islands of the Philippines arose millions of years ago, independently of the Asian mainland (with the exception of Palawan), and these islands have moved towards Asia as a result of the northward-moving Australian continent (Heaney 1998). According to Roeder (1977) the Philippines has a composite origin. The western parts are regarded as fragments rifted during the Late Mesozoic from the continental margin of South China while the eastern parts are considered to be an intraoceanic arc that collided with the rifted fragments during the Oligocene. The fact that most of the islands remained isolated during the recent Ice Age in South East Asia (the Pleistocene Epoch: 15-20 thousands of years ago) could have led to a high level of endemism (Agoo 1996). However, the islands also harbour several groups of *Alpinia* (e.g. section *Alpinia* subsection *Catimbium*) which are common in Eastern Asia, the South East Asian mainland, the Indo-Malaysian region as well as New Guinea and the surrounding islands (Smith 1990a). Thus it appears that the Philippines may have been an important stepping-stone for the dispersal of *Alpinia* in many regions earlier than the Pleistocene Epoch (Agoo 1996). The next migratory routes could be to Borneo as well as China, Taiwan and Japan. Three species (*A. glabra*, *A. nieuwenhuizii* and *A. ligulata*) are found endemic to Borneo. In Eastern Asia (China, Taiwan and Japan) advanced groups of *Alpinia* species exist; many of them have small flowers (*A. coriacea*, *A. polyantha*, *A. pumila*, *A. japonica*, *A. intermedia*, *A. suishaensis* and *A. maclurei*), while the others have large showy flowers (*A. formosana*, *A.* aff. *shimadai*, *A. kwangsiensis*, *A. zerumbet*, *A. blepharocalyx* var. *glabrior* and *A. blepharocalyx* var. *blepharocalyx*). The

biogeographical interpretation of *Alpinia* has become more complicated with the same plants which co-occur in different areas. These plants, including *A. malaccensis* (Indo-Malaysian region, India and China), *A. latilabris* (Malaysia and India), *A. officinarum* (Hainan but also widely cultivated in South East Asia), *A. calcarata* (India and China) and *A. mutica* (Malaysia and India), possess the latter type of flower. The wide occurrence of these species could be merely a result of domestication.

6.4 THE MORPHOLOGICAL CHARACTERS OF ZINGIBERACEAE EXAMINED IN THIS STUDY

6.4.1 The stomata

The stomata are small pores located between two guard cells in the epidermis which have two basic functions that allow (i) gas exchange necessary for photosynthesis, and (ii) transpiration which may prevent the plant from over-heating, particularly in direct sunlight. The fact that the leaves of the Zingiberaceae under study were amphistomatic but with more stomata on the abaxial surface indicates that the plants show some adaptation to water loss. Most of these leaves also had a thick waxy layer of the cuticle which was waterproof and in some species of *Alpinia* such as, *A. rafflesiana* and *A. javanica*, the leaves possessed short hairs which restrict air movement and hence evaporation. In both species there was no significant difference in the stomatal densities on the two surfaces of the epidermis. They are common throughout Malaya in the lowlands, but the fact that *A. rafflesiana* is found up to 1219.2 m (4000 feet) on the hills and *A. javanica* is found in open places in the forest may result in the frequency of the stomata being different depending on where the plants are located. In this case, sampling from different areas may be necessary to find out if the nearly equal densities of the stomata on

both surfaces of the leaf are related to the habitats of the plants or to their genetic inheritance.

6.4.2 The rhizomes

In many families of monocotyledons (e.g. Iridaceae, Liliaceae, Araceae, Cyperaceae) plants possess rhizomes which serve as a food-storage or a resting organ (the rhizomes persist underground during unfavourable seasons while the leafy shoots wither). However, as most Zingiberaceae are confined to regions of the world with a warm and relatively uniform climate, the rhizomes merely act as a means for continuous vegetative growth of the plants (exceptions are commonly found in *Kaempferia*, *Curcuma* and *Zingiber* whose rhizomes are fleshy and certainly adapted for the storage of food) (Holttum 1955).

All rhizomes of the Zingiberaceae examined in this study had the direction of growth transverse to the plane of distichy of the leaves, hence the plants were classified in the tribe Alpineae. This type of growth provides ample space for a branching component. In contrast, in the tribes Hedychieae, Globbeae and Zingibereae, the rhizomes are parallel to the plane of distichy of the leaves and lateral meristems develop mostly from lower rhizomes and successive rhizome segments are predisposed to grow in the same direction as the parent axis (an example in *Hedychium* was given by Bell and Tomlinson 1980). This type of growth is less spacious for the branching component. If plants cannot change the direction of their growth due to limited space, by having linear rhizomes they can increase their chance of occupying less crowded ground and potentially enhance their fitness (Routledge 1986). For most *Alpinia* under study, Y-shaped rhizomes were prevalent. Although they did not form hexagonal grids, the Y-shaped pattern could be viewed as a good way of making use of available space which also help to avoid "clashes" of aerial shoots. Hutchings and de Kroon (1994) suggested that

plasticity in the morphology of the rhizome enables a plant to exploit local resources more efficiently.

6.4.3 The stigmas

It has been found that the most widespread stigma form in angiosperms is wet and papillate. Wet and non-papillate stigmas are rare and largely restricted to concave receptive surfaces such as in some species of Leguminosae and Marantaceae (Endress 1994). This latter type of stigma is found in *Alpinia zerumbet*, and Müller (1888) observed that flowers of this species are pollinated by large bees (euglossines). The stigma (**Figure 34 H**) was covered with dense hairs and it may be that the hairs hold a drop of stigmatic secretion or while touching the pollinator's body or wings, they generate electrostatic forces that attract adherent pollen (an example in *Globba* (Zingiberaceae) was given by Müller 1931).

The stigma size and shape may be influenced by natural selection generated by pollinators and other factors. The well expanded stigmas which are common among *Alpinia* species presumably evolved as a way to increase the quantity of pollen deposited on the stigmas. The structure and position of the orifice may largely depend on the type of pollinators and/or the type of pollen grains.

6.4.4 The pollen

In the Zingiberaceae under study the pollen grains were inaperturate and they possessed a very thin exine layer which was either smooth or spiny. The benefit of a thin exine is that the grains have multiple sites for pollen tube exit and, therefore, apertures are not necessary (Kress 1986; Stone 1987). Although the thin exine may not be suitable for performing functions such as protecting the gamete (Stanley and Linskens 1974) and preventing desiccation of the gametophyte (Wodehouse 1935;

Payne 1972, 1981; Muller 1979), it may still serve as a store of germination and self-incompatibility compounds (Heslop-Harrison 1975a, b) and act to facilitate transport during pollination (Muller 1979). According to Kress (1986), the structure of the exine in Zingiberales has most likely evolved in response to selective pressures exerted by pollen-stigma interaction and not pollen transport vectors. Zavada (1984, 1990) gave some examples (although not directly applicable to *Alpinia* and its outgroup) which illustrated that pollen morphology is strongly correlated with the compatibility system of species of angiosperms at the broadest levels. He concluded that reticulate-perforate exine sculpturing is correlated with sporophytic self-incompatibility while imperforate and microperforate exine sculpturing is correlated with gametophytic self-incompatibility. Gibbs and Ferguson (1987) however, urged that Zavada's conclusions should be treated with considerable caution.

In my study of the character evolution using MacClade, **Figure 44** shows the existence of *A. vulcanica* (with smooth pollen) in the *A. aquatica* clade (with spiny pollen). This could be due to a reversal of the character towards the ancestral state (smooth pollen).

6.5 THE MORPHOLOGICAL CHARACTERS OF ZINGIBERACEAE USED IN MACCLADE ANALYSES

6.5.1 The presence/absence and the type of the bract

The bracts arise from the main axis of an inflorescence and they protect floral buds and immature fruit. Large, stout bracts which are colourful may increase the display of small flowers, as suggested for the enlarged extrafloral bracts in some Verbenaceae (Classen 1986) and *Etlingera elatior* (Classen 1987). Such bracts occur in several species of *Alpinia* such as *A. purpurata* and *A. vittata.* In *Hornstedtia* the tightly overlapping bracts contain aqueous solution secreted by the

plants which functions as a sticky trap to reduce herbivory on reproductive floral parts (Ippolito and Armstrong 1993). In an advanced group of *Alpinia* subsection *Catimbium* (Smith 1990a) the bracts have been lost during the course of evolution. The bracts have become unnecessary, probably because the inflorescence develops almost to maturity inside the protective leaf sheaths (Holttum 1950) and bracteoles usually enclosing the flower buds, thereby protecting them. Other species of *Alpinia* possess bracts which are soon deciduous (or soon after anthesis). Their protective function is no longer required after anthesis.

Figure 39 of the MacClade analysis shows the existence of *A. officinarum* (bracts present) within the *A. zerumbet* clade which contains subsection *Catimbium* (bracts absent). This occurrence could be interpreted as a reversal.

6.5.2 The presence/absence and the type of the bracteole

The bracteoles are bracts which subtend individual flowers in a cincinnus. As with the bracts, the bracteoles also protect floral buds in an early stage of development. However, in some species of *Alpinia* (in Smith's (1990a) subsections *Catimbium* and *Probolocalyx*) the bracteoles enclose the floral buds until the later stage of development and are usually pushed off by the opening flowers. The absence of the bracteoles in subsection *Cenolophon* (Smith 1990a) may be related to the fact that each cincinnus is reduced to a single flower, while in subsection *Paniculatae* (Smith 1990a), protective calyptrate bracts are present. Based on the ITS cladograms *Alpinia pumila* (bracteoles absent) (Smith's (1990a) section *Didymanthus*) is closely related to members of Smith's (1990a) section *Alpinia* subsection *Alpinia* (bracteoles present). The lack of the bracteole in *A. pumila* could be due to its unique character of terminal inflorescences on a very short stem and also the presence of the persistent bract.

Many *Alpinia* species with tubular bracteoles often have tightly congested inflorescences (e.g. in Smith's (1990a) sections *Pycnanthus* and *Dieramalpinia*). These bracteoles are usually persistent and protect mature flowers as well as fruit. They may function to collect rain or as sticky traps to guard against predators. In **Figure 42** it is indicated that for *Alpinia* the non-tubular bracteole character was derived from the more primitive tubular bracteole character. This may suggest that the functions of the tubular bracteole may not be absolutely necessary for the plants and therefore the tubular structure can be lost through the course of evolution.

6.5.3 The type of the fruit and seed

Although the anatomical characters of the fruit and seed of Chinese *Alpinia* (see **Table 9**) are highly congruent with the results based on ITS data, there are a few points which indicate that these characters should be used with caution. First, Liao and Wu (1996a, b) did not cite voucher specimens, so the species identification may be uncertain and therefore results obtained from the studies are unreliable. Second, many of the fruit and seed characters, especially those concerned with the volume of the cells and the amount of pigment bodies in the cells, are not distinctive. They appear to be very subjective. In order to use these characters for phylogenetic studies of *Alpinia*, the species need to be identified with certainty and the characters re-examined. More samples of both Chinese and non-Chinese *Alpinia* species should be included.

However, the grouping of *A. galanga* with *A. nigra* and *A. conchigera* (suggested by molecular data) is strongly supported by the following fruit and seed characters (see **Table 9**; fruit characters: (i)-(ii), seed characters: (iii)-(vii)): (i) hypodermis present in mesocarp, (ii) the innermost cell layer of mesocarp is composed of a row of roundish parenchymatous cells with extremely large volume, (iii) number of cell layers of seed coat is less than eight, (iv) parenchymatous cells

of pigment cell layers contain pigment cells only, (v) mesophyll contains some pigment cells and translucent cells with large volume, (vi) positions of gap of endotesta and chalazal pigment cell group are at dorsal side and near the top, and (vii) seeds are slightly asymmetrical.

6.6 CONFLICTS BETWEEN MOLECULAR DATA AND MORPHOLOGICALLY BASED CLASSIFICATIONS OF *ALPINIA*

Although this present study of the infrageneric classification of *Alpinia*, based on molecular data, represents only a small sample, the results reveal some conflicts between the molecular and the morphologically based analyses. The molecular data partly indicate that Smith's (1990a) taxonomic categories are misleading at all levels including subgenera (e.g. the strongly supported *A. eubractea* clade contains members of both subgenera *Alpinia* and *Dieramalpinia*), sections (e.g. the polyphyly of section *Allughas*) and subsections (e.g. the unnatural grouping of subsection *Alpinia*). These conflicts may be attributed to both the morphological and the molecular characters used, and result from the apparent or real lack of congruence between them. Factors which may be involved in such conflicts are as follows.

(i) Unequal rates of morphological evolution (the rate of change in the morphology is highly variable, while that of the DNA is usually fairly constant). A dramatic increase in the rate of morphological divergence often gives rise to a discrepancy between traditional and molecular data as seen in the example of *Heterogaura* (Onagraceae), a monotypic genus which was closely related to *Clarkia* based on floral morphology, but molecular analyses (cp DNA and nrDNA analyses) indicated that *Heterogaura* was actually derived from within *Clarkia* (Sytsma and Gottlieb 1986a, b).

(ii) Convergence and/or parallelism of morphological and molecular characters. This problem can result in a high level of homoplasy, thus misleading phylogenetic relationships are obtained. An example is given in subtribe Oncidiinae (Orchidaceae) where a cpDNA phylogeny of the subtribe (based on comparative cpDNA restriction site mapping) revealed that strong regional evolution in this neotropical group of orchids produced similar floral morphologies in parallel among relatively distantly related species (Palmer *et al.* 1988). This type of floral parallelism had previously misled taxonomists into assuming that several sections of *Oncidium* in subtribe Oncidiinae, although geographically widespread, were closely related on the basis of floral synapomorphies. Convergence also occurs in molecular characters, in some instances to a considerable extent as shown by Hori *et al.* (1985) and Bremer *et al.* (1987) in 5S rDNA sequence data. However, the convergence in the molecular data can be dealt with more easily due to the present knowledge of the genetic basis for convergences in molecules. For example, the bias towards transitions rather than transversions in most DNA sequences allows reseachers to carry out additional analysis on sequence data with high levels of convergences.

(iii) Ancient hybridisation. More recent hybridisation and/or introgression may have not occurred in the species of Zingiberaceae under study because to some extent the ITS and the cpDNA spacer phylogenies are congruent. However, in this study, not all species which amplified well for the ITS region amplified with the cpDNA spacer so congruence between the two sets of data cannot be entirely demonstrated. It could be that ancient hybridisation may still have occurred but in the ITS region it was followed by concerted evolution and lineage sorting which homogenised different types of nrDNA repeat from different parental origin so that only one type of the repeat may be seen in the hybrid. The use of hybrid derivatives in the molecular analyses could then lead to discordance between molecular phylogenies and morphology (e.g. in Wendel *et al.* 1995a, b). In addition, it has

been shown in allopolyploid *Gossypium* that bidirectional interlocus concerted evolution in the ITS region (a possibility whenever two or more divergent repeat types are maintained through at least one cladogenetic (speciation) event) when followed by interlocus homogenisation to alternative repeat types, a strongly supported, but positively misleading phylogeny was obtained (Wendel *et al.* 1995a).

In *Alpinia* the conflicts between different data sets may involve more than one factor. However, it seems most likely that convergence and/or parallelism of both the morphological and the molecular characters are the major factors. Classifications by Schumann, Holttum and Smith are based on only a few characters which may be related to biological functions and hence are not independent. The use of a single, highly adaptive and homoplastic character such as the labellum (Smith 1990a) can undoubtedly result in conflict. The infrageneric classification of *Alpinia* may be more reliable using molecular data which contain neutral characters. However, the ITS sequence data are also found to exhibit a certain amount of homoplasy.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDY

The putative phylogenies of the Zingiberaceae under study obtained from ITS analyses suggest that *Alpinia* in fact, may not be a natural group. In addition, the results based on both nrDNA and cpDNA spacers (the ITS region and the region between *trn*L (UAA) 3' exon and *trn*F (GAA), respectively) largely disagree with Smith's (1990a) infrageneric classification of *Alpinia*. My data are insufficient to allow a complete reclassification of the genus. However, the molecular and morphological results do suggest the following modifications should be made to Smith's (1990a) system. First, the dividing line between subgenera *Alpinia* and *Dieramalpinia* is not distinct. Second, *Alpinia abundiflora* and *A. fax* may be transferred to *Renealmia*. Third, subgenus *Alpinia* should be recast because the type species of the genus (*A. galanga*) is currently misclassified. Finally, a recognition of the fact that section *Allughas* and section *Alpinia* subsections *Alpinia* and *Cenolophon* are not monophyletic.

Based on ITS analyses a sectional classification of *Alpinia* is suggested as shown in **Table 13**.

This molecular work provides good support for the separation of *Pleuranthodium*. It also serves as an interesting model system to explore several important topics in evolutionary biology, especially when combined with the morphological and biogeographical data.

For further studies of *Alpinia*, there are several aspects to be considered. First, the non-monophyletic nature of the genus should be confirmed by adding more samples of African *Renealmia* and samples of Tropical American *Renealmia* as well as other closely related genera (in terms of floral morphology) such as *Riedelia*, *Geostachys* and *Vanoverbergia* Merr. in molecular analyses. Secondly, more work is required on alpha taxonomy of *Alpinia* species in poorly collected

areas such as the Philippines, New Guinea and the surrounding islands, and to include these species which mainly belong to Smith's (1990a) subgenus *Dieramalpinia* in the molecular analyses. Thirdly, other molecules such as the non-transcribed portion of 5S rDNA gene which may be more variable than the ITS region, and the *mat*K gene (encoding a maturase enzyme) which has been found to evolve more rapidly than the most frequently used intergenic spacer, *trn*L-*trn*F of cpDNA (reviewed in Sang *et al.* 1997) should be sequenced to obtain more resolved branches within *Alpinia*. Fourthly, the hunt for new morphological characters can include re-examination and follow-up of the study of fruit and seed anatomy of *Alpinia* by Liao and Wu (1996a, b). Other pieces of work which are related to the study of ovary structure and anatomy, and which may be used for phylogenetic analyses are found, for instance, in Heliconiaceae and Musaceae (Kirchoff 1992) and in Costaceae (Winnell *et al.* 1992). Next, studies involving pollination mechanisms and pollination syndromes may be carried out for *Alpinia*. Finally, the type of polyploidy (allopolyploidy or autopolyploidy) in the tetraploid species of *Alpinia* and its outgroup should be determined. If these species are allopolyploid, it is possible that they may have experienced similar events as demonstrated in allopolyploid *Gossypium* (Wendel *et al.* 1995a), resulting in misleading ITS phylogenies.

Table 13. A sectional classification of *Alpinia* suggested by ITS analyses.

1. Section *Fax* (BS = 99%)
(or may be transferred to *Renealmia*)
A. abundiflora
A. fax
2. Section *Alpinia* (BS = 100%)
A. galanga
A. conchigera
A. nigra
3. Section *Javanica* (BS = 100%)
A. rafflesiana
A. javanica
4. Section *Pycnanthus* (BS = 99%)
A. carolinensis
A. boia
A. coeruleoviridis
5. Section *Guillainia* (BS = 100%)
A. eubractea
A. elegans
A. luteocarpa (*scorpoidea*)
A. arctiflora
A. arundelliana
A. modesta
A. caerulea
A. purpurata
A. oceanica
A. vittata
6. Section *Presleia* (BS = 95%)
A. vulcanica
A. aquatica
A. foxworthyi
A. flabellata
A. brevilabris
7. Section *Catimbium* (BS = 90%)
Subsection *Catimbium*
A. formosana
A. aff. *shimadai*
A. kwangsiensis
A. zerumbet
A. blepharocalyx var. *glabrior*
A. blepharocalyx var. *blepharocalyx*
A. malaccensis
A. latilabris
A. officinarum
A. calcarata
A. mutica
Other members of Section *Catimbium*
A. oxyphylla
A. glabra
A. nieuwenhuizii
A. ligulata
A. coriacea
A. polyantha
A. pumila
A. japonica
A. intermedia
A. suishaensis
A. maclurei
8. Incertae Sedis (BS < 50%)
A. oxymitra

Figure 46. Colour plates illustrating the variation within *Alpinia* and some of its outgroup. Taxonomic treatment of *Alpinia* follows Smith's (1990a) system.

A. *Burbidgea schizocheila* Hackett. Photo: A. Rangsiruji.

B. *Elettariopsis triloba* (Gagnep.) Loes. Photo: A. Rangsiruji.

C. *Alpinia* sp. (subg. *Alpinia* sect. *Alpinia* subsect. *Alpinia*). Photo: A. Rangsiruji.

D. *Alpinia aquatica* (Retz.) Roscoe (subg. *Alpinia* sect. *Alpinia* subsect. *Presleia*). Photo: J. Mood.

E. *Alpinia nieuwenhuizii* Valeton (subg. *Alpinia* sect. *Alpinia* subsect. *Paniculatae*). Photo: J. Mood.

F. *Alpinia hansenii* R.M.Sm. (subg. *Alpinia* sect. *Alpinia* subsect. *Cenolophon*). Photo: A. Lamb.

G. *Alpinia zerumbet* (Pers.) B.L.Burtt & R.M.Sm. (subg. *Alpinia* sect. *Alpinia* subsect. *Catimbium*). Photo: A. Rangsiruji.

H. *Alpinia glabra* Ridl. (subg. *Alpinia* sect. *Alpinia* subsect. *Probolocalyx*). Photo: The Royal Botanic Garden Edinburgh.

I. *Alpinia abundiflora* B.L.Burtt & R.M.Sm. (subg. *Alpinia* sect. *Fax*). Photo: The Royal Botanic Garden Edinburgh.

J. *Alpinia purpurata* (Vieill.) K.Schum. (subg. *Alpinia* sect. *Guillainia*). Photo: A. Rangsiruji.

K. *Alpinia javanica* Blume (subg. *Alpinia* sect. *Allughas* subsect. *Allughas*). Photo: The Royal Botanic Garden Edinburgh.

L. *Alpinia conchigera* Griff. (subg. *Alpinia* sect. *Allughas* subsect. *Strobidia*). Photo: The Royal Botanic Garden Edinburgh.

M. *Alpinia monopleura* K.Schum. (subg. *Dieramalpinia* section *Myriocrater*). Photo: The Royal Botanic Garden Edinburgh.

N. *Alpinia vittata* W.Bull (subg. *Dieramalpinia* section *Dieramalpinia*). Photo: The Royal Botanic Garden Edinburgh.

A.

B.

C.

D.

E.

F.

G.

H.

I.

J.

K.

L.

M.

N.

REFERENCES

Agoo, E.M.G. (1996). Philippine *Alpinia*. In Wu, T-L., Wu, Q-G. & Chen, Z-Y. (eds.): *Proceedings of the second symposium on the family Zingiberaceae*, pp. 28-30. Zhongshan University Press: Guangzhou, China.

Ahamad, P.Y.A. & Ahmed, S.M. (1991). Potential of some rhizomes of Zingiberaceae family as grain protectants against storage insect pests. *J. Food Sci. Technol.* (Mysore) **28**: 375-377.

Aldrich, J., Cherney, B.W., Merlin, E. & Christopherson, L. (1988). The role of insertions/deletions in the evolution of the intergenic region between *psb*A and *trn*H in the chloroplast genome. *Curr. Genet.* **14**: 137-146.

Appels, R. & Dvorak, J. (1982). The wheat ribosomal DNA spacer region: its structure and variation in populations and among species. *Theor. Appl. Genet.* **63**: 337-348.

Appels, R., McIntyre, C.L. & Clarke, B.C. (1986). Alien chromatin in wheat: ribosomal DNA spacer probes for detecting specific nucleolar organizer region loci introduced into wheat. *Canad. J. Genet. Cytol.* **28**: 665-672.

Arnheim, N., Krystal, M., Schmickel, R., Wilson, G., Ryder, O. & Zimmer, E. (1980). Molecular evidence for genetic exchanges among ribosomal genes on non-homologous chromosomes in man and apes. *Proc. Natl. Acad. Sci. USA* **77**: 7323-7327.

Baker, J.G. (1890). Scitamineae. In Hooker, J.D. (ed.): The Flora of British India, vol. 6, pp. 198-257. Reeve & Co.: Kent, England.

Baldwin, B.G. (1992). Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molec. Phylogenet. Evol.* **1**: 3-16.

Baldwin, B.G. (1993). Molecular phylogenetics of *Calycadenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: chromosomal and morphological evolution reexamined. *Amer. J. Bot.* **80**: 222-238.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S. & Donoghue, M.J. (1995). The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Gard.* **82**: 247-277.

Baum, D.A., Systma, K.J. & Hoch, P.C. (1994). A phylogenetic analysis of *Epilobium* (Onagraceae) based on nuclear ribosomal DNA sequences. *Syst. Bot.* **19**: 363-388.

Bell, A.D. (1974). Rhizome organisation in relation to vegetative spread in *Medeola virginiana*. *J. Arnold Arbor.* **55**: 458-468.

Bell, A.D. (1976). Computerised vegetative mobility in rhizomatous plants. In Lindenmayer, A. & Rozenberg, G. (eds.): *Automata, Languages, Development*. North Holland Publishing Company: Amsterdam.

Bell, A.D. (1979). The hexagonal branching pattern of rhizomes of *Alpinia speciosa* L. (Zingiberaceae). *Ann. Bot.* **43**: 209-223.

Bell, A.D. & Bryan, A. (1991). *Plant form: an illustrated guide to flowering plant morphology*, pp. 130-131. Oxford University Press: Oxford.

Bell, A.D. & Tomlinson, P.B. (1980). Adaptive architecture in rhizomatous plants. *Bot. J. Linn. Soc.* **80**: 125-160.

Beltran, I.C. & Kiew, K.Y. (1984). Cytotaxonomic studies in the Zingiberaceae. *Notes Roy. Bot. Gard. Edinburgh* **41**: 541-559.

Bentham, G. & Hooker, J.D. (1883). *Genera plantarum*, vol. 3. L. Reeve & Co.; Williams & Norgate: London.

Birky, C.W. (1988). Evolution and variation in plant chloroplast and mitochondrial genomes. In Gottlieb, L.D. & Jain, S.K. (eds.): *Plant evolutionary biology*, pp. 23-53. Chapman and Hall: London.

Blume, C.L. (1827). *Enumeratio plantarum Javae*. Lugduni Batavorum.

Böhle, U-R., Hilger, H., Cerff, R. & Martin, W.F. (1994). Non-coding chloroplast DNA for plant molecular systematics at the infrageneric level. In Schierwater, B., Streit, B., Wagner, G.P. & DeSalle, R. (eds.): *Molecular ecology and evolution*: approaches and applications, pp. 391-403. Birkhauser Verlag: Basel, Switzerland.

Bot, P.V.M., Holton, R.W., Stam, W.T. & van den Hoek, C. (1989a). Molecular divergence between North Atlantic and Indo-West Pacific *Cladophora albida* (Cladophorales, Chlorophyta) isolates as indicated by DNA-DNA hybridisation. *Mar. Biol.* **102**: 307-313.

Bot, P.V.M., Stam, W.T., Boele-Bos, S.A., van den Hoek, C. & van Delden, W. (1989b). Biogeographic and phylogenetic studies of *Cladophora* (Cladophorales, Chlorophyta) using DNA-DNA hybridisation. *Phycologia* **28**: 159-168.

Boulter D., Thompson, E.W., Ramshaw, J.A.M. & Richardson, M. (1970). Higher plant cytochrome *c*. *Nature* (London) **228**: 552-554.

Boulter, D., Haslett, B.G., Peacock, D., Ramshaw, J.A.M. & Scawen, M.D. (1977). Chemistry, function and evolution of plastocyanin. In Northcote, D.H. (ed.):

International review of biochemistry, plant biochemistry II, vol. 13, pp. 1-40. University Park Press: Baltimore, USA.

Boulter, D., Peacock, D., Guise, A., Gleaves, J.T. & Estabrook, G. (1979). Relationships between partial amino acid sequences of plastocyanin from members of ten families of flowering plants. *Phytochemistry* **18**: 603-608.

Boulter, D., Ramshaw, J.A.M., Thompson, E.W., Richardson, M. & Brown, R.H. (1972). A phylogeny of higher plants based on the amino acid sequences of cytochrome *c* and its biological implications. *Proc. Roy. Soc. London, Ser. B.* **181**: 441-455.

Bremer, K. (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42**: 795-803.

Bremer, K., Humphries, C.J., Mishler, B.D. & Churchill, S.P. (1987). On cladistic relationships in green plants. *Taxon* **36**: 339-349.

Briggs, J.C. (1987). *Biogeography and plate tectonics*. Elsevier Science Publishers B.V.: Amsterdam.

Brown, D.D., Wensink, P.C. & Jordan, E. (1972). A comparison of the ribosomal DNAs of *Xenopus laevis* and *Xenopus mulleri*; the evolution of tandem genes. *J. Molec. Biol.* **63**: 57-73.

Brown, J.W.S. & Shaw, P.J. (1998). Small nucleolar RNAs and pre-rRNA processing in plants. *Pl. Cell* **10**: 649-657.

Brown, R. (1830). Remarks on apostasia. *Plantae Asiaticae Rariores* **1**: 76.

Bruneau, A. (1996). Phylogenetic and biogeographical patterns in *Erythrina* (Leguminosae: Phaseoleae) as inferred from morphological and chloroplast DNA characters. *Syst. Bot.* **21**: 587-605.

Buckler IV, E.S. & Holtsford, T.P. (1996a). *Zea* systematics: ribosomal ITS evidence. *Molec. Biol. Evol.* **13**: 612-622.

Buckler IV, E.S. & Holtsford, T.P. (1996b). *Zea* ribosomal repeat evolution and substitution patterns. *Molec. Biol. Evol.* **13**: 623-632.

Buckler IV, E.S., Ippolito, A. & Holtsford, T.P. (1997). The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. *Genetics* **145**: 821-832.

Burtt, B.L. & Olatunji, O.A. (1972). The limits of the tribe Zingibereae. *Notes Roy. Bot. Gard. Edinburgh* **31**: 167-169.

Burtt, B.L. & Smith, R.M. (1972a). Key species in the taxonomic history of Zingiberaceae. *Notes Roy. Bot. Gard. Edinburgh* **31**: 177-227.

Burtt, B.L. & Smith, R.M. (1972b). Monoecism in *Alpinia* (Zingiberaceae), with descriptions of six new species. *Notes Roy. Bot. Gard. Edinburgh* **32**: 29-43.

Burtt, B.L. & Smith, R.M. (1976). Notes on the collection of Zingiberaceae. *Fl. Males. Bull.* **29**: 2599-2601.

Cain, M.L. & Cook, R.E. (1988). Growth in *Medeola virginiana* clones. II. Stochastic simulation of vegetative spread. *Amer. J. Bot.* **75**: 732-738.

Campbell, C.S., Donoghue, M.J., Baldwin, B.G. & Wojciechowski, M.F. (1995). Phylogenetic relationships in Maloideae (Rosaceae): Evidence from sequences of the internal transcribed spacers of nuclear ribosomal DNA and its congruence with morphology. *Amer. J. Bot.* **82**: 903-918.

Carr, G.D. (1987). Beggar's ticks and tarweeds: masters of adaptive radiation. *Trends Ecol. Evol.* **2**: 192-195.

Chandler, M.E.J. (1964). The Lower Tertiary floras of Southern England. IV. A summary and survey of findings in the light of recent botanical observations. *Bull. Brit. Mus.* (*Nat. Hist.*), *Geol.* **12**: 1-151.

Chase, M.W. & Hills, H.H. (1991). Silica gel: an ideal material for field preservation of leaf samples for DNA studies. *Taxon* **40**: 215-220.

Chase, M.W., Soltis, D.E., Olmstead, R.G., Morgan, D., Les, D.H., Mishler, B.D., Duvall, M.R., Price, R.A., Hills, H.G., Qiu, Y-L., Kathleen, A.K., Rettig, J.H., Conti, E., Palmer, J.D., Manhart, J.R., Sytsma, K.J., Michael, H.J., Kress, W.J., Karol, K.G., Clark, W.D., Hedrén, M., Gaut, B.S., Jansen, R.K., Kim, K-J., Wimpee, C.F., Smith, J.F., Furnier, G.R., Strauss, S.H., Xiang, Q-Y., Plunkett, G.M., Soltis, P.S., Swensen, S.M., Williams, S.E., Gadek, P.A., Quinn, C.J., Eguiarte, L.E., Golenberg, E., Learn (Jr.), G.H., Graham, S.W., Barrett, S.C.H., Dayanandan, S. & Albert, V.A. (1993). Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. *Ann. Missouri Bot. Gard.* **80**: 528-580.

Chatterson, N.J., Hsiao, C., Asay, K.H., Wang, R.R-C. & Jensen, K.B. (1992a). Nucleotide sequences of the internal transcribed spacer region of rDNA in diploid wheat, *Triticum speltoides* L. (Tausch) Gren. ex Richter (Gramineae). *Pl. Molec. Biol.* **20**: 157-158.

Chatterson, N.J., Hsiao, C., Asay, K.H., Wang, R.R-C. & Jensen, K.B. (1992b). Nucleotide sequences of the internal transcribed spacer region of rDNA in wheat, *Triticum aestivum* L. (Gramineae). *Pl. Molec. Biol.* **20**: 159-160.

Chatterson, N.J., Hsiao, C., Asay, K.H., Wang, R.R-C. & Jensen, K.B. (1992c). Nucleotide sequences of the internal transcribed spacer region of rDNA in mountain rye, *Secale montanum* Guss. (Gramineae). *Pl. Molec. Biol.* **20**: 161-162.

Chatterson, N.J., Hsiao, C., Asay, K.H., Wang, R.R-C. & Jensen, K.B. (1992d). Nucleotide sequences of the internal transcribed spacer region of rDNA in the primitive oat species, *Avena longiglumis* Durieu (Gramineae). *Pl. Molec. Biol.* **20**: 163-164.

Chatterson, N.J., Hsiao, C., Asay, K.H., Wang, R.R-C. & Jensen, K.B. (1992e). Nucleotide sequences of the internal transcribed spacer region of rDNA in barley, *Hordeum vulgare* L. (Gramineae). *Pl. Molec. Biol.* **20**: 165-166.

Chen, Z-Y. (1989). Evolutionary patterns in cytology and pollen structure of Asian Zingiberaceae. In Holm-Nielsen, L.B., Nielsen, I.C. & Balslev, H. (eds.): *Tropical forests: botanical dynamics, speciation and diversity*, pp. 185-191. Academic Press: London.

Chen, Z-Y. & Huang, X-X. (1996). Cytotaxonomy of the tribe Alpinieae. In Wu, T-L., Wu, Q-G. & Chen, Z-Y. (eds.): *Proceedings of the second symposium on the family Zingiberaceae*, pp. 112-121. Zhongshan University Press: Guangzhou, China.

Choi, B-H. & Kim, J-H. (1997). ITS sequences and speciation on Far Eastern *Indigofera* (Leguminosae). *J. Pl. Res.* **110**: 339-346.

Chopra, R.N., Nayar, S.L. & Chopra, I.C. (1956). *Glossary of Indian medicinal plants*. Council of Scientific & Industrial Research: New Delhi.

Classen, R. (1986). Organization und funktion der blumenbilden den hochblatthiillen bei Symphoremoideae (Verbenaceae). *Beitr. Biol. Pflanzen.* **60**: 383-402.

Classen, R. (1987). Morphological adaptations for bird pollination in *Nicolaia elatior* (Jack) Horan. (Zingiberaceae). *Gard. Bull. Singapore* **40**: 37-43.

Clegg, M.T. (1989). Molecular diversity in plant populations. In Brown, A.H.D., Clegg, M.T., Kahler, A.L. & Weir, B.S. (eds.): Plant population genetics, breeding and genetic resources, pp. 98-115. Sinauer Associates: Sunderland, Massachusetts.

Clegg, M.T. & Zurawski, G. (1991). Chloroplast DNA and the study of plant phylogeny: present status and future prospects. In Soltis, P.M., Soltis, D.E. & Doyle, J.J. (eds.): *Molecular systematics of plants*, pp. 1-13. Chapman and Hall: New York.

Clegg, M.T., Learn (Jr.), G.H. & Golenberg, E.M. (1991). Molecular evolution of chloroplast DNA. In Selander, R.K., Clark, A.G. & Whittam, T.S. (eds.): *Evolution at the molecular level*, pp. 135-149. Sinauer Associates: Sunderland, Massachusetts.

Clegg, M.T., Gaut, B.S., Learn (Jr.), G.H. & Morton, B.R. (1994). Rates and patterns of chloroplast DNA evolution. *Proc. Natl. Acad. Sci. USA* **91**: 6795-6801.

Clifford, R.P. & Wendel, J.F. (1990). Molecular divergence between Asian and North American species of *Liriodendron* (Magnoliaceae) with implications for interpretation of fossil floras. *Amer. J. Bot.* **77**: 1243-1256.

Collinson, M.E., Fowler, K. & Boulter, M.C. (1981). Floristic changes indicate a cooling climate in the Eocene of Southern England. *Nature* **291**: 315-317.

Council of Scientific & Industrial Research India (1948). *The wealth of India: a dictionary of Indian raw materials and industrial products*. Raw materials, vol. 1: A (revised). Publications & Information Directorate Council of Scientific & Industrial Research: New Delhi.

Cowley, J. (1982). A revision of *Roscoea* (Zingiberaceae). *Kew Bull.* **36**: 747-777.

Crawford, D.J. (1990). *Plant molecular systematics*. John Wiley: New York.

Crisp, M.D. (1994). Evolution of bird-pollination in some Australian legumes (Fabaceae). In Eggleton, P. & Vane-Wright, R.I. (eds.): *Phylogenetics and ecology*, pp. 281-309. The Linnaean Society of London and Academic Press: London.

Cronquist, A. (1981). *An integrated system of classification of flowering plants*. Columbia University Press: New York.

Dahlgren, R.M.T. (1977). A commentary on a diagramatic presentation of the angiosperms in relation to the distribution of character states. In Kubitzki, K. (ed.): *Flowering plants: evolution and classification of higher categories*, pp. 253-283. Springer-Verlag: New York.

Dahlgren, R.M.T. & Clifford, H.T. (1982). *The monocotyledons: a comparative study*. Academic Press: London.

Dahlgren, R. & Rasmussen, F.N. (1983). Monocotyledon evolution: characters and phylogenetic estimation. In Hecht, M.K., Wallace, B. & Prance, G.T. (eds.): *Evolutionary Biology* **16**: 255-395.

Dahlgren, R., Clifford, H.T. & Yeo, P.F. (1985). *The families of the monocotyledons*. Springer-Verlag: Berlin.

Debener, T., Salamini, F. & Gebhardt, C. (1990). Phylogeny of wild and cultivated *Solanum* species based on nuclear restriction fragment length polymorphisms (RFLPs). *Theor. Appl. Genet.* **79**: 360-368.

Doebley, J.F., Ma, D.P. & Renfroe, W.T. (1987). Insertion/deletion mutations in the *Zea* chloroplast genome. *Curr. Genet.* **11**: 617-624.

Doebley, J.F., Durbin, M., Golenberg, E.M., Clegg, M.T. & Ma, D.P. (1990). Evolutionary analysis of the large subunit of carboxylase (*rbc*L) nucleotide sequence among the grasses (Gramineae). *Evolution* **44**: 1097-1108.

Donoghue, M.J. & Baldwin, B.G. (1993). Phylogenetic analysis of *Viburnum* based on ribosomal DNA sequences from the internal transcribed spacer regions. *Amer. J. Bot.* **80** (supplement, abstract): 145.

Donoghue, M.J., Olmstead, R.G., Smith, J.F. & Palmer, J.D. (1992). Phylogenetic relationships of *Dipsacales* based on *rbc*L sequences. *Ann. Missouri Bot. Gard.* **79**: 333-345.

Dover, G.A. (1982). Molecular drive: A cohesive mode of species evolution. *Nature* **299**: 111-117.

Dover, G.A. (1986). Molecular drive in multigene families: how biological novelties arise, spread and are assimilated. *Trends Genet.* **6**: 159-165.

Downie, S.R. & Katz-Downie, D.S. (1996). A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal spacer sequences. *Amer. J. Bot.* **83**: 234-251.

Downie, S.R. & Palmer, J.D. (1992a). Restriction site mapping of the chloroplast DNA inverted repeat: a molecular phylogeny of the Asteridae. *Ann. Missouri Bot. Gard.* **79**: 266-283.

Downie, S.R. & Palmer, J.D. (1992b). Use of chloroplast DNA rearrangements in reconstructing plant phylogeny. In Soltis, P.S., Soltis, D.E. & Doyle, J.J. (eds.): *Molecular systematics of plants*, pp. 14-35. Chapman and Hall: New York.

Doyle, J.J. (1992). Gene trees and species trees: molecular systematics as one-character taxonomy. *Syst. Bot.* **17**: 144-163.

Doyle, J.J. & Dickson, E.E. (1987). Preservation of plant samples for DNA restriction endonuclease analysis. *Taxon* **36**: 715-722.

Doyle, J.J. & Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**: 11-15.

Doyle, J.J. & Doyle, J.L. (1993). Chloroplast DNA phylogeny of the papilionoid legume tribe Phaseoleae. *Syst. Bot.* **18**: 309-327.

Drouin, G. & Dover, G.A. (1990). Independent gene evolution in the potato actin gene family demonstrated by phylogenetic procedures for resolving gene conversions and the phylogeny of angiosperm actin genes. *J. Molec. Evol.* **31**: 132-159.

Efron, B. (1979). Bootstrap methods: another look at the jackknife. *Ann. Stat.* **7**: 1-26.

Eichler, A.W. (1875). Bluthendiagramme construirt und erlautert. Leipzig, Germany.

Eldenäs, P., Anderberg, A.A. & Källersjö, M. (1998). Molecular phylogenetics of the tribe Inuleae *s. str.* (Asteraceae) based on ITS sequences of nuclear ribosomal DNA. *Pl. Syst. Evol.* **210**: 159-173.

Elmer, A.D.E. (1919). Zingiberaceae of the Sorsogon Peninsula. *Leafl. Philipp. Bot.* **8:** 2963-2995.

Endress, P.K. (1994). *Diversity and evolutionary biology of tropical flowers.* In Ashton, P.S., Hubbel, S.P., Janzen, D.H., Raven, P.H. & Tomlinson, P.B. (eds.). Cambridge University Press: Great Britain.

Farris, J.S. (1969). A successive approximations approach to character weighting. *Syst. Zool.* **18**: 374-385.

Farris, J.S. (1989). The retention index and homoplasy excess. *Syst. Zool.* **38**: 406-407.

Federoff, N.V. (1979). On spacers. *Cell* **16**: 697-710.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

Fischer, H. & Jensen, U. (1990). Phytoserological investigation of the tribe Carolueae *s.l.* (Compositae). *Pl. Syst. Evol.* (supplement 4): 99-111.

Fitch, W.M. (1970). Distinguishing homologous from analogous proteins. *Syst. Zool.* **19**: 99-113.

Fitch, W.M. & Margoliash, E. (1967). Construction of phylogenetic trees. *Science* **155**: 279-284.

Flavell, R.B. & O'Dell, M. (1979). The genetic control of nucleolus formation in wheat. *Chromosoma* **71**: 135-152.

Fogel, S. & Mortimer, R.K. (1969). Information transfer in meiotic gene conversion. *Proc. Natl. Acad. Sci. USA* **62**: 96-103.

Gagnepain, F. (1908). Zingibéracées. *Flore Générale de l'Indo-Chine* **5**: 25-121.

Gentzbittel, L., Perrault, A. & Nicolas, P. (1992). Molecular phylogeny of the *Helianthus* genus, based on nuclear restriction-fragment-length polymorphism (RFLP). *Molec. Biol. Evol.* **9**: 872-892.

Giannasi, D.E., Zurawski, G., Learn (Jr.), G. & Clegg, M.T. (1992). Evolutionary relationships of the Caryophyllidae based on comparative *rbc*L sequences. *Syst. Bot.* **17**: 1-15.

Gibbs, P.E. & Ferguson, I.K. (1987). Correlations between pollen exine sculpturing and angiosperm self-incompatibility systems - a reply. *Pl. Syst. Evol.* **157**: 143-159.

Gielly, L. & Taberlet, P. (1994). Chloroplast DNA polymorphism at the infrageneric level: implications for the establishment of plant phylogenies. *C.R. Acad. Sci. Paris, Sciences de la Vie/Life Sciences* **317**: 685-692.

Gielly, L. & Taberlet, P. (1996). A phylogeny of the European gentians inferred from chloroplast *trn*L (UAA) intron sequences. *Bot. J. Linn. Soc.* **120**: 57-75.

Gielly, L., Yuan, Y-M., Küpfer, P. & Taberlet, P. (1996). Phylogenetic use of noncoding regions in the genus *Gentiana* L.: chloroplast *trn*L (UAA) intron versus nuclear ribosomal internal transcribed spacer sequences. *Molec. Phylogenet. Evol.* **5**: 460-466.

Gonzalez, I.L., Sylvester, J.E., Smith, T.F., Stambolian, D. & Schmickel, R.D. (1990). Ribosomal RNA gene sequences and hominoid phylogeny. *Molec. Biol. Evol.* **7**: 203-219.

Gottlieb, L.D. (1984). Genetics and morphological evolution in plants. *Amer. Naturalist.* **123**: 681-709.

Gregory, P.J. (1936). The floral morphology and cytology of *Elettaria cardamomum* Maton. *J. Linn. Soc., Bot.* **50**: 363.

Hallé, F. & Oldeman, R.A.A. (1970). Essai sur l'architecture et la dynamique de croissance des arbres tropicaux. *Collection de Monographies de Botanique et de Biologie Vegetale*, No. 6. Masson et Cie: Paris. (English translation by Stone, B.C. 1975. University Press: Kuala Lumpur, Malaysia).

Hallé, F., Oldeman, R.A.A., Tomlinson, P.B. (1978). *Tropical trees and forests: an architectural analysis*. Springer-Verlag: Berlin.

Hallé, N. (1967). *Aframomum polyanthum* (K.Schum.) K.Schum. et *Costus dinklagi* K.Schum. Deux Zingibéracées peu connues et nouvelles pour le Gabon, présentées avec leurs parties souterraines d'après des sujets vivants. *Adansonia* (sér. 2) **7**: 73-80.

Hallé, N. (1979). Architecture du rhizome chez quelques Zingibéracées d'Afrique et d'Océanie. *Adansonia* (sér. 2) **19**: 127-144.

Hallick, R.B., Ling, H., Drager, R.G., Favreau, M.R., Monfort, A., Orsat, B., Spielmann, A. & Stutz, E. (1993). Complete sequence of *Euglena gracilis* chloroplast DNA. *Nucl. acids Res.* **21**: 3537-3544.

Hamby, R.K. & Zimmer, E.A. (1992). Ribosomal RNA as a phylogenetic tool in plant systematics. In Soltis, P.S., Soltis, D.E. & Doyle, J.J. (eds.): *Molecular systematics of plants*, pp. 50-91. Chapman and Hall: New York.

Havey, M.J. & Muehlbauer, F.J. (1989). Variability for restriction fragment lengths and phylogenies in lentil. *Theor. Appl. Genet.* **77**: 839-843.

Heaney, L.R. (1998). The origins and dimensions of biodiversity in the Philippines. In Dorfman, R. (ed.): *Vanishing treasures of the Philippine rain forest.* The Field Museum: Chicago.

Hepper, F.N. (1968). Zingiberaceae. *Flora of West Tropical Africa*, 2 nd. ed., vol. 3, pp. 69-79. Whitefriars press Ltd.: Great Britain.

Hershkovitz, M.A. & Lewis, L.A. (1996). Deep-level diagnostic value of the rDNA-ITS region. *Molec. Biol. Evol.* **13**: 1276-1295.

Heslop-Harrison, J. (1975a). The physiology of the pollen grain surface. *Proc. Roy. Soc. London, Ser. B.* **190**: 275-299.

Heslop-Harrison, J. (1975b). The adaptive significance of the exine. In Ferguson, I.K. & Muller, J. (eds.): *The evolutionary significance of the exine*, pp. 27-37. Academic Press: New York & London.

Hesse, M. & Waha, M. (1982). The fine structure of the pollen wall in *Strelitzia reginae* (Musaceae). *Pl. Syst. Evol.* **141**: 285-298.

Heywood, V.H. (1971). The characteristics of the SEM and their importance in biological studies. In Heywood, V.H. (ed.): *Scanning electron microscopy*, pp. 1-16. Academic Press: London.

Heywood, V.H. (1984). The current scene in plant taxonomy. In Heywood, V.H. & Moore, D.M. (eds.): *Current concepts in plant taxonomy*, pp. 3-21. Academic Press: London.

Hickey, L.J. (1981). Land plant evidence compatible with gradual, not catastrophic, change at the end of the Cretaceous. *Nature* **292**: 529-531.

Hickey, L.J. & Peterson, R.K. (1978). *Zingiberopsis*, a fossil genus of the ginger family from late Cretaceous to early Eocene sediments of Western Interior North America. *Canad. J. Bot.* **56**: 1136-1152.

Hillis, D.M. & Huelsenbeck, J.P. (1992). Signal, noise, and reliability in molecular phylogenetic analyses. *J. Heredity* **83**: 189-195.

Hillis, D.M., Moritz, C., Porter, C.A. & Baker, R.J. (1991). Evidence for biased gene conversion in concerted evolution of ribosomal DNA. *Science* **251**: 308-310.

Hiratsuka, J., Shimada, H., Whitter, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C.R., Meng, B-Y., Li, Y-Q., Kanno, A., Nishizawa, Y., Hirai, A., Shinozaki, K. & Sugiura, M. (1989). The complete sequence of rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. *Molec. Gen. Genet.* **217**: 185-194.

Holmquist, R. (1983). Transitions and transversions in evolutionary descent: an approach to understanding. *J. Molec. Evol.* **19**: 134-144.

Holttum, R.E. (1950). The Zingiberaceae of the Malay Peninsula. *Gard. Bull. Singapore* **13**: 1-250.

Holttum, R.E. (1955). Growth-habits of monocotyledons - variations on a theme. *Phytomorphology* **5**: 399-413.

Hopkins H.C.F., Huxley, C.R., Pannell, C.M., Prance, G.T. & White, F. (1998). *The biological monograph: the importance of field studies and functional syndromes for taxonomy and evolution of tropical plants*. Continental Printing: Belgium.

Hori, H., Lim, B-L. & Osawa, S. (1985). Evolution of green plants as deduced from 5S rRNA sequences. *Proc. Natl. Acad. Sci. USA* **82**: 820-823.

Hsiao, C., Chatterson, N.J., Asay, K.H. & Jensen, K.B. (1994). Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. *Genome* **37**: 112-120.

Hsiao, C., Chatterson, N.J., Asay, K.H. & Jensen, K.B. (1995). Molecular phylogeny of the Pooideae (Poaceae) based on nuclear rDNA (ITS) sequences. *Theor. Appl. Genet.* **90**: 389-398.

Hsü, J. (1983). Late Cretaceous and Cenozoic vegetation in China, emphasizing their connections with North America. *Ann. Missouri Bot. Gard.* **70**: 490-508.

Huelsenbeck, J.P. (1991). Tree-length distribution skewness - an indicator of phylogenetic information. *Syst. Zool.* **40**: 257-270.

Huelsenbeck, J.P. & Hillis, D.M. (1993). Success of phylogenetic methods in the four-taxon case. *Syst. Biol.* **42**: 247-264.

Hutchings, M.J. & de Kroon, H. (1994). Foraging in plants: the role of morphological plasticity in resource acquisition. In Begon, M. & Fitter, A.H. (eds.): *Advances in ecological research*, vol. 25, pp. 159-238. Academic Press: London.

Hutchinson, J. (1934). *The families of flowering plants*, vol. 2: monocotyledons. Macmillan & Co.: London.

Ippolito, A. & Armstrong, J.E. (1993). Floral biology of *Hornstedtia scottiana* (Zingiberaceae) in a lowland rain forest of Australia. *Biotropica* **25**: 281-289.

Itokawa, H., Morita, H., Sumitomo T., Totsuka, N. & Takeya, K. (1987). Antitumour principles from *Alpinia galanga*. *Pl. Med.* **1**: 32-33.

Jansen, R.K., Michaels, H.J. & Palmer, J.D. (1991). Phylogeny and character evolution in the Asteraceae based on chloroplast DNA restriction site mapping. *Syst. Bot.* **16**: 98-115.

Jansen, R.K., Holsinger, K.E., Michaels, H.J. & Palmer, J.D. (1990). Phylogenetic analysis of chloroplast DNA restriction site data at higher taxonomic levels: an example from the Asteraceae. *Evolution* **44**: 2089-2105.

Jarvie, J.K. & Barkworth, M.E. (1990). Isozyme similarity in *Thinopyrum* and its relatives (Triticeae: Gramineae). *Genome* **33**: 885-891.

Jeandroz, S., Roy, A. & Bousquet, J. (1997). Phylogeny and phylogeography of the circumpolar genus *Fraxinus* (Oleaceae) based on internal transcribed spacer sequences of nuclear ribosomal DNA. *Molec. Phylo. Evol.* **7**: 241-251.

Jitoe, A., Masuda, T., Tengah, I.G.P., Suprapta, D.N., Gara, I.W. & Nakatani N. (1992). Antioxidant activity of tropical ginger extracts and analysis of the contained curcuminoids. *J. Agric. Food Chem.* **40**: 1337-1340.

Johns, R.J. (1995). Malesia - an introduction. *Curtis's Botanical Magazine* **12**: 52-62.

Jorgensen, R.A. & Cluster, P.D. (1988). Modes and tempos in the evolution of nuclear ribosomal DNA: new characters for evolutionary studies and new markers for genetic and population studies. *Ann. Missouri Bot. Gard.* **75**: 1238-1247.

Jorgensen, R.A., Cuellar, R.E. & Thompson, W.F. (1982). Modes and tempos in the evolution of nuclear encoded ribosomal RNA genes in legumes. Carnegie Institution Washington Year Book **81**: 98-101.

Källersjö, M., Farris, J.S., Kluge, A.G. & Bult, C. (1992). Skewness and permutation. *Cladistics* **8**: 275-287.

Kam, Y-K. (1982). The genus *Elettariopsis* (Zingiberaceae) in Malaya. *Notes Roy. Bot. Gard. Edinburgh.* **40**: 139-152.

Kardolus, J.P., Herman, J. van Eck & Ronald, G. van den Berg (1998). The potential of AFLPs in biosystematics: a first application in *Solanum* taxonomy (Solanaceae). *Pl. Syst. Evol.* **210**: 87-103.

Kavanagh, T.A. & Timmis, J.N. (1988). Structure of melon rDNA and nucleotide sequence of the 17S-25S spacer region. *Theor. Appl. Genet.* **76**: 673-680.

Kazan, K. & Muehlbauer, F.J. (1991). Allozyme variation and phylogeny in annual species of *Cicer* (Leguminosae). *Pl. Syst. Evol.* **175**: 11-21.

Kim, K.J. & Jansen, R.K. (1994). Comparison of phylogenetic hypotheses among different data sets in dwarf dandelions (*Krigia*, Asteraceae): additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. *Pl. Syst. Evol.* **190**: 157-185.

Kirchoff, B.K. (1992). Ovary structure and anatomy in the Heliconiaceae and Musaceae (Zingiberales). *Canad. J. Bot.* **70**: 2490-2508.

Kiss, T., Mihaly, K., Steffen, A. & Solymosy, F. (1988). Nucleotide sequence of the 17S-25S spacer region from tomato rDNA. *Nucl. Acids Res.* **16**: 7179.

Kluge, A.G. & Farris, J.S. (1969). Quantitative phyletics and the evolution of anurans. *Syst. Zool.* **18**: 1-32.

Koenig, J.F. (1783). Observationes botanicae, vol. 3. Erlangae.

Kohorn, B.D. & Rae, P.M.M. (1982). Non-transcribed spacer sequences promote *in vitro* transcription of *Drosophila* ribosomal DNA. *Nucl. Acids Res.* **10**: 6879-6886.

Kohorn, B.D. & Rae, P.M.M. (1983). Localization of DNA sequences promoting RNA polymerase I activity in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **80**: 3265-3268.

Kolberg, J., Barre, A. & Rougé, P. (1994). Use of a monoclonal antibody as a confirmational and phylogenetical probe for leguminosae lectins. *Biochem. Syst. & Ecol.* **22**: 317-322.

Kress, W.J. (1985). Bat pollination of an old world *Heliconia*. *Biotropica* **17**: 302-308.

Kress, W.J. (1986). Exineless pollen structure and pollination systems of tropical *Heliconia* (Heliconiaceae). In Blackmore, S. & Ferguson, I.K. (eds.): *Pollen and spores: form and function*, pp. 329-345. Academic Press: London.

Kress, W.J. (1990). The phylogeny and classification of the Zingiberales. *Ann. Missouri Bot. Gard.* **77**: 698-721.

Kress, W.J. & Stone, D.E. (1982). Nature of the sporoderm in monocotyledons, with special reference to the pollen grains of *Canna* and *Heliconia*. *Grana* **21**: 129-148.

Kress, W.J. & Stone, D.E. (1983). Morphology and phylogenetic significance of exine-less pollen of *Heliconia* (Heliconiaceae). *Syst. Bot.* **8**: 149-167.

Kron, P. & Stewart, S.C. (1994). Variability in the expression of a rhizome architecture model in a natural population of *Iris versicolor* (Iridaceae). *Amer. J. Bot.* **81**: 1128-1138.

Kubitzki, K. & Krutzsch, W. (1998). Origins of East and South East Asian plant diversity. In Aoluo, Z. & Sugong, W. (eds.): *Floristic characteristics and diversity of East Asian plants*, pp. 56-70. Springer-Verlag: Berlin.

Larsen, K. (1996). The Zingiberaceae in the Malesian region. In Wu, T-L., Wu, Q-G. & Chen, Z-Y. (eds.): *Proceedings of the second symposium on the family Zingiberaceae*, pp. 10-14. Zhongshan University Press: Guangzhou, China.

Lee, S.B. & Taylor, J.W. (1992). Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molec. Biol. Evol.* **9**: 636-653.

Liang, Y-H. (1988). Pollen morphology of the family Zingiberaceae in China - pollen types and their significance in the taxonomy. *Acta Phytotax. Sin.* **26**: 265-286.

Liao, J-P. & Wu, Q-G. (1996a). Fruit anatomy of Chinese *Alpinia* and its taxonomic significance. In Wu, T-L., Wu, Q-G. & Chen, Z-Y. (eds.): *Proceedings of the second symposium on the family Zingiberaceae*, pp. 82-90. Zhongshan University Press: Guangzhou, China.

Liao, J-P. & Wu, Q-G. (1996b). The significance of the seed anatomy of Chinese *Alpinia* in taxonomy and systematics. In Wu, T-L., Wu, Q-G. & Chen, Z-Y. (eds.): *Proceedings of the second symposium on the family Zingiberaceae*, pp. 91-106. Zhongshan University Press: Guangzhou, China.

Linnaeus, C. (1753). *Species plantarum*, 1st ed. Holmiæ.

Linnaeus, C. (1762). *Species plantarum*, 2nd ed. Holmiæ.

Linnaeus, C. [Linn. f.] (1781). *Supplementum plantarum systematis vegetabilium.* Brunsvigæ.

Liston, A., Robinson, W.A., Oliphant, J.M. & Alvarez-Buylla, E.R. (1996). Length variation in the nuclear ribosomal DNA internal transcribed spacer region of non-flowering seed plants. *Syst. Bot.* **21**: 109-120.

Liu, J-S. & Schardl, C.L. (1994). A conserved sequence in internal transcribed spacer 1 of plant nuclear rRNA genes. *Pl. Molec. Biol.* **26**: 775-778.

Loesener, T.H. (1930a). Zingiberaceae. In Engler, A. & Prantl, K. (eds.): *Die Natürlichen Pflanzenfamilien*, 2nd ed., vol. 15a, pp. 541-640. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Loesener, T.H. (1930b). Marantaceae. In Engler, A. & Prantl, K. (eds.): *Die Natürlichen Pflanzenfamilien*, 2nd ed., vol. 15a, pp. 654-693. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Long, E.O. & Dawid, I.B. (1980). Repeated genes in eukaryotes. *Annual Rev. Biochem.* **49**: 727-764.

Maddison, D.R. (1991). The discovery and importance of multiple islands of most parsimonious trees. *Syst. Zool.* **40**: 315-328.

Maddison, W.P. & Maddison, D.R. (1992). MacClade, version 3.01: analysis of phylogeny and character evolution. Sinauer Associates: Sunderland, Massachusetts.

Mahanty, H.K. (1970). A cytological study of the Zingiberales with special reference to their taxonomy. *Cytologia* **35**: 13-49.

Mangaly, J.K. & Nayar, J. (1990). Palynology of South Indian Zingiberaceae. *Bot. J. Linn. Soc.* **103**: 351-366.

Mangaly, J.K. & Sabu, M. (1992). A taxonomic revision of South Indian *Alpinia* Roxb. (Zingiberaceae). *Rheedea* **2**: 38-51.

Manos, P.S. (1997). Systematics of *Nothofagus* (Nothofagaceae) based on rDNA spacer sequences (ITS): taxonomic congruence with morphology and plastid sequences. *Amer. J. Bot.* **84**: 1137-1155.

Martin, P.G. & Dowd, J.M. (1984a). The study of plant phylogeny using amino acid sequences of ribulose-1,5-biphosphate carboxylase. III. Addition of Malvaceae and Ranunculaceae to the phylogenetic tree. *Austral. J. Bot.* **32**: 283-290.

Martin, P.G. & Dowd, J.M. (1984b). The study of plant phylogeny using amino acid sequences of ribulose-1,5-biphosphate carboxylase. IV. Proteaceae and Fagaceae and the rate of evolution of the small subunit. *Austral. J. Bot.* **32**: 291-299.

Martin, P.G. & Dowd, J.M. (1984c). The study of plant phylogeny using amino acid sequences of ribulose-1,5-biphosphate carboxylase. V. Magnoliaceae, Polygonaceae and the concept of primitiveness. *Austral. J. Bot.* **32**: 301-309.

Martin, P.G. & Dowd, J.M. (1986). A phylogenetic tree for some monocotyledons and gymnosperms derived from protein sequences. *Taxon* **35**: 469-475.

Martin, P.G. & Jennings, A.C. (1983). The study of plant phylogeny using amino acid sequences of ribulose-1,5-biphosphate carboxylase. I. Biochemical methods and the patterns of variability. *Austral. J. Bot.* **31**: 395-409.

Martin, P.G., Dowd, J.M. & Stone, S.J.L. (1983). The study of plant phylogeny using amino acid sequences of ribulose-1,5-biphosphate carboxylase. II. The analysis of small subunit data to form phylogenetic trees. *Austral. J. Bot.* **31**: 411-419.

Maxam, A.M. & Gilbert, W. (1977). A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA* **74**: 560-564.

McClure, F.A. (1966). *The bamboos - a fresh perspective*. Harvard University Press: Cambridge, Massachusetts.

McIntyre, C.L. (1988). Variation at isozyme loci in Triticeae. *Pl. Syst. Evol.* **160**: 123-142.

McMullen, M.D., Hunter, B., Phillips, R.L. & Rubinstein, I. (1986). The structure of the maize ribosomal DNA spacer region. *Nucl. Acids Res.* **14**: 4953-4968.

Miller, J.C. & Tanksley, S.D. (1990). RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon*. *Theor. Appl. Genet.* **80**: 437-448.

Mindell, D.P. (1991). Aligning DNA sequences: homology and phylogenetic weighting. In Miyamoto M.M. & Cracraft, J. (eds.): *Phylogenetic analysis of DNA sequences*, pp. 73- 89. Oxford University Press: New York.

Mitsui, S., Kobayashi, S., Nagahovi, H. & Ogiso, A. (1976). Constituents from seeds of *Alpinia galanga* (L.) Willd. and their antiulcer activities. *Chem. Pharm. Bull.* **24**: 2377-2382.

Möller, M. & Cronk, Q.C.B. (1997a). Origin and relationships of *Saintpaulia* (Gesneriaceae) based on ribosomal DNA internal transcribed spacer (ITS) sequences. *Amer. J. Bot.* **84**: 956-965.

Möller, M. & Cronk, Q.C.B. (1997b). Phylogeny and disjunct distribution: evolution of *Saintpaulia* (Gesneriaceae). *Proc. Roy. Soc. London, Ser. B.* **264**: 1827-1836.

Monte, J.V., McIntyre, C.L. & Gustafson, J.P. (1993). Analysis of phylogenetic relationships in the Triticeae tribe using RFLPs. *Theor. Appl. Genet.* **86**: 649-655.

Müller, F. (1888). Zweimännige Zingiberaceen-blumen. *Ber. Deutsch. Bot. Ges.* **6**: 95-100.

Müller, L. (1931). Ueber den Bau und die Mechanik der Blüte von *Globba atrosanguinea*. *Oesterr. Bot. Z.* **80**: 149-161.

Muller, J. (1979). Form and function in angiosperm pollen grains. *Ann. Missouri Bot. Gard.* **66**: 593-632.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* **51**: 263-273.

Musters, W., Boon, K., van der Sande, C.A.F.M., van Heerikhuizen, H. & Planta, R.J. (1990). Functional analysis of transcribed spacers of yeast ribosomal DNA. *E.M.B.O. J.* **9**: 3989-3996.

Nakai, T. (1941). Notulae ad Plantas Asiae Orientalis (XVI). *Jap. J. Bot.* **17**: 189-203.

Neigel, J.E., and Avise, J.C. (1986). Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. In Karlin, S. & Nevo, E. (eds.): *Evolutionary processes and theory*, pp. 515-534. Academic Press: New York.

Newman, M.F. (1997). *Elettariopsis unifolia* (Gagnep.) M.F. Newman, comb. nov. (Zingiberaceae). *Edinburgh J. Bot.* **54**: 110-111.

Nickrent, D.L., Schuette, K.P. & Starr, E.M. (1994). A molecular phylogeny of *Arceuthobium* (Viscaceae) based on nuclear ribosomal DNA internal transcribed spacer sequences. *Amer. J. Bot.* **81**: 1149-1160.

Nimzyk, R., Schondorf, T. & Hachtel, W. (1993). In-frame length mutations associated with short tandem repeats are located in unassigned open reading frames of *Oenothera* chloroplast DNA. *Curr. Genet.* **23**: 265-270.

O'Donnell, K. (1992). Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Curr. Genet.* **22**: 213-220.

Ogihara, Y., Terachi, T. & Sasakuma, T. (1988). Intramolecular recombination of chloroplast genome mediated by short direct-repeat sequences in wheat species. *Proc. Natl. Acad. Sci. USA.* **85**: 8573-8577.

Ohta, T. (1984). Some models of gene conversion for treating the evolution of multigene families. *Genetics* **106**: 517-528.

Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesonso, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H. & Ozeki, H. (1986). Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. *Nature* **322**: 572-574.

Olatunji, O.A. (1970). Taxonomic studies in the Zingiberaceae with special reference to vegetative characters. Unpublished Ph.D. thesis, University of Edinburgh.

Olatunji, O.A. (1980). The structure and development of stomata in some Zingiberales. *Notes Roy. Bot. Gard. Edinburgh* **38**: 499-516.

Olmstead, R.G. & Palmer, J.D. (1992). A chloroplast DNA phylogeny of the Solanaceae: subfamilial relationships and character evolution. *Ann. Missouri Bot. Gard.* **79**: 346-360.

Olmstead, R.G. & Palmer, J.D. (1994). Chloroplast DNA systematics: a review of methods and data analysis. *Amer. J. Bot.* **81**: 1205-1224.

Olmstead, R.G., Michaels, H.J., Scott, K.M. & Palmer, J.D. (1992). Monophyly of the Asteridae and identification of their major lineages inferred from DNA sequences of *rbc*L. *Ann. Missouri Bot. Gard.* **79**: 249-265.

Olsen-Stojkovich, J., West, J.A. & Lowenstein, J.M. (1986). Phylogenetics and biogeography in the Cladophorales complex (Chlorophyta): some insights from immunological distance data. *Bot. Mar.* **29**: 239-249.

Oxelman, B. & Lidén, M. (1995). Generic boundaries in the tribe Sileneae (Caryophyllaceae) as inferred from nuclear rDNA sequences. *Taxon* **44**: 525-542.

Palmer, J.D. (1985a). Evolution of chloroplast and mitochondrial DNA in plants and algae. In MacIntyre, R.J. (ed.): *Molecular evolutionary genetics*, pp. 131-240. Plenum: New York.

Palmer, J.D. (1985b). Chloroplast DNA and molecular phylogeny. *Bioessays* **2**: 263-267.

Palmer, J.D. (1987). Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *The Amer. Naturalist* **130** (Supplement): S6-S29.

Palmer, J.D. (1991). Plastid chromosomes: structure and evolution. In Vasil, I.K. (ed.): *Molecular biology of plastids and the photosynthetic apparatus*, pp. 5-53. Academic Press: New York.

Palmer, J.D. & Thompson, W.F. (1982). Chloroplast DNA rearrangements are more frequent when a large inverted repeated sequence is lost. *Cell* **29**: 537-550.

Palmer, J.D., Nugent, J.M. & Herbon, L.A. (1987). Unusual structure of geranium chloroplast DNA: a triple-sized inverted repeat, extensive gene duplications, multiple inversions, and two repeat families. *Proc. Natl. Acad. Sci. USA* **84**: 769-773.

Palmer, J.D., Jansen, R.K., Michaels, H.J., Chase, M.W. & Manhart, R.J. (1988). Chloroplast DNA variation and plant phylogeny. *Ann. Missouri Bot. Gard.* **75**: 1180-1206.

Patriat, P. & Achache, J. (1984). India-Eurasia collision chronology has implications for crustal shortening and driving mechanism for plates. *Nature* **311**: 615-621.

Payer, J.B. (1857). *Traité d'Organogenie de la Fleur*. Paris.

Payne, W.W. (1972). Observations of hamomegathy in pollen of Anthophyta. *Grana* **12**: 93-98.

Payne, W.W. (1981). Structure and function in angiosperm pollen wall evolution. *Rev. Palaeobot. Palynol.* **35**: 39-59.

Perry, L.M. (1980). *Medicinal plants of East and South East Asia: attributed properties and uses*. The MIT Press: Massachusetts.

Petersen, O.G. (1889a). Musaceae. In Engler, A. & Prantl, K.: *Die Natürlichen Pflanzenfamilien*, 2nd ed., vol. 2, part 6, pp. 1-10. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Petersen, O.G. (1889b). Zingiberaceae. In Engler, A. & Prantl, K.: *Die Natürlichen Pflanzenfamilien*, 2nd ed., vol. 2, part 6, pp. 10-30. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Petersen, O.G. (1889c). Cannaceae. In Engler, A. & Prantl, K.: *Die Natürlichen Pflanzenfamilien*, 2nd ed., vol. 2, part 6, pp. 30-32. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Petersen, O.G. (1889d). Marantaceae. In Engler, A. & Prantl, K.: *Die Natürlichen Pflanzenfamilien*, 2nd ed., vol. 2, part 6, pp. 33-43. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Porter, J.M. (1993a). Phylogeny of Polemoniaceae based on nuclear ribosomal internal transcribed spacer sequences. *Amer. J. Bot.* **80** (supplement, abstract): 171.

Porter, J.M. (1993b). Phylogenetic systematics of *Gilia* section *Giliandra*. Unpublished Ph.D. dissertation, University of Arizona, Tucson.

Potter, D. & Doyle, J.J. (1992). Origins of the African yam bean (*Sphenostylis stenocarpa*, Leguminosae): evidence from morphology, isozymes, chloroplast DNA, and linguistics. *Econ. Bot.* **46**: 276-292.

Prakash, U. (1972). Palaeoenvironmental analysis of Indian Tertiary floras. *Geophytology* **2**: 178-205.

Pridgeon, A.M., Bateman, R.M., Cox, A.V., Hapeman, J.R. & Chase, M.W. (1997). Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Intergeneric relationships and polyphyly of *Orchis sensu lato*. *Lindleyana* **12**: 89-109.

Primack, R.B. (1973). Growth patterns of five species of *Lycopodium*. *Amer. Fern J.* **63**: 3-7.

Qiagen (1998). DNA from difficult plant and fungal species using DNeasy Plant Kits. *Qiagen News* (issue 2): 14-17.

Quenouille, M. (1956). Notes on a bias in estimation. *Biometrika* **43**: 353-360.

Randall, J.B., Hufford, L. & Soltis D.E. (1996). Phylogenetic relationships in Sarraceniaceae based on *rbc*L and ITS sequences. *Syst. Bot.* **21**: 121-134.

Rao, V.S. & Gupte, K. (1961). The floral anatomy of some Scitamineae. Part IV. *J. Univ. Bombay* **29**: 134-150.

Rao, V.S. & Pai, R.M. (1959). The floral anatomy of some Scitamineae. Part II. *J. Univ. Bombay* **28**: 82-114.

Rao, V.S. & Pai, R.M. (1960). The floral anatomy of some Scitamineae. Part III. *J. Univ. Bombay* **28**: 1-19.

Rao, V.S., Karnik, H. & Gupte, K. (1954). The floral anatomy of some Scitamineae. Part I. *J. Indian Bot. Soc.* **33**: 118-147.

Rathgeber, J. & Capesius, I. (1989). Nucleotide sequence of the 18S-25S spacer region from mustard rDNA. *Nucl. Acids Res.* **17**: 7522.

Raubeson, L.A. & Jansen, R.K. (1992). A rare chloroplast-DNA mutation is shared by all conifers. *Biochem. Syst. & Ecol.* **20**: 17-24.

Raven, P.H. & Axelrod, D.I. (1974). Angiosperm biogeography and past continental movements. *Ann. Missouri Bot. Gard.* **61**: 539-673.

Reeder, R.H. (1984). Enhancers and ribosomal gene spacers. *Cell* **38**: 349-351.

Reeder, R.H., Roan, J.G. & Dunaway, M. (1983). Spacer regulation of *Xenopus* ribosomal gene transcription: competition in oocytes. *Cell* **35**: 449-456.

Rendle, A.B. (1930). *The classification of flowering plants*, vol. 1, pp. 332-337. Cambridge University Press: UK.

Retzius, A.J. (1791) Observationes botanicae, vol. 6. Erlangae.

Ridley, H.N. (1899). The Scitamineae of the Malay Peninsula. *J. Roy. Asiat. Soc. Brit.* **32**: 85-184.

Rieseberg, L.H. (1991). Homoploid reticulate evolution in *Helianthus* (Asteraceae): evidence from ribosomal genes. *Amer. J. Bot.* **78**: 1218-1237.

Rieseberg, L.H. & Soltis, D.E. (1991). Phylogenetic consequences of cytoplasmic gene flow in plants. *Evol. Trends Pl.* **5**: 65-84.

Rieseberg, L.H., Soltis, D.E. & Palmer, J.D. (1988). A. re-examination of introgression between *Helianthus annuus* and *H. bolanderi* (Compositae). *Evolution* **42**: 227-238.

Ritland, C.E., Ritland, K. & Straus, N.A. (1993). Variation in the ribosomal internal transcribed spacers (ITS1 and ITS2) among eight taxa of the *Mimulus guttatus* species complex. *Molec. Biol. Evol.* **10**: 1273-1288.

Roeder, D. (1977). Philippine arc system - collision of flipped subduction zones. *Geology* **5**: 203-206.

Rogers, S.O. & Bendich, A.J. (1987). Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. *Pl. Molec. Biol.* **9**: 509-520.

Roscoe, W. (1807). A new arrangement of the plants of the Monandrian class usually called Scitamineae. *Trans. Linn. Soc. London* **8**: 330-357.

Routledge, R.D. (1986). Rhizome architecture for dispersal in *Eleocharis palustris*. *Canad. J. Bot.* **65**: 1218-1223.

Roxburgh, W. (1812). Descriptions of several of the monandrous plants of India, belonging to the natural order called Scitamineae by Linnaeus, Cannae by Jussieu and Drimyrhizae by Ventenat. *Asiat. Res.* **11**: 318-362.

Roxburgh, W. (1832). *Flora Indica or descriptions of Indian plants*, vol. 1. Serampore, W. Thacker and Co.: Calcutta, India.

Rumphius, G.E. (1747). *Galanga major* and *minor*. *Herb. Amb.* **5**: 143-147, t. 63.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. & Allard, R.W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* **81**: 8014-8018.

Saiki, R.K., Gyllensten, U.B. & Erlich, H.A. (1988). The polymerase chain reaction. In Davies, K. (ed.): *Gene technology: a practical approach*, pp. 141-151. Information Press Ltd.: Oxford, England.

Sanderson, M.J. & Doyle, J.J. (1992). Reconstruction of organismal and gene phylogenies from data on multigene families: concerted evolution, homoplasy, and confidence. *Syst. Biol.* **41**: 4-17.

Sang, T., Crawford, D.J. & Stuessy, T.F. (1995). Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: inplications for biogeography and concerted evolution. *Proc. Natl. Acad. Sci. USA*. **92**: 6813-6817.

Sang, T., Crawford, D.J. & Stuessy, T.F. (1997). Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *Amer. J. Bot.* **84**: 1120-1136.

Sanger, F., Nicklen, S. & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467.

Sauer, P., Welters, S., Himmelreich, R., Möstl, D., Düsterhöft, A. & Kang, J. (1998). Effects of PCR fragment quality on fluorescent sequencing reactions. *Qiagen News* (issue 1): 13-17.

Savard L., Michaud, M. & Bousquet, J. (1993). Genetic diversity and phylogenetic relationships between birches and alders using ITS, 18S rRNA, and *rbc*L gene sequences. *Molec. Phylogenet. Evol.* **2**: 112-118.

Savolainen, V., Cuénoud, P., Spichiger, R., Martinez, M.D.P., Crèvecoeur, M. & Manen, J-F. (1995). The use of herbarium specimens in DNA phylogenetics: evaluation and improvement. *Pl. Syst. Evol.* **197**: 87-98.

Schiebel, K. & Hemleben, V. (1989). Nucleotide sequence of the 18S-25S spacer region from rDNA of mung bean. *Nucl. Acids Res.* **17**: 2852.

Schlötterer, C., Hauser, M.T., von Haeseler, A. & Tautz, D. (1994). Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*. *Molec. Biol. Evol.* **11**: 513-522.

Schnabel, A. & Wendel, J.F. (1998). Cladistics biogeography of *Gleditsia* (Leguminosae) based on *ndh*F and *rpl*16 chloroplast gene sequences. *Amer. J. Bot.* **85**: 1753-1765.

Schumann, K. (1900). Musaceae. In Engler, A. (ed.): *Das Pflanzenreich* IV, vol. 45. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Schumann, K. (1902). Marantaceae. In Engler, A. (ed.): *Das Pflanzenreich* IV, vol. 48. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Schumann, K. (1904). Zingiberaceae. In Engler, A. (ed.): *Das Pflanzenreich* IV, vol. 46. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Scogin, R. (1981) Amino acid sequence studies and plant phylogeny. In Young, D.A. & Seidler, D.S. (eds.): *Phytochemistry and angiosperm phylogeny*, pp. 229-290. Praeger: New York.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H. & Sugiura, M. (1986). The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. *Pl. Molec. Biol. Reporter* **4**: 110-147.

Skvarla, J.J. & Rowley, J.R. (1988). Adaptability of scanning electron microscopy to studies of pollen morphology. *Aliso* **12**: 119-175.

Smirnova, O.B. (1967). Growth structure of *Carex pilosa* Scop. and *Aegopodium podagraria* L. In Uranove, A.A. (ed.): *Ontogenesis and the age composition of the populations of flowering plants*. Moscow, USSR.

Smith, G.P. (1976). Evolution of repeated DNA sequences by unequal crossover. *Science* **191**: 528-535.

Smith, R.L. & Sytsma, K.J. (1990). Evolution of *Populus nigra* (sect. *Aigeiros*): introgressive hybridisation and the chloroplast contribution of *Populus alba* (sect. *Populus*). *Amer. J. Bot.* **77**: 1176-1187.

Smith, R.M. (1975). A. preliminary review of the large bracteate species of *Alpinia*. *Notes Roy. Bot. Gard. Edinburgh* **34**: 149-182.

Smith, R.M. (1977). Additional notes on *Alpinia* sect. *Myriocrater*. *Notes Roy. Bot. Gard. Edinburgh* **35**: 195-208.

Smith, R.M. (1981). *Synoptic keys to the genera of Zingiberaceae pro parte*. Royal Botanic Garden, Edinburgh, departmental publication series, No. 2.

Smith, R.M. (1985). A review of Bornean Zingiberaceae: I (Alpineae p.p.). *Notes Roy Bot. Gard. Edinburgh* **42**: 261-314.

Smith, R.M. (1986). A review of Bornean Zingiberaceae: II (Alpineae, concluded). *Notes Roy. Bot. Gard. Edinburgh* **43**: 439-466.

Smith, R.M. (1990a). *Alpinia* (Zingiberaceae): a proposed new infrageneric classification. *Edinburgh J. Bot.* **47**: 1-75.

Smith, R.M. (1990b). *Psychanthus* (K.Schum.) Ridley (Zingiberaceae): its acceptance at generic level. *Edinburgh J. Bot.* **47**: 77-82.

Smith, R.M. (1991). *Pleuranthodium* replaces the illegitimate *Psychanthus* (Zingiberaceae). *Edinburgh J. Bot.* **48**: 63-68.

Soltis, D.E. & Kuzoff, R.K. (1995). Discordance between nuclear and chloroplast phylogenies in the *Heuchera* group (Saxifragaceae). *Evolution* **49**: 727-742.

Soltis, D.E., Leigh, A. J. & Looney, C. (1996). Discordance between ITS and chloroplast topologies in the *Boykinia* group (Saxifragaceae). *Syst. Bot.* **21**: 169-185.

Soltis, D.E., Soltis, P.S. & Bothel, K.D. (1990). Chloroplast DNA evidence for the origins of the monotyptic *Bensoniella* and *Conimitella* (Saxifragaceae). *Syst. Bot.* **15**: 349-362.

Stam, W.T., Bot, P.V.M., Boele-Bos, S.A., van Rooij, J.M. & van den Hoek, C. (1988). Single-copy DNA-DNA hybridisation among five species of *Laminaria* (Phaeophyceae): phylogenetic and biogeographic implications. *Helgoländer Meeresuntersuch* **42**: 251-267.

Stanley, R.G. & Linskens, H.F. (1974). *Pollen: biology, biochemistry, management*. Springer-Verlag: New York.

Stebbins, G.L. & Khush, G.S. (1961). Variation in the organisation of the stomatal complex in the leaf epidermis of monocotyledons and its bearing on their phylogeny. *Amer. J. Bot.* **48**: 51-59.

Stein J. von & Hachtel, W. (1988). Deletions/insertions, short inverted repeats, sequences resembling att-lambda, and frame-shift-mutated open reading frames are involved in chloroplast DNA differences in the genus *Oenothera* subsection *Munzia*. *Molec. Gen. Genet.* **213**: 513-518.

Stoll, P., Egli, P. & Schmid, B. (1998). Plant foraging and rhizome growth patterns of *Solidago altissima* in response to mowing and fertilizer application. *J. Ecol.* **86**: 341-354.

Stone, D.E. (1987). Developmental evidence for the convergence of *Sassafras* (Laurales) and *Heliconia* (Zingiberales) pollen. *Grana* **26**: 179-191.

Sugiura, M. (1989). The chloroplast chromosomes in land plants. *Annual Rev. Cell Biol.* **5**: 51-70.

Suh, Y., Thien, L.B., Reeve, H.E. & Zimmer, E.A. (1993). Molecular evolution and phylogenetic implications of internal transcribed spacers of nuclear ribosomal DNA in Winteraceae. *Amer. J. Bot.* **80**: 1042-1055.

Sun,Y., Skinner, D.Z., Liang, G.H. & Hulbert, S.H. (1994). Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. *Theor. Appl. Genet.* **89**: 26-32.

Susana, A., Jacas, N.G., Soltis, D.E. & Soltis, P.S. (1995). Phylogenetic relationships in tribe Cardueae (Asteraceae) based on ITS sequences. *Amer. J. Bot.* **82**: 1056-1068.

Swartz, O. (1791). *Observationes botanicae*, vol. 6. Erlangae.

Swofford, D.L. (1991). PAUP: Phylogenetic Analysis Using Parsimony, version 3.0s. Illinois Natural History Survey: Champaign, Illinois.

Swofford, D.L. (1993). PAUP 3.1 User's Manual. Illinois Natural History Survey: Champaign, Illinois.

Swofford, D.L. (1998). PAUP*: Phylogenetic Analysis Using Parsimony, test version 4.0d64. Sinauer Associates: Sunderland, Massachusetts.

Swofford, D.L. & Maddison, W.P. (1987). Reconstructing ancestral character states under Wagner parsimony. *Math. Biosci.* **87**: 199-229.

Sytsma, K.J. & Gottlieb, L.D. (1986a). Chloroplast DNA evolution and phylogenetic relationships in *Clarkia* sect. *Peripetasma* (Onagraceae). *Evolution* **40**: 1248-1261.

Sytsma, K.J. & Gottlieb, L.D. (1986b). Chloroplast DNA evidence for the origin of the genus *Heterogaura* from a species of *Clarkia* (Onagraceae). *Proc. Natl. Acad. Sci. USA* **83**: 5554-5557.

Sytsma, K.J. & Schaal, B.A. (1985). Phylogenetics of the *Lisianthius skinneri* (Gentianaceae) species complex in Panama utilizing DNA restriction fragment analysis. *Evolution* **39**: 594-608.

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Pl. Molec. Biol.* **17**: 1105-1109.

Takaiwa, F. & Sugiura, M. (1982). Nucleotide sequence of the 16S-23S spacer region in a rRNA gene from tobacco chloroplast DNA. *Nucl. Acids Res.* **10**: 2665-2676.

Takaiwa, F., Oono, K. & Sugiura, M. (1985). Nucleotide sequence of the 17S-25S spacer region from rice rDNA. *Pl. Molec. Biol.* **4**: 355-364.

Takhtajan, A.L. (1969). *Flowering plants: origin and dispersal.* Smithsonian Institution Press: Washington, D.C.

Takhtajan, A.L. (1980). Outline of the classification of flowering plants (Magnoliophyta). *Bot. Rev.* (Lancaster) **46**: 225-359.

The Institute of Chinese Materia Medica China Academy of Traditional Chinese Medicine (1989). *Medicinal plants in China.* World Health Organisation Regional Publications Western Pacific Series No. 2: Manila, the Philippines.

Theilade, I., Mærsk-Møller, M.-L., Theilade, J. & Larsen, K. (1993). Pollen morphology and structure of *Zingiber* (Zingiberaceae). *Grana* **32**: 338-342.

Thompson, J.M. (1933). *Studies in advancing sterility.* Part VI.: The theory of Scitaminean flowering. Publ. No. 11. Hart. Bot. Lab.: Liverpool.

Thompson, J.M. (1934). *Studies in advancing sterility.* Part VII.: The state of flowering known as Angiospermy. Publ. No. 12. Hart. Bot. Lab.: Liverpool.

Thompson, J.M. (1936). *On the floral morphology of Elettaria cardamomum Maton.* Publ. No. 14. Hart. Bot. Lab.: Liverpool.

Tiffney, B.H. (1985). The Eocene North Atlantic land bridge and its importance in Tertiary and modern phytogeography of the Northern Hemisphere. *J. Arnold Arbor.* **66**: 243-273.

Tomlinson, P.B. (1956). Studies in the systematic anatomy of the Zingiberaceae. *J. Linn. Soc., Bot.* **55**: 547-592.

Tomlinson, P.B. (1962). Phylogeny of Scitamineae - morphological and anatomical considerations. *Evolution* **16**: 162-213.

Tomlinson, P.B. (1969). Classification of the Zingiberales (Scitamineae) with special reference to anatomical evidence. In Metcalfe, C.R. (ed.): *Anatomy of the monocotyledons*, vol. 3, pp. 295-302. Clarendron Press: Oxford.

Tomlinson, P.B. (1970). Monocotyledons - towards an understanding of their morphology and anatomy. In Preston, R.D. (ed.): *Advances in botanical research*, vol. 3, pp. 207-292. Academic Press: London, New York.

Tomlinson, P.B. & Esler, A.E. (1973). Establishment growth in woody monocotyledons native to New Zealand. *New Zealand J. Bot.* **11**: 627-644.

Torres, R.A., Ganal, M. & Hemleben, V. (1990). GC balance in the internal transcribed spacers ITS1 and ITS2 of nuclear ribosomal RNA genes. *J. Molec. Evol.* **30**: 170-181.

Tsudzuki, J., Nakashima, K., Tsudzuki, T., Hiratsuka, J., Shibata, M., Wakasugi, T. & Sugiura, M. (1992). Chloroplast DNA of black pine retains a residual inverted repeat lacking rRNA genes: nucleotide sequence of *trn*Q, *trn*K, *psb*A, *trn*I, and *trn*H and the absence of *rps* 16. *Molec. Gen. Genet.* **232**: 206-214.

Vairapandi, M. & Duker, N.J. (1994). Excision of ultraviolet-induced photoproducts of 5-methylcytosine from DNA. *Mutat. Res.* **315**: 85-94.

Valeton, T.H. (1904). Ueber neue und unvollständig bekannte Zingiberaceae aus West-Java und Buitenzorg. *Bull. Inst. Bot. Buitenzorg* **20**: 1-99.

Valeton, T.H. (1913). Zingiberaceae. *Nova Guinea, Bot.* **8**: 923-988.

Valeton, T.H. (1914). Die Zingiberaceen Deutsch-Neu-Guinea. *Bot. Jahrb. Syst.* **52**: 40-100.

Valeton, T.H. (1917). Zingiberaceae. In Merrill, E.D. (ed.): *An interpretation of Rumphius's herbarium amboinense*, pp. 151-165. Department of Agriculture and Natural Resources, Bureau of Science: Manila, the Philippines.

Valeton, T.H. (1918). New notes on the Zingiberaceae of Java and Malaya. *Bull. Jard. Bot. Buitenzorg* ser. 2, **27**: 1-167.

van der Sande, C.A.F.M., Kwa, M., van Nues, R.W., van Heerikhuizen, H., Raue, H.A. & Planta, R.J. (1992). Functional analysis of transcribed spacer 2 of *Saccharomyces cerevisiae* ribosomal DNA. *J. Molec. Biol.* **223**: 899-910.

van Ham, R.C.H.J., 't Hart, H., Mes, T.H.M. & Sandbrink, J.M. (1994). Molecular evolution of noncoding regions of the chloroplast genome in the Crassulaceae and related species. *Curr. Genet.* **25**: 558-566.

van Tiegham, P.H. (1868). Recherches sur la stucture du pistil. *Ann. Sci. Nat., Bot.* sér. 5, **9**: 127-226.

van Tiegham, P.H. (1871). Recherches sur la stucture du pistil et sur l'anatomie comparée de la fleur. *Mem. Savants a l'Institute* **2**: 1.

Venkateswarlu, K. & Nazar, R. (1991). A conserved core structure in the 18-25S rRNA intergenic region from tobacco, *Nicotiana rustica*. *Pl. Molec. Biol.* **17**: 189-194.

von Borstel, R.C. (1994). Origins of spontaneous base substitutions. *Mutat. Res.* **307**: 131-140.

Wakeley, J. (1996). The excess of transitions among nucleotide substitutions: new methods estimating transition bias underscore its significance. *Trends Ecol. Evol.* **11**: 158-163.

Wallace, R.S. & Jansen, R.K. (1990). Systematic implications of chloroplast DNA variation in the genus *Microseris* (Asteraceae, Lactuceae). *Syst. Bot.* **15**: 606-616.

Walsh, J.B. (1986). Selection and biased gene conversion in a multigene family: consequences of interallelic bias and threshold selection. *Genetics* **112**: 669-716.

Watt, G. (1883). *Economic products of India*, part V: medicinal products. The Superintendent of Government Printing: Calcutta, India.

Weisse, A. (1932). Zur Kenntnis der Blattstellungsverhältnisse bei den Zingiberaceen. *Ber. Deutsch. Bot. Ges.* **50**A: 327-366.

Weisse, A. (1933). Die Art den Distiche an dem Achseesprossen von Zingiber. *Ber. Deutsch. Bot. Ges.* **51**: 13-20.

Wen, J. & Zimmer, E.A. (1996). Phylogeny and biogeography of *Panax* L. (the ginseng genus, Araliaceae): inferences from ITS sequences of nuclear ribosomal DNA. *Molec. Phylogenet. Evol.* **6**: 167-177.

Wendel, J.F., Schnabel, A., & Seelanan, T. (1995a). Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). *Proc. Natl. Acad. Sci. USA* **92**: 280-284.

Wendel, J.F., Schnabel, A., & Seelanan, T. (1995b). An unusual ribosomal DNA sequence from *Gossypium gossypioides* reveals ancient, cryptic intergenomic introgression. *Molec. Phylogenet. Evol.* **4**: 298-313.

Wendel, J.F., Stewart, J.M. & Rettig, J.W. (1991). Molecular evidence for homoploid reticulate evolution among Australian species of *Gossypium*. *Evolution* **45**: 694-711.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (eds.): *PCR protocols: a guide to methods and applications*, pp. 315-322. Academic Press: San Diego, USA.

Whitfeld, P.R. & Bottomley, W. (1983). Organization and structure of chloroplast genes. *Annual. Rev. Pl. Physiol.* **34**: 279-310.

Willdenow, C.L. (1797). S*pecies plantarum*, 1st ed. Berolini.

Willis, J.C. (1985). A dictionary of the flowering plants and ferns, 8 th ed., revised H.K. Airy Shaw. Student edition. Cambridge University Press: UK.

Winkler, H. (1930a). Musaceae. In Engler, A. & Prantl, K. (eds.): *Die Natürlichen Pflanzenfamilien*, 2nd ed., vol. 15a, pp. 505-541. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Winkler, H. (1930b). Cannaceae. In Engler, A. & Prantl, K. (eds.): *Die Natürlichen Pflanzenfamilien*, 2nd ed., vol. 15a, pp. 640-654. Verlag von Wilhelm Engelmann: Leipzig, Germany.

Winnell, S., Newman, H. & Kirchoff, B.K. (1992). Ovary structure in the Costaceae (Zingiberales). *Int. J. Pl. Sci.* **153**: 471-487.

Wodehouse, R.P. (1935). *Pollen grains: their structure, identification and significance in medicine*. McGraw-Hill.: New York.

Wojciechowski, M.F., Sanderson, M.J., Baldwin, B.G. & Donoghue, M.J. (1993). Monophyly of aneuploid *Astragalus* (Fabaceae): evidence from nuclear

ribosomal DNA internal transcribed spacer sequences. *Amer. J. Bot.* **80**: 711-722.

Wolfe, J.A. (1978). A paleobotanical interpretation of Tertiary climates in the Northern Hemisphere. *Amer. Sci.* **66**: 694-703.

Wolfe, K.H., Morden, C.W. & Palmer, J.D. (1992). Functions and evolution of a minimal plastid genome from a nonphotosynthetic parasitic plant. *Proc. Natl. Acad. Sci. USA* **89**: 10648-10652.

Wolfson, R., Higgins, K.G. & Sears, B.B. (1991). Evidence for replication slippage in the evolution of *Oenothera* chloroplast DNA. *Molec. Biol. Evol.* **8**: 709-720.

Wu, T-L. (1981). Zingiberaceae. *Flora Reipublicae Popularis Sinicae* **16**, pp. 22-152. Science Press: Beijing.

Yakura, K., Kato, A. & Tanifuji, S. (1984). Length heterogeneity of the large spacer of *Vicia faba* rDNA is due to the differing numbers of a 326 bp repetitive sequence element. *Molec. Gen. Genet.* **193**: 400-405.

Yokota, Y., Kawata, T., Iida, Y., Kato, A. & Tanifuji, S. (1989). Nucleotide sequences of the 5.8S rDNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. *J. Molec. Evol.* **29**: 294-301.

Zavada, M.S. (1984). The relation between pollen exine sculpturing and self-incompatibility mechanisms. *Pl. Syst. Evol.* **147**: 63-78.

Zavada, M.S. (1990). Correlations between pollen exine sculpturing and angiosperm self-incompatibility systems - a rebuttal. *Taxon* **39**: 442-447.

Zentgraf, U., King, K. & Hemleben, V. (1992). Repetitive sequences are valuable as molecular markers in studies of phylogenetic relationships within the genus *Cucumis*. *Acta. Bot. Neerl.* **41**: 397-406.

Zimmer, E.A., Hamby, R.K., Arnold, M.L., Leblanc, D.A. & Theriot, E.C. (1989). Ribosomal RNA phylogenies and flowering plant evolution. In Fernholm, B., Bremer, K. & Jornvall, H. (eds.): *The hierachy of life*, pp. 205-214. Elsevier Science: Amsterdam.

Zurawski, G., Clegg, M.T. & Brown, A.H.D. (1984). The nature of nucleotide-sequence divergence between barley and maize chloroplast DNA. *Genetics* **106**: 735-749.