SYSTEMATIC STUDY OF *CURCUMA* L.: TURMERIC AND ITS ALLIES

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"Behold, in the creation of the heavens and the earth, in the alteration of night and day, in the sailing of the ships through the ocean for the profit of mankind, in the rain which Allah sends down from the skies and the life which He gives therewith to an earth that is dead, in the beasts of all kinds that he scatters through the earth, in the change of the winds and the clouds which they trail like their slaves between the sky and the earth, here indeed are signs for a people that are wise". (Al-Baqarah: 164)

For my husband, my daughter, my son,
and my family
thanks for your support, love,
and sacrifice

ABSTRACT

The genus *Curcuma* L. comprises more than 80 species distributed from India, Burma, Thailand, Southeast Asia, to Queensland and the Pacific Islands. One of its two subgenera, subgenus *Curcuma* (Baker) K. Schum., contains many morphologically highly similar polyploid taxa, which have been treated at specific level by previous authors, contributing to taxonomic confusion in the genus. I have studied this problem using a sample of the genus which includes all the Javanese *Curcuma* (16 species) as well as 5 species from India, 7 species from Thailand and one from Australian/New Guinea. The closely related genera *Smithatris* W.J. Kress & K. Larsen and *Stahlianthus* O. Kuntze have also been sampled. *Roscoea* Sm. and *Cautleya* (Royle ex Benth.) Hook.f. were used as outgroups.

A phylogenetic study of *Curcuma* was carried out using 35 gross morphological characters and molecular data from the nuclear ribosomal DNA (ITS; internal transcribed spacer region) and plastid DNA of the *trn*L-F region. The tree that resulted from the morphological approach was not well resolved, especially in subgenus *Curcuma*. The molecular data gave a more resolved tree. Both trees are almost congruent except that *C. aurantiaca* Zijp and *C. cf. australasica* Hook.f., which were placed between *C. ecomata* Craib and the *C. thorelii* Gagnep. clade in the molecular analyses, were shifted to nodes between *C. roscoeana* Wall. and *C. petiolata* Roxb. in the tree constructed from morphological data. Molecular phylogenetic analysis shows that *Curcuma* is not monophyletic. *Smithatris* and *Stahlianthus* are nested within *Curcuma* and need to be transferred to *Curcuma* to make it monophyletic. Subgenera *Hitcheniopsis* (Baker) K. Schum. and *Curcuma* are phylogenetically distinct. Subgenus *Curcuma* is monophyletic with good support in the ITS data (BS=95; DI=+4). Current attempted sectional level classification within subgenus *Curcuma* should be abandoned, as it is mainly based on inflorescence position, which is homoplasious. Analysis of ITS polymorphisms in subgenus *Curcuma* reveals indel (one to four bp) polymorphisms within an individual, suggesting possible hybrid origin for some species in subgenus *Curcuma*.

Anatomical study of epidermal characters, leaf transverse section and SEM of seeds revealed patterns of similarity among species. Principal Component Analysis of epidermal and stomatal cell measurements did not reveal obvious clusters.

Floral diversity in *Curcuma* was examined using Principal Component Analysis. Three floral types, ie. complex, small, and simple flowers, suggest three putative pollination syndromes. Mapping morphological characters (especially those characters used in the existing classification) onto the molecular tree gave some insight to the evolutionary history of *Curcuma*. Mapping the floral types show that the simple flower in subgenus *Curcuma* was probably derived from the more complex flower characteristic of subgenus *Hitcheniopsis*.

Isozyme electrophoresis reveals isozyme polymorphism in populations of *C. colorata* Valeton, *C. heyneana* Valeton & Zijp , *C. longa* L., and *C. zanthorrhiza* Roxb. The pattern of polymorphism is interpreted as possibly indicating a multiple origin of the 'species'. Chromosome count results from *Curcuma* subgenus *Curcuma* were mostly triploid 2n=63; while counts for subgenus *Hitcheniopsis* was different (eg. *C. thorelii*; 2n=c.38). Revision of Javanese *Curcuma* is presented with a proposed new classification, key, and descriptions.

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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.

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CHAPTER 1: INTRODUCTION

1.1 ORDER ZINGIBERALES, FAMILY ZINGIBERACEAE AND TRIBE HEDYCHIEAE

1.1.1 Order Zingiberales

The natural order Zingiberales, which is phenetically distinctive in many correlated characters (Tomlinson 1962; Cronquist 1981; Dahlgren *et al.* 1985; Kress 1990), combined with the order Bromeliales, forms the subclass Zingiberidae Cronquist in the class Liliopsida Cronquist or Monocotyledons (Cronquist 1978, 1981). In the past, the order used to be called Scitamineae or Arillatae (Engler 1892). Recent study (Kress *et al.* 2001) using combined morphological and molecular data shows the monophyly of the order Zingiberales which is placed on terminal in the cladogam among the monocots (**Figure 1.1**).

The history of the classification of the order Zingiberales (see **Table 1.1)** was compiled and discussed by Tomlinson (1962) and then by Kress (1990). They highlighted five different systems and Kress proposed another system of classification. The first system was that of Bentham & Hooker (1883) which recognized Scitamineae (later called Zingiberales) as a family consisting of four tribes, namely, Museae Bentham & Hooker, Zingibereae Bentham & Hooker, Maranteae Bentham & Hooker, and Canneae Bentham & Hooker. Then, in 1889, Petersen in Engler & Prantl, who gave the rank Reihe to the Scitamineae, recognized the four tribes of Bentham & Hooker at family rank. He divided the Musaceae A.L.Jussieu into two tribes, namely, Museae Petersen and Heliconieae Petersen. The refinement and division was continued by raising the Museae from tribal to subfamily rank, Musoideae K. Schum., and by raising *Strelitzia* W. Aiton from the tribe Museae to the subfamily Strelitzioideae K. Schum. New ranks were given to the tribe Heliconieae K. Schum, and the subfamilies Zingiberoideae K. Schum and Costoideae K. Schum. Subfamily Lowioideae K. Schum was later included in the system (Schumann in Engler 1900, 1902, 1904; Winkler in Engler & Prantl 1930; Loesener in Engler & Prantl 1930). Hutchinson (1934, 1959) raised the Strelitzioideae to family level and separated it from the Musaceae resulting in

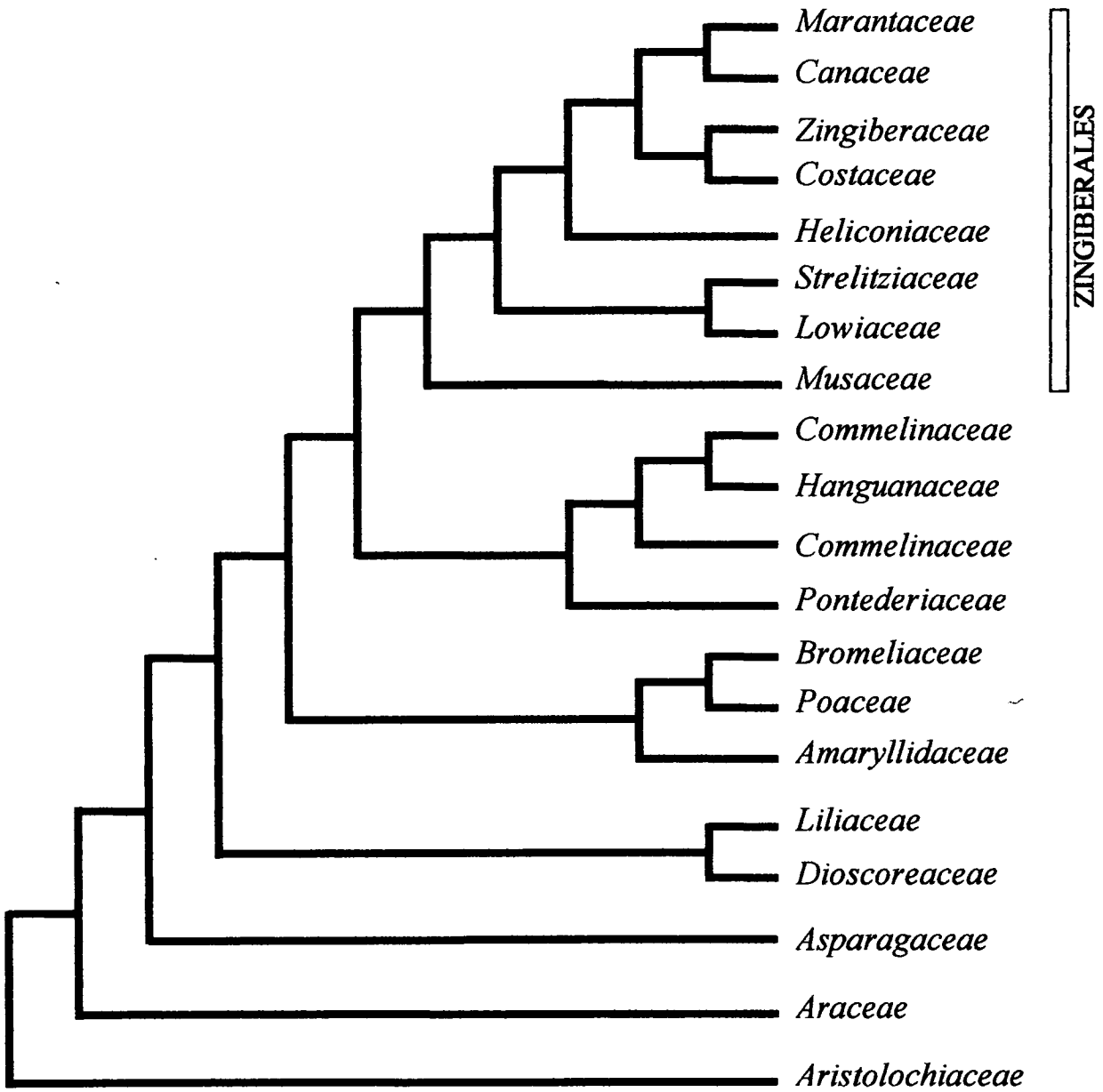


Figure 1.1 Cladogram showing relationships of Zingiberales to monocots (Modified from Kress *et al.* 2001)

Table 1.1 Systems of classification of Zingiberales (modified from Tomlinson 1962, and Kress 1990)

Bentham & Hooker 1883	**Petersen (Engler & Prantl 1889)**	**Schumann (Engler 1900, 1902, 1904, 1912), Winkler (Engler & Prantl 1930), Loesener (Engler & Prantl 1930)**
Family Scitamineae Bentham & Hooker	Reihe Scitamineae Petersen	Order Scitamineae K. Schum.
Tribe 1. Museae Bentham & Hooker	Family 1. Musaceae A.L.Jussieu	Family 1. Musaceae A.L.Jussieu
Musa L., *Ravenala* Adanson, *Strelitzia* W. Aiton	Tribe 1. Museae Petersen	Subfamily 1. Musoideae K. Schum.
Heliconia L.	*Musa* L.,	*Musa* L.
	Strelitzia W. Aiton, *Ravenala* Adanson	Subfamily 2. Strelitzioideae K. Schum.
		Tribe 1. Strelitzieae K. Schum.
		Strelitzia W. Aiton, *Ravenala* Adanson
	Tribe 2. Heliconieae Petersen	Tribe 2. Heliconieae K. Schum.
	Heliconia L.	*Heliconia* L.
		Subfamily 2. Lowioideae K. Schum.
	Orchidantha N. E. Brown not placed	*Lowia* Scortechini, *Orchidantha* N. E. Brown
Tribe 2. Zingibereae Bentham & Hooker	Family 2. Zingiberaceae Lindley including *Costus* L., *Tapeinochilus* Miq.	Family 2. Zingiberaceae Lindley
		Subfamily 1. Zingiberoideae K. Schum.
		Subfamily 2. Costoideae K. Schum.
Tribe 3. Canneae Bentham & Hooker	Family 3. Cannaceae A.L. Jussieu	Family 3. Cannaceae A.L. Jussieu
Tribe 4. Maranteae Bentham & Hooker	Family 4. Marantaceae Petersen	Family 4. Marantaceae Petersen

Table 1.1 (continued) Systems of classification of Zingiberales (modified from Tomlinson 1962, and Kress 1990)

Hutchinson (1934)	Nakai (1941), Tomlinson (1962), Takhtajan (1980), Cronquist (1981), Dahlgreen et al. (1985), Takhtajan (1997)	Kress 1990
Order Scitamineae (later Zingiberales)	Order Zingiberales Nakai	Order Zingiberales Nakai
Family 1. Musaceae A.L. Jussieu	Family 1. Musaceae A.L.Jussieu *Musa* L., *Ensete* Horan.	Suborder 1. Musineae Kress Family 1. Musaceae A.L.Jussieu
Family 2. Strelitziaceae Hutchinson *Strelitzia* W. Aiton, *Ravenala* Adanson, *Phenakospermum* Endlicher, *Heliconia* L.	Family 2. Strelitziaceae Hutchinson *Strelitzia* W. Aiton, *Ravenala* Adanson, *Phenakospermum* Endlicher Family 3. Heliconiaceae Nakai *Heliconia* L.	Suborder 2. Strelitziineae Kress Family 2. Strelitziaceae Hutchinson Suborder 3. Heliconiineae Kress Family 3. Heliconiaceae Nakai
Family 3. Lowiaceae Ridley *Lowia* Scortechini, *Orchidantha* N. E. Brown	Family 4. Lowiaceae Ridley	Suborder 4. Lowineae Kress Family 4. Lowiaceae Ridley
Family 4. Zingiberaceae Lindley Tribe 1. Zingibereae Meisner Tribe 2. Hedychieae Horan. Tribe 3. Globbeae Meisner	Family 5. Zingiberaceae Lindley	Suborder 5. Zingiberineae Kress Superfamily 1. Zingiberariae Kress Family 5. Zingiberaceae Lindley
Tribe 4. Costeae Meisner	Family 6. Costaceae Nakai	Family 6. Costaceae Nakai
Family 5. Cannaceae A.L. Jussieu	Family 7. Cannaceae A.L. Jussieu	Superfamily 2. Cannariae Kress Family 7. Cannaceae A.L. Jussieu
Family 6. Marantaceae Petersen	Family 8. Marantaceae Petersen	Family 8. Marantaceae Petersen

six families in the order. Nakai (1941) raised the rank of the Costeae Meisner and separated it from the Zingiberaceae Lindley. This was followed by Tomlinson (1962, 1969), who accepted Nakai's system after investigating the distribution of anatomical characters in the order. Takhtajan (1980) also followed Nakai. Nakai also separated the Heliconieae from the Musaceae and raised it to family level resulting in the establishment of eight families in the order, which is commonly followed by modern taxonomists and phylogeneticists (Kress 1990).

1.1.2 Taxonomy, habitat and distribution of Zingiberaceae

The name Zingiber is derived from the Greek zingiberis which comes from the Sanskrit name of the spice singabera. The family comprises about 49 genera and 1,300 species (Heywood 1993). The main genera are *Alpinia* Roxb., with approximately 227 species (Smith 1990), followed by *Zingiber* Boehm. (80-90 species), *Curcuma* L. (70 species), *Kaempferia* L. (55 species), and *Hedychium* J. G. König with about 50 species (Heywood 1993). In earlier classifications, the family Costaceae was included in Zingiberaceae. However, with a number of distinctive characters such as lack of aromatic oils, branched aerial stems, and spiral monostichous phyllotaxy, Costaceae are now generally classified in a separate family.

Most Zingiberaceae like shady places and are normally found on the forest floor under a rather open canopy. They are often found on river banks. Some genera such as *Curcuma* prefer a monsoon climate with markedly dry and rainy seasons. Some other cultivated members of the family are deciduous, resting as rhizomes during the dry season. In Malaya, with no dry season, some of these grow indefinitely, but others (e.g. *Kaempferia rotunda* Blanco) are deciduous in spite of the climate. Native Malayan species are all evergreen (Holttum 1951).

The family is distributed throughout the tropics (**Figure 1.2**) from Africa to South America with its greatest concentration in tropical Asia (Holttum 1951) and relatively few representatives in America (Tomlinson 1969). *Roscoea* Sm., *Cautleya* (Royle ex Benth.) Hook.f., and *Hedychium* are anomalous in their distribution by

reaching temperate regions of the mountains of China, Burma, North India and the Himalaya (Cowley 1982, Ngamriabsakul *et al.* 2000).

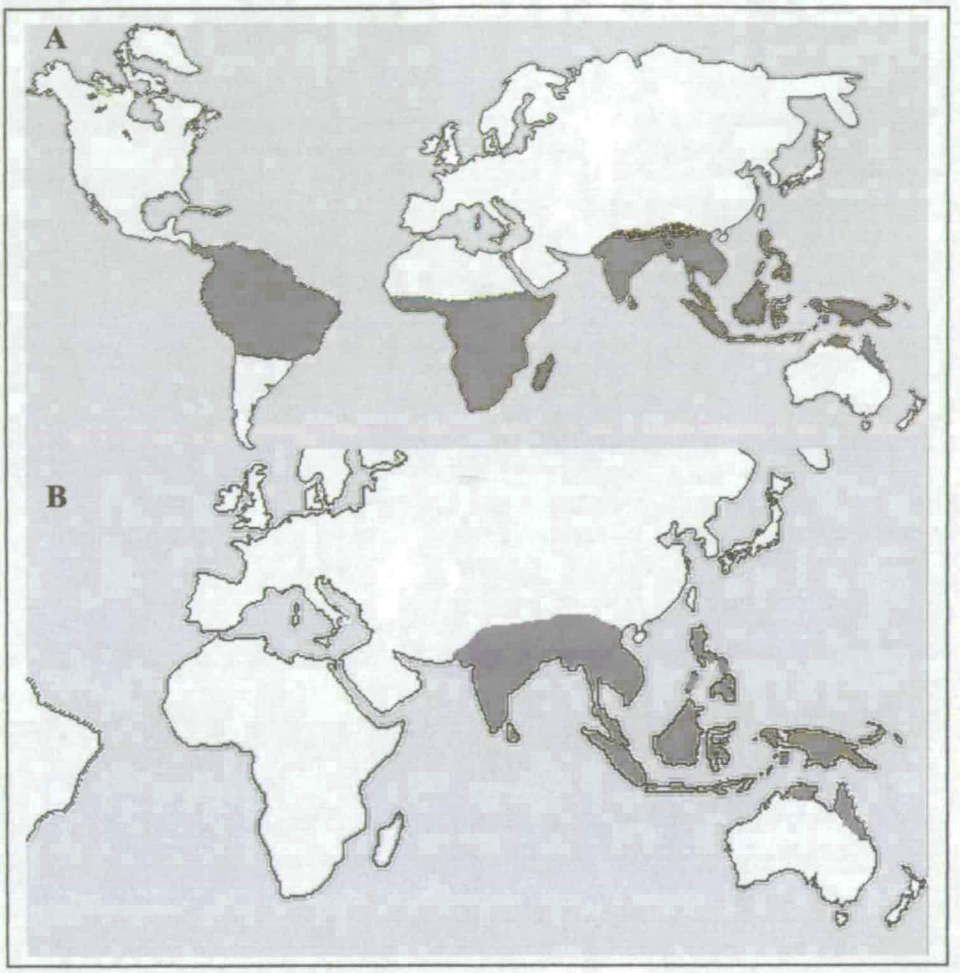


Figure 1.2 Map of distribution of Zingiberaceae and *Curcuma*
A. Distribution of Zingiberaceae B. Distribution of *Curcuma*

1.1.3 The Uses of Zingiberaceae

The family is economically a very important group being rich in volatile oils. It would be inappropriate here to mention all the uses of Zingiberaceae. Several papers that deal with uses are Burkill 1935, Quisumbing 1951, Ochse 1931, Heyne 1950, Perry 1980, Holttum 1951, Humphries in Heywood 1993, Chopra *et al.* 1956, Burch *et al.* 1987. Gingers are used as spices (condiments, herbs), sources of starch, flavourings for food, vegetables, dyes, perfumes, medicines, and tropical and greenhouse ornamentals (e.g. *Hedychium, Costus* L.). The rhizome is the most commonly used part followed by the seeds and the flower buds.

The rhizomes of *Zingiber officinale* Roscoe are ground up to produce ginger, and those of *Curcuma longa* L. provide the spice and yellow dye called turmeric, which is a common ingredient of curry powder. Cardamom is obtained from the whole or ground dried fruit or seeds of various spp. of *Amomum* Roxb. and *Elettaria* Maton, especially *E. cardamomum* (L.) Maton. East Indian arrowroot starch is made from the rhizomes of several species of *Curcuma* (Cronquist 1981), for example from *Curcuma angustifolia* Roxb. Abir is a perfumed powder obtained from the rhizome of *Hedychium spicatum* Sm. Zedoary is a spice, tonic, and perfume made from the rhizomes of *C. zedoaria* (Christm.) Roscoe. Galangal from the rhizomes of *Alpinia officinale* of Hainan and *A. galanga* (L.) Sw. of the Moluccas is used as medicine and flavouring. The spice Meleguetta pepper is produced from *Aframomum melegueta* (Humphrey in Heywood 1993). In Indonesia, rhizomes from several species are used as spices such as *kencur* from *Kaempferia galanga* L., *temu kunci* from *Boesenbergia rotunda* (L.) Mansf., and some also are used as salad such as *C. mangga* Valeton & Zijp and *C. longa.* In Java, several species of Zingiberaceae are used as a component of traditional medicines called *jamu* (see Erdelen *et al.* 1999).

1.1.4 Tribe Hedychieae

The classification of the Zingiberaceae into tribes has undergone several changes in its division and concept. Petersen's tribe *Zingibereae* (Petersen in Engler & Prantl 1889, Schumann in Engler & Prantl 1904, Loesener in Engler & Prantl 1930) consisted

of, e.g. *Zingiber, Alpinia,* and *Amomum,* on the basis of the character of the lateral staminodes which are very small or absent. However, if we consider *Zingiber* as having petaloid lateral staminodes deeply adnate to the labellum, we will exclude it from the tribe.

Holttum (1950) revised this by excluding the genus from Petersen's tribe *Zingibereae*, shifting it to the tribe *Hedychieae* on the basis of the lateral staminodes that are free from the labellum or deeply adnate to the labellum in *Zingiber*. His tribe *Hedychieae* was not legitimate according to the International Code for Botanical Nomenclature as it contains *Zingiber* which is the type name for the family, the order, etc. (Burtt & Olatunji 1972).

After intensive study, Olatunji (1970), and Burtt & Olatunji (1972) proposed a new tribe *Zingibereae* which includes *Zingiber* alone (see **Table 1.2**), leaving *Hedychieae* otherwise unchanged. They separated *Zingibereae* and *Hedychieae* on the basis of the following characters: lateral staminodes adnate to the labellum in *Zingibereae* vs. free in *Hedychieae*; style extended beyond anther-thecae, the upper part wrapped round by the elongate anther-crest in *Zingibereae* vs. style not extended beyond anther-thecae and stigma protruding at top of these, anther crest if present flat in *Hedychieae*; petiole swollen and pulvinus-like in *Zingibereae* vs. not swollen nor pulvinus-like in *Hedychieae*; vascular bundle with collenchymatous sheath in *Zingibereae* vs. sclerenchymatous in *Hedychieae.*

Smith (1981) constructed a key to the tribes with the characters of the *Hedychieae* as follows: plane of distichy of the leaves parallel to the rhizome (*Zingibereae, Hedychieae*); lateral staminodes free from the lip (*Globbeae, Hedychieae*); ovary trilocular with axile placentation or with unilocular placentation with basal or free columnar placentation (*Hedychieae*). The character of plane of distichy in relation to the axis of the rhizome was first described by Weisse in 1931, 1933 (cited in Burtt & Olantunji 1972).

However, the delimitation is still not wholly clear (Newman 1988) as some species in one tribe possess characters that fit the criteria of another tribe. An example is given here from Newman (1988). *Gagnepainia* K. Schum., which is the member of

Table 1.2 Characteristics and tribes of Zingiberaceae
(After Schumann 1904, Holttum 1950, Burt & Smith 1972, Larsen 1998)

Character	**Alpinieae** A. Rich.	**Hedychieae** Horan.	**Globbeae** Meisn.	**Zingibereae** Meisn
Plane of distichy of leaves	Perpendicular to rhizome	Parallel to rhizome	Parallel to rhizome	Parallel to rhizome
Lateral staminodes	Small or absent, never petaloid	Petaloid, free from labellum	Petaloid, free from labellum and sometimes connate to filament	Petaloid, adnate to labellum
Labellum	Not connate to filament	Not connate to filament	Connate to filament in slender tube	Not connate to filament
Stamen	Medium length	Short length	Long with arching filament	Anther crest elongated and wrapped aroud style
Ovary	3-locular (sometimes incompletely so)	3-locular (sometimes incompletely so)	1-locular	3-locular
Placentation	axial or free central	axial, basal, or free columnar	parietal	axial
Style	not extended beyond anther-thecae	not extended beyond anther-thecae	not extended beyond anther-thecae	extended beyond anther-thecae
Stigma	expanded	expanded	not expanded	not expanded
E.g.	*Alpinia, Amomum*	*Hedychium, Curcuma, Cautleya*	*Globba, Mantisia*	*Zingiber*

Globbeae, has a short and not long exserted filament. On the other hand, *Hedychium*, which is a member of *Hedychieae,* has a long exserted filament. The question was also faced when Smith (cited in Newman 1988) discovered a new genus of which most of the characters fit the description of tribe *Hedychieae* but lacking lateral staminodes. This genus, *Stadiochilus* R. M. Smith, was subsequently placed in *Alpineae* and the possibility was noted that species with no lateral staminodes may be derived from those with petaloid ones (Smith cited in Newman 1988). The illustration of the petaloid lateral staminodes can be seen in **Figure 1.3**.

1.2 *THE GENUS* CURCUMA *L.*

The genus *Curcuma* comprises more than 80 species. The exact number has not been reported yet since the genus is still being worked on by people from various places. It has been reported that there are more than 40 species in Thailand (Sirirugsa, unpublished data), 31 species in India (Velayudhan *et al.* 1996) of which 12 are endemic (Jain & Prakash 1995), and more than 20 species in Malesia.

1.2.1 *What does the word* Curcuma *mean?*

The word *Curcuma* has been adopted from a presumed Arabic name. The word curcum or kurkum, which is the original term, does not denote turmeric but saffron. The word kurkum in Arabic means yellow, however turmeric in Arabic is Uruku's sufr or Uruku's sabaghin or Carcumaa Avicenna. Kurkum is Persian according to Richardson, Arabic in the dictionary of Golius and Meninski, Hebrew in Parkhurst lexicon, but Syriac according to the author of Mekhzenu'l Adviyeh (Roxburgh 1812). It is probably derived from the same source as the Sanscrit Cuncuma (not Curcuma), with the Greek Crocos and Crocon and with the Latin Crocus and Crocum all denoting saffron. Rumphius had already remarked on the affinity of these names (Roxburgh 1812). He derives the name Curcum from a Chaldaic word, to wash or anoint (Graham 1839).

Can the word tell the place of origin of *Curcuma*? It is hard to judge if the word kurkum, which is more from West or South Asia, would relate to the place of origin of

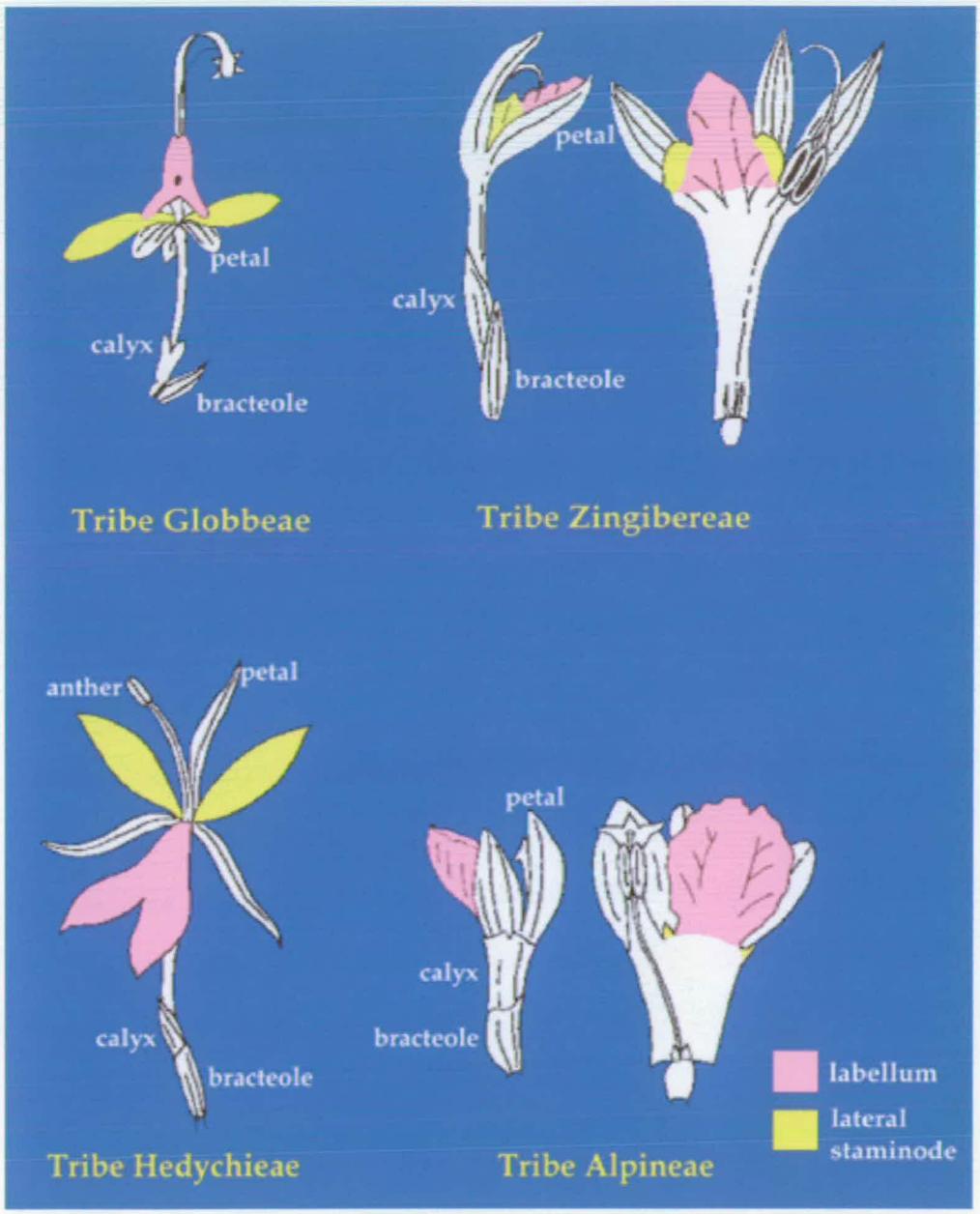


Figure 1.3 Flower structure of the tribes in Zingiberaceae (After Smith 1981).

Curcuma. In other places, for example Thailand to Malaysia and Indonesia, there is a uniformity of people in calling turmeric as "yellow" in their own words. This is almost similar to the case of people saying kurkum. However, there is a sign that kurkum which may refer to turmeric has been long developed and cultivated in the region of West and South Asia rather than in South East Asia. The history of turmeric will be covered in more detail in Chapter Eight.

1.2.2 Morphological characteristics

The genus is differentiated by (**Figure 1.4**) the conspicuous bracts which are connate to each other in the lower part to the backs of those above, forming pouches. The top bracts of the inflorescence are usually sterile and differently coloured, called the coma (Smith 1981).

The characteristics below are taken from several related publications and my own observations. The underground parts consist of the *main rhizome,* called *mother rhizome* or *primary rhizome* or *primary tuber*. Some species produce *sessile tubers* that spring out from the primary rhizome. The inflorescence comes from the primary rhizome or from an old sessile tuber. The position is central when it is terminal on the leafy shoot, whereas lateral when it is separated from the leafy shoot. The colour and smell of the rhizome are diagnostically important in distinguishing some "species".

The leaf is composed of a sheath, which wraps around the other sheaths forming a *false stem*, a petiole, which can be very short, and blade which is entire, elliptic to lanceolate. A *purple streak* on the leaves can stretch along the midrib, sometimes only in the basal part of the lamina. The midrib is green or brownish. The *base* is acute decurrent or almost rounded, while the *apex* is normally acuminate. The *surface* is glabrous, pubescent, or with a few hairs along the nerves towards the apex or hairy on the midrib.

The inflorescence consists of a *peduncle* that is usually hairy, and the *compound cincinnus* (**Figure 1.5**), which is cylindrical or strobilaceous, consisting of numerous concave pouched bracts. The pouched bracts subtend a cincinnus of 2-7 *flowers*. Each flower is embraced laterally by a *bracteole*, which is hyaline and boat-shaped. The

Figure 1.4 Illustration of *Curcuma* subgenus *Curcuma* (A & B) and subgenus *Hitcheniopsis* (C & D).
A. *C. ferruginea* Roxb., **B**. *C. longa*, **C**.*C. oligantha* Trimen, **D**. *C. roscoeana* Wall. (A & C taken from Roscoe 1828; B & D photographed by D. White)

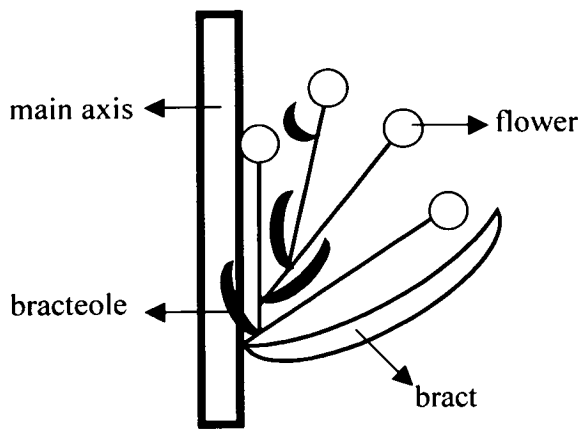


Figure 1.5 Diagram of compound cincinnus flower

flower which is bisexual consists of the *ovary* which is inferior, hairy or not, unilocular with basal placentation or trilocular with axile placentation. From the top of the ovary, two slim cylindrical *epigynous glands* stand out. A few species lack these glands. The glands are supposed to produce *nectar*.

The flower is composed of sepals, petals, labellum, lateral staminodes, pistil and stamen. The *sepals* are tubular and three-toothed (one rather deeply toothed and two shallowly toothed). The *petals* are tubular or infundibular (long stalked-cupped shaped) at the base and three-lobed at the apex. The dorsal petals are normally slightly bigger than the lateral ones. The *lateral staminodes* consist of two petaloid structures adnate to and flanking the stamen, fused at the base with the petals. These clasping staminodes nearly hide the stamen and pistil. In some species, the lateral staminodes are free and not clasping. The *labellum* which is petaloid obovate or almost circular or elongated, slightly bilobed, and conspicuous is adnate at the base on the side with lateral staminodes. It is formed by the inner whorl of the androecium. It has a thickened longitudinal bar in the centre and in some species, the sides of the bar towards the base are slightly erect. The side lobes are erect so as to form a wide channel whereas the apical lobe is recurved or protruded. In some species, the side lobes are very short and the lobe is more elongated so the channel is not wide.

The *filament* is short and broad, constricted at the top and connected to the base of the *anther* or the back of the connective making it versatile. The anther has two *thecae* which are parallel with the connective at the back. They embrace the *style* that is filiform and support it. The dehiscence is towards the front, while the back- and sidewalls are very thick and fleshy. These fleshy walls end in short or long awlshaped *spurs* in most species.

The *pollen* is ovoid, smooth, three sulcate under SEM, rather large and adhering by means of a glutinous substance, not soluble in water. The *fruit* is ellipsoid, thin-walled, and dehiscent. It releases the seeds in the mucilage of the bract-pouch. The *seed* is ellipsoid with a lacerate aril of few segments, which are free to the base.

1.2.3 Habitat and distribution

Curcuma is most often found in undergrowth at the tropical and subtropical margins of primary forest, open grasslands, secondary forest and plantations in plains, or in coconut and arecanut groves (Mangaly & Sabu 1993). They are found in many soil types in several rainfall regions, from wet to seasonally dry.

The genus is distributed (**Figure 1.2**) from India, southeast through Malesia to Queensland and the Pacific Islands (Smith 1981). In India, the main centres of distribution are southwest and northeast India. Many species are cultivated and naturalized (Mangaly & Sabu 1993).

1.2.4 Importance of Curcuma to humans

The uses of *Curcuma* can be found in the literature for the uses of the Zingiberaceae in general as discussed previously. Some other references discuss the uses of *Curcuma* such as Flückiger & Hanbury 1879, Bentley & Trimen 1880, Dey 1896, Lassak & McCarthy 1997.

The rhizomes are the most widely used part of the plant. There are various kinds of colours, smells and tastes. These features are hard to explain in words. The rhizomes of many species of *Curcuma* are used as spices, dyes, tonic, medicines, and as a source of starch. Turmeric from the rhizome of *C. longa* is well known not only for colouring foods but also for giving a taste and eliminating bad smells. In Java, rhizomes from almost all species are used in medicine. Burkill (1935), Ochse 1931, and Heyne 1950 discuss this in detail.

The young leaves of some species are used as flavouring. Again, the most widely known is *C. longa.* They are used in curry (in Malaysia and Indonesia), and for wrapping fish before it is cooked. Young inflorescences are also used for cooking. For example, the young inflorescence of *C. angustifolia*, and *C. zedoaria* are used to make soup or as flavouring for food.

1.3 CLASSIFICATION OF CURCUMA

The history of the classification of *Curcuma* begins as early as Roxburgh in 1812. It was continued by Horaninow in 1862, Baker in 1894, Schumann in 1904, Valeton in 1918, and recently by Velayudhan *et al.* in 1996. The classifications are summarized and tabulated in **Table 1.3**.

Roxburgh (1812) divided the genus into two sections, one with lateral spikes which appear before or with the leaves, and the other with central spikes. He recorded *C. rubescens* having a lateral inflorescence, but he also said *"and sometimes from the centre of the leaves"*. It is therefore concluded that *C. rubescens* has both inflorescence positions, lateral in May and then central in September (Roxburgh 1812).

Horaninow (1862) had the same idea as Roxburgh in dividing the genus into sections *Exantha* (for species that produce a lateral inflorescence) and *Mesantha* (for those species that produce a central inflorescence). However, he also added a new section *Amphiantha* for species that produce both positions of inflorescence, such as *C. rubescens* and *C. decipiens*.

Baker (1894) excluded section *Amphiantha* but accepted the two sections *Exantha* and *Mesantha*. He added a new section *Hitcheniopsis* (see **Table 1.4**). He included *C. parviflora* Wall., *C. strobilifera* Wall., *C. grandiflora* Wall., *C. petiolata* Roxb., and *C. roscoeana* Wall. in section *Hitcheniopsis*. Sections *Exantha* and *Mesantha* (see **Table 1.4**) were maintained.

Schumann (1904) raised the sections to subgeneric level resulting in subgenera *Eucurcuma* and *Hitcheniopsis*. In *Hitcheniopsis*, he added that the bracts are adnate to each other almost for their whole length and that the anther is spurless. His original description is tabulated in **Table 1.4**. He put together sections *Exantha* and *Mesantha* under subgenus *Eucurcuma* (see **Table 1.4**)

Valeton (1918) criticized Baker's section *Hitcheniopsis*, saying

"this last section was based principally in Curcuma Roscoeana, Wall (1830 t.9), which according to Bentham in Genera Plantarum (1880, 643), ought to be transferred to Hitchenia, as has been done by Petersen 1868 II 6.16".

Table 1.3 Classification of *Curcuma*.

Roxburgh 1812	Horaninow 1862	Baker 1894	Schumann 1904	Valeton 1918	Velayudhan *et al.* 1996
Section with lateral spike	Section *Exantha*	Section *Exantha*	Subgenus *Eucurcuma* (*Curcuma*)	Subgenus *Eucurcuma* (*Curcuma*)	Subgenus *Eucurcuma* (*Curcuma*)
					Section *Tuberosa*
			Section *Exantha*	Section	Subsection 1
Section with central spike	Section *Mesantha*	Section *Mesantha*	Section *Mesantha*	*Exantha* Section *Mesantha*	Subsection 2
					Section *Nontuberosa*
					Subsection 1
					Subsection 2
					Subsection 3
					Section *Stolonifera*
		Section *Hitcheniopsis*	Subgenus *Hitcheniopsis*	Subgenus *Paracurcuma*	Subgenus *Paracurcuma*
	Section *Amphianta*				

He also criticized Schuman's subgenus *Hitcheniopsis* mentioning

"He takes however in this subgenus among others C. petiolata Roxb., notwithstanding this species has calcarate anthers as may be seen in the 4 figures quoted by himself, and, according to Hooker (Bot. Mag. 5431), the bracts are adnate to the middle, not to the top".

Valeton stated that the proportion of the adnate part of the bracts to the free part is very vague and useless in practice. He excluded subgenus *Hitcheniopsis* and coined a new subgenus *Paracurcuma* which contained *C. aurantiaca, C. petiolata, C. cordifolia, C. meraukensis,* and *C. latifolia.* His descriptions of subgenera *Paracurcuma* and *Eucurcuma* are tabulated in **Table 1.4**.

Though Valeton criticized Schumann's use of length of adnation of the bracts, one of his descriptions still mentions this feature. He says that, in subgenus *Paracurcuma,* the bracts are adnate at least partly beyond the middle. This character is not constant throughout the subgenus. *C. alismatifolia* has bracts that connect to each other almost at the base, therefore this should match subgenus *Eucurcuma* according to Valeton's concept. This is probably because he excluded *C. alismatifolia* from the genus *Curcuma*. To bear in mind, his description is only based on several *Curcuma* especially in Malesian region (*C. aurantiaca, C. petiolata, C. cordifolia, C. meraukensis,* and *C. latifolia*).

Velayudhan *et al.* (1996) proposed a new classification of *Curcuma.* Their classification at subgeneric level is basically the same as the previous classification which divided the genus into two subgenera, *Curcuma* and *Paracurcuma.* However, the sectional level classification of subgenus *Curcuma* is very different. Previous authors divided subgenus *Curcuma* into sections *Exantha* and *Mesantha* mainly based on the position of inflorescence. Velayudhan *et al.* proposed a sectional classification of subgenus *Curcuma* on the basis of rhizome structures and the place on the tuber from which the flower spikes arise (from tip or side). They proposed three sections and five subsections. The first section is section *Tuberosa* for species in which the main root stock or secondary root stock gives rise to sessile fingers. The second is section *Nontuberosa* for species lacking the sessile tubers but producing stipitate tubers in large

Table 1.4 Original description of sections or subgenera in *Curcuma*.

Section/Subgenus *Hitcheniopsis*	Section/Subgenus *Curcuma*
Baker (1894) – Section *Hitcheniopsis* "spike autumnal, from the centre of the tuft of leaves; bracts very obtuse, adnate at the sides and spreading at the tip".	**Baker (1894) – Section *Mesantha*** "spike autumnal, from the centre of the tuft of leaves; bracts very obtuse, adnate at the sides and spreading at the tip". **Baker (1894) – Section *Exantha*** "flower-spike vernal or aestival, distinct from the leaves, and usually developed before they appear; peduncle sheathed by scariose bracts-leaves".
Schumann (1904) - Subgenus *Hitcheniopsis* "spica autumnalis e medio foliorum, bracteae tota longitudine lateraliter adnatae apice liberae et divaricantes et recurvatae, antherae basi ecalcaratae".	**Schumann (1904) – Subgenus *Eucurcuma*** "bracteae basi tantum axi et contiguis adnatae, apice haud anguste recurvatae antherae calcaratae".
Valeton (1918) – Subgenus *Paracurcuma* "bracts often very numerous, connected at least partly beyond the middle. Spike cylindrical, with comparatively short bracts of the coma. Bracteoles small, staminodia straight, larger than the dorsal petal which is somewhat cucullate, obtuse or with a short concave top, not clasping the staminodes, except in *C. cordifolia* Wall. Anthers attached near the base, not or very shortly calcarate, spur no longer than a quarter of the anther, grooved on the face, as a continuation of the loculi; appendix of the connective forming a short cup which encloses the stigma entirely or its base. Stem short; leaves spreading, short-or long stalked, the base mostly rounded. Ligule large, forming an ovate auricle on both sides of the base of the petiole. Rhizome short or wanting, bulbs or tubers in groups".	**Valeton (1918) – Subgenus *Eucurcuma*** "bracts mostly not adnate over the middle; only in *C. colorata* Val. this is the case with the lowest floral bracts. Bracts of the coma mostly extant far beyond the floral bracts. Staminodia longitudinally grooved, folded under the cucullate and pointed dorsal lobe. Anthers calcarate; spur attached with a fleshy base to the back of the cells. Connective rounded or narrowed towards the top, not lengthened to a cup, sometimes slightly produced between the loculi; anther attached to the filament at the back about the middle; outer wall of thecae prolonged at the lower end to a small tubercle, the cell not continuous along the lower side, or in some species of the *Exantha*, only as a narrow furrow, not containing pollen. Full-grown leaves acuminate at the base. Ligule without elongated auricles. Rhizomes lengthened, consisting of merithalia and forming lateral branches. Fourteen species in Java and two in Sumatera"

numbers. And the last is section *Stolonifera* for species which have stoloniferous tubers arising from the rhizomes.

Section *Tuberosa* is subdivided into two subsections. The first is a subsection in which flower spikes arise from the tips of sessile tubers of the preceding year's growth during the off season (e.g. *C. aeruginosa, C. zedoaria*). The second is a subsection in which flower spikes arise from the tip of the primary mother stock (primary mother rhizome) or secondary stock (secondary mother rhizome) during the main growing season, e.g. *C. longa* (Velayudhan *et al.* 1996). Sessile tubers, however, can grow to form a mother rhizome which will produce another clone.

Section *Nontuberosa,* is divided into three subsections on the basis of the position of the flower spike on the root stocks. In the first subsection the spikes arise from the side of the root stocks; while, in the second subsection the spikes arise from the tip of the root stocks. Third is a subsection in which the flower spikes arise both from the tip and from the sides of the mother rhizomes in different seasons.

Section *Tuberosa* subsection 1 and section *Nontuberosa* subsection 1 (e.g. *C. zedoaria* and *C. neilgherrensis* respectively) correspond with section *Exantha*, while section *Tuberosa* subsection 2 and section *Nontuberosa* subsection 2 (e.g. *C. longa* and *C. pseudomontana* respectively) correspond with section *Mesantha* (Velayudhan *et al.* 1996).

1.4 WAS THE CLASSIFICATION NATURAL?

Valeton excluded some of Schumann's species that are included in his subgenus *Hitcheniopsis.* They are *C. roscoeana, C. parviflora, C. alismatifolia, C. sparganifolia, C.gracillima, C. sylvestris, C. lanceolata,* and *C. kunstlerii.* Together, according to him, they *"do not constitute a natural group"*. He added that they have in common with *Curcuma* their strobiliform inflorescence, but the structures of coma bracts, petals, lateral staminodes, labellum, and anther, are very different from those of *Curcuma.*

The reason why he tends to exclude those species from *Curcuma* is as follows. ***C. roscoeana*** was thought to be not *Curcuma* according to Valeton because it has:

"no coma, all bracts rigid, red, erect with a much recurved top (free, according to Wallich, except at the broad base, adnate with the edges, according to Baker);dorsal lobe not cucullate; staminodes not lobed, not connate with the filament (?); labellum simple, not lobe, not concave, with two elevated lines in the centre, including a median groove; anther terminal, articulate to the filament with a broad base, thecae distant much shorter than the large connective which ends in a membranaceous, ciliate crest".

Valeton agreed with Bentham in reducing it to *Hitchenia.*

C. parviflora has *"petals converge behind the stamen and staminodes; staminodes free from filament and seem to be placed in exterior cycle; labellum patent, recurved, not lobed not concave, without erect side parts and central bar, without a median groove; anther terminal subarticulate and nutant with a broad base, very short thecae (opening by pores?), a very large fleshy connective prolonged into a considerable crest. The violet lip radiating white lines shows more relation to Gastrochilus".*

In ***C. alismatifolia*** the *".... narrow parallel theca of the rather long crested anther are attenuate at their base into a kind of spurs, and the connection with filament is at the backside near the base, probably it is nutant"..*

In ***C. sparganifolia*** the *"bracts of the spike are quite free one from another; anther with shortly pointed thecae is evidently terminal; staminodes are free from filament labellum entire".*

In ***C.gracillima*** the *"the bracts are all alike, erect with extant subacute tips; anther terminal or versatile?".*

He added that no stylodes were seen in the four species above (*C. parviflora, C. alismatifolia, C. sparganifolia, C.gracillima*). My observation of those species, except *C. sparganifolia,* agrees with Valeton that there are no epigynous glands on the top of the ovary.

C. sylvestris has a *"slender creeping rhizome; anther terminal with a recurved violet crest, and emarginate lip with a yellow central spot and violet streaks on the lobes".*

Valeton thought this was probably *Gastrochilus* (now *Boesenbergia*). He added that *C. lanceolata* and *C. kunstlerii* are supposed to be *Gastrochilus.*

Valeton predicted that the species which he excluded from Curcuma, would form a group distinct from *his other Curcuma.* He said *"Provisionally I think they must remain together forming a rather dubious group, Hitcheniopsis, which might be put as*

an Appendix to Gastrochilus. None of them occur in the Archipel".

Investigation should be carried out to prove whether all *Curcuma* species form a natural group or some must be transferred to other genera as Valeton proposed. At the moment, I will include all species of *Curcuma* that Valeton excluded for convenience and treat them as in Schumann's classification (subgenus *Hitcheniopsis* instead of *Paracurcuma*). The results of this study should be able to answer this question and solve the problem.

If we finally decide to include species that Valeton excluded, we must redefine either Valeton's subgenus *Paracurcuma* or Schumann's subgenus *Hitcheniopsis*. This is in order to accommodate all the species and to reflect their natural relationships. Both concepts at subgeneric level still overlap. Velayudhan *et al.*'s concept at subgeneric level should be examined, but the material for my study is limited so that I would not be able to investigate their subgeneric classification.

Subgenus *Eucurcuma* should simply be called *Curcuma* if we follow the current International Code of Botanical Nomenclature. The subgenus is divided into two sections, namely *Exantha* (with lateral inflorescence) and *Mesantha* (with central inflorescence). However, some species produce both positions of inflorescence, making it difficult to assign them to either of the sections. Roxburgh was the first to note both inflorescence positions in *C. decipiens*. Later on, Santapau reported *C. pseudomontana* as having both inflorescence positions (Santapau 1945, Santapau 1945, Mangaly & Sabu 1987). Several other species, namely *C. amada, C. decipiens, C. inodora, C. neilgherrensis,* and *C. oligantha* were reported by Mangaly & Sabu (1993) as having both positions.

Therefore, the sectional level classification causes even more conflict. All concepts from Roxburgh to Velayudhan *et al.* are vague. When the soil or environment is very favourable for the species to grow, they can produce lateral inflorescences followed by central ones. The sections therefore seem unnatural. Even if we establish a section for species that produce inflorescences in both positions, as was put forward by Horaninow (1862), it would just make life uneasy. The position of the inflorescence can also be affected by developmental genes.

It is not just difficult to devise a natural classification of *Curcuma.* It is even difficult to identify them to species. Roxburgh (1812) said:

"The plants of this genus, are the most easily distinguished of all the Scitaminean tribe..... But unfortunately, this uncommonly great similarity extends to almost every other part; which renders it so difficult to distinguish the species; that without the aid of colour, I should despair of making their specific characters discriminative. From daily habit I find no difficulty in recognizing them, yet it is by no means easy to find words that will convey that knowledge to others".

Baker (1894) also thought that the genus was difficult of determination. He put forward a hypothesis that most species of section *Exantha* were probably just varieties.

Curcuma is a taxonomically difficult genus, a nightmare to plant hunters, herbarium technician, and taxonomist (Mangaly & Sabu 1993). This is because they are not easy to identify either from fresh materials or herbarium specimens. It is probable that natural crossing to produce natural hybrids has occurred -probably in several of Valeton's species (Holttum 1951). It is no wonder that Backer & Bakhuizen van den Brink (1968) arrived at the conclusion that there were only two collective species within subgenus *Curcuma.* However, their two collective species are based on the position of inflorescence which is, again, not a good character as discussed previously. Their first collective species is *C. zedoaria sensu lato,* which consists of infraspecific taxa *C. zedoaria sensu stricto* (*s.s.*), *C. heyneana, C. phaeocaulis, C. xanthorrhiza, C. aeruginosa, C. mangga,* and *C. sylvatica.* The second collective species is *C. viridiflora s.l.* (older name is probably *C. montana*) consisting of *C. viridiflora s.s., C. longa, C. purpurascens, C. colorata, C. euchroma, C. brog, C. soloensis,* and *C. ochrorhiza.* The segregation at infraspecific level into "species" was on the basis of several characters but all were colours, such as colour of rhizome, colour of leaves along the midrib, colour of leaf sheath, colour of coma bracts, and colour of flower (Backer & Bakhuizen van den Brink 1968). Therefore, the key is not very easy to use, for example the key to species of *C. brog* and *C. soloensis* is as follows: rhizome citron-yellow in *C. brog*, versus rhizome orange-yellow in *C. soloensis* (Backer & Bakhuizen van den Brink 1968). Valeton is right in emphasising the importance of assigning colour using a colour chart. He made use of the "Code des couleurs" by Klincsiek *et* Valette (1908) and

"Chromotaxia" of Saccardo.

Why are some species so similar to each other, disregarding colour? If we look at some other species such as most of the Thai species of subgenus *Hitcheniopsis*, they are fertile and we can easily distinguish them even if we fail to see the colour. We can distinguish them by the shapes of their various organs such as bract and flower shape. However, if we look at all the sterile species of subgenus *Curcuma,* we shall very likely arrive at the same conclusion as Backer & Bakhuizen van den Brink. Herbarium specimens are therefore useless unless the colour is recorded precisely or the smell of the rhizome is well described.

1.5 THE USE OF SUBGENERA AND SECTIONS

There are two types of rank in taxonomic hierarchy. First, the principal ranks. The principal ranks of taxa, according to the International Code of Botanical Nomenclature, in descending sequence are kingdom, division or phylum, class, order, family, genus, and species. Every individual plant is treated as belonging to an indefinite number of taxa of consecutively subordinate rank, among which the rank of species is basic. Second, the secondary ranks. These are ranks that may be used or not. The secondary ranks are generally used to subdivide large groups. Thus, a large family may be divided into tribes, a large genus into sections, large sections in series, etc. If that is not enough, one can always create additional ranks immediately below any or all of the principal or secondary ranks by adding the prefix "sub-" to the rank concerned. For example, subfamily is a rank immediately below a family but above a tribe. Subgenera is a rank immediately below a genus. Similarly, one can insert ranks above any of the recognized ranks, e.g., a superorder or superdivision. The advantages of these secondary ranks are, for instance, to ease of reference and identification, to call attention to variation or correlation with geography.

1.6 AIMS OF THE PROJECT

The project is first set up to revise the Malesian species. As herbarium materials are difficult to study, and as the living materials are not all to hand, it will be very difficult to achieve the goal. Fieldwork was carried out in Java to get living material and add them to a living collection. Good living collections in the glasshouses of the Royal Botanic Garden Edinburgh, allowed me to observe flowering of several species between 1996 and 2000.

Other species from places outside Malesia were also studied in order to obtain general results as to the subgeneric and sectional boundaries in the genus. As some triploid sterile species from subgenus *Curcuma* are difficult to work out using morphological investigation, a molecular study was also carried out as part of the project. It is hoped that, by phylogenetic analysis using a different source of data, I would be able to draw conclusions as to whether the present classifications are natural or not. At the end we need to search for and define boundaries at subgeneric level that reflects natural relationships.

1.7 THESIS STRUCTURE

1.7.1 Introduction

Chapter One gives an introduction to the thesis, the background of *Curcuma*, its position in the plant kingdom in general and in family Zingiberaceae and tribe Hedychieae, its taxonomical history, its structure, and its problem. The aim of the project is also noted.

1.7.2 Molecular investigations using DNA sequences

Chapter Two is a phylogenetic study using DNA sequences data from two different organelle of cell. First is DNA sequences from nuclear DNA, i.e. the Internal Transcribed Spacer. The second is DNA sequences from plastid DNA of region *trn*L-F.

1.7.3 Morphological approach

Chapter Three dealt with phylogenetic study using gross morphological (vegetative underground and above ground parts, and inflorescence and flower characters) and some anatomical characters (epidermal structures and leaf transverse section for some species represented the two subgenera, and SEM of seed coat in four species represented the two subgenera). SEM of pollen was tried but was not successful. The pollen were sticky one to another. I have limited time to go through the process of avoiding these sticky pollen for SEM study.

1.7.4 Morphometric investigations of flowers

Chapter Four is about the attempt to classify the floral diversity in *Curcuma* based on an investigation on the morphometric of flower. Floral types could hint putative pollinator guilds. Mapping the floral character onto molecular tree was also carried out.

1.7.5 Chromosomes analysis

Chapter Five is about the attempt to understand the meiotical divisions in triploid species. The work tried also to confirm the chromosome numbers of some species of *Curcuma*.

1.7.6 Finding genetic variations using isozyme technique

Chapter Six concerns about the discovery of genetic variations among populations of some species of *Curcuma* using isozymes as the marker.

1.7.7 Polymorphisms of ITS

Chapter Seven is about the attempt to sort out the polymorphic sequences of ITS in some species of *Curcuma*.

1.7.8 *Revision of Javanese* Curcuma

Chapter Eight is a preliminary study for the preparation of the Revison of the genus for Flora Malesiana.

1.7.9 *General discussion, conclusions and suggestion for further study*

Chapter Nine is a discussion of the whole aspects carried out in the study, conclusion and suggestion for future study.

CHAPTER 2: PHYLOGENETIC STUDY USING MOLECULAR DATA FROM NUCLEAR TRANSCRIBED SPACER (RIBOSOMAL DNA) AND *TRN*L-F PLASTID DNA SEQUENCES

2.1 INTRODUCTION

2.1.1 Internal Transcribed Spacer (ITS) region of ribosomal DNA

Ribosomal DNA (rDNA) is a multigene family, occuring as one or several clusters in the haploid chromosome set. Cytologically, we recognize these clusters as the nucleolar organizer (NOR) in the chromosomes (Long & Dawid 1980). rDNA occurs in many copies, ranging from 200 copies in *Linum usitassimum* to 22,000 copies per haploid genome in *Vicia faba* (Long & Dawid 1980, Rogers & Bendich 1987). This copy number varies not only between distantly related species, but also among members of the same genus and a population of a single species (Rogers & Bendich 1987).

The many copies of rDNA exist in large arrays of tandem repeats (**Figure 2.1**). The repeats consist of a gene region (pre-rRNA gene) and a spacer that separates one gene from the next. The 5', 16-18S, 5.8S, 25S, 3' are transcribed as a single large precursor which is processed subsequently to the mature 16-18S, 5.8S, and 25-28S RNA molecules (Jorgensen & Cluster 1988). In some cases the repeating unit also codes for 5S rRNA, but in general pre-rRNA and 5S RNA genes in eukaryotes are not linked. The transcription unit of pre-rRNA has a size of 8 kb in most eukaryotes. The configuration of ribosomal genes is usually repeated in tandem in a head-to-tail configuration. (Long & Dawid 1980).

Transcription units alternate with spacers called nontrancribed spacers (NTS). NTS is later called Intergenis Spacer (IGS). This spacer is called NTS or IGS because regions which are transcribed into nonconserved parts of the pre-rRNA are called transcribed spacers. These transcribed spacers are subdivided into external (ETS) and internal (ITS) regions.

The family of rRNA coding region sequences contained within the arrays is generally highly conserved, whereas the spacer regions often exhibit extensive

intraspecific variablity in both sequence and length (Polans *et al.* 1986). IGS can vary extensively in length among species, at the population level, or even within a single individual (Schaal & Learn 1988 in Baldwin 1992). In contrast to the IGS, ITS is evolutionarily conservative in length. As a result of the short length, too few restriction sites generally occur within the ITS.

The rDNA repeat units of an individual plant are highly homogenous. This homogeneity is presumably the result of concerted evolution of rDNA repeat units as explained by Arnheim *et al.* (1980).

Several mechanisms have been thought to be the factor induced concerted evolution such as saltatory replication hypothesis (Britten & Kohne 1968, Buongiorno-Nardelli *et al.* 1972 in Li 1997), unequal crossing-over (Edelman & Gally 1970, Smith 1974, 1976 in Li 1997), replication slippage (Dover 1986), gene conversion (Edelman & Gally 1970, Birky & Skavaril 1976 in Li 1997), and duplicative transposition (Dover 1982 in Li 1997) or all called as molecular drive. The generality of concerted evolution in multigene families have been confirmed using restriction enzyme analysis and DNA sequencing (reviewed in Ohta 1980, Dover 1982, Arnheim 1983). Gene conversion and unequal crossing over are probably the ultimate mechanisms for the occurrence of concerted evolution (Li 1997). The genes evolve together through gene conversion, unequal crossing over, and probably repeat amplification (Baldwin *et al.* 1995).

2.1.2 Functions of the ITS

Study in *Saccharomyces cerevisiae* ribosomal DNA shows that small deletions in the 5'-terminal portion of ITS2 completely block maturation of 26S rRNA at the level of the 29 SB precursor (5.8S rRNA-ITS2-26S rRNA). However, various deletions in the 3'-terminal part, though severely reducing the efficiency of processing, still allow some mature 26S rRNA to be formed. The deletions of ITS2 do not affect the production of mature 17S rRNA (van der Sande et al 1992). Van der Sande *et al.* 1992 also concluded that the precise (secondary and/or primary) structure at the lower end of helix V, but excluding the loop, is of crucial importance for efficient removal of ITS2.

The study suggests that ITS is under evolutionary constraint as a result of an important role in processing mature rRNAs from primary trancripts. Secondary, "crucifex or tRNA-like-core" structures assumed by both ITS units in the primary rRNA transcripts may be critical to rRNA maturation by bringing the ends of the 18S, 5.8S, and 26S rRNA regions into close proximity for processing (Venkateswarlu & Nazar 1991).

2.1.3 Advantages and disadvantages of using the ITS

Nuclear ribosomal DNA has proven to be a powerful phylogenetic tool because it is ubiquitous in all organisms and occurs as repeated units in high copy number. One of the advantages of rDNA as a phylogenetic tool is that the repeat unit consists of several regions that have different rates of sequence change. Therefore, different regions of the molecule can be used to examine lineages with different levels of divergence. The 18S and 26S coding regions have been used to address phylogenetic questions at the family level or higher taxonomic levels in plants (Zimmer *et al.* 1989, Hamby & Zimmer 1991). On the other hand, ITS sequences appear to be useful for assessing relationships at lower taxonomic levels such as among genera or species because the sequences of spacer regions evolve more rapidly than the coding regions in general (Suh *et al.* 1993).

The tandem repeat structure and extremely high copy numbers of nrDNA make it especially easy to detect or clone in the laboratory. The two spacers of the region, ITS1 and ITS2, which is flanking with conserved regions (18S, 5.8S and 26S), can be readily amplified by Polymerase Chain Reaction (PCR) and sequenced using universal primers, even from DNA of herbarium specimens. High alignability and minimal length variation among ITS 1, 5.8S and ITS2 sequences make it easy to determine the positional homology of nucleotide sites.

Schaal & Learn (1988) discussed the prospect of using ribosomal DNA in the study of microevolutionary processes. Although ITS are present as numerous copies, but since molecular drive is put on to trigger a concerted evolution, the copies will be homogenized and therefore will be possible to use in phylogenetic study.

Problems in nrDNA genes includes polymorphisms within the arrays. Homogenization of nrDNA is not instantaneous and individual plants may contain a mixture of older and more-derived alleles. Recombination can also result in individual alleles with multiple lineages (Buckler 1996). Buckler (1996) modelled these polymorphisms at the infraspecific level with polymorphisms parsimony that accounts for a high probability of polymorphism persistence.

2.2 TRNL-F OF CHLOROPLAST DNA

Chloroplast DNA or cpDNA (**Figure 2.2**) has been used as an important source of characters for phylogeny reconstruction in plants (Palmer *et al.* 1988). However, maternal-inherited property of this genes accounts for significant error from hybridization and introgession events or lineage sorting (Doyle 1992). However, comparison of cpDNA and nuclear DNA in phylogenetic studies helps solving such problems (Smith & Systma 1990, Rieseberg *et al.* 1988, Baldwin 1992). Moreover, it also helps verifying species relationships and better understanding of the origin of polyploid species (Soltis & Soltis 1991). It can also be used to confirm the phylogenetic tree built on the basis of other genes especially from other compartment of the cells, such as nuclear ribosomal DNA. The *trn*L-F genes can be seen in **Figure 2.3**. It has been successfully used for phylogenetic reconstruction (Gielly *et al.* 1996, Sang *et al.* 1997). The region of *trn*L (UAA) 5' exon to *trn*F (GAA) in divided into two subregions, ie. *trn*L intron and *trn*L-F spacer (Taberlet *et al.* 1991).

2.3 MOLECULAR APPROACH USED IN THIS STUDY

Analyses of ITS sequences have provided phylogenetic resolutions of infra- and intergeneric relationships. Many groups of plant have been sequenced for their ITS, to name a few, in Fabaceae (Wojciechowski *et al.* 1993), grass species (Hsiao *et al.* 1993), Onagraceae (Baum & Sytsma 1994), Araliaceae (Wen & Zimmer 1996), Asteraceae (Baldwin 1992; Baldwin 1993; Kim & Jansen. 1994; Susanna *et al.* 1995, Eldenäs *et al.* 1998), Winteraceae (Suh *et al.* 1993), Saxifragaceae (Soltis *et al.* 1996), *Zea* (Buckler &

Holtsford 1996), Rubiaceae (Persson 2000), and Gesneriaceae (Möller & Cronk 1997a; Möller & Cronk 1997b; Denduangboripant & Cronk 2000). In Zingiberaceae, it has been used to study the classification of *Curcuma* (Ardiyani 1997), and the phylogeny of *Alpinia* (Rangsiruji 2000), *Kaempferia* Group (Searle & Hedderson 2000), *Roscoea* (Ngamriabsakul *et al.* 2000), and *Hedychium* (Wood *et al.* 2000).

Molecular approach has been widely used in phylogenetic study. The product of this study is a gene tree which hypothesises relationships among genes or genomes. The tree resulted from this approach may not be congruent with the true species phylogeny. This may be due to biological phenomena such as introgression, lineage sorting, and gene duplication. In such situations, all of the nucleotides or restriction sites of a gene or genome may be necessarily correlated as a single species tree character. The robustness of phylogenetic hypothesis is then meaningless. However, as with other characters, a gene tree can be combined with other characters such as non molecular characters to be tested best by parsimony analysis (Doyle 1992).

Morphological approaches to *Curcuma* taxonomy continue to be significant and probably vital. However, this cannot help to solve the problems in subgenus *Curcuma*. Therefore, a molecular systematic study will be attempted. A preliminary study of the genus has already been carried out (Ardiyani 1997). Attempts to sequence more species in *Curcuma* using the ITS have been made in this study.

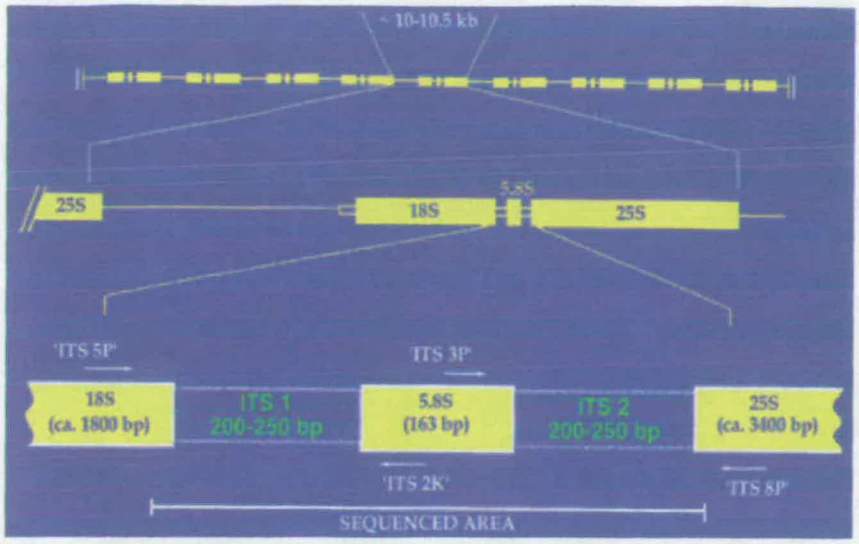


Figure 2.1 Repeat units of the nuclear ribosomal DNA and the organization of the ITS region. Arrows denote orientation and approximate position of primer sites. Primer names in quotation marks are modofied from White *et al.* 1990. Primer "ITS2K" was designed by Rangsiruji (1999)

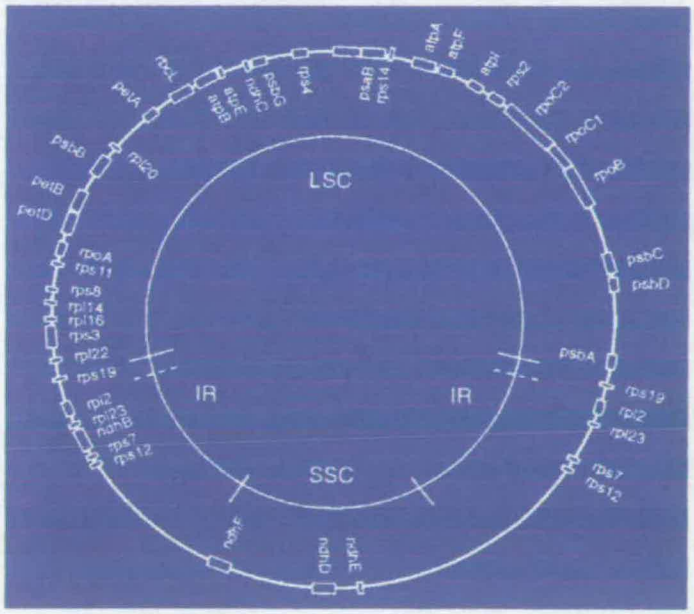


Figure 2.2 Chloroplast DNA
LSC: large single copy region; IR: inverted region;
SSC: small single copy region

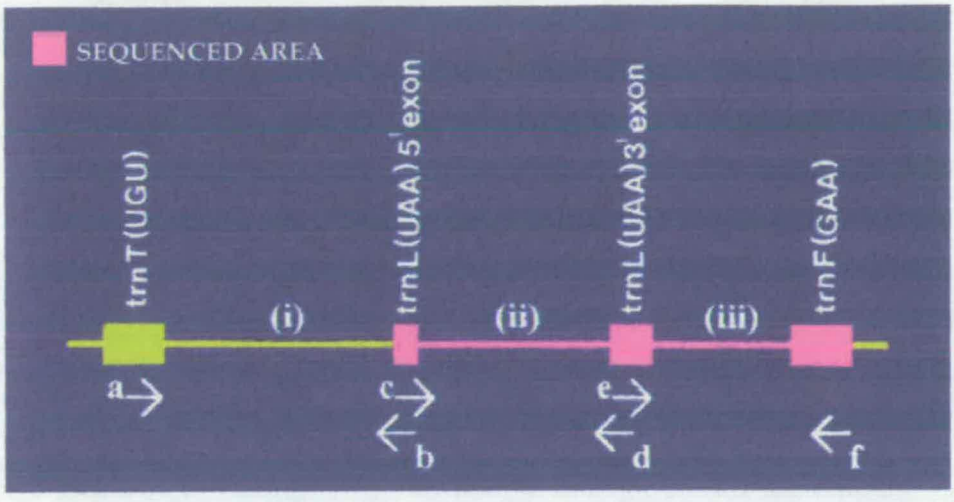


Figure 2.3 The chloroplast DNA region between the *trn*T (UGU) and the *trn*F (GAA) genes. (i): intergenic spacer between *trn*T (UGU) and *trn*L (UAA) 5' exon; (ii): *trn*L (UAA) intron; (iii): another intergenic spacer between *trn*L (UAA) 3' exon and *trn*F (GAA). The arrows with small letters indicate positions and directions of universal primers a to f (After Taberlet *et al.* 1991).

2.4 MATERIALS

The materials used are summarized in **Table 2.1**.

2.4.1 Ingroup and outgroup taxa

A phylogenetic study of the Zingiberaceae using ITS1-5.8s-ITS2 sequences by Wood *et al.* (2000), and a similar study using combined ITS and *mat*K sequence data by Kress *et al.* (2000) show that the nearest taxa to *Curcuma* are *Stahlianthus* and *Hitchenia* (not available for this study). *Stahlianthus involucratus* (Baker) Loes. and *Smithatris supraneanae* W.J. Kress & K. Larsen (another related species) were therefore included in the analysis. Two species of *Roscoea* (*R. auriculata* and *R. schneideriana*) and two species of *Cautleya* (*Ca. spicata* and *Ca. gracilis*) were used as the outgroup to assess the monophyly of *Curcuma* in respect of *Stahlianthus* and *Smithatris*. Either their ITS or *trn*L-F sequences meet the necessity for alignment. Likewise, they are distantly enough related to enable unequivocal rooting of the tree. The sequence data of *Roscoea*, and *Cautleya* were obtained from Ngamriabsakul *et al.* (2000), while those of *Stahlianthus* and *Smithatris* were obtained from Ngamriabsakul *et al.* (unpublished data).

Ten species representing subgenus *Hitcheniopsis*, namely *C. ecomata, C. aurantiaca, C. australasica., C. harmandii, C. thorelii, C. alismatifolia, C. gracillima, C. parviflora, C. roscoeana, C. petiolata*, and 15 species representing subgenus *Curcuma*, namely *C. phaeocaulis, C. aeruginosa, C. zedoaria, C. zanthorrhiza, C. amarissima, C. heyneana, C. elata, C. aromatica, C. soloensis, C. colorata, C. longa, C. amada, C. mangga, C. ochrorhiza,* and *C. purpurascens* were studied in the investigation. It is presumed that these 25 species of *Curcuma* represent about 25% of species in the genus which includes almost 100 species.

Apart from the widely cultivated *C. longa, C. zanthorrhiza, C. zedoaria,* and *C. aeruginosa*, the rest are representative of *Curcuma* from Java and Thailand. It is likely

that most, if not all, of Javanese *Curcuma* are not wild species, but are cultivated or escaped from cultivation and naturalized.

Verification of the species of *Curcuma* was accomplished by referring to the literature on *Curcuma* (Roscoe 1807; Roxburgh 1812, 1832; Blume 1827; Horaninow 1862; Baker 1894; Schumann 1904; Gagnepain 1908; Valeton 1918; Holttum 1950, 1951; Backer & Bakhuizen van den Brink 1968).

Apart from *C. cf. australasica, C. petiolata, C. elata,* and *C. aromatica,* which are herbarium specimens stored in Royal Botanic Garden Edinburgh (RBGE) herbarium (E), all other species are cultivated in the RBGE research glass house. I brought some Javanese species from a field expedition to Java, Indonesia. Some other Thai species were brought by M.F. Newman & C. Ngamriabsakul from Thailand.

The DNA of *C. australasica, C. petiolata, C. elata,* and *C. aromatica* were extracted from herbarium materials, while the DNA of the rest of the species was extracted from silica gel dried leaves. Voucher specimens for species which are kept in the glass house were made, and when the plants were flowering, inflorescences and flowers were collected and preserved in Copenhagen mixture (water 5.5 units; methanol 3.5 units; glycerol 0.5 units). They are deposited at E. The colours of the rhizomes and inflorescences were matched to the Royal Horticultural Society colour chart and were noted. Some photographs or slides were taken by myself besides those which were taken professionally by Debbie J. White. Slides were stored in RBGE library slide collection by D.J. White.

Table 2.1 50 accessions representing 31 taxa of used in the molecular study (ITS and *trn*L-F).

Species	Source	Origin	Voucher
Ca. gracilis (Sm.) Dandy*	RBGE 19820532	not known	C. Ngamriabsakul 11 (E)
Ca .spicata (Sm.) Baker*	E00061739 (E)		E00061739 (E)
R. auriculata K.Schum.*	RBGE 19699652	not known	C. Ngamriabsakul 14 (E)
R. schneideriana (Loes.) Cowley*	RBGK 19903345	Yunnan	-
St. involucratus (Baker) Loes.	RBGE 19981701	Thailand	C. Ngamriabsakul 34 (E)
Sm. supraneanae W. J. Kress & K. Larsen*	Y. Paisooksantivatana (BK)	Thailand	Y. Paisooksantivatana 00081101 (BK)
C. parviflora Wall.	RBGE 19851661	Sukhothai, Thailand	M. Ardiyani 31 (E)
C. thorelii 1 Gagnep.	RBGE 19973659	Thailand	M. Ardiyani 82 (E)
C. thorelii 2	M945	Thailand	M.F. Newman 945 (E)
C. roscoeana Wall.	RBGE 19973658	Thailand	M. Ardiyani 83 (E)
C. alismatifolia 1 Gagnep.	RBGE 19973657	Thailand	M. Ardiyani 84 (E)
C. alismatifolia 2	M944	Thailand	M.F. Newman 944 (E)
C. gracillima Gagnep.	CNG60	Phetchabun, Thailand	C. Ngamriabsakul 60 (E)
C. ecomata Craib	CNG38	Chiang Mai, Thailand	C. Ngamriabsakul 38 (E)
C. harmandii Gagnep.	CNG46	Chachoengsao, Thailand	C. Ngamriabsakul 46 (E)
C. petiolata Roxb.	K.M. Nagata 3688 (E)		K.M. Nagata 3688 (E)
C. cf. australasica Hook.f.	K.M. Nagata 2312 (E)		K.M. Nagata 2312 (E)
C. mangga Valeton & Zijp	RBGE 19780191	Java, Indonesia	M. Ardiyani 75 (E)
C. ochrorhiza 1 Valeton	54MA	Central Java, Indonesia	M. Ardiyani 54 (E)
C. ochrorrhiza 2	57MA	Central Java, Indonesia	M. Ardiyani 57 (E)
C. aurantiaca 1 Zijp	35MA	West Java, Indonesia	M. Ardiyani 35 (BO)
C. aurantiaca 2	67MA	East Java, Indonesia	M. Ardiyani 67 (E)
C. longa 1 L.	RBGE 19931919	not known	M. Ardiyani 33 (E)
C. longa 2	RBGE 19782126	not known (cultivated)	M. Ardiyani 85 (E)
C. longa 3	RBGE 19711837	not known (cultivated)	M. Ardiyani 86 (E)
C. longa 4	60MA	Central Java, Indonesia (cultivated)	M. Ardiyani 60 (E)
C. longa 5	W81p246	cultivated	W81p246
C. longa 6	RBGE 19721701	not known (cultivated)	M. Ardiyani 87 (E)

Table 2.1 (continued) 50 accessions representing 31 taxa of used in the molecular study (ITS and *trn*L-F).

Species	Source	Origin	Voucher
C. cf. longa	37MA	West Java, Indonesia (cultivated)	M. Ardiyani 37 (E)
C. elata Roxb.	J. Lau & Cong 2213 (E)		J. Lau & Cong 2213 (E)
C. amada Roxb.	RBGE 19810001	Kerala, India	M. Ardiyani 27 (E)
C. cf. amada	RBGE 19710261	not known	M. Ardiyani 88 (E)
C. zanthorrhiza 1 Roxb.	RBGE 19740965	not known	M. Ardiyani 80 (E)
C. zanthorrhiza 2	RBGE 19771295	West Java, Indonesia	M. Ardiyani 89 (E)
C. zanthorrhiza 3	46MA	Central Java, Indonesia	M. Ardiyani 46 (E)
C .zanthorrhiza 4	RBGE 19780194	Indonesia	M. Ardiyani 90 (E)
C. zanthorrhiza 5	RBGE 19780187	Java, Indonesia	M. Ardiyani 91 (E)
C. zedoaria 1 (Christm.) Roscoe	RBGE 19771296	East Java, Indonesia	M. Ardiyani 92 (E)
C. zedoaria 2	W78p242	India	M. Ardiyani 93 (E)
C. zedoaria 3	RBGE 19730871	Sri Lanka	M. Ardiyani 28 (E)
C. cf. zedoaria	38MA	West Java, Indonesia	M. Ardiyani 38 (E)
C. heyneana Valeton & Zijp	RBGE 19780189	Java, Indonesia	M. Ardiyani 30 (E)
C. cf. heyneana	42MA	Central Java, Indonesia	M. Ardiyani 42 (E)
C. aeruginosa Roxb.	RBGE 19780186	Indonesia	M. Ardiyani 26 (E)
C. phaeocaulis Valeton	RBGE 19771293	Java, Indonesia	M. Ardiyani 73 (E)
C. aromatica Salisb.	R.C. Joshi *s.n.* (E)	India	R.C. Joshi *s.n.* (E)
C. soloensis Valeton	47MA	Central Java, Indonesia	M. Ardiyani 47 (BO)
C. amarissima Roscoe	RBGE 19871252	Thailand	M. Ardiyani 74 (E)
C. purpurascens Blume	RBGE 19780193	Java, Indonesia	M. Ardiyani 32 (E)
C. colorata Valeton	RBGE 19771290	West Java, Indonesia	M. Ardiyani 34 (E)

Notes:

- indicates that material is obtained from Ngamriabsakul *et al.* (2000 and unpublished); (E) is Edinburgh herbarium. In source of samples: MA is Marlina Ardiyani; M: Mark Newman; CNG: Chatchai Ngamriabsakul; RBGE: Royal Botanic Garden Edinburgh; RBGK: Royal Botanic Gardens Kew; W: Waimea Arboretum and Botanical Garden, Hawaii, USA.

2.5 METHOD AND ANALYSIS

Methods are summarized in **Figure 2.4**.

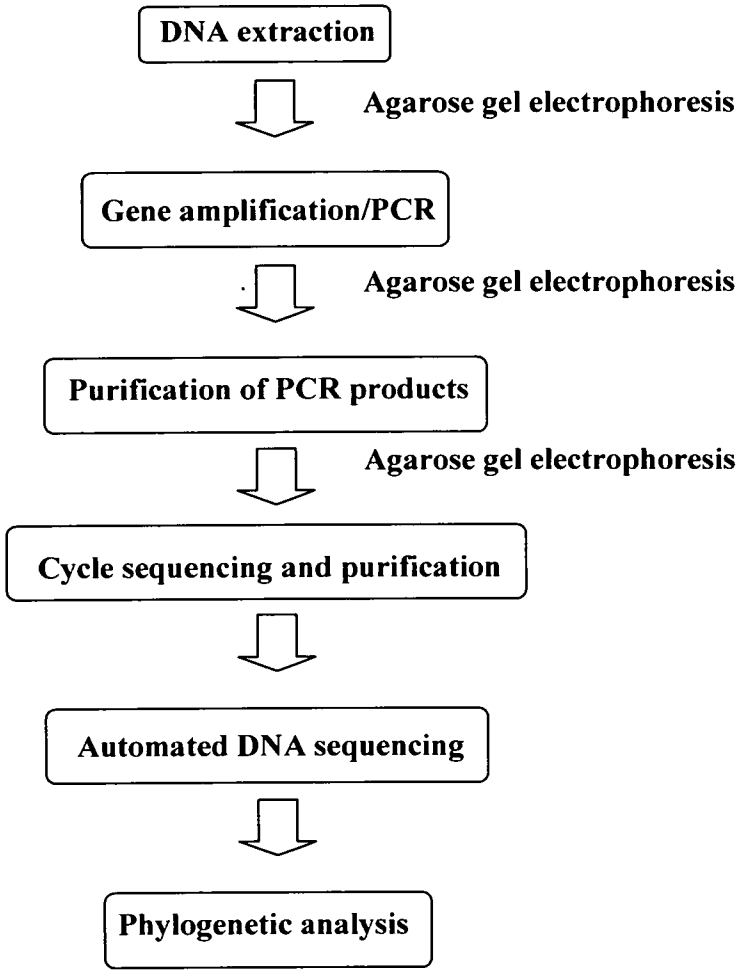


Figure 2.4 Flowchart of the methods used in the molecular study.

Chemicals used are compiled in **Appendix 1**.

2.5.1 Total genomic DNA extraction

The method for total genomic DNA extraction (see **Figure 2.5**) is modified from Doyle & Doyle (1987). The 2x "hot" CTAB method was used throughout. Preheated CTAB was used instead of cold CTAB. This "hot" CTAB method is preferred (M.

Hollingsworth, pers. com.) as it works better and faster. The method is also good for isolating DNA from herbarium specimens.

The 2x CTAB contains CTAB, sodium chlorides, EDTA, beta-mercaptoethanol, and PVPP. The function of this extraction buffer, the 2x CTAB, is mostly to protect the DNA from degradation by native enzymes and secondary plant metabolites. CTAB is a cationic detergent that helps to lyse the cell membranes and will form complexes with nucleic acids. Sodium chlorides help the formation of nucleic acid-CTAB complexes. EDTA chelates divalent ions, especially Ca^{2+} and Mg^{2+}, and prevents the activity of metal-dependent nucleases. Beta-mercaptoethanol is a reducing agent that protects the DNA against quinones, disulphides, peroxidases, and polyphenol oxidases. Finally, PVPP which has a similar function to PVP-40T (polyvinyl pyrrolidone), will form complexes with secondary plant products, in particular complex polyphenols, tannins, and quinones.

The first process is to disrupt the cell material. One circle cut out by punching fresh healthy leaves or silica gel dried material was obtained using 1.5 ml Eppendorf tube lid per sample. A pinch of sand was added, then all the tubes containing material for DNA extraction were placed on a rack. In another 1.5 ml Eppendorf tube (one tube was needed for each DNA sample), 500 µl 2xCTAB was preheated with 0.2% mercaptoethanol (1 µl) at 65°C in a water bath. Using forceps, the tube that contains leaf sample was submerged in liquid nitrogen (the lid of the tube must be opened or pierced if the lid was closed). Using a small plastic pestle, the tissue (sample) inside the tube was ground to a fine dry powder. The sample should be maintained cold by submerging it back in liquid nitrogen. The cooling and grinding step was repeated two to three times.

The powder obtained was dissolved in 400 µl preheated 2xCTAB, and then just a pinch of PVPP was added. The tube containing the mixture was placed in a vortex for a few seconds, then incubated for an hour at 65°C in a water bath to allow the cell to lyse for DNA liberation. After that, the tubes were taken out of the water bath and were allowed to cool for 10 minutes. Then, the samples were centrifuged at 13,000 rpm

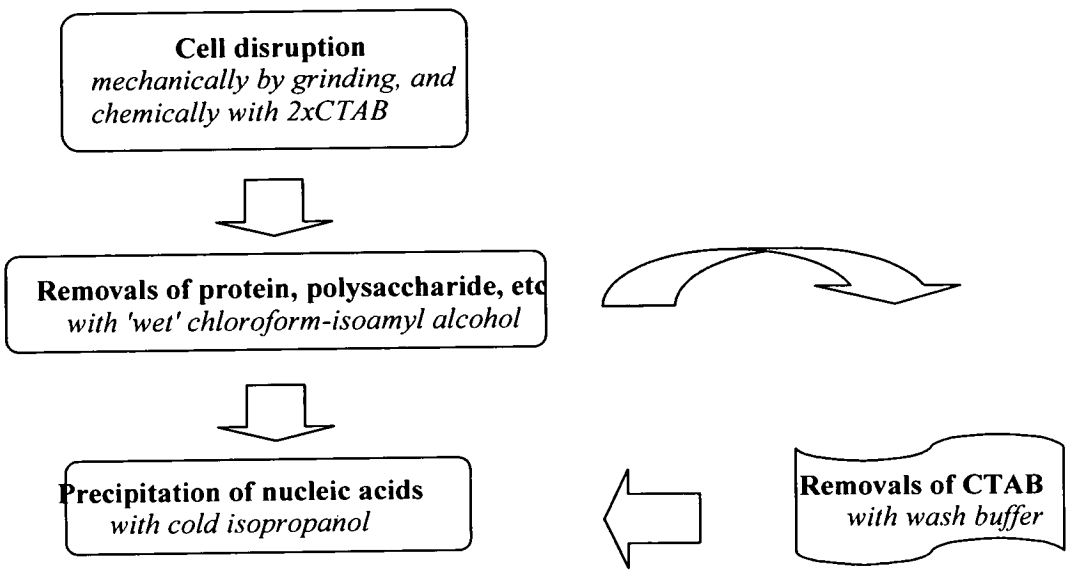


Figure 2.5 Flowchart to show simplified method of extracting nucleic acids.

for 10 minutes at room temperature. Gently, the aqueous (upper) phase was removed without removing any of the particulate matter, and was placed in a clean 1.5 ml Eppendorf tube.

400 µl of "wet" chloroform:iso-amyl alcohol (24:1) were added and mixed well by inversion. The chloroform is referred to as "wet" by which addition will change the mixtures to be slightly more hydrophilic (attracted to water). This will be able to precipitate proteins and polysaccharides more effectively, and therefore will extract nucleic acids better. The samples were placed on a shaker for 20 minutes for more effectiveness instead of only inverting them by hands for a few times (M. Hollingsworth, pers. com.). Next, the samples were centrifuged at 13,000 rpm for 10 minutes at room temperature. The supernatant was removed gently being careful not to pick up any of the bottom layer, and was placed in a clean, 1.5 ml Eppendorf tube. The above steps were repeated once again to re-extract the supernatant.

To precipitate the nucleic acids, 2/3 volume (300 µl) freezer cold isopropanol (also known as propan-2-ol) were added and mixed well by gentle inversion. The mixing by inversion was continued until the oily appearance of the mixture had gone. The samples were then left at -20°C overnight. After that, they were centrifuged at 13,000 rpm for 10 minutes at room temperature. At this stage, the concentration of salt in the extraction buffer is reduced. Therefore the CTAB-nucleic acid complex is precipitated.

In the final stage, the supernatant was poured off (being careful not to pour out the pellet), the tube was inverted and the pellet was dried in a vacuum drier for 10 minutes. It is important not to over-dry the pellet as it will stick hard to the tube and will be difficult to dissolve it. To obtain the nucleic acid solution, the pellet was resuspended in 50 µl of TE buffer by flicking the tube with a finger. The genomic nucleic acids can be stored in the freezer until required.

Prior to the final stage, the pellet formed can be washed by wash buffer before it is agitated to release the pellet from the bottom of the tube. This is to dissolve the CTAB-nucleic acid complex and to remove the CTAB. However, throughout my study wash buffer was not used as the DNA obtained was already clean.

Freezing is suitable for long-term storage. However, constant freezing and thawing will induce shearing of the DNA. The final nucleic acid sample contains a mixture of RNA, nuclear DNA, chloroplast DNA, and mitochondrial DNA.

2.5.2 Agarose gel electrophoresis to check DNA quality and quantity

The next step is to check the quality and quantity of the DNA obtained by running each sample on a 1% agarose gel (0.5 g agarose dissolved in 50 ml 1xTBE buffer) electrophoresis stained with Ethidium bromide.

The agarose in TBE buffer was heated in a microwave until all the particles had dissolved. It is best to wait until the bubbles get bigger (M. Hollingsworth, pers. com.)

to avoid having small bubbles in the set gel. When it was cooled, 1 µl Ethidium bromide was added. Then, it was poured to a gel mould fitted with a gel comb. When the gel was set, the comb was removed so wells were formed.

Five µl of extracted total genomic DNA were mixed with 3 µl loading solution and then loaded in a well. Five µl DNA size marker (DNA Hyperladder) was loaded in one side well of the samples to compare with the total genomic DNA. They were run in an electrophoretic field at 60-80V for 1-1.5 hours.

The negatively charged DNA will move to the positive electrode at a certain speed which depends upon the size of the molecules. Observation of the bands of ethidium bromide corporated-DNA was done under UV light. The results were documented with a digital camera and printed out.

2.5.3 Gene amplification

Gene amplification to produce identical DNA copies was obtained via the Polymerase Chain Reaction (PCR) technique. Three basic stages were involved, i.e. denaturation, annealing, and synthesis or primer extension (**Figure 2.6**). The denaturation phase (high temperature) splits the double-stranded DNA into single-stranded DNA. The annealing phase involves lowering the temperature. In this phase, the oligonucleotide primers will bind to the single-stranded DNA. The third stage, synthesis stage, involves the binding of polymerase enzyme (*Taq= Thermus aquaticus* polymerase) to deoxyribonucleotide triphosphates (dNTPs) and catalyze a reaction by attaching the nucleotides to the single-stranded DNA.

The ITS region was amplified using primers "ITS 5P" (5'-GGA AGG AGA AGT CGT AAC AAG G-3') and "ITS 8P" (5'-CAC GCT TCT CCA GAC TAC A-3') (Möller & Cronk 1997) which yielded double-stranded DNA of approximately 800 bp (**Figure 2.1**). The *trn*L (UAA) 5' exon- *trn*F (GAA) region (**Figure 2.3**) was amplified using universal primers "c" (5'-CGA AAT CGG TAG ACG CTA CG-3') and "f" (5'-ATT TGA ACT GGT GAC ACG AG-3') (Taberlet *et al.* 1991). PCR amplification was

performed in the thermal cycle (GeneAmp PCR System 9600, Perkin Elmer, USA or DNA Engine Peltier Thermal Cycler 200, Gradient Cycler, GRI).

The PCR reaction mixtures of total volume 50 µl in 0.2 ml PCR contained 34.5 µl sterile distilled water, 5.0 µl of 2 mM deoxyribonucleoside triphosphate (dNTP) mix (Sigma Chemicals, Poole, Dorset, UK), 5.0 µl of 10x Bioline taq™ reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris HCl pH 8.8 at 25° C, 0.1% Tween-20), 2.5 µl of 50 mM $MgCl_2$, two pairs of 10 mM primers ("ITS5P" and "ITS8P" for ITS region; and "c" and "f" for *trn*L-F region) each 1.5 µl (Oswel DNA Service, Southampton, UK), 0.25 µl of 5U/µl Dynazyme™ II thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland), and 2 µl DNA template from aliquots of total genomic DNA. Sterile distilled water was used instead of DNA template for the negative control. PCR cycle parameters for ITS amplification were as follows: initial denaturation for 3 min at 94°C; denaturation of template DNA for 1 min at 94°C; primer annealing for 1 min at 55°C; primer extension for 1.5 min at 72°C. After 30 cycles, a final extension step of 5 min at 72°C was added. This extension was meant to allow completion of unfinished strands. PCR cycle parameters for *trn*L-F region were as follow: initial denaturation for 4 min at 94°C; denaturation of template DNA for 0.45 min at 94°C; primer annealing for 0.45 min at 54°C; primer extension for 2 min at 72°C. A final extension step of 10 min at 72°C was added after 35 cycles. Gel electrophoresis (method described previously) at 60-80 V for 1-1.5 hours using 1.5 µl of PCR products was carried out to check successful amplification and quantity of PCR products. DNA size marker 123 bp ladder or 1KB ladder was sometimes used for comparison of amplified DNA obtained.

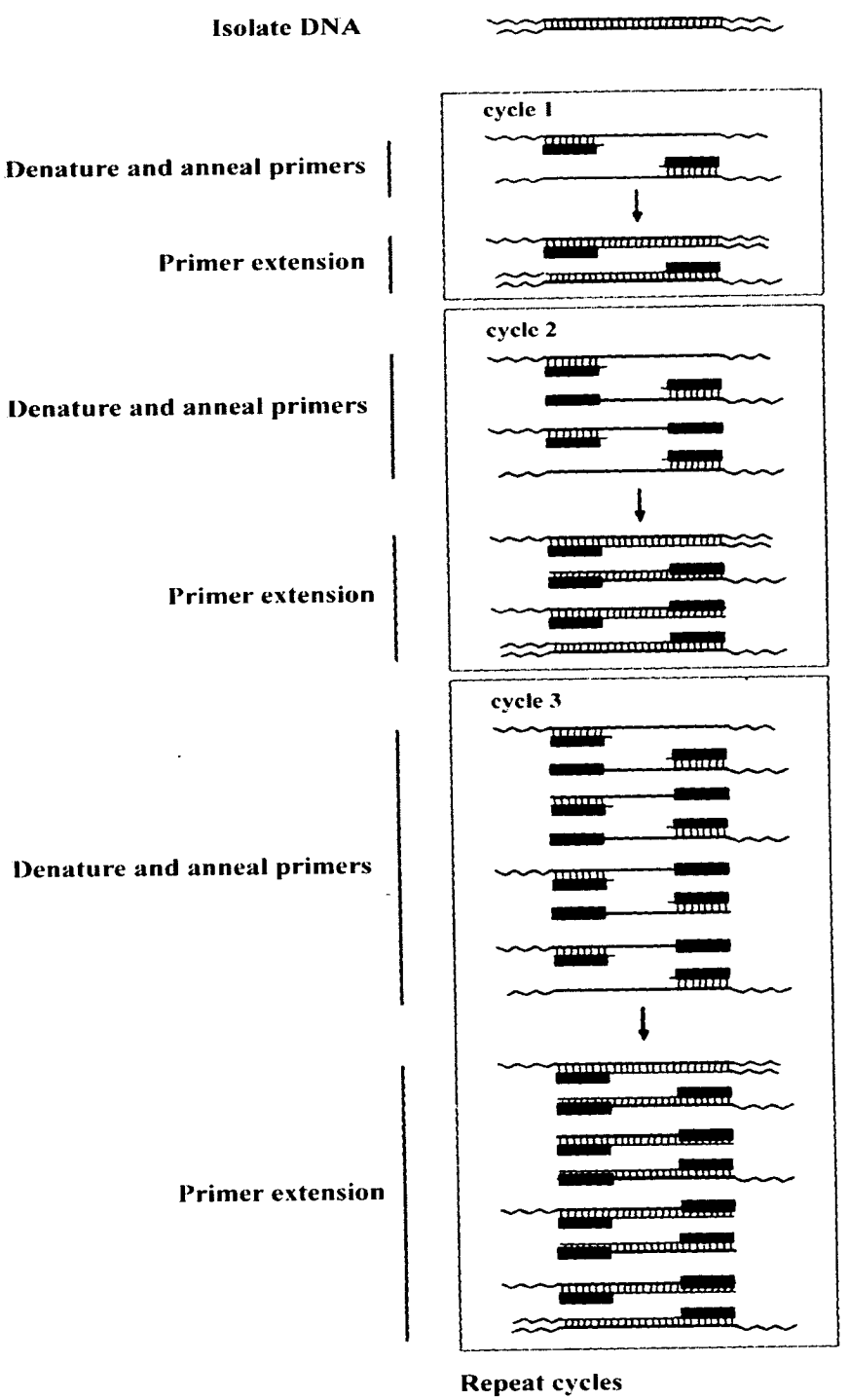


Figure 2.6 General protocol of the polymerase chain reaction for amplifying DNA (after Oste 1988 in Avise 1994)

2.5.4 Purification of PCR products

Purification means to purify the DNA obtained from any unwanted artefacts from PCR such as primers, unincorporated nucleotides, polymerases, and salts. The PCR products of ITS region and region between *trn*L (UAA) 5' exon and *trn*F (GAA) were purified using the QIAquick™ PCR Purification Kit with a unique silica gel membrane technology. The protocol from the manufacturer was as follow (see **Figure 2.7**).

One volume (50 µl) of PCR products was added to five volumes (250 µl) of buffer PB. A QIAquick™ spin column was placed in a provided 2 ml collection tube. The samples were then applied to the QIAquick™ column and were centrifuged at ~13,000 rpm for 30-60 sec to bind the DNA. The flow-through was discarded, and the column was placed back into the same tube. 0.75 ml Buffer PE was added to the column in order to wash the DNA. This was centrifuged at the same speed as before. The flow-through was discarded, and the column was again placed back in the same tube. An additional 1 min centrifugation was applied to the column. The next step was to place the column in a clean 1.5 ml microfuge tube. Finally, 30 µl elution buffer were added to the centre of the QIAquick™ column and it was allowed stand for 1 min to elute the DNA. This was centrifuged to allow the DNA to drop down. To check the purified PCR products, gel electrophoresis as previously described was carried out again.

2.5.5 Cycle sequencing and purification of sequencing reactions

Cycle sequencing was performed prior to automated sequencing. The 20 µl sequencing PCR mixture contained: 13 µl of sterile distilled water, 4 µl of Thermo Sequenase II reagent Premix, 1 µl of 5 mM of one primer type, 2 µl of DNA template (from purified PCR products). The samples were placed in a thermal cycler and run for 25 cycles with the following PCR conditions: denaturation step for 10 sec at 94°C; primer annealing for 5 sec at 50°C; and primer extension for 4 min at 60°C.

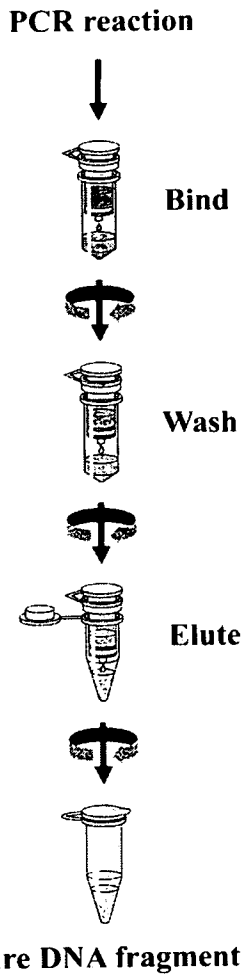


Figure 2.7 The QIAquick spin purification procedure (modified from QIAquick Spin Handbook)

In PCR cycle sequencing of the ITS region, two external primers identical to those used in normal PCR, i.e. forward external primer "ITS5P" and reverse external primer "ITS8P", were applied. In addition, two more internal primers for shorter sequences, i.e. a reverse internal primer "ITS2K" (Rangsiruji 1999) which starts from the far end of 5.8S, and a forward internal primer "3P" (Möller & Cronk 1997) which starts from the beginning of 5.8S, were also employed. "ITS2K" was 5'-GGC ACA ACT TGC GTT CAA AG-3', and "ITS3P" was 5'-GCA TCG ATG AAG AAC GTA

GC-3'. For cycle sequencing of the region between *trn*L (UAA) 5' exon and *trn*F (GAA), two external primers identical to those in normal PCR were employed, i.e. forward external primer "c" and reverse external primer "f". Two more internal primers were also used to obtain shorter sequences, namely internal reverse primer "d" (Taberlet *et al.* 1991) and internal forward primer "e" (Taberlet *et al.* 1991). Primer "d" was 5'-GGG GAT AGA GGG ACT TGA AC-3', while primer "e" was 5'-GGT TCA AGT CCC TCT ATC CC-3'. The primers used are summarized in **Table 2.2.**

Table 2.2 Primers used in PCR and cycle sequencing.

Primer	Location	Direction	Sequence
"ITS5P"	18S	forward	5'-GGA AGG AGA AGT CGT AAC AAG G-3'
"ITS8P"	25S	reverse	5'-CAC GCT TCT CCA GAC TAC A-3'
"ITS2K"	5.8S	reverse	5'-GGC ACA ACT TGC GTT CAA AG-3'
"ITS3P"	5.8S	forward	5'-GCA TCG ATG AAG AAC GTA GC-3'
"c"	*trn*L (UAA) 5' exon	forward	5'-CGA AAT CGG TAG ACG CTA CG-3'
"f"	*trn*F (GAA)	reverse	5'-ATT TGA ACT GGT GAC ACG AG-3'
"d"	*trn*L (UAA) 3' exon	reverse	5'- GGG GAT AGA GGG ACT TGA AC-3'
"e"	*trn*L (UAA) 3' exon	forward	5'-GGT TCA AGT CCC TCT ATC CC-3'

The results of cycle sequencing were purified according to the following procedure. The 20 µl of PCR cycle sequencing products were transferred to a fresh 0.5 ml tube containing 2 µl of sodium acetate/EDTA buffer. 55 µl of 100% cold (-20°C) ethanol was added to each reaction. They were mixed briefly with a vortex mixer and were placed on ice for 15-20 min to precipitate the DNA. Then, they were centrifuged in a microcentrifuge for 15 min at ~13,000 rpm. The supernatant formed was removed as much as possible. 250 µl of cold 70% ethanol were added to wash the pellet which

was then centrifuged at the same speed for 5 min. The supernatant formed was again removed as much as possible. Finally, the pellet that remained at the bottom of the tube was vacuum-dried in a vacuum centrifuge for 2-5 min.

2.5.6 Automated DNA sequencing

Gel preparation and loading for automated DNA sequencing was performed by M. Hollingsworth on an ABI Model 377 Prism Automatic DNA Sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA), in the molecular laboratory of the Royal Botanic Garden Edinburgh, according to the manual supplied.

2.5.7 Sequence analyses

The sequence boundaries of each region of ITS (ITS1 and ITS2) were compared with the results of Rangsiruji *et al.* (2000) and Ngamriabsakul *et al.* (2000). Each region was confirmed from forward and reverse sequences, for instance to verify the ITS1 region, the sequence obtained from the "ITS5P" primer was compared with that obtained from the "ITS2K" primer. The same thing was applied to the *trn*L (UAA) 5' exon and *trn*F (GAA) comparing with the results of Rangsiruji *et al* (2000). These were done using Factura™ version 2.0 (a program in Sequence Navigator™ package). Another program AutoAssembler™ version 2.1 (Applied Biosystems) is able to assemble the complementary strands automatically from the four forward and reverse sequences obtained prior to editing. A consensus sequence was then built for the whole region using this AutoAssembler (**Figure 2.8**).

Sequence alignment was carried out using the CLUSTAL option in the multiple alignment program Sequence Navigator™ version 1.0.1 software package (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA). These alignments were subsequently refined by eye. The G+C content and the number and size of insertion/deletion events (indels) were examined and determined manually. Sequence divergence was obtained from unambiguous aligned region using PAUP* version 4.0b4a (Swofford 2000) in the DISTANCE MATRIX option. Other sequence characteristics such as number of constant sites, variable sites, informative sites and autapormophic

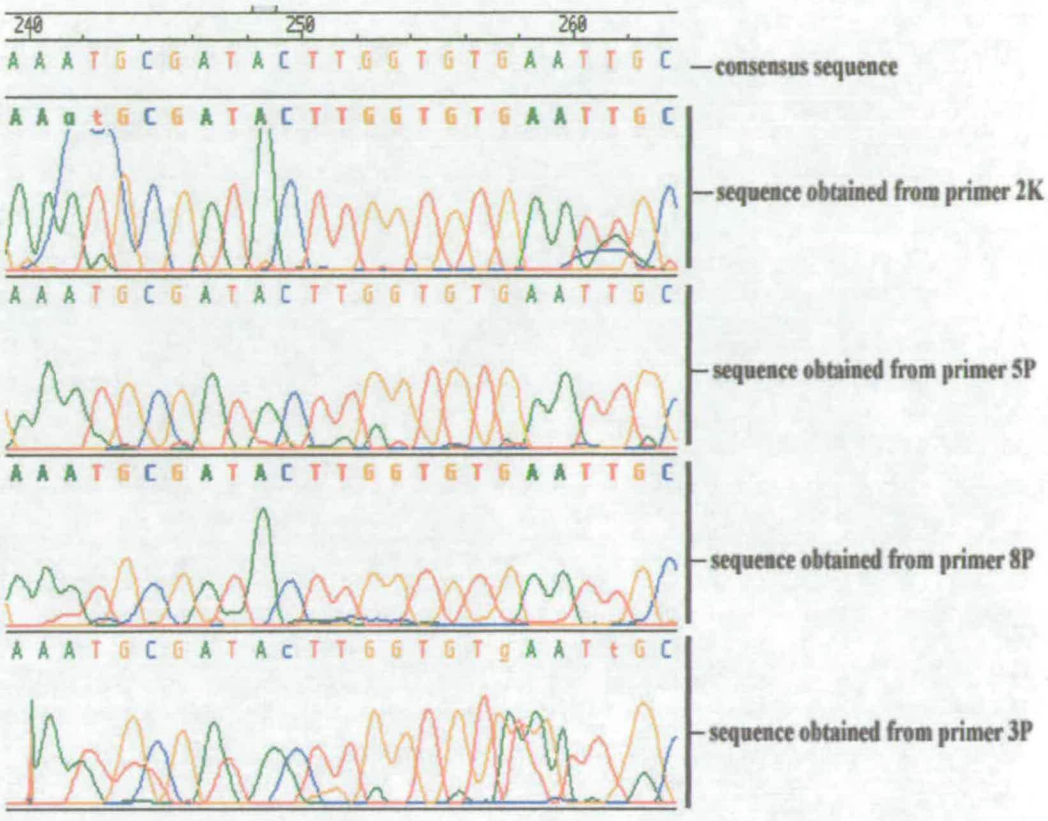


Figure 2.8 AutoAssembler assemble the complementary strands automatically from the whole four forward and reverse sequences.

sites were calculated in PAUP* 4.0b4a as well. The numbers of transitions and transversions were determined using MacClade version 3.08a (Maddison & Maddison 1999).

2.5.8 Phylogenetic analysis

Phylogenetic analysis to produce phylogenetic trees was carried out using PAUP* version 4.0b4a and run on an iMac DV 400 MHz computer. The method for heuristic search followed Möller & Cronk (1997). The large amount of data hamper the

analysis using exhaustive search, which guarantees to find the shortest tree or trees, due to the excessive computational time. Branch-and-bound search was applied when the data was not too large (i.e. data of only non-polymorphic ITS sequences). This method guarantees to find the shortest tree or trees. When the data set is large (i.e. data of polymorphic and non-polymorphic ITS sequences), heuristic search was employed, which does not guarantee optimality of the trees obtained but is relatively fast and efficient.

Cladistic terminology in bold font is explained in **Table 2.3**.

For branch-and-bound search, **MulTrees** and **FURTHEST addition sequence** options were selected. For heuristic search, the following strategies were applied: **SIMPLE addition sequence** with **TBR (Tree Bisection-Reconnection)** swapping, and **RANDOM addition sequence** of 500 replicates with no swapping. The resulting trees were subjected to TBR swapping. The application of random addition sequence has been suggested as a means to detect any multiple islands of most parsimonious trees (Maddison 1991). The options **COLLAPSE,** MulTrees, **STEEPEST DESCENT**, and **ACCTRAN** optimization were in effect.

The robustness of the phylogenetic trees was calculated using **Bootstrap** values (Felsenstein 1985) and **Decay indices** or **Bremer support** (Bremer 1988, Donoghue *et al.* 1992). Bootstrap analyses were performed in PAUP* 4.0b4a with HEURISTIC option and SIMPLE addition sequence using 1000 replicates with MAXTREE set to 1000. Decay indices or Bremer support were performed using the program Autodecay version 4.02 (Eriksson 1998) and PAUP* 4.0b4a. The Bremer support trees were viewed using the program TreeViewPPC (Page 2000).

All characters are unordered and equally weighted except for a separate analysis with characters weighted by transition/transversion ratio (MacClade). Gaps were treated as missing data and multistate were interpreted as uncertain. A separate analysis was conducted with coded gaps over insertion and deletion. Ambiguous regions from the alignment, which cause alternative alignment interpretations, were excluded from the analysis (Wojciechowski *et al.* 1993, Downie & Katz-Downie 1996, Möller & Cronk

1997). Analyses of ITS and *trn*L-F sequences were conducted separately as well as an analysis of the combined data sets.

Descriptive statistics in the parsimony analysis were given by the **Consistency Index=CI** (Kluge & farris 1969), **Retention Index=RI** (Farris 1989), and **Rescaled Consistency Index=RC** (Swofford 1993). A measure of the phylogenetic signal in the data matrix based on skewness of a tree length distribution, called the $\mathbf{g_1}$ statistic (Huelsenbeck 1991, Hillis & Huelsenbeck 1992), was made in PAUP* with 10000 random trees search options. A successive weighting approach (Farris 1969) by re-weighting on a rescaled index was applied to reduce the effects of homoplasious characters. This was done in PAUP* using heuristic search with TBR swapping and re-weighted characters on CI value.

Table 2.3 Cladistic terms and their definition or function, used in phylogenetic analysis.

Terminology	Definition or function
MulTrees	When selected will save all minimal trees found during branch swapping; when not selected will save only one of the best trees found. This option is synonymous with MulPars option in earlier versions of PAUP.
TBR swapping	Tree Bisection-Reconnection swapping is a method of branch-swapping that clips off subcladograms from the main cladogram and re-roots them before attaching them in a new position elsewhere on the remnant main cladogram.
COLLAPSE	When selected will collapse any zero-length branches into polytomies for all trees and then keep only those trees that are unique after the collapsing is accomplished.
STEEPEST DESCENT	When selected will not abandon a round of swapping until all input trees from the previous round have been examined by the swapping algorithm.
ACCTRAN	ACCelerates the TRANsformation will affect to favor reversals over palellism when the choice is equally parsimonious.
Bootstrap	A statistical method applied to place confidence intervals on phylogenies. It involves resampling points from one's own data, replacement
Decay indices or Bremer support	The number of extra steps required before a clade is lost from the strict consensus tree of near-minimum length cladograms.
consistency index=ci	A measure of the amount of homoplasy (extra steps) in a character relative to a given cladogram. ci=m/s, where m is minimum amount of change or steps that a character can show on any tree; and s is minimum number of steps the same character can exhibit on the cladogram in question.
retention index=ri	A measure of the amount of implied synapomorphy in the data matrix that is retained as synapomorphy on the tree. r=(g-s)/(g-m), where g is the greatest number of steps a character can exhibit on any cladogram; m is the minimum number of steps a character can exhibit on any cladogram; s is the number of steps the same character can exhibit on the cladogram in question.
rescaled consistency index=rc	The product of the consistency index and the retention index of a character.
g_1	Tree length distribution skewness.

2.6 RESULTS

2.6.1 Total genomic DNA extraction

The "Hot" CTAB method with liquid nitrogen grinding method was very good for extracting DNA in almost all the samples studied (samples either from silica gel dried leaf material or from herbarium material). Contamination of the RNA was also very low. **Figure 2.9** shows the result of genomic DNA extraction from silica gel dried leaves.

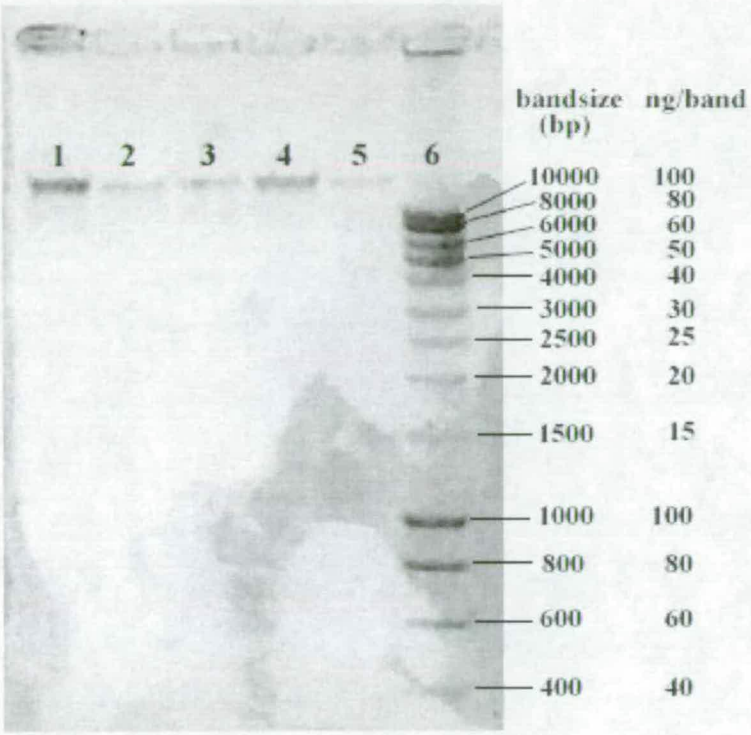


Figure 2.9 Gel electrophoresis from total genomic DNA extraction. Lane 1: *C. thorelii*, lane 2: *C. zedoaria*, lane 3: *C. roscoeana*, lane 4: *C. alismatifolia*, lane 5: *C. longa*, and lane 6: DNA size marker (Hyperladder).

After comparing the total genomic DNA band with that of DNA size and quantity marker (DNA Hyperladder), total genomic DNA concentration obtained was more than 10,000 bp with approximately 15-100 ng/band or 4-20 ng/µl varied from one sample to another.

DNA extractions from very brown leaf herbarium material of around 17 years old were not successful. No band came out on the gel electrophoresis. The reason for this is probably that the DNA is degraded or there are too many inhibitors. Degradation of DNA is probably due to the way of preserving the material, for example the use of alcohol in preparation of herbarium specimens.

On another experiment using the same method, but applied to herbarium material with a greenish brown colour of also 17 years old, successful results were gained. Though the intensity of the bands was not very strong, subsequent PCR resulted in a good DNA amplification. Unfortunately, a picture of this result (DNA extraction from herbarium material) was not available, but PCR result from herbarium samples will be shown later (**Figure 2.12**).

2.6.2 Gene amplification and purification of PCR products

Amplification of ITS region using primers "ITS5P" and "ITS8P" resulted in successful amplicons of one single band for both PCR from silica gel dried leaf material and herbarium material. The amplicons of ITS regions are approximately 850 bp (**Figure 2.10**).

Amplifications of *trn*L (UAA) 5' exon and *trn*F (GAA) region using primers "c" and "f" in some species, for instance *C. soloensis, C. zanthorrhiza, C. phaeocaulis,* and *C. ochrorrhiza*, resulted in successful amplicons of one single band of approximately 984 bp (**Figure 2.10**). However in most of other species, for example *C. roscoeana* and *C. longa*, instead of one single band of ~984bp, another band reflecting smaller sized-amplicons of approximately 400 bp appeared (**Figure 2.11**).

After more observation, the same species from different accession numbers resulted in different amplicon lengths. For example, *C. longa* accession 19931919 resulted in one single band, while *C. longa* accession 60MA resulted in double bands. However, amplification of the same species from the same accession number (but at different-timed DNA extractions and PCR) even resulted in different amplicon lenghts too. The explanation for this is probably related to the chance of the primers to bind to

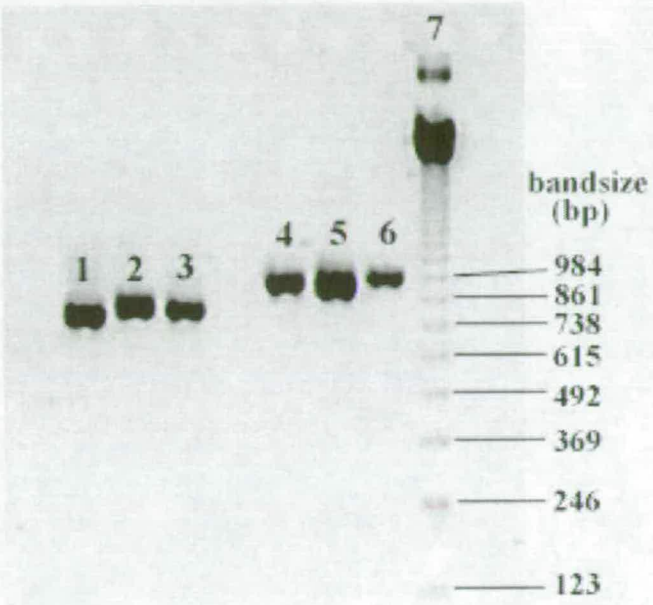


Figure 2.10 Gel electrophoresis result from PCR of ITS and region between *trn*L (UAA) 5' exon and *trn*F (GAA).
Lane 1: *C. amada*, lane 2: *C. heyneana*, lane 3: *C. zanthorrhiza*, lane 4: *C. soloensis*, lane 5: *C. zanthorrhiza*, lane 6: *C. phaeocaulis*, and lane 7: DNA size marker (123bp ladder). Lanes 1-3 are from PCR (of ITS region) subjected to primers "5P" and "8P". Lanes 4-6 are from PCR (of *trn*L-F region) subjected to primers "c" and "f".

region ~984 bp amplicon or ~400 bp amplicon. The amplicon of ~984 bp are more stable and are expected to be the right region. It is reasonable to use this amplicon as the template for the next step, the sequencing step.

To obtain one single band for those species or accessions with double bands, PCR was carried out in two separate reactions, first with primers "c" and "d", and second with primers "e" and "f". The combination of primers "c" and "d" resulted in region of *trn*L (UAA) 5' exon to *trn*L (UAA) 3' exon. The combination of primers "e" and "f" resulted in region of *trn*L (UAA) 3' exon and *trn*F (GAA). These methods of

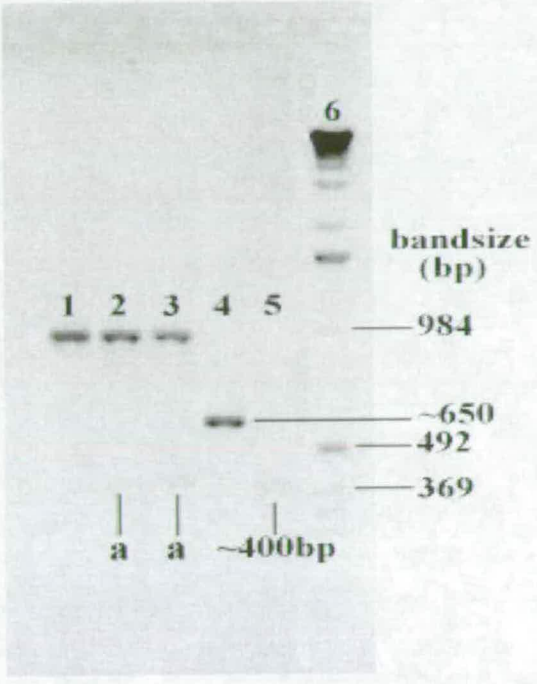


Figure 2.11 Gel electrophoresis result from PCR of region between *trn*L (UAA) 5' exon and *trn*F (GAA). Lane 1: *C. ochrorhiza*, lane 2: *C. roscoeana*, lanes 3-5: *C. longa*, lane 6: DNA size marker (123bp ladder). Lane 1 are from PCR subjected to primers "c" and "f" which produce one single band of ~984bp. Lanes 2-3 are from PCR subjected the same primers ("c" and "f"), but produce additional band (a) of ~400bp. Lane 4 is from PCR subjected to primers "c" and "d" resulted in one single band of ~650bp. Lane 5 is from PCR subjected to primers "e" and "f" resulted in one single band of ~400bp.

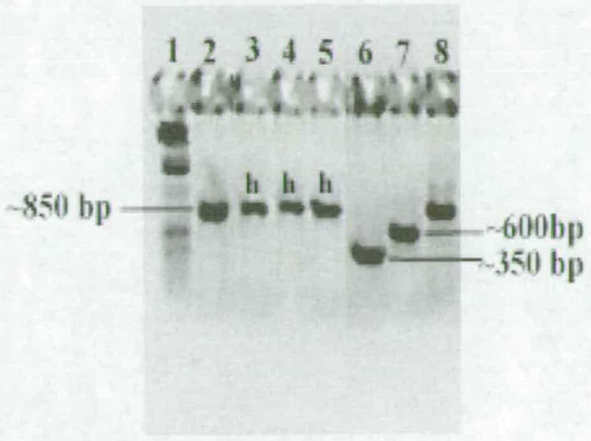


Figure 2.12 Gel electrophoresis result from PCR of ITS region.
Lane 1: DNA size marker (123bp ladder), lane2: *C. thorelii*, lane 3: *C.cf.australasica*, lane 4: *C. elata*, lane 5: *C. petiolata*, lanes 6-7: *C. roscoeana*, lane 8: *C. heyneana*. Lanes 1-4 and 8 are from PCR subjected to primers "5P" and "8P". Lane 6 is from PCR subjected to primers "5P" and "2K" or individual ITS1 (~350bp). Lane 7 is from PCR subjected to primers "3P" and "8P" or individual ITS2 (~600bp). Lanes 3-4 were obtained from herbarium material (h).

separating PCR in two different reactions using different sets of primers were successful in giving a single band of ~650 bp and ~ 400 bp respectively.

Internal primers of ITS, i.e. primers "2K" and "3P" were also tried in PCR before further sequencing PCR was carried out. A set of primers "5P" and "2K" in PCR resulted in one band of approximately 350 bp, while another set of primers "3P" and "8P" resulted in one band of approximately 600 bp (**Figure 2.12**).
Purification of PCR products using QIAquick™ PCR Purification Kit gave a clean result throughout the work.

2.6.3 Cycle sequencing and automated DNA sequencing

Automated DNA sequencing resulted in non polymorphic and polymorphic sequence (**Figure 2.13**) below. Polymorphic sequences are referred to in Chapter 7 p.180.

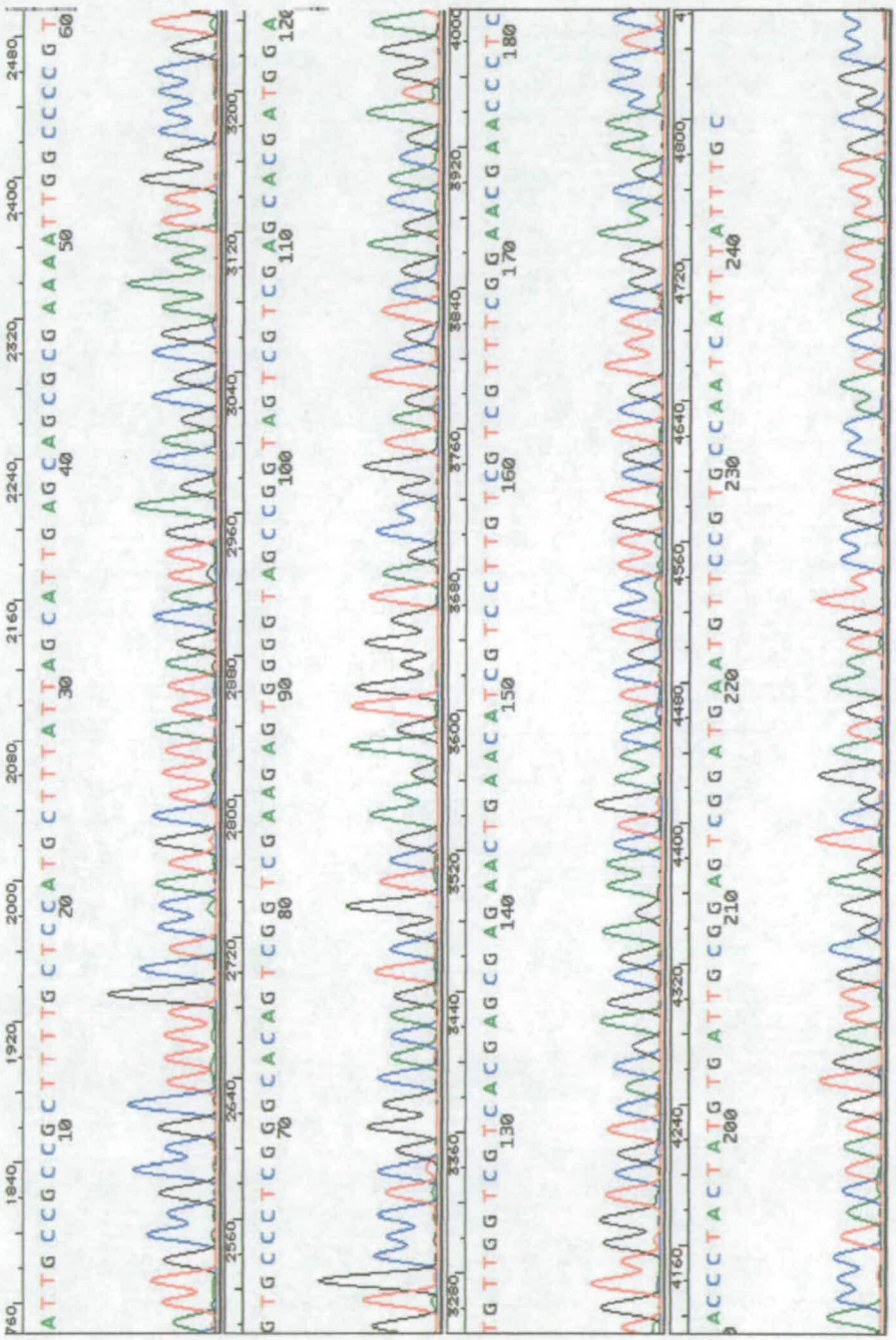


Figure 2.13 The electropherogram an automated DNA sequencing of ITS2 region of *C. aurantiaca* showing clean non-polymorphic sequence.

Base 182-190 is not shown. The height of each of the four coloured lines indicates the relative intensity of fluorescence that corresponds to each of the four labeled dideoxynucleotides. Therefore, the peaks may be read directly as DNA sequences (bases indicated above the electropherogram).

2.6.4 Sequence analysis

The ITS regions from the two accessions of *C. thorelii, C. alismatifolia,* and *C. aurantiaca* are identical, so only one is shown and used for the analysis. The alignment of ITS (**Appendix 2**) resulted in in a sequence 471 bp long, composed of 214 bp of ITS1 and 257 bp of ITS2. The mean lengths of ITS1 and ITS2 of *Curcuma* were 193.5 and 235.5 respectively. These are slightly longer than those in *Alpinia* (Rangsiruji *et al.* 2000) and *Roscoea* (Ngamriabsakul *et al.* 2000) which were 187.7 and 228.0; and 188.93 and 224.53, respectively. The sequence characteristics are summarized in **Table 2.4**. The alignment of *trn*L-F (**Appendix 3**) resulted in a sequence 911 bp long. The sequence characteristics are summarized in **Table 2.5**.

The alignment of all taxa required insertion of 35 gaps of size 1 to 4 bases, 19 gaps in ITS1 and 16 in ITS2. Eighteen of these are plesiomorphies. Two gaps of one bp size are synapomorphies uniting *C. cf. australasica* and *C. aurantiaca,* while one gap synapomorphy of two bp size unite the Thai species (*C. thorelii, C. alismatifolia, C. gracillima, C. parviflora,* and *C. harmandii).* Two clades in subgenus *Curcuma* are affected by a one bp sized gap synapomorphy at 472 bp grouping *C. soloensis, C. aromatica, C. elata, C. longa, C. phaeocaulis,* while another one bp sized gap synapomorphy at 486 bp groups the remaining species in the subgenus.

The alignment of all taxa required insertion of 12 gaps of size 1 to 15 bases into the *trn*L-F sequence. Four of these are plesiomorphies, while five are autapomorphies for *Ca. spicata* (two gaps of six and one bp size), *R. humeana* (one gap of five bp), *C. gracillima* (one gap of one bp), and *C. harmandii* (one gap of 15 bp). One gap of one bp (921 bp) is a synapomorphy of *Sm. supraneae* and *C. roscoeana.*

Pairwise comparison between the ingroups showed sequence divergence of 0-10.55%, 0-17.33%, and 0-1.02 for ITS1, ITS2, and *trn*L-F respectively. The ITS1 was less variable compared to 0-16.1% in *Alpinia* (Rangsiruji *et al.* 2000) and 0-13.86% in *Roscoea* (Ngamriabsakul *et al.* 2000), but the ITS2 was more variable than 0-14.6% in *Alpinia* and 0-7.58% in *Roscoea.* The G+C content of the ITS of the species studied had

almost the same average as that of the *Alpinia* and *Roscoea* studies, i.e. 51.94-56.65%, 53.3-57.5%, and 51.55-57.35% respectively. *trn*L-F contains less G+C, 32.71-33.33%.

2.6.5 Phylogenetic analysis

Phylogenetic analysis of aligned ITS sequences with equally weighted characters including uninformative characters resulted in 613 equally most parsimonious trees (CI=0.7143, RI=0.8238, RC=0.5884) with a length of 280 steps (**Figure 2.14**). The exclusion of uninformative characters reduced the tree length to 213 steps. Analysis with coded gaps added to the data matrix with inclusion of uninformative characters resulted in 48 most parsimonious trees (CI=0.6801, RI=0.8147, RC=0.5541) with a length of 322 steps, longer than that of excluding coded gaps (**Figure 2.15**). Re-weighting characters to the transition/ transversion ratio resulted in the same trees with much longer steps both for coded data indels or data exluding coded indels. However, re-weighting characters to CI, RI or RC index resulted in similar but shorter and slightly more resolved trees in the *C. parviflora, C. alismatifolia, C. gracillima* group, and the *C. soloensis, C. longa, C. phaeocaulis, C. aromatica, C. elata* group.

Phylogenetic analysis of aligned *trn*L-F sequences with equally weighted characters including uninformative characters resulted in one most parsimonious tree (CI=1.000, RI=1.000, RC=1.000) with a length of 29 steps (**Figure 2.16**). The exclusion of uninformative characters reduced the tree length to 5 steps. Analysis with coded gaps added to the data matrix with inclusion of uninformative characters resulted in 55 most parsimonious trees (CI=0.7531, RI=0.7692, RC=0.5793) with a length of 53 steps, longer than that excluding coded gaps (**Figure 2.17**). Re-weighting characters to CI, RI or RC index resulting in shorter length of similar topology.

The trees resulting from ITS data are more resolved than those resulting from *trn*L-F data. Yet, this *trn*L-F can be used as a support for ITS data. For example, the consensus tree from coded gap of *trn*L-F data is almost congruent with that of ITS. The subgenus *Curcuma* clade is separate from the *Hitcheniopsis* clade.

The combined data sets resulted in trees similar to those of ITS data alone (**Figure 2.18-2.19**). Subgenus *Curcuma* forms a clade which is monophyletic supported

by BS=82 and DI=4. Two subclades (supported by BS=71, 82 and DI=1, 2 respectively) are detected within this clade. No morphological character corresponding to each subclade has been found. Subgenus *Hitcheniopsis* is paraphyletic, with *Sm. supraneae* nested with *C. ecomata* or at least separated from the *C. ecomata* clade but nested within the *Curcuma* clade. Several clades from subgenus *Hitcheniopsis* are well supported such as *C. petiolata* (BS=89, DI=3), *C. roscoeana* (BS=89, DI=3), the *C. thorelii, C. gracillima, C. alismatifolia, C. harmandii* group (BS=100, DI=14), and the *C. cf. australasica, C. aurantiaca* group (BS=100, DI=10). Further study with added taxa is needed to get a meaningful evolutionary history of these clades.

Table 2.4 Sequence characteristics of ITS1 and ITS2 regions of 28 taxa of Zingiberaceae.

Parameter	ITS1	ITS2	ITS1 and ITS2
Length range (total) (bp)	184-203	224-246	409-447
Length mean (total) (bp)	193.5	235	428
Length range (ingroup) (bp)	184-203	225-246	409-447
Length mean (ingroup) (bp)	193.5	235.5	428
Length range (outgroup) (bp)	188-190	224-225	412-415
Length mean (outgroup) (bp)	189	224.5	413.5
Aligned length (bp)	214	257	471
G+C content range (%)	48.44-55.28	52.19-60.69	51.07-57.60
G+C content mean (%)	51.94	56.65	54.42
Sequence divergence (ingroup) (%)	0-10.55	0-17.33	0-13.13
Sequence divergence (total) (%)	3.78-14.48	12.05-23.72	5.64-18.07
Number of indels (ingroup)	15	14	29
Number of indels (total)	19	16	35
Size of indels (ingroup) (bp)	1-4	1-4	1-4
Size of indel (total) (bp)	1-4	1-4	1-4
Number of sites	214	257	471
Number of variable sites (%)	69(32.24)	104(40.47)	173(36.73)
Number of constant sites (%)	145(67.76)	153(59.53)	298(63.27)
Number of informative sites (%)	41(19.16)	67(26.07)	108(22.93)
Number of autapomorphic sites (%)	28(13.08)	37(14.40)	65(13.90)
Transitions (minimum)	19	79	113
Transversions (minimum)	13	33	51
Transitions/transversions (ts/tv) ratio	1.46	2.39	2.22
Skewness of tree length distribution (g_1 value for 10,000 random trees)	-0.63	-0.86	-0.80

Table 2.5 Sequence characteristics of chloroplast regions between *trn*L (UAA) 5' exon and *trn*F (GAA) of 25 taxa of Zingiberaceae.

Parameter	The spacer between *trn*L (UAA) 5' exon and *trn*F (GAA)
Length range (total) (bp)	881-908
Length mean (total) (bp)	899.20
Length range (ingroup) (bp)	886-903
Length mean (ingroup) (bp)	899.59
Length range (outgroup) (bp)	881-908
Length mean (outgroup) (bp)	894.50
Aligned length (bp)	911
G+C content range (%)	32.71-33.33
G+C content mean (%)	33.10
Sequence divergence (ingroup) (%)	0-1.02
Sequence divergence (total) (%)	0.1-1.78
Number of indels (ingroup)	10
Number of indels (total)	12
Size of indels (ingroup) (bp)	1-15
Size of indel (total) (bp)	1-15
Number of sites	911
Number of variable sites (%)	61(6.70)
Number of constant sites (%)	850(93.3)
Number of informative sites (%)	14(1.54)
Number of autapomorphic sites (%)	47(5.16)
Transitions (minimum)	2
Transversions (minimum)	2
Transitions/transversions (ts/tv) ratio	1
Skewness of tree length distribution (g_1 value for 10,000 random trees)	-3.26

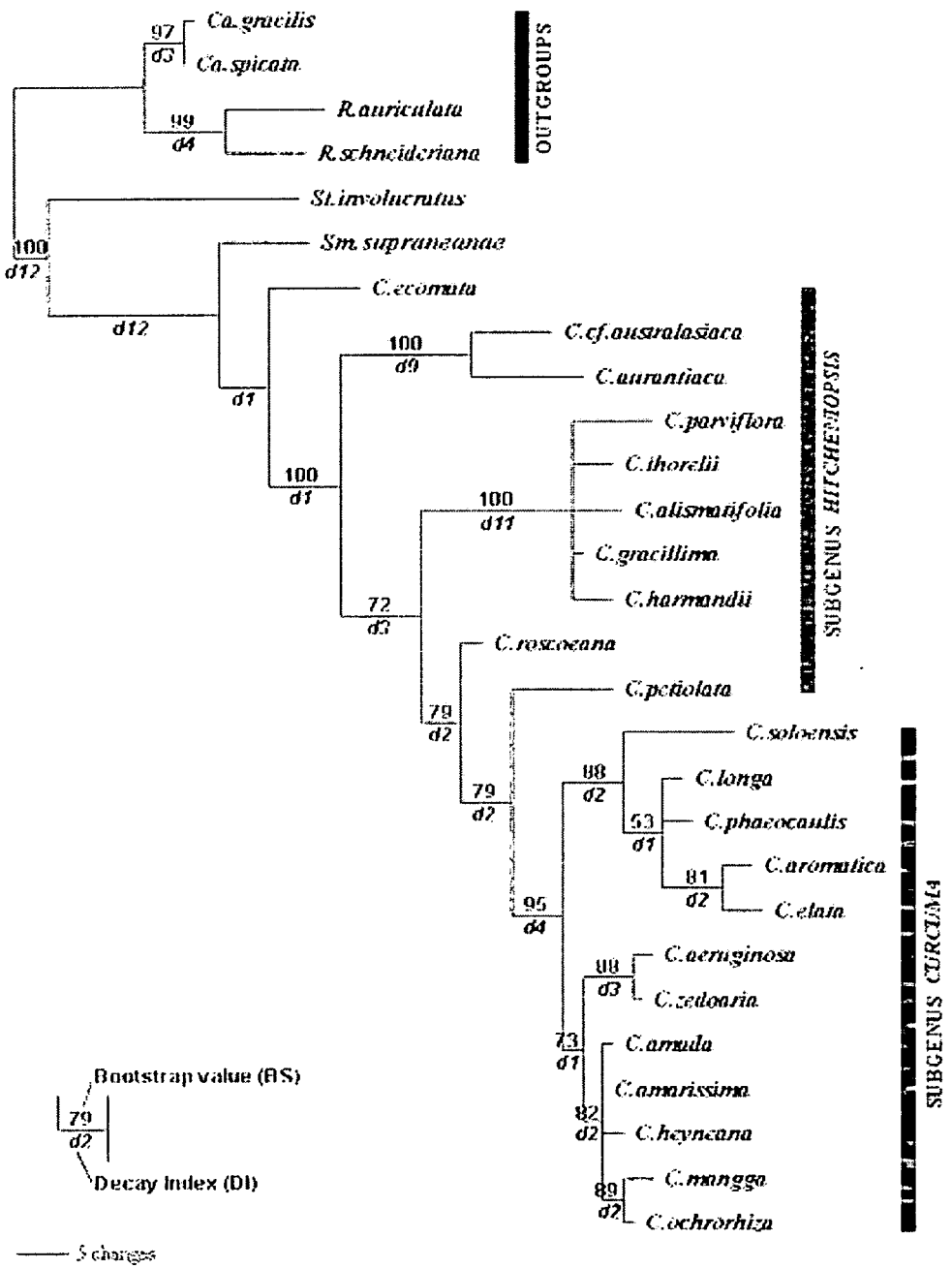


Figure 2.14 Strict consensus tree obtained from 613 equally most parsimonious trees of length 280 steps resulting from equally weighted parsimony analysis of the combined ITS1 and ITS2 data with gaps treated as missing data (CI=0.7143; RI=0.8238; RC=0.5884).

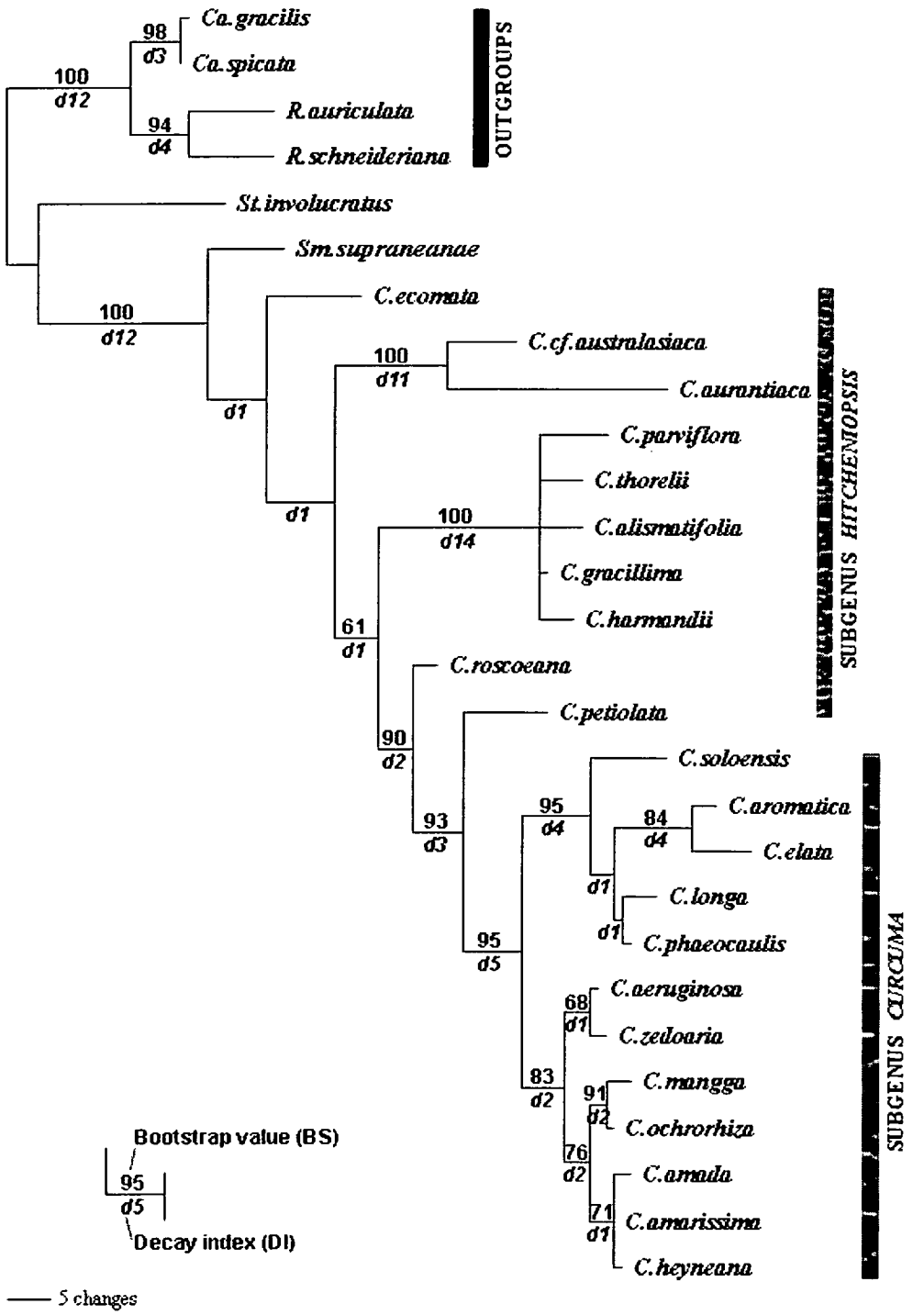


Figure 2.15 Strict consensus tree obtained from 48 equally most parsimonious trees of length 322 steps resulting from equally weighted parsimony analysis of the combined ITS1 and ITS2 data with indels treated as coded present absent (CI=0.6801; RI=0.8147; RC=0.5541).

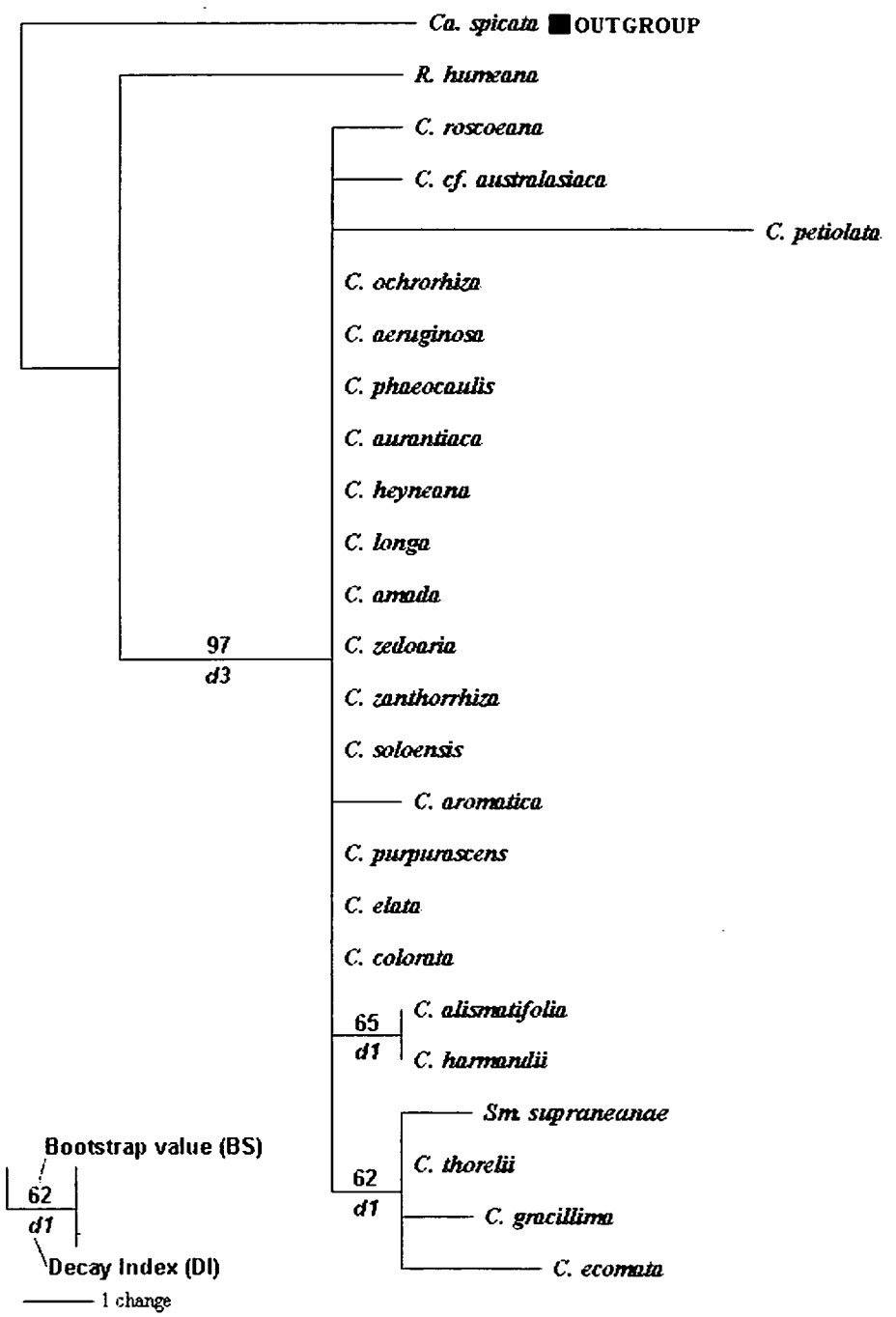


Figure 2.16 One most parsimonius tree of length 29 steps resulting from equally weighted parsimony analysis of the chloroplast DNA between *trn*L (UAA) 3' exon and *trn*F (GAA) data with gaps treated as missing data (CI=1.000; RI=1.000; RC=1.000).

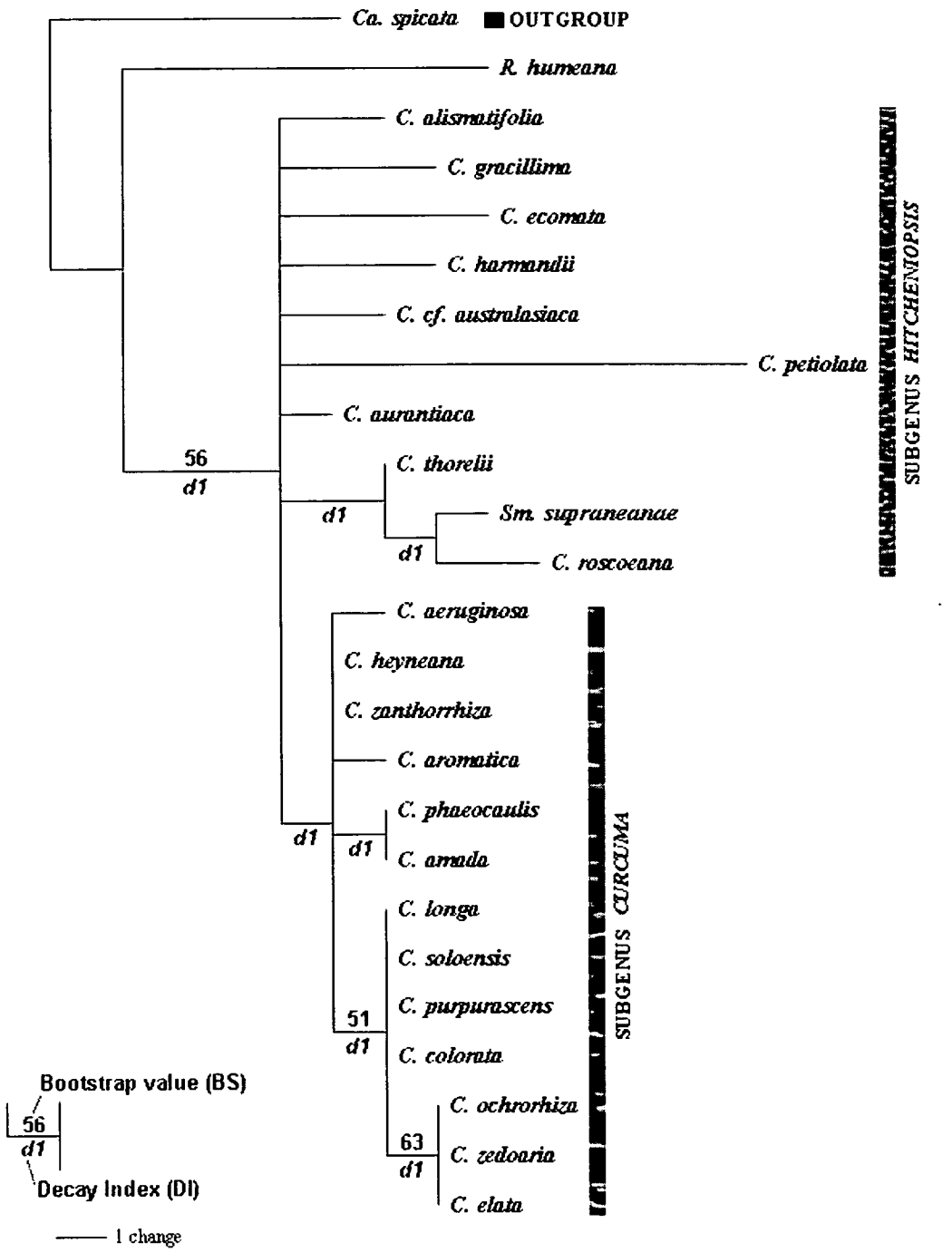


Figure 2.17 Strict consensus tree obtained from 55 equally most parsimonious trees of length 53 steps resulting from equally weighted parsimony analysis of the chloroplast DNA between *trn*L (UAA) 3' exon and *trn*F (GAA) data with indels treated as coded present absent (CI=0.7531; RI=0.7692; RC=0.5793).

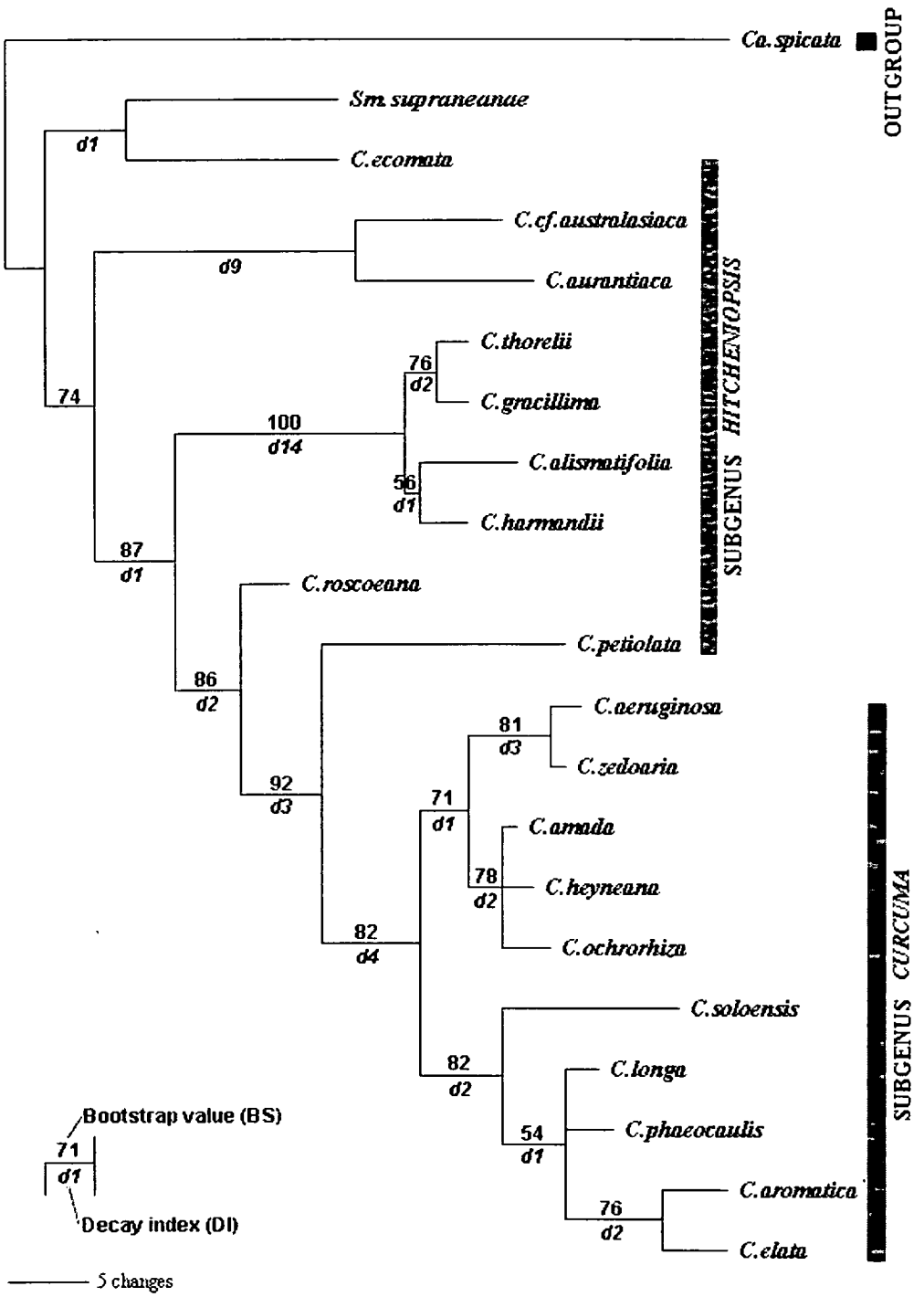


Figure 2.18 Strict consensus tree obtained from 9 equally most parsimonious trees of length 243 steps resulting from equally weighted parsimony analysis of the combined ITS and chloroplast DNA between *trn*L (UAA) 3' exon and *trn*F (GAA) data with gaps treated as missing data (CI=0.6775; RI=0.7265; RC=0.4922).

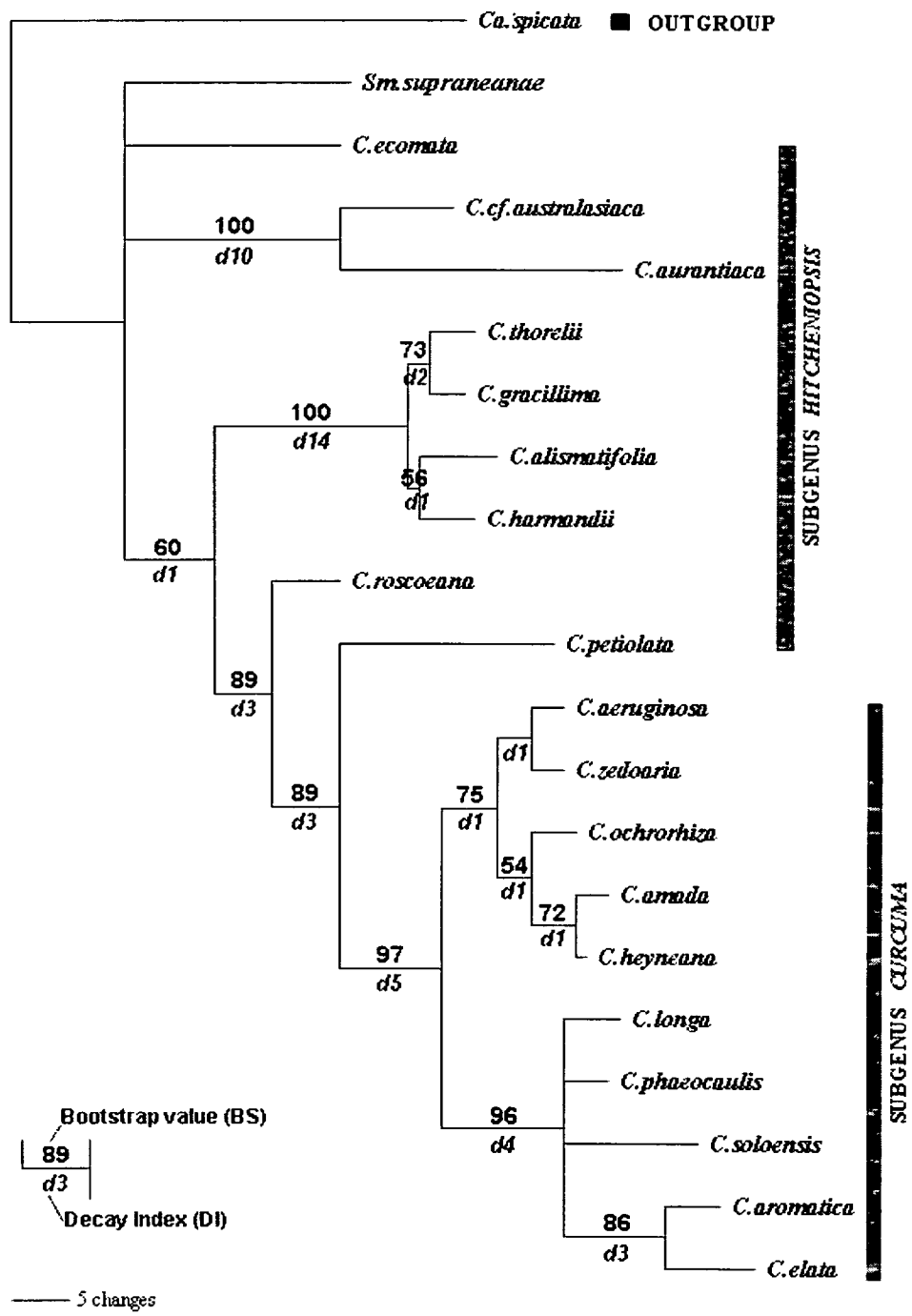


Figure 2.19 Strict consensus tree obtained from 8 equally most parsimonious trees of length 307 steps resulting from equally weighted parsimony analysis of the combined ITS and chloroplast DNA between *trn*L (UAA) 3' exon and *trn*F (GAA) data with indels treated as coded present absent (CI=0.6775; RI=0.7265; RC=0.4922).

2.7 DISCUSSION

2.7.1 PHYLOGENETIC RELATIONSHIPS WITHIN CURCUMA AND BETWEEN CURCUMA AND ITS OUTGROUP

2.7.1.1 *Basal clade* – Stahlianthus involucratus, Smithatris supraneanae, *and* C. ecomata

Three taxa, namely *Stahlianthus involucratus, Smithatris supraneanae,* and *C. ecomata*, are nested at the base next to the outgroups. All these species come from Thailand. The well-supported clade, which includes *Smithatris* and all *Curcuma* species, shows that *Curcuma* is not a monophyletic group. *Smithatris* and *Curcuma* share a common character of the multiple bracts or pouches. However, the bracts are free, not connate in *Smithatris,* while they are adnate at the sides in *Curcuma. Stahlianthus* is closely related to *Smithatris* and *Curcuma*. This genus, however, has a single involucre bract which is considered to be derived from two bracts joined together (Wood *et al* 2000).

Analysis of the ITS region shows that *C. ecomata* is nested at the base in relation to other species from subgenus *Hitcheniopsis*. It is nested far from the subgenus *Curcuma* clade. Gagnepain (1908), however, placed *C. ecomata* in subgenus *Curcuma,* probably because it has a spurred anther. Analysis of the *trn*L-F region also shows that *C. ecomata* is nested out of the subgenus *Curcuma* clade in the analysis with coded gaps. Gagnepain's grouping, therefore, is not supported by either molecular analysis of the ITS or the *trn*L-F regions. *C. ecomata* shares many other morphological characters with subgenus *Hitcheniopsis* despite the difference of the anther spurs. They share common characters, such as the hardly developed rhizome, the free (not clasping) lateral staminodes, and the crested anther. These are synapomorphic characters. This would made *Hitcheniopsis* paraphyletic. In the molecular tree (p.71), the node of *Smithatris* and *Curcuma* is not resolved. A wider outgroup sampling would have enabled a more accurate base to the limits of *Curcuma.*

2.7.1.2 *The* C. aurantiaca *clade*

Analysis of the ITS region showed strong support for the *C. aurantiaca* and *C. cf. australasica* clade. The clade is characterized by the hardly developed rhizome, the leaves which are rounded at the base, the lateral staminodes which are not clasping, and the ligules which are auriculate. *C. aurantiaca* and *C. cf. australasica* are also the only Malesian species studied which set seeds. One character which does not unite them is the spur on anther. *C. aurantiaca* has no spur, while *C. cf. australasica* has a spur on its anther.

The existence of the *C. aurantiaca* and *C. cf. australasica* clade indicates that Valeton's (1918) subgenus *Paracurcuma* may be a natural group. However, *C. petiolata,* which was placed in subgenus *Paracurcuma* by Valeton, is not nested within the *C. aurantiaca* and *C. cf. australasica* clade. Schumann (1904) placed *C. australasica* within subgenus *Curcuma* on the basis of the rhizomes which were big with many sessile tuber. More samples of *C. australasica* are needed for confirmation of the rhizome character.

C. aurantiaca is distributed from Continental Asia to the Malesian archipelago, while *C. australasica* occurs in New Guinea and northern Australia. The clade, which is strongly supported by the ITS data, suggests a close relationship between the two species. *C. australasica* may have escaped from the continental region of Asia to New Guinea and northern Australia. The inclusion of other species of New Guinea, such as *C. meraukensis* and *C. latiflora*, would give more insight into the relationships among the New Guinea species.

2.7.1.3 *The* C. parviflora *clade*

This clade, strongly supported by molecular analysis of the ITS region, contains five species namely *C. parviflora, C. thorelii, C. alismatifolia, C. gracillima,* and *C. harmandii.* The clade reflects some degree of correlation between *Curcuma* phylogeny and biogeography. All the samples come from Thailand though the species are

distributed more widely from Indo-China to Thailand (continental Asia). One species, *C. parviflora,* reaches the Malay Peninsula.

C. parviflora was placed in section *Hitcheniopsis* by Baker (1894) in which was raised to subgenus *Hitcheniopsis* by Schumann (1904). Schumann also included *C. gracillima* and *C. alismatifolia* in subgenus *Hitcheniopsis.* However, Valeton (1918) excluded those species from the genus *Curcuma* altogether. The reasons he excluded the species, as already mentioned in Chapter One, are that the structures of coma bracts, petals, lateral staminodes, labellum, and anther, are very different from those of *Curcuma.* The exclusion of these species from the genus would make *Curcuma* a polyphyletic group. The *C. parviflora* clade therefore should be maintained in the genus *Curcuma* in order to keep it monophyletic.

The species in the *C. parviflora* clade share common characters, such as rhizomes which are hardly developed and are not ginger scented though this is subjective; the ligules are auriculate; the labellum which is purple or white and elongated; the petals which are whitish (greenish in *C. harmandii*); the lateral staminodes which are whitish, free (not clasping), and are without groove; the spurless but crested anther; and the stylar growth which is absent.

2.7.1.4 *The subgenus* Curcuma *clade*

This clade, strongly supported by molecular analysis of the ITS and the *trn*L-F regions, reveals the natural grouping of Baker's (1894) sections *Exantha/Mesantha,* Schumann's (1904) and Valeton's (1918) subgenus *Curcuma.* It is characterized by the well-developed rhizomes, the narrow leaves at the base, the spurred anther, the folded lateral staminodes, and the ligules which are not auriculate.

All of the taxa within this clade were treated at specific level by previous authors. They show polymorphic ITS sequences. All of them are triploid and sterile. Seeds have not been reported except in *C. mangga* from Java which were reported by Valeton (1918). However, most of the herbarium specimens of *C. mangga* have no record on the presence of seeds. The morphological characteristics of the taxa in this clade are very highly similar so that people cannot distinguish one from another only by

looking at the herbarium specimens. Therefore, they should not be assigned at specific level. They should be treated at a lower rank in the classification.

Two subclades, well supported by molecular analysis of the ITS, are found within the subgenus *Curcuma* clade. The first subclade contains *C. soloensis, C. aromatica, C. elata, C. longa* and *C. phaeocaulis.* The second clade contains *C. aeruginosa, C. zedoaria, C. mangga, C. ochrorhiza, C. amada, C. amarissima,* and *C. heyneana.* These subclades, however, do not exist in the *trn*L-F trees due to unresolved branches in many 'species'. At present there are no morphological data which support the grouping into the two subclades. The reason for this may relate to the polymorphism of the ITS. The polymorphic ITS sequences might have been the result of hybridization of different sequences from different 'species'. Further study to clone the polymorphic ITS would probably be able to verify this. However, no further study was made because of time shortage.

The tree from molecular analysis of *trn*L-F does not fully support the subclades within the subgenus *Curcuma* clade from molecular analysis of the ITS. *C. ochrorhiza, C. zedoaria,* and *C. elata* which are in one subclade of the tree resulting from molecular analysis of the *trn*L-F are nested in different subclades of the tree built from molecular analysis of the ITS. *C. phaeocaulis* and *C. amada* which are in one subclade with weak support from the *trn*L-F, are also nested at different subclades of the tree resulting from the ITS sequence data. Lack of conference probably results from different source of DNA (nuclear versus chloroplast DNA). Analysis with more DNA regions may help to verify this. The tree resulting from combined ITS and *trn*L-F data resembles that resulting from the ITS. Use of polymorphisms of ITS is discussed in Chapter 7.

The existing sectional level classification of *Curcuma* is not supported either by molecular analysis of the ITS or the *trn*L-F regions. Section *Exantha,* those 'species' with a lateral inflorescence, come out mixed up with section *Mesantha,* those 'species' with a central inflorescence. The sectional level classification, therefore, should be abandoned as it is only based on the position of inflorescence character which is homoplasious.

2.7.1.5 C. roscoeana *and* C. petiolata

C. roscoeana is nested between the *C. petiolata* and the *C. parviflora* clades, a placement which is well supported by molecular analysis of the ITS. *C. petiolata* is nested between *C. roscoeana* and the subgenus *Curcuma* clade, which is also well supported by molecular analysis of the ITS. The *C. roscoeana* clade supports the placement of the species in Baker's (1894) section *Hitcheniopsis* and Schumann's (1904) subgenus *Hitcheniopsis*. Valeton (1918) excluded *C. roscoeana* from the genus *Curcuma* because several characters, according to him, did not fit the delimitation of the genus. Those characters are mentioned in Chapter One (p.22). The *C. petiolata* clade supports the placement of the species in Baker's (1894) section *Hitcheniopsis*, Schumann's (1904) and Valeton's (1918) subgenus *Hitcheniopsis*. *C. petiolata* has intermediate characters between the two subgenera (*Hitcheniopsis* and *Curcuma*), such as the spur on the anther which is very short (no spur or very short spur in subgenus *Hitcheniopsis* vs. spurred anther in subgenus *Curcuma*), and the rhizomes which are short (hardly developed rhizomes in subgenus *Hitcheniopsis* vs. well developed rhizome in subgenus *Curcuma*). This is supported by the position of *C. petiolata* in the tree which is nested at the border of subgenus *Hitcheniopsis* next to subgenus *Curcuma*.

C. roscoeana is distributed in Burma and Thailand, while *C. petiolata* is distributed in Burma, Thailand, the Malay Peninsula, and Java. In Java, there are only two species from subgenus *Hitcheniopsis*, namely *C. petiolata* and *C. aurantiaca*. *C. petiolata* may have been introduced to Java with *C. aurantiaca* from the continental Asia. The teak forest, which is their habitat in Java, is also introduced.

2.7.2 MOLECULAR EVOLUTION OF THE ITS AND THE TRNL-F REGIONS

In *Curcuma*, the length of the ITS1 (184-203 bp) is shorter than that of the ITS2 (225-246 bp). The same phenomenon occurs in two other genera of the Zingiberaceae, *Alpinia* (ITS1: 187-188 bp, ITS2: 226-235 bp; Rangsiruji *et al.* 2000) and *Roscoea* (ITS1: 188-190 bp, ITS2: 224-225 bp Ngamriabsakul *et al.* 2000). The length variation

is due to the occurrence of several indel events. In *Saintpaulia* and *Streptocarpus* (Gesneriaceae), the length of the ITS1 is slightly longer than that of the ITS2 (ITS1: 235-249 bp, ITS2: 196-245 bp; Möller & Cronk 1997). This contradicts the findings from the three genera in the Zingiberaceae mentioned above. However, unlike *Saintpaulia* and *Streptocarpus, Aeschynanthus,* another genus in the Gesneriaceae, has a shorter ITS1 than ITS2 (ITS1: 217-229 bp, mean 225.0, ITS2: 206-246 bp, mean 239.5; Denduangboripant & Cronk 2000).

The GC contents of the ITS1 and the ITS2 are more or less uniform (approximately 50-60%) in *Curcuma, Alpinia, Roscoea,* and in the Gesneriaceae (*Saintpaulia, Streptocarpus,* and *Aeschynanthus*). This may reflect some degree of coevolution between the two spacers. The sequence divergence between the two spacers is almost uniform too (ITS1: 0-10.55%, ITS2: 0.17.33% in *Curcuma;* ITS1: 0-16.1%, ITS2: 0-14.6% in *Alpinia;* ITS1: 0-13.86, ITS2: 0-.7.58% in *Roscoea;* ITS1: 0.45-15.26%, ITS2: 0.41-15.57 in *Aeschynanthus*). This indicates the same rate of evolution between the two spacers.

The length of the chloroplast spacer between the *trn*L (UAA) 5' exon and *trn*F (GAA) (mean 899.59 bp) is almost twice the length of the ITS region (mean 428 bp). The length variation is also due to the occurrence of several indel events. The GC contents of the *trn*L-F region are more or less uniform (approximately 30%) in *Curcuma, Alpinia,* and *Roscoea.* These are much lower than those of the ITS region (approximately 50-60%). The sequence divergence of the *trn*L-F region in *Curcuma* (0-1.02%) is much lower than that of the ITS region (ITS1: 0-10.55%, ITS2: 0-17.33%). The same phenomena occur in *Alpinia* and *Roscoea.* The much lower percentage of the sequence divergence in the *trn*L-F region indicates the much slower rate of evolution of the spacer. The tree constructed from the *trn*L-F sequence data is much less resolved than that constructed from the ITS sequence data. Thus, the chloroplast spacer of *trn*L-F provides very limited phylogenetic information for the present study of *Curcuma.* Nevertheless, this chloroplast spacer contributes a useful confirmation of the results based on the ITS region.

CHAPTER 3: PHYLOGENETIC STUDY USING MORPHOLOGICAL DATA

3.1 INTRODUCTION

Morphology has been used as the basis for the classification of *Curcuma* since Roxburgh (1812) and the later authors, Horaninow (1862), Baker (1894), Schumann (1904), Valeton (1918). Inflorescence position, a character that is used for separating sections by Roxburgh (1812), Horaninow (1862), Baker (1894), Schumann (1904), and Valeton (1918), has proved to be unreliable or unstable and has been criticised by some authors (see Chapter One). Some other characters that are used at the sectional level by Baker (1894) or at the subgeneric level by Schumann (1904) and Valeton (1918) are still in question. This study aims to test whether the existing classification of *Curcuma,* at the subgeneric level, reflects its evolutionary history.

Morphological study has been carried out along side a molecular study. Trees were built from morphological data and were compared with those from molecular sequence data. Morphological characters were also mapped onto molecular phylogenetic trees to elucidate the possible evolutionary history of *Curcuma.* The present classification of *Curcuma* will therefore be tested to determine whether it is natural. The limited sampling (only around 30% of *Curcuma* species were examined) makes the result of this study merely a stepping stone for a future broader study.

3.1.1 Characters used in the existing infrageneric classification of Curcuma

3.1.1.1 Rhizomes

Valeton (1918) used rhizome characters for separating subgenus *Paracurcuma,* which has a short or absent rhizome, from subgenus *Curcuma.* Subgenus *Curcuma* has a long rhizome which forms lateral branches.

3.1.1.2 Leaves

One of several types of character that Valeton (1918) used for separating subgenus *Paracurcuma* from subgenus *Curcuma* was leaf characters, i.e. base of leaves mostly rounded, stem short, and leaves spreading. However, other species within subgenus *Hitcheniopsis* (subgenus *Paracurcuma sensu* Valeton), such as *C. alismatifolia,* have an acuminate base and narrow lanceolate leaves. This species, however, was excluded from the genus *Curcuma* by Valeton (1918).

3.1.1.3 Ligules

The ligule forms an ovate auricle, on both sides of the base of the petiole, in subgenus *Paracurcuma* while it is without elongated auricle in subgenus *Curcuma* (Valeton 1918). This character is easily observed from fresh material. Where observation is not possible, data were extracted from the literature.

3.1.1.4 Fertile bracts

Baker (1894) was the first to note bract characters in separating his sectional level groups (*Exantha, Mesantha* and *Hitcheniopsis*). Section *Hitcheniopsis* has very obtuse bracts, which are adnate at the sides and spreading at the tip. The bracts of the rest of the sections are not recurved at the tip.

Schumann (1904) also noted bract characters in his subgeneric level groups (*Eucurcuma* and *Hitcheniopsis*). His subgenus *Eucurcuma* has bract, which is adnate only near the base while the greater part is free. *Hitcheniopsis* has bract, which is adnate for a large portion of their length, the free tips are recurved.

Valeton (1918) criticized Schumann's note on these bracts. He said that the adnation of bracts in Schumann's classification is not reliable. The bracts of *C. petiolata* (subgenus *Hitcheniopsis*) are in fact adnate to the middle and not to the top. Nevertheless, Valeton did mention the adnation of bracts for his subgenera *Eucurcuma* and *Hitcheniopsis.* He described subgenus *Eucurcuma* having bracts which are

connected at least beyond the middle, and *Hitcheniopsis* having bracts which are mostly not adnate over the middle.

3.1.1.5 Coma bracts

Valeton (1918) recognized subgenus *Eucurcuma* as having coma bracts that are mostly extended far beyond the floral bracts. Subgenus *Paracurcuma* has coma bracts that are relatively short, described as without coma bracts by most authors.

3.1.1.6 Staminode

Two characters of the staminode are useful at the subgeneric level. Subgenus *Eucurcuma* has longitudinally grooves and folded or clasped staminodes under the cucullate pointed dorsal lobe, while subgenus *Paracurcuma* has straight staminodes and the dorsal petal does not clasp the staminodes (Valeton 1918).

3.1.1.7 Anther

Schumann (1904) and Valeton (1918) used anther characters in their classifications. Schumann found spurless anthers in subgenus *Hitcheniopsis,* and calcarate anther in subgenus *Eucurcuma.* However, Schumann included *C. petiolata,* which in fact has a very short spur on its anther, in his subgenus *Hitcheniopsis.* This received criticism from Valeton.

Valeton made meticulous observations of anther characters to be used in his subgeneric classification. Those are mentioned here: spur attached near the base (*Paracurcuma*) versus spur attached at the back about the middle (*Eucurcuma*); spur not or very shortly calcarate (*Paracurcuma*) versus spur calcarate (*Eucurcuma*); anther grooved on the face as a continuation of the loculi (*Paracurcuma*) versus anther not grooved on the face (*Eucurcuma*); appendix of the connective forming a short cup which encloses the stigma entirely or its base (*Paracurcuma*) versus connective rounded or narrowed towards the top, not lengthened to a cup, sometimes slightly produced between

the loculi (*Eucurcuma*). This over-reliance on one character (anther character) is dangerous to be used in classification.

3.1.2 Are there any other characters defining infrageneric classification in Curcuma?: a search for them

3.1.2.1 Leaf anatomy

Epidermal characters using transverse sections and epidermal peels were used to recognize species in *Zingiber* (Husin & Widjaja 1987). However, the degree of the difference is quite subtle. In *Aframomum,* different sets of leaf anatomical characters determine different groups of species (David Harris, *pers. comm.*). Therefore, work on leaf anatomy was carried out in this study.

3.1.2.2 Stigma

The stigma is unique to sectional or subgeneric level in *Alpinia* (Rangsiruji 1999). It was Smith (1990) who first examined the stigmas in *Alpinia* and found different types of stigmas at different levels of classification. Valeton did not observe stigma type in *Curcuma* (1918).

3.1.2.3 Flower

In *Streptocarpus* several types of flower shape and size are found. This is connected to different types of pollinators (Harrison *et al.* 1999). Different shapes and sizes of flower in *Curcuma* are also observed. The morphological, anatomical and physiological characters of flowers do not vary randomly. However, certain characteristics tend to occur together. This is known as pollination syndromes. Red flowers are often odourless, long-tubular, and pendant. This is the syndrome of bird pollination. Determining the pollination syndrome of a particular flower can be a useful method of analysing floral diversity (van der Pijl 1971).

3.1.2.4 Seeds

Liao and Wu (1996) studied seed anatomy in *Alpinia*. So far, one type of seeds has been found in *Curcuma*. However, nobody has looked at the ultrastructure of the surface of seeds using SEM technique. This could be different among species. The SEM of seed has been used to study the taxonomy of *Cyanastroideae* (Brummit *et al.* 1999).

3.2 MATERIALS

3.2.1 Origin of plant materials, outgroup and ingroup taxa

Origin of plant material can be seen in Chapter Two. The rest are from herbarium specimens which are loans from Bogor-BO, Leiden-L, and Kew-K. Living collections (some are from rhizomes collected from field work in Java, Indonesia) are maintained at the RBGE glass house. All but 9 accessions are vouchered at RBGE. They will be mounted there eventually.

3.3 METHOD AND ANALYSIS

3.3.1 CODED MORPHOLOGICAL CHARACTERS AND PHYLOGENETIC ANAYSIS

3.3.1.1 Gross morphological characters (external morphology and light microscopy)

Morphological data was taken from herbarium specimens (including spirit material) as well as living collections. Measurements and also characters on shape, colour, odour, etc were recorded. Photographs were also taken.

3.3.1.2 Phylogenetic analysis

Detail of phylogenetic analysis can be seen in Chapter Two. Morphological data contain binary to multistate character. The multistate character is treated as unordered character (the order has not been determined). In an unordered character, transformation

between any two states costs the same number of steps. Molecular data contain multistate character of four bases. They are treated as unordered character.

3.3.2 NON CODED MORPHOLOGICAL CHARACTERS

3.3.2.1 Epidermis and stomata (light microscopy)

A very simple preparation was made from the middle part of the leaf between midrib and edge and between apex and base. The method used partly followed Olatunji (1980). The leaf portion was boiled in 2% sodium hydroxide solution for 5-10 minutes in order to induce detachment of the epidermis from the other cells/tissue. Using a scalpel, the unwanted tissue was scraped off. The epidermis tissue was then mounted on a slide in distilled water for temporary preparation. Observation was carried out by light microscope Axiophot Zeiss. Images were captured using Optimas 6.2. Measurements were taken by using rules produced by taking an image of graticules in Optimas. Epidermal observations on the upper and lower surfaces of the leaves (epidermal cells: shape and size; stomata: shape, size, type including subsidiary cells, stomatal index; modified epidermis like hairs).

3.3.2.2 Leaf transverse section using Freezing Microtome (light microscopy)

A portion of fresh leaf (as for epidermis and stomata study) was directly put in a freezing microtome. If herbarium material was used, it was boiled in detergent solution until it was almost back to its normal shape. Then it was put in the microtome. A tissue of 20 μm thick was sliced on each cut. After that, it was put in a slide dropped with stain. It was then covered by a cover glass. For permanent preparation, a solution was dropped before covering the tissue with cover glass.

3.3.2.3 Scanning electron microscopy of seeds

Seeds from herbarium specimens were mounted, sputter-coated and then plotted in the scanning electron microscope (SEM) for examination and photography. Three

samples (*C. aurantiaca, C. petiolata* and *C. australasica*) represent subgenus *Hitcheniopsis*. One sample, *C. aromatica*, represents subgenus *Curcuma* (**Figure 3.24**). Pollen obtained from spirit collections were critical point dried prior to the steps mentioned.

Pollen observation was finally excluded from the work. Preliminary work (critical point drying was carried out by Frieda Christie) indicated that the pollen was sticky. Further pre treatment was needed to get rid of this problem. Due to limited time, I decided to omit this part and concentrate on other work.

3.3.2.3.1 Mounting the specimens

The surface of seeds from only four species of *Curcuma* was tested for their ultra structure. The seeds were selected, then the good ones were mounted onto SEM aluminium stubs with carbon discs or dyes (silver in methyl isobutyl ketone). The discs or the dye will not only hold up the seeds but also will lessen the background effect.

3.3.2.3.2 Sputter-coating

The mounted specimens were transferred into the sputter coater chamber. Argon was then glowed through for 10 sec. The chamber was purged to 9.3-13.3 N/m^2 (0.07-0.1 Torr) after the purge light came on for 1 min. The specimens were coated at a preset deposition rate (25 mA) and for a preset time (2.5 min). The coating generates a conductive surface, which prevents the build-up of negative charge that would interfere in causing image distortion.

3.3.2.3.3 Scanning electron microscopy (Zeiss 962 SEM)

The system was ventilated with nitrogen, and then evacuated for several times before the high voltage and filament buttons were switched on. Mounted specimens were placed in the SEM chamber. High voltage (5 kV) with working distance (12 mm) was adjusted to get the optimum resolution of the image. The magnification, focus, and contrast/brightness were adjusted in the scanning mode. The images were stored in

Study of the literature reveals that the small species in the study that are diploid belong to subgenus *Hitcheniopsis*. Most medium sized to large species that are triploid belong to subgenus *Curcuma*. Chromosome numbers of *Curcuma* species are listed in **Table 5.2** (Chapter Five). Before it is concluded that subgenus *Hitcheniopsis* contains diploid and small habit species, and subgenus *Curcuma* contains mostly triploid large species, other *Curcuma* species especially Indian ones, should be examined. There is a possibility that subgenus *Curcuma* contains diploid and small habit species.

Does triploidy account for the large habit feature? Polyploidy can contribute to gigantism. The doubling of chromosomes or the doubling of genes within chromosomes may cause the increase in the cell size which in turn makes the whole plants to be gigantic. Environmental factors can contribute significant change to plant size. However, under the same environment the triploid *Curcuma* are bigger than the diploid one. The genetic factor may cause the large habit on triploid *Curcuma*.

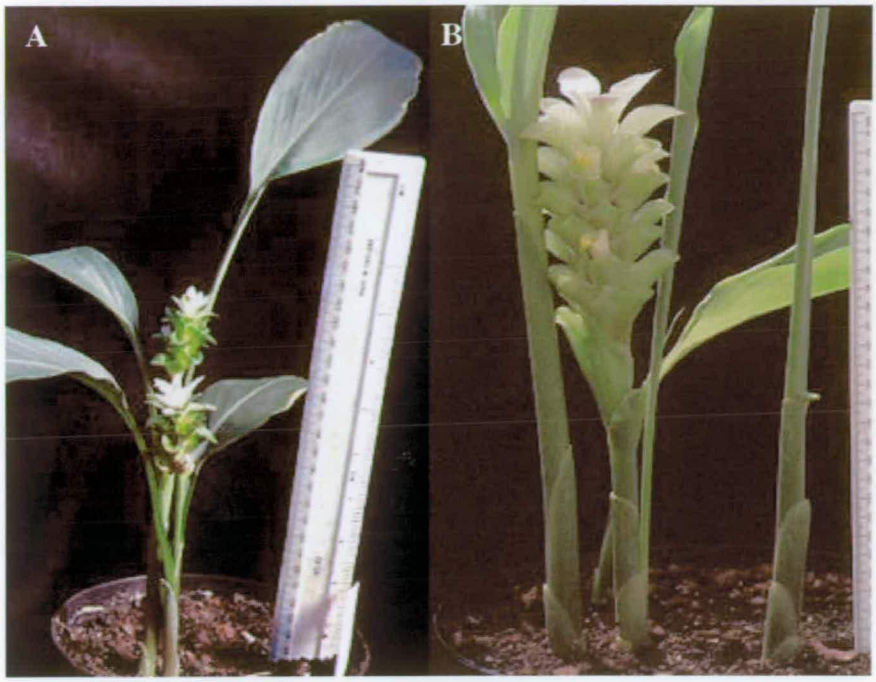


Figure 3.1 The habit of *Curcuma.*

A. Small habit of *C. parviflora* (subgenus *Hitcheniopsis*)
B. Large habit of *C. amada* (subgenus *Curcuma*). B is photographed by D. White.

Character states:

0) 30 to 50 cm

1) 1 to 3 m

3.4.1.1.2 Rhizome structure

Subgenus *Hitcheniopsis* comprises species with rather small primary rhizome and almost wholly reduced secondary rhizomes. On the other hand, subgenus *Curcuma* consists of species with well-developed secondary rhizomes. The structure is shown in **Figure 3.2**.

The primary rhizomes tend to terminate in a vegetative shoot or flowering shoot. Whenever the flowering shoot springs out from the side of the rhizome, the plant will produce a lateral inflorescence. The other way, whenever the flowering shoot comes out from the tip, the plant will produce a central inflorescence.

The roots, that are long filiform, spring out from the primary tuber. During the flowering period (Valeton 1918) or by the end of the rainy season (Santapau 1945), tubers called root-tubers form at the end of the roots. The shape may be ovate, pearshaped, spindle-shaped or ellipsoidal. Sometimes, from the end of this root-tuber springs fibrous roots again (Valeton 1918). The internal colour of this tuber varies from pearl-grey to yellow or orangish in colour. **Figure 3.2** describes the root-tubers in some species. In the case of *Boesenbergia rotunda* the pendulous clavate tubers are actually the roots (Valeton 1918).

Santapau (1952) observed four types of rhizomes in *Curcuma*, (i) a small rhizome (primary rhizome) without any tubers (secondary rhizomes); (ii) primary rhizomes posseses sessile tubers which are generally globose or ellipsoid; (iii) primary

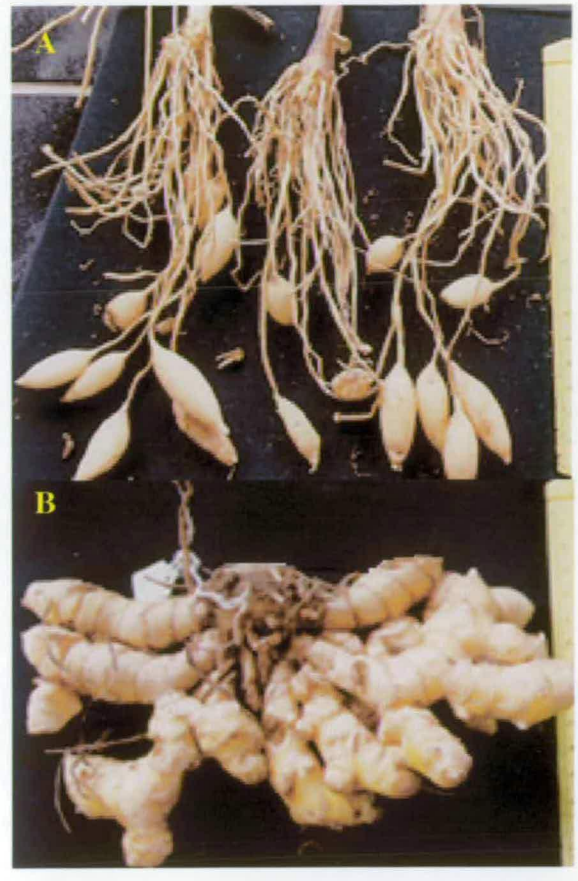


Figure 3.2 Rhizome structure in *Curcuma*.

A. Undeveloped rhizome in *C. aurantiaca* (subgenus *Hitcheniopsis);* **B**. Well developed rhizome in *C. soloensis* (subgenus *Curcuma*)

rhizomes posseses sessile palmate (pinnate is more proper according to Valeton) tubers; (iv) Tubers are at the end of long fibrous roots.

Compared to the rhizome architecture of other genera, such as *Alpinia, Aframomum,* and *Hornstedtia, Curcuma* possesses a relatively simple design of rhizomes. As a view, rhizomes of *Alpinia zerumbet* show architecture of nearly hexagonal form as a primary functional requirement for exploitation of the substrate (Bell 1979).

Why is the rhizome well developed and why it is not?

The fact that most sterile triploid species have well-developed rhizomes, and on the other hand, fertile diploid species possess reduced rhizomes, probably indicates that the sterility relates to the development of the rhizomes. The other theory is vice versa where the merely vegetative reproduction has induced the ability of well-developed rhizome propagation and induced the lost of generative reproduction. The rhizomes serve not only as food reservoir, but also function as reproduction of the plants. Species with reduced or undeveloped rhizomes is already successful to reproduce generatively.

Character states:

0) Hardly developed

1) Well-develeloped

3.4.1.1.3 Colour of internal rhizome

The colour of the rhizome of *Curcuma* varies from whitish to orange or bluish white. **Figure 3.3** shows the section and the colour of the rhizomes in some representative *Curcuma.* **Table 3.1** shows the colour of the rhizomes quoted from Valeton (1918) and from self-observation using the RHS (Royal Horticultural Society) colour chart as "Code des coulers" by Klincksick and Valette (1908 cited in Valeton 1918), which were used by Valeton, are not available. Colour and smell characters are subjective. Presence or absence of pigment character, such as the yellow pigment of curcumin, will be a better basis for codification of the characters into states.

Character states:

0) orange

1) yellow
2) white
3) bluish

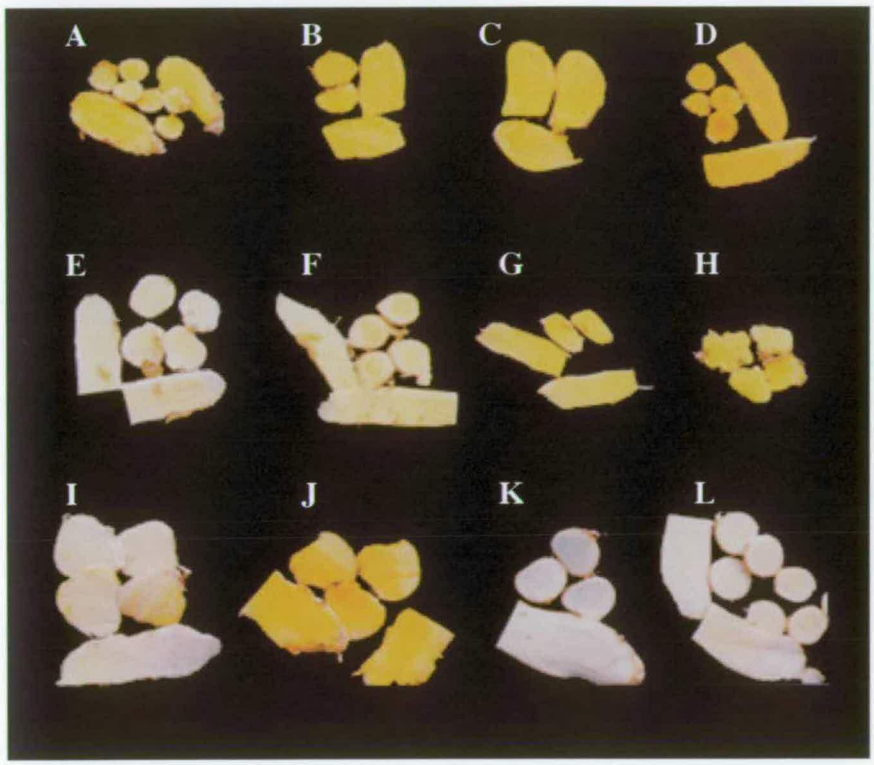


Figure 3.3 Colour of rhizome section in *Curcuma* subgenus *Curcuma*.

A. *C. purpurascens*, **B**. *C. soloensis*, **C**. *C. euchroma*, **D**. *C. longa*, **E**. *C. ochrorhiza*, **F**. *C. mangga*, **G**. *C. heyneana*, **H**. *C. cf. longa*, **I**. *C. zedoaria*, **J**. *C. zanthorrhiza*, **K**. *C. aeruginosa*, **L**. *C. phaeocaulis*

Table 3.1 Colour of the internal rhizome of *Curcuma*.

Species	Colour of the internal rhizome from Valeton	Colour of the internal rhizome using RHS colour chart
C. petiolata	Pale sulphureous	-
C. longa	Orange yellow to orange 151-126	Deep orange 21A or 22A
C. aeruginosa	Greenish blue 386 or pale greenish blue 396 light blue 396	Yellow 20C (centre), white/cream (peripheral) with blue tinged
C. brog	Pale sulphureous 206-216	Orange 25A
C. colorata	Deep orange 156	Orange 25B
C. euchroma	Orange 156	Orange 25A or 25B
C. heyneana	Pale sulphureous 226-236	Yellow 7A
C. mangga	Pale sulphureous 236-241	Light yellow 25B (centre), 20B (peripheral)
C. ochrorhiza	White with sulphur tinge	Light yellow 1B
C. phaeocaulis	Pale greenish blue 386	Yellow 20C (centre), white/cream (peripheral) with blue tinged
C. purpurascens	Orange yellow to pure yellow 156-161	Yellow 17C
C. soloensis	Orange 176-156	Orange 25A
C. viridiflora	Orange 176	-
C. zanthorrhiza	Orange yellow to orange 151-126	Orange 21A or 22A
C. zedoaria	Pale sulphureous 241-246; old rhizome pale ambercoloured 153D	Light yellow 20C

3.4.1.1.4 Smell of rhizome

The smell also varies from pungent, turmeric-smell to young mango smell. Colour and smell are important characters for designing at "species" (Valeton 1918, Holttum 1950). There is a degradation of the colour too, which makes it difficult to assign a specific colour to each "species".

The colour or smell may be due to the chemical constituents of the rhizomes. An example is the yellow colour of turmeric, which is affected by curcumin, one of the chemical compounds in the rhizome.

Character states:

0) not fragrant or not pungent

1) pungent

2) mango-smell

3.4.1.1.5 Leaf shape (Figure 3.4)

Most *Curcuma* species have elliptic to lanceolate leaves in one individual. An anomaly is, for instance, in *C. alismatifolia* that has linear leaves.

Character states:

0) elliptic to elliptic oblong

1) linear

3.4.1.1.6 Shape of ligule (Figure 3.5)

A limited number of species was observed from living collections. It is hard to see from herbarium material. The ligule is formed as an elongation of the internal sheath at the junction of the leaf blade. Data of this character for subgenus *Hitcheniopsis*, except *C. parviflora*, are adopted from Valeton (1918) where I could not see it.

Character states:

0) not auriculate

1) auriculate

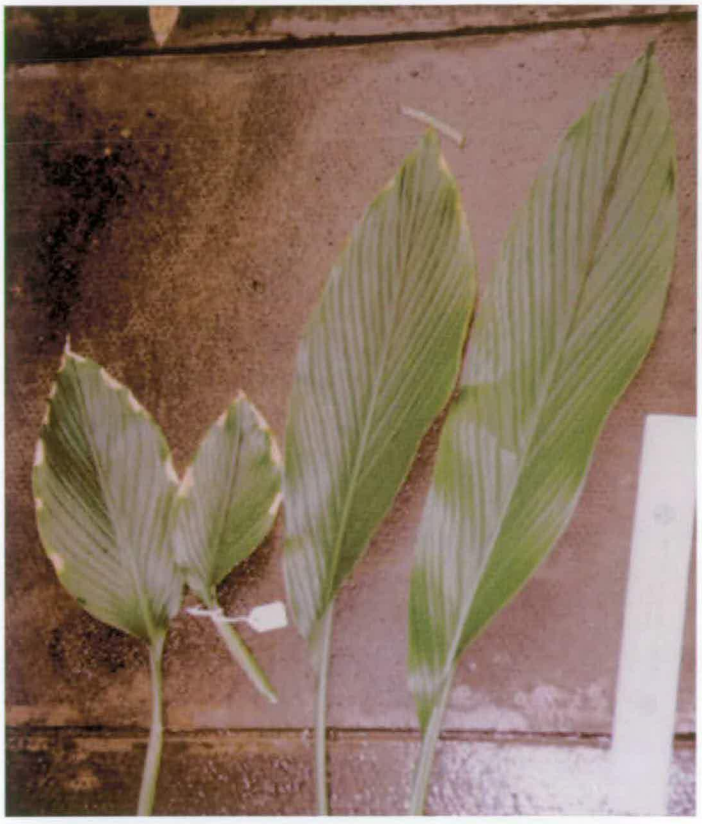


Figure 3.4 Variation of leaf shape in an individual of *C. aeruginosa*

The very left, which is the oldest leaf, is elliptic while the very right, which is the youngest leaf, is lanceolate.

3.4.1.1.7 *Colour of midrib* (Figure 3.6)

Valeton (1918) also notes the colour of the midrib.

Character states:

0) green

1) brownish

3.4.1.1.8 *Colour of sheath* (Figure 3.7)

The colour of the sheath is a diagnostic character in some species, such as *C. phaeocaulis,* and *C. elata.*

Character states:

0) green

1) brownish

3.4.1.1.9 *Purple flush on leaves* (Figure 3.8)

The leaves are green ranging from dark to pale green. There is a brownish reddish flush along both sides of the midrib. In some species, this flush will fade in the old leaves. Ridley (1947) observed that the environment effect this coloration. Poor environment affects the absence of production of the flush. However, in greenhouse conditions, these differences were maintained.

The leaves, whether they are plain green without purple flush or not, in some case may help in assigning "species". Species without having any purple flush include for example *C. longa,* and *C. heyneana.*

Character states:

0) present

1) absent

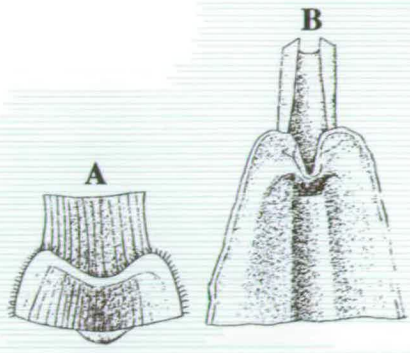


Figure 3.5 Ligule shape in *Curcuma*

(**A**. not auriculate, **B**. auriculate. After Valeton 1918)

Figure 3.6 Colour of midrib in *Curcuma*

(**A**. Green midrib in *C. longa*, **B**. Purple midrib in *C. colorata*).

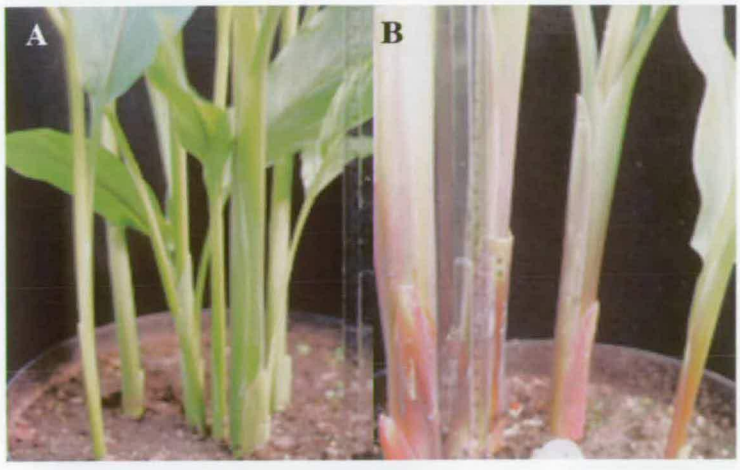


Figure 3.7 Colour of sheath in *Curcuma.*

(**A**. Green leaf sheath in *C. colorata;* **B**. Brown leaf sheath in *C. phaeocaulis*)

Figure 3.8 Variation of colour flushes on leaves of *Curcuma.*

(**A**. Purple flush 2/3 length of the midrib towards the base in *C. aeruginosa,* **B**. purple flush along the midrib in *C. zanthorrhiza*. Photographed by D. White)

Figure 3.9 Position of inflorescence in *Curcuma.*

(**A**. Central inflorescence in *C. aurantiaca,* **B**. Lateral inflorescence in *C. zedoaria.* B is photographed by D. White)

3.4.1.1.10 Position of inflorescence

Curcuma has two types of inflorescence, which are lateral or central (**Figure 3.9**). Lateral inflorescence comes out before or with leaves. Species of this type produce the inflorescence just after the rainy season starts. Central inflorescence comes out after the plants produced many leaves. Species and inflorescence position are tabulated in **Table 3.2** below. As the character of the position of inflorescence is used to distinguish the existing sectional level classification, I took this in my character records to proof that this character is homoplasy.

Despite this, the position of inflorescence is not stable in one species. Roxburgh (1812) was the first to note both inflorescence positions in *C. decipiens*. This is why Horaninow (1862) coined a section *Amphiantha*. However, this makes a difficult genus even more difficult. It takes the whole season to observe both positions (Burtt 1982). Moreover, in the case of *C. amada*, they produce both positions at unspecific times.

Santapau (1945, 1952) confirmed both inflorescence positions in *C. pseudomontana* after he spent three years working in the field. *C. pseudomontana* has lateral spikes at the beginning of the rainy season, and central spikes at the end of the season. He also did careful dissection and found a diminutive inflorescence from the centre of the leaf tufts. Later on, Mangaly & Sabu (1987) reported their studies for over 8 years in South India and observed some species produce only lateral (*C. zedoaria*) and some only terminal (*C. longa, C. ecalcarata*) and some others produce both positions. *C. amada, C. decipiens, C. inodora, C. neilgherrensis*, and *C. oligantha* are reported by Mangaly & Sabu (1993) as having both positions. *C. amada* that was described as having central inflorescence was reported to produce only lateral ones in certain regions (Mangaly & Sabu 1987). My observation in the research glasshouse in the Royal Botanic Garden Edinburgh is that *C. amada* produce lateral inflorescences. Santapau (1945) was of the opinion that the classification based on inflorescence positions.

It is critical to consider that Valeton described a new species *C. mangga* for the sole reason that it is different from *C. amada* in the position of its inflorescence. He wrote, "This species is not *Curcuma amada*, Roxb. as I took it to be formerly (Heyne l.c.) before I had seen the lateral scape.". *C. mangga* therefore could be reduced to *C.*

Table 3.2 Frequent inflorescence position in *Curcuma spp.*

Species in subgenus *Hitcheniopsis*	**Infl. position**	**Species in subgenus *Curcuma***	**Infl. position**	**Species in subgenus *Curcuma***	**Infl. position**
C. alismatifolia	central	*C. aeruginosa*	lateral	*C. colorata*	central
C. aurantiaca	central	*C. amarissima*	lateral	*C. euchroma*	central
C. australasiaca	central	*C. aromatica*	lateral	*C. longa*	central
C. ecomata	lateral	*C. elata*	lateral	*C. mangga*	central
C. gracillima	central	*C. heyneana*	lateral	*C. purpurascens*	central
C. harmandii	central	*C. leucorrhiza*	lateral	*C. soloensis*	central
C. parviflora	central	*C. ochrorhiza*	lateral	*C. viridiflora*	central
C. petiolata	central	*C. phaeocaulis*	lateral	*C. amada*	lateral or central
C. roscoeana	central	*C. zanthorrhiza*	lateral	*C. decipiens*	both
C. thorelii	central	*C. zedoaria*	lateral	*C. inodora*	both
				C. neilgherrensis	both
				C. oligantha	both

Note: Infl.= inflorescence

amada as *C. amada* has been found to produce lateral inflorescence too.

Character states:

0) central

1) lateral

3.4.1.1.11 Shape of bracts (Figure 3.10)

The bracts that enclose the flower conform generally to two types. They also vary in shape or colour. Most *Curcuma* species have elliptic to broad lanceolate bracts with blunt apices to the fertile bracts. However, *C. harmandii* has very elongated narrow fertile bracts with sharp apices. Therefore, the character is an autoapomorphy in *C. harmandii* and should have been omitted.

Character states:

0) almost linear

1) elliptic or lanceolate

3.4.1.1.12 Length of coma compared to fertile bracts

In most *Curcuma,* the coma bracts are differently coloured and longer than the fertile bracts. However, in some other species, the coma can be the same colour as the fertile bracts and shorter or as long as the fertile bracts (**Figure 3.10**). This condition referred to as having no coma, but I take it as two states, i.e. coma is shorter or as long as fertile bracts; longer than fertile bracts.

Character states:

0) shorter or as long as fertile bracts

1) longer than fertile bracts

3.4.1.1.13 Colour of coma and fertile bracts

The upper bracts called the coma can be sterile and are usually differently coloured (**Figure 3.10**). These coma bracts are normally narrower and longer than the

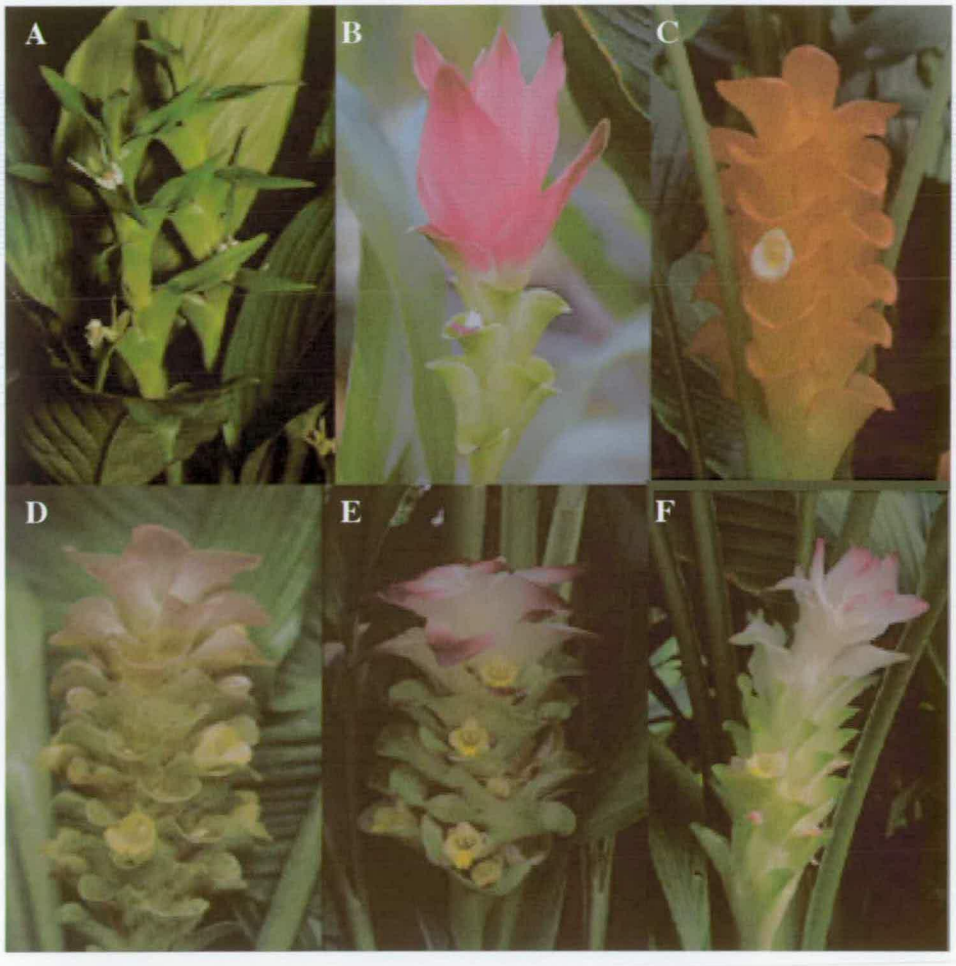


Figure 3.10 Variation of bract shape and colour in *Curcuma*.

(**A**. *C. harmandii,* **B**. *C. alismatifolia,* **C**. *C. roscoeana* **D**. *C. australasica,* **E**. *C. longa,* **F**. *C. longa.* **A** & **B** are taken from S. Wannakrairoj; **C-F** are photographed by D. White)

fertile bracts. In *C. roscoeana* the coma bracts are not differently coloured from the fertile bracts. The coma and fertile bracts are orange.

Santapau (1945) reported that the colours of the coma bracts are very variable even within a single species that he was observing in Khandala, India. The coma bract colour of *C. pseudomontana* can be (i) uniform pink with various degradation; (ii) pure white; (iii) white with pink tips, (iv) white with pink tips and a broad stripe or pink stripes running down along the centre of the bracts; (v) white with several green stripes running longitudinally downwards and parallel to each other; and (vi) pink with very deep purple that is almost black (Santapau 1945). Various authors also reported at least two variations in the colour of the coma bracts of *C. longa,* white or pink. The colour of the coma bracts or the whole bracts, therefore, I think is not a reliable character defining species.

Nakayama *et al.* (2000) demonstrated that malvidin 3-rutinoside is responsible for the pigment of the coma bracts in *C. alismatifolia.* It was the only anthocyanin identified from pink bracts of *Curcuma alismatifolia* cultivars. The concentration of malvidin 3-rutinoside increased as the intensity of the pink colour in the bracts increased. Does the environment affect the production of malvidin 3-rutinoside? If yes, it could help to explain the reason why the colour of the coma bracts varies in *C. pseudomontana* and *C. longa.*

The shape of the bracts is more stable within a species than the colour. Environment will have less effect on this than on colour. The extreme shape is between that of *C. harmandii* and other subgenus *Curcuma* species such as *C. zedoaria, C. zanthorrhiza, C.longa.* The shape of the bracts within subgenus *Curcuma* is more uniform than that of subgenus *Hitcheniopsis.* Valeton was of the opinion to exclude some species of *Curcuma* which should be placed in subgenus *Hitcheniopsis.* One of his reasons is on the basis of the different structures of the bracts (Valeton 1918).

Character states:

0) uniform

1) different

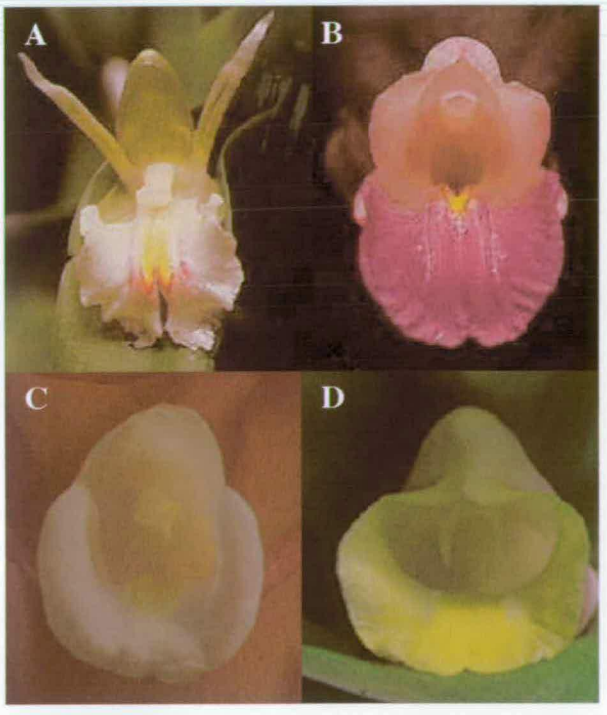


Figure 3.11 Variation of flower shape in *Curcuma*

The three flower types in *Curcuma:* simple (**D**), complex (**A & C**), and small (**B**)

3.4.1.1.14 Flower structure

The flower comprises three types, viz. simple, complex, and small (Chapter Four). Most species of *Curcuma* subgenus *Curcuma* have simple type flowers. Some species from subgenus *Hitcheniopsis* have complex or small type flowers. **Figure 3.11** shows the three types of flower. The variation of floral shape and structure may relate to different pollinators. In Chapter Four I will discuss this in detail.

Character states:

0) simple

1) complex

2) small

Flower types, however, cannot be considered as individual characters. Those character states are not comparable states. There might be too many different homologies. Therefore, this character is very dubious and should be omitted from the analyses.

3.4.1.1.15 Hair on ovary

Character states:

0) hairy

1) glabrous

3.4.1.1.16 Shape of petals

Petals are mostly elliptic or broad lanceolate (**Figure 3.12**).

Character states:

0) lanceolate

1) elliptic

3.4.1.1.17 Colour of petals

The corolla tube (**Figure 3.13**) is usually hidden by the pouched bracts. The corolla lobes emerge from the bracts with their colour ranging from pink or red (*C. zanthorrhiza, C. aeruginosa*) to yellow or whitish.

Figure 3.12 Variation of petal shape in *Curcuma*
A. Elliptic petals in *C. mangga*, **B**. Lanceolate petals in *C. stenochila*.
B is taken from S. Wannakrairoj.

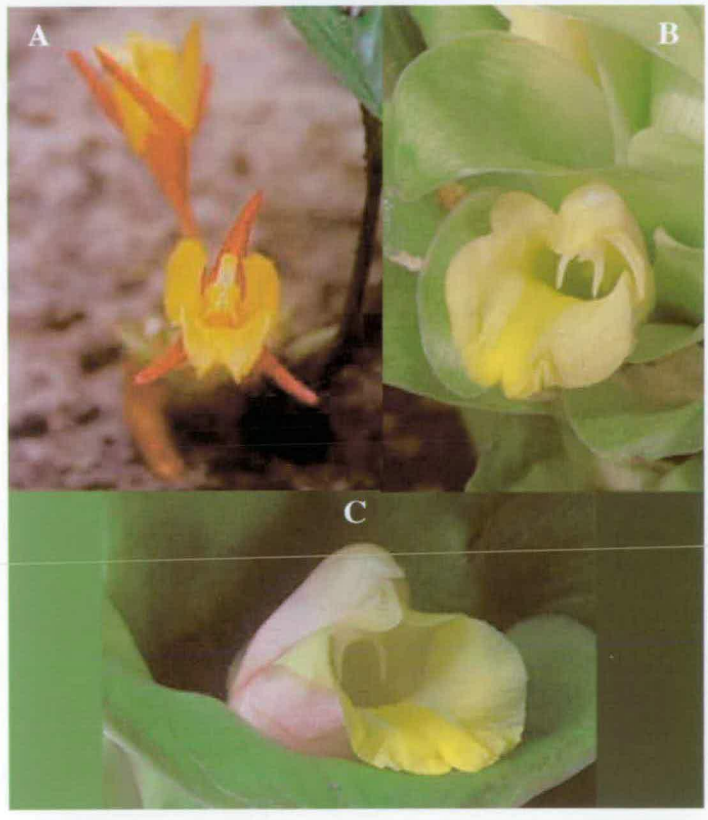


Figure 3.13 Petal colours in *Curcuma*

(**A**. *C. stenochila* with red petals, **B**. *C. longa* with light yellow petals
C. *C. mangga* with pink petal. **A** is taken from S. Wannakrairoj.
B & C are photographed by D. White).

Character states:

0) reddish or purplish

1) whitish

3) greenish

4) yellowish or orangish

3.4.1.1.18 Hair on dorsal petal

The abaxial dorsal petal is mostly glabrous or hairy at tip.

Character states:

0) glabrous

1) hairy at tip

2) hairy throughout

3.4.1.1.19 Cucullate on dorsal petal

The dorsal petal is mostly cucullate.

Character states:

0) not cucullate

1) cucullate

3.4.1.1.20 Shape of labellum

The labellum varies from elongate (e.g. *C. alismatifolia, C. gracillima)* to obovate (e.g. *C. zedoaria, C. longa*) or rounded shape (e.g. *C. roscoeana*). **Figure 3.14** shows the variation of this shape.

Character states:

0) larger than wide

1) wider than long

3.4.1.1.21 Colour of labellum

The labellum varies from yellow with a darker yellow thickened band in the middle to whitish or purplish.

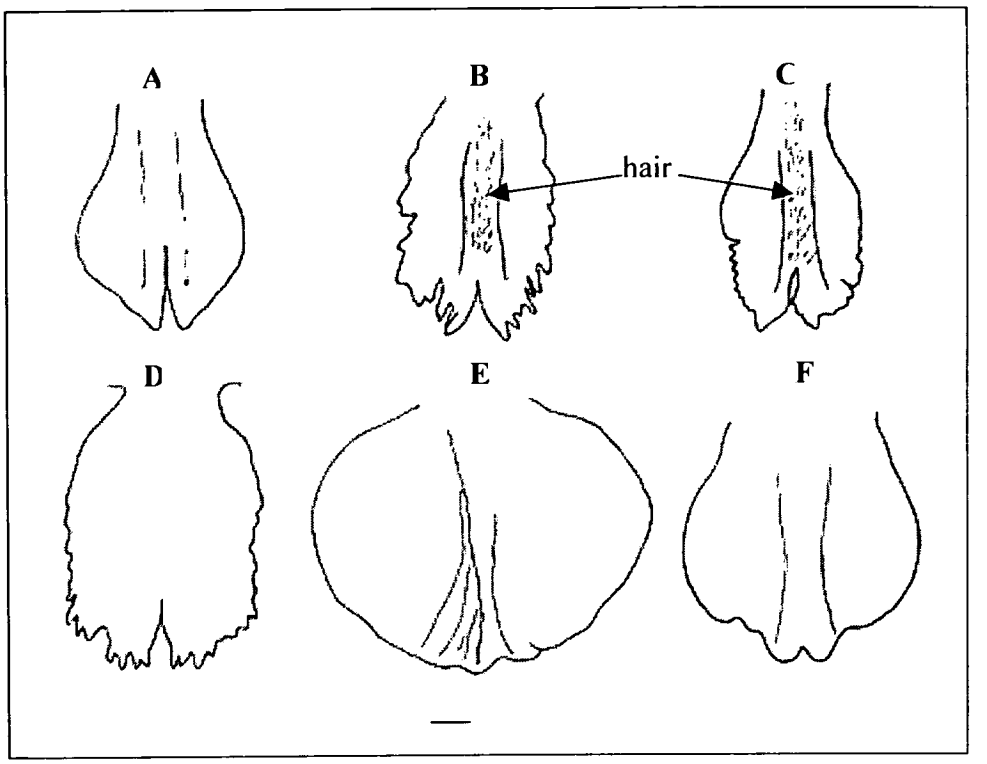


Figure 3.14 Labellum shape, and hair on the middle band of the labellum in *Curcuma*
(**A**. *C. ecomata*, **B**. *C. gracillima*, *C. parviflora*, **C**. *C. harmandii*
D. *C. thorelii*, **E**. *C. roscoeana*, **F**. *Curcuma* subgenus *Curcuma*)

Character states:

0) purple or white and purple

1) yellow or orange

3.4.1.1.22 Hair on labellum blade

The labellum is mostly glabrous, but hair is found in some species outside the band boundary.

Character states:

0) present

1) absent

3.4.1.1.23 Hair on the middle band of the labellum

On the middle band of the labellum, hair is present (**Figure 3.14**) in some species.

Character states:

0) present

1) absent

3.4.1.1.24 Shape of lateral staminodes

The lateral staminodes also vary from linear to elliptic or oblong (**Figure 3.15**). They cover the stigma and stamen or are free (e.g. in *C. harmandii*).

Character states:

0) linear

1) obovate to oblanceolate

3.4.1.1.25 Arrangement of lateral staminodes

Lateral staminodes can be free, and can be folded or clasped under the dorsal petal (**Figure 3.15**) in some species.

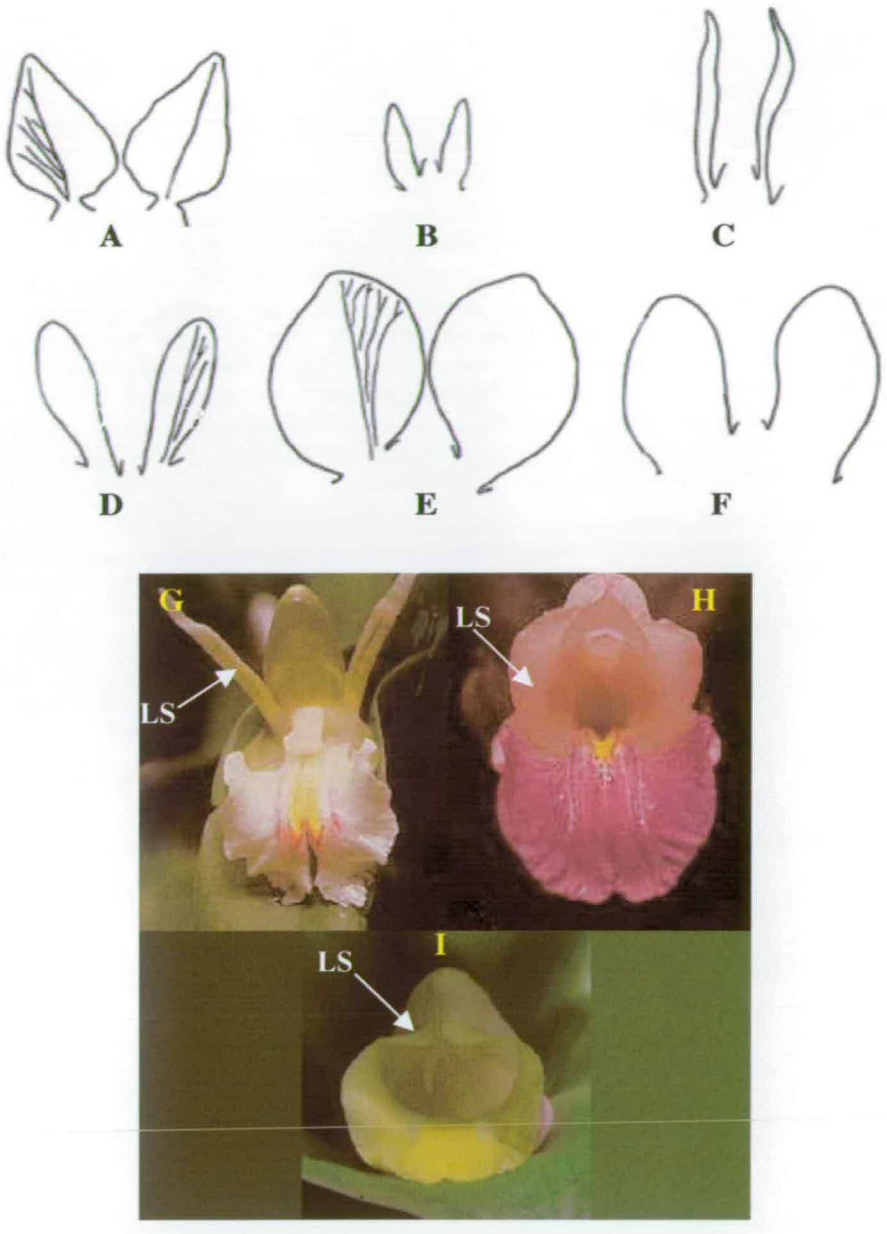


Figure 3.15 Shape and orientation of lateral staminodes in *Curcuma*.

(**A**. *C. ecomata,* **B**. *C. gracillima, C. parviflora*, **C**. *C. harmandii,* **D**. *C. thorelii,* **E**. *C. roscoeana,* **F**. *Curcuma* subgenus *Curcuma*, **G**. *C. harmandii* with free lateral staminodes; **H**. *C. alismatifolia* with free lateral staminodes; **I**. *C. longa* with clasped lateral staminodes. Arrow with LS indicates lateral staminode. G & H are taken from S. Wannakrairoj; I is photographed by D. White)

Character states:

0) free

1) clasping

3.4.1.1.26 Colour of lateral staminodes

0) reddish or purplish

1) whitish

2) greenish

3) yellowish or orangish

3.4.1.1.27 Groove on lateral staminodes

Lateral staminodes posseses a groove that makes it uneven. They can be smooth and flat (**Figure 3.15**).

Character states:

0) absent

1) present

3.4.1.1.28 Patch of granules at apex of lateral staminodes

A patch of granules can be found under high magnification (light microscope) from spirit material. These may be cells containing colour pigments.

Character states:

0) absent

1) present

3.4.1.1.29 Length of anther (Figure 3.16 & 3.17)

Character states:

0) 4 - 7 mm or more

1) 0 - 4 mm

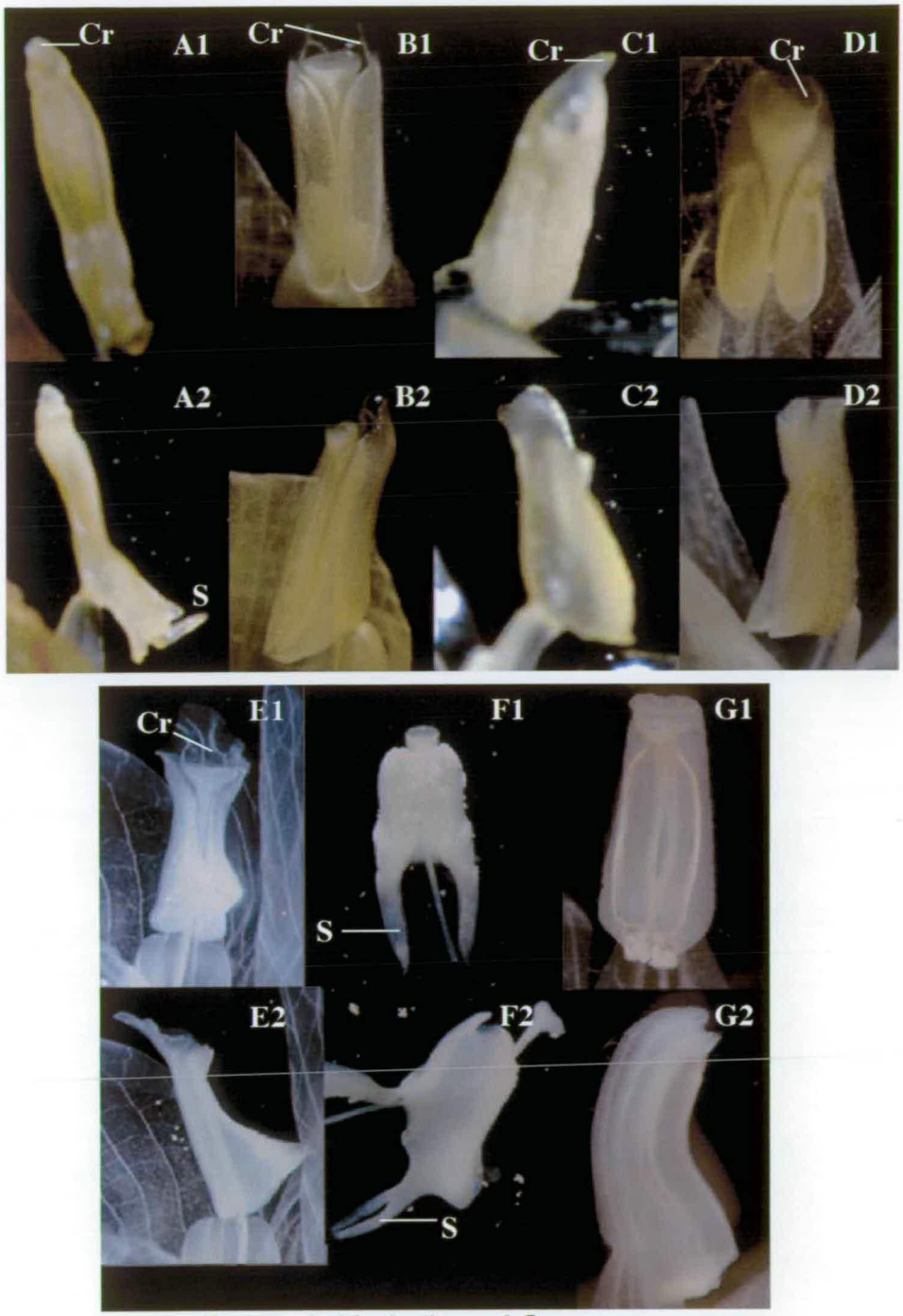


Figure 3.16 Anther of *Curcuma.*

(**A**. *C. ecomata,* **B**. *C. alismatifolia,* **C**. *C. thorelii,* **D**. *C. gracillima,* **E**. *C. roscoeana,* **F**. *C. longa,* and **G**. *C. aurantiaca.* Number 1 indicates front view, 2 indicates lateral view. **F** is from subgenus *Curcuma.* The rest are from subgenus *Hitcheniopsis.* S is spur, Cr is crest)

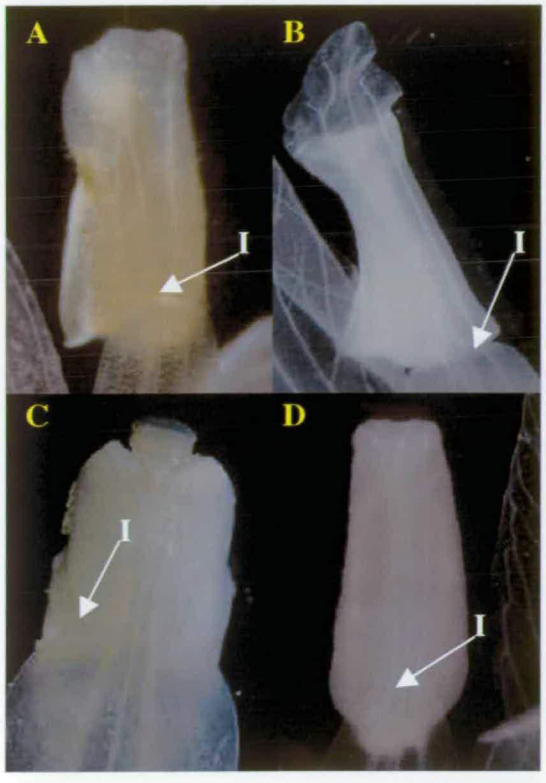


Figure 3.17 Anther of *Curcuma* from the back.

(**A**. *C. gracillima;* **B**. *C. roscoeana*; **C**. *C. longa*; **D**. *C. aurantiaca*. **C** is from subgenus *Curcuma,* and the rest are from subgenus *Hitcheniopsis.* Arrow with I indicates insertion of filament on the anther).

3.4.1.1.30 Spur on anther (Figure 3.16)

Character states:

0) absent

1) present

3.4.1.1.31 Crest on anther (Figure 3.16)

Character states:

0) present

1) absent

3.4.1.1.32 Anther dehiscence (Figure 3.16)

Character states:

0) along locules up to the base

1) only along locules

3.4.1.1.33 Stigma type (Figure 3.18)

Stigma emerges just above the anther. Its stylus is supported by the locules of the anther.

Character states:

0) inflated

1) funnel-shaped

3.4.1.1.34 Stylar growth

Stylodes grow on the septa of ovary in some species. They, two stylodes, secrete nectar.

Character states:

0) absent

1) present

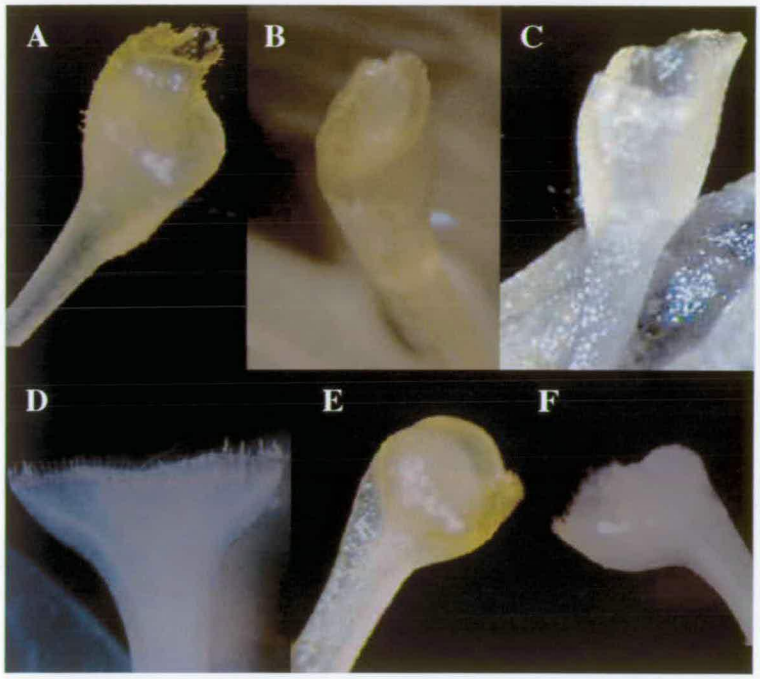


Figure 3.18 Stigma type in *Curcuma*.

Inflated type (**A**. *C. ecomata;* **E**. *C. longa*; **F**. *C. aurantiaca*) and funnel shape (**B**. *C. alismatifolia*; **C**. *C. thorelii*; **D**. *C. roscoeana*). **E** is in subgenus *Curcuma,* and the rest are in subgenus *Hitcheniopsis.*

3.4.1.1.35 Ring of hair on corolla tube

The bunch of hair on the throat of the corolla tube makes a ring in some species, but it is spread randomly in other species.

Character states:

0) absent

1) present

Table 3.3 Character coding for morphological data of *Curcuma* spp.

SPECIES	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Ca. spicata	0	0	2	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	1	1	1	1	0
Ca. gracilis	0	0	2	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	1	1	1	1	0
R. auriculata	0	0	2	0	1	1	0	0	1	0	0	1	0	1	1	0	0	0	1	1	0	1	1	1	1	0	1	0	0	1	1	1	0	1	0
C.ecomata	0	0	2	0	0	1	0	0	1	1	1	0	0	1	0	0	3	1	0	0	1	0	1	1	0	3	0	0	0	1	0	1	1	1	0
C.parviflora	0	0	2	0	0	1	0	0	1	0	1	1	1	2	1	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0	1	1	0	0
C.gracillima	0	0	2	0	0	1	0	0	1	0	1	0	1	2	1	1	1	0	0	0	0	1	0	1	0	1	0	0	1	0	0	1	1	0	0
C.alismatifolia	0	0	2	0	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	0	1	1	0	0
C.thorelii	0	0	2	0	0	1	0	0	1	0	1	1	1	2	1	1	1	0	0	0	0	1	0	1	0	0/1	0	0	1	0	0	1	1	0	0
C.harmandii	0	0	2	0	0	1	0	0	1	0	0	0	0	1	1	1	2	0	0	0	0	1	0	0	0	2	0	0	1	0	0	1	1	0	0
C.aurantiaca	0	0	2	0	0	1	0	0	1	0	1	1	1	0	0	1	3	2	0	1	1	1	1	1	0	3	0	0	1	0	0	0	0	1	1
C.cf.australasica	0	0	0	0	0	1	0	0	1	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	0	3	0	1	1	1	1	1	1	1	1
C.roscoeana	0	0	2	0	0	1	0	0	1	0	1	0	0	1	1	1	3	2	0	1	1	1	1	1	0	3	0	0	1	0	0	1	1	1	0
C.petiolata	0	1	2	1	0	1	0	0	1	0	1	0	1	0	0	1	3	1	1	1	1	1	1	1	0	3	0	1	1	1	1	1	0	1	1
C.longa	1	1	0	1	0	0	0	0	1	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.aromatica	1	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.elata	1	1	3	1	0	0	0	1	0	1	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.leucorrhiza	1	1	2	1	0	0	0	0	1	1	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.amarissima	1	1	3	1	0	0	0	1	1	1	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.amada	1	1	2	2	0	0	0	0	1	0/1	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.viridiflora	1	1	0	1	0	0	0	0	1	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.soloensis	1	1	0	1	0	0	0	0	1	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.euchroma	1	1	0	1	0	0	1	0	1	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.brog	1	1	0	1	0	0	0	0	1	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	0	1	1	1	1	1	0	1	1
C.ochrorhiza	1	1	2	1	0	0	0	0	1	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.colorata	1	1	1	1	0	0	1	0	1	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.purpurascens	1	1	1	1	0	0	0	0	0	0	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1

Table 3.3 (continued) Character coding for morphological data of *Curcuma* spp.

SPECIES	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
C.zanthorrhiza	1	1	0	1	0	0	1	0	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.aeruginosa	1	1	3	1	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.zedoaria	1	1	1/2	1	0	0	0	0	0	1	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.heyneana	1	1	1	1	0	0	0	0	1	1	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.mangga	1	1	1	2	0	0	0	0	1	1	1	1	1	0	0	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1
C.phaeocaulis	1	1	3	1	0	0	0	1	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	0	1	1

Notes: 1-35 in bold: Character (see next page)
0-3 : Character states (see next page)

1) Habit
0: small
1: tall

2) Rhizomes structure
0: hardly developed
1: well-developed

3) Colour of internal rhizome
0: orange
1: yellow
2: white
3: bluish

4) Smell of rhizome
0: not fragrant or not pungent
1: pungent
2: mango-smell

5) Leaf shape
0: elliptic to elliptic oblong
1: linear

6) Shape of ligule
0: not auriculate
1: auriculate

7) Colour of midrib
0: green
1: brownish

8) Colour of sheath
0: green
1: brownish

9) Purple flush on leaves
0: present
1: absent

10) Position of inflorescence
0: central
1: lateral

11) Shape of bracts
0: almost linear
1: elliptic or lanceolate

12) Length of coma compared to fertile bracts
0: shorter or as long as fertile bracts
1: longer than fertile bracts

13) Colour of coma and fertile bracts
0: uniform
1: different

14) Flower structure
0: simple
1: complex
2: small

15) Hair on ovary
0: hairy
1: glabrous

16) Shape of petals
0: lanceolate
1: elliptic

17) Colour of petals
0: reddish or purplish
1: whitish
2: greenish
3: yellowish or orangish

18) Hair on dorsal petal
0: glabrous
1: hairy at tip
2: hairy throughout

19) Cucullate on dorsal petal
0: not cucullate
1: cucullate

20) Shape of labellum
0: larger than wide
1: wider than long

21) Colour of labellum
0: purple or white and purple
1: yellow or orange

22) Hair on labellum blade
0: present
1: absent

23) Hair on labellum band
0: present
1: absent

24) Shape of lateral staminodes
0: linear
1: obovate to oblanceolate

25) Arrangement of lateral staminodes
0: free
1: clasping

26) Colour of lateral staminodes
0: reddish or purplish
1: whitish
2: greenish
3: yellowish or orangish

27) Groove on lateral staminodes
0: absent
1: present

28) Patch of granules at apex of lateral staminodes
0: absent
1: present

29) Length of anther
0: 4-7 mm or more
1: 0-4 mm

30) Spur on anther
0: absent
1: present

31) Crest on anther
0: present
1: absent

32) Anther dehiscence
0: along locules up to the base
1: only along locules

33) Stigma type
0: inflated head
1: not inflated head

34) Stylar growth
0: absent
1: present

35) Ring of hair on corolla tube
0: absent
1: present

3.4.1.2 Phylogenetic analysis

Phylogenetic analysis of 35 morphological characters with equally weighted characters including uninformative characters resulted in 16383 equally most parsimonious trees (CI=0.579, RI=0.862, RC=0.499) with a length of 76 steps (**Figure 3.19**). Excuding of uninformative characters reduced the tree length to 73 steps. Re-weighting characters to CI, RI or RC index resulted in shorter but similar trees. These were slightly more resolved in the *C. parviflora, C. alismatifolia,* and *C. gracillima* group, and the *C. soloensis, C. longa, C. phaeocaulis, C. aromatica,* and *C. elata* group.

The trees resulting from molecular data are more resolved than those resulting from morphological data, especially in subgenus *Curcuma*. Despite these trees can be compared and used as support for the molecular data or vice versa. Both trees are almost congruent except that *C. aurantiaca* and *C. cf. australasica*, which were placed between *C. ecomata* and the *C. thorelii* clade in the molecular analyses, were shifted to nodes between *C. roscoeana* and *C. petiolata* in the tree constructed from morphological data (**Figure 9.1**).

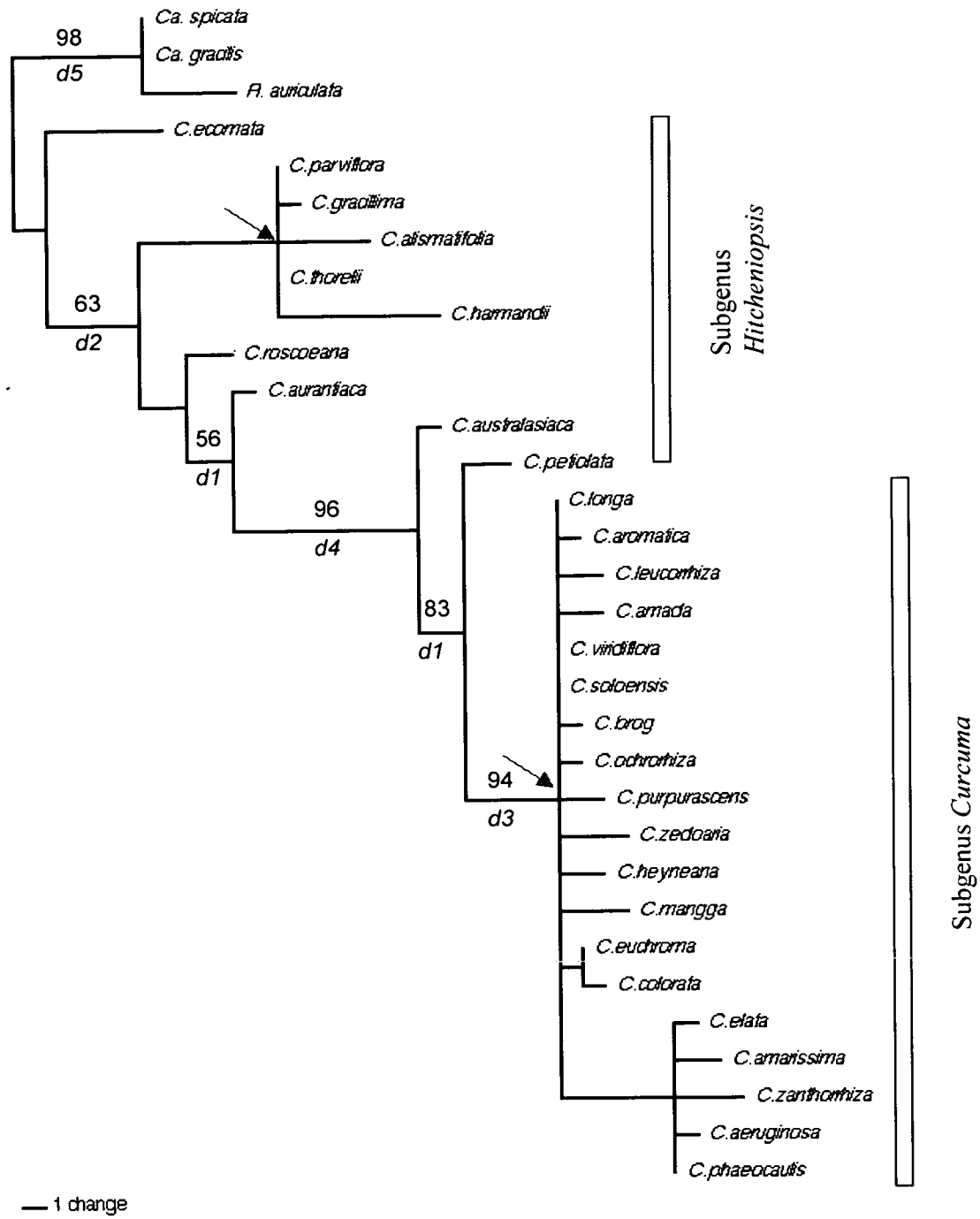


Figure 3.19 Strict consensus tree obtained from 1000 equally most parsimonious trees of length 75 steps resulted from equally weighted parsimony analysis of morphological (CI=0.5789; RI=0.8621; RC=0.4991).

3.4.2 NON CODED MORPHOLOGICAL CHARACTERS

3.4.2.1 Epidermis and stomata (Figure 3.20-3.22)

Adaxial epidermis are transversely elongated (the longest cell is perpendicular to the vein) and hexagonal shape (**Figure 3.20**). The epidermal cells are about 39-125 μm long and 18-68 μm wide. The arrangement is in regular straight rows parallel to the direction of the veins. Stomata consist of guard cells 30-60 μm long and 43-95 μm wide, lateral subsidiary cells, and two or three terminal subsidiary cells. The longest side of guard cell runs in parallel with the veins. Lateral subsidiary cells flanks the guard cells, while terminal subsidiary cells flank both guard cells and lateral subsidiary cells. Stomatal density is the percentage of the number of stomata to the number of epidermal cells per cm^2. Stomatal density is about 1.13-2.43% in adaxial epidermis. The terminal subsidiary cells run in parallel with the longest part of the epidermal cells. Epidermal cells under the vein is smaller than those that are not under the vein (**Figure 3.20**).

Abaxial epidermis are mixed longitudinally elongated (the longest cell is perpendicular to the vein), transversely elongated and isodiametric (**Figure 3.21**). The epidermal cells are 17-96 μm long and 18-102 μm wide. They are randomly arranged and are not like the adaxial epidermall cells which are in almost regular straight rows. Stomata consist of guard cells (32-56 μm long and 35-72 μm wide), lateral subsidiary cells, and two or three terminal subsidiary cells. The arrangement of this stomatal complex is similar with those in adaxial. Stomatal density is 8.15-13.98% in abaxial epidermis. Epidermal cells under the vein is also smaller than the others which are not under the vein (**Figure 3.21**).

There are some differences in the epidermal characters of *C. amada* and *C. mangga* (**Figure 3.22**). The abaxial epidermal cells of *C. amada* are mostly transversely elongated with some isodiametric while in *C. mangga* they are mostly longitudinally elongated with some isodiametric. The arrangement is in regular straight rows in *C. mangga* whereas as other *Curcuma* this is not so in *C. amada.* The abaxial epidermis are hairy or pubescent in *C. amada* while they are glabrous in *C. mangga.* However, the

character of presence of hair is sometimes inconsistent in this case. Some *C. amada* can have glabrous abaxial surface of leaves (Sirirugsa 1996). To confirm the different shape of the epidermal cells between those of *C. amada* and *C. mangga,* more samples are needed.

C. amarissima is quite unique in having more than two terminal subsidiary cells (**Figure 3.20-3.21**). However, two terminal subsidiary cells are also found in one slide preparation. Again, more samples are needed to confirm this.

The stomatal density is slightly lower in those from subgenus *Hitcheniopsis* (sampled species are *C. alismatifolia, C. parviflora*). Environment could affect this, but before any conclusion is drawn, more samples should be examined. Epidermal characters are not included in the phylogenetic analysis as morphometric analysis did not support any groupings.

3.4.2.2 Leaf: transverse section (Figure 3.23)

A few samples were taken for leaf transverse section investigation. From six species, there are two types of transverse sections disregarding sectional level or even subgeneric level. The first type has hypodermis on both sides (adaxial and abaxial), and sclerenchyme fibres that never interrupt the lower hypodermis. The second type has hypodermis on abaxial, but no hypodermis or one layer on adaxial. The sclerenchyme fibres in this type sometimes interrupt the lower hypodermis. The first type *comprises C. zedoaria, C. heyneana*, and *C. purpurascens*, while the second comprises *C. longa, C. zanthorrhiza* and *C. roscoeana.* The epidermis of *C. roscoeana* looks larger than the others. Sections have been attempted in other species, but the results were not good. **Figure 3.23** shows the transverse sections of some *Curcuma* spp. mentioned above. Leaf transverse sections are not included in the phylogenetic analysis.

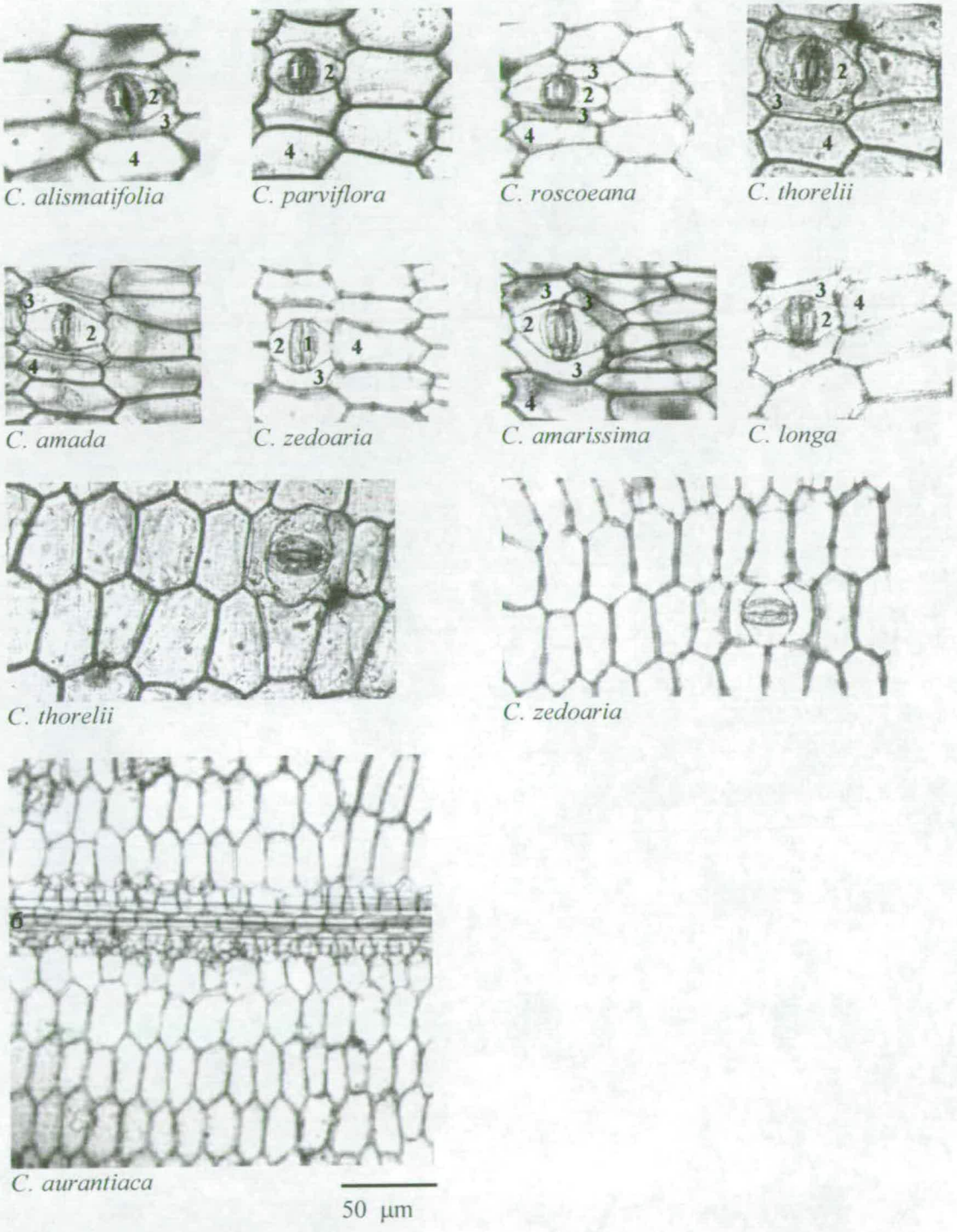


Figure 3.20 Adaxial epidermis in some representative *Curcuma* spp.

1= Guard cells; 2= Lateral subsidiary cells; 3= Terminal subsidiary cells; 4= Epidermis; 5= Hair; 6= Epidermis under the veins.

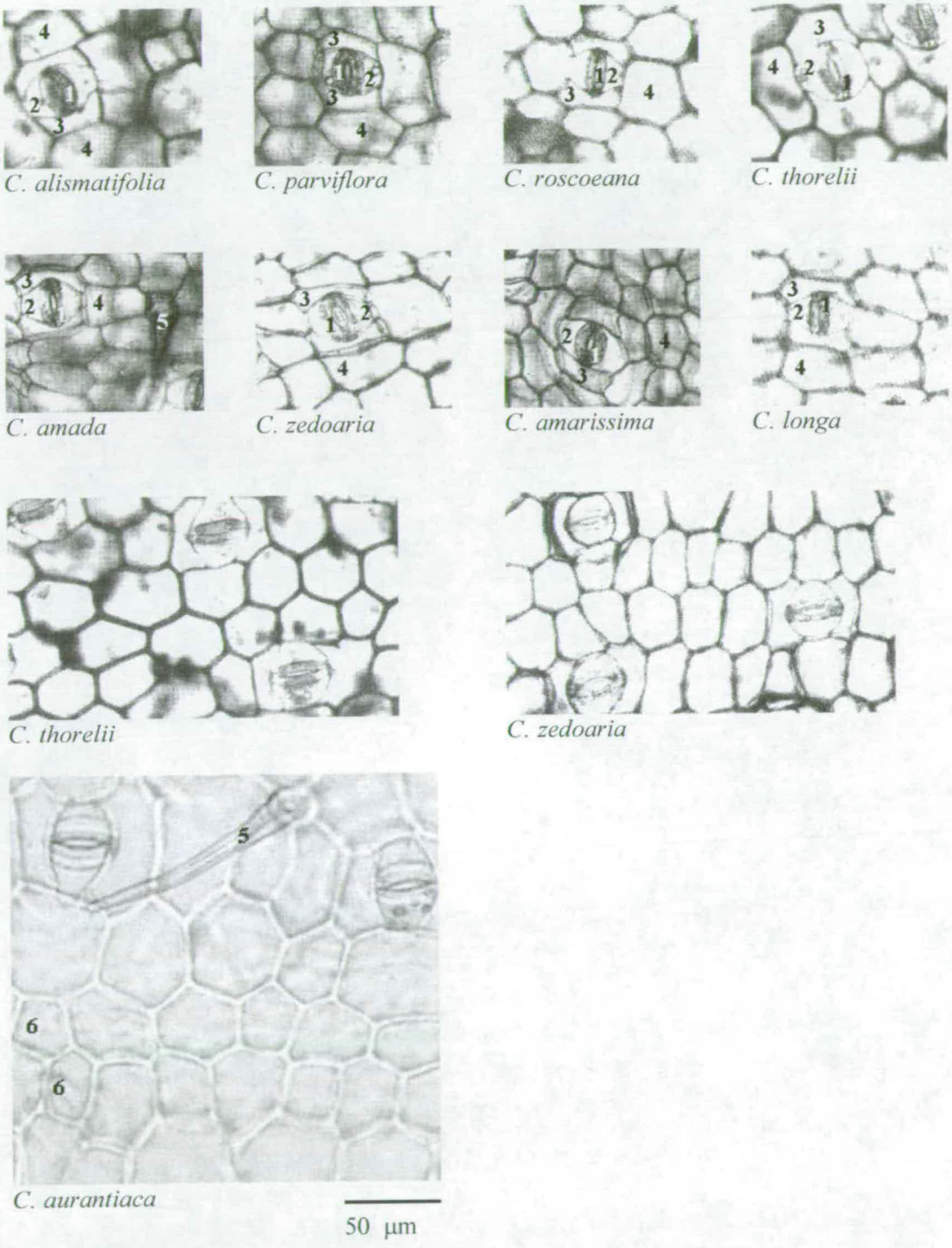


Figure 3.21 Abaxial epidermis in some representative *Curcuma* spp.

1= Guard cells; 2= Lateral subsidiary cells; 3= Terminal subsidiary cells; 4= Epidermis; 5= Hair; 6= Epidermis under the veins.

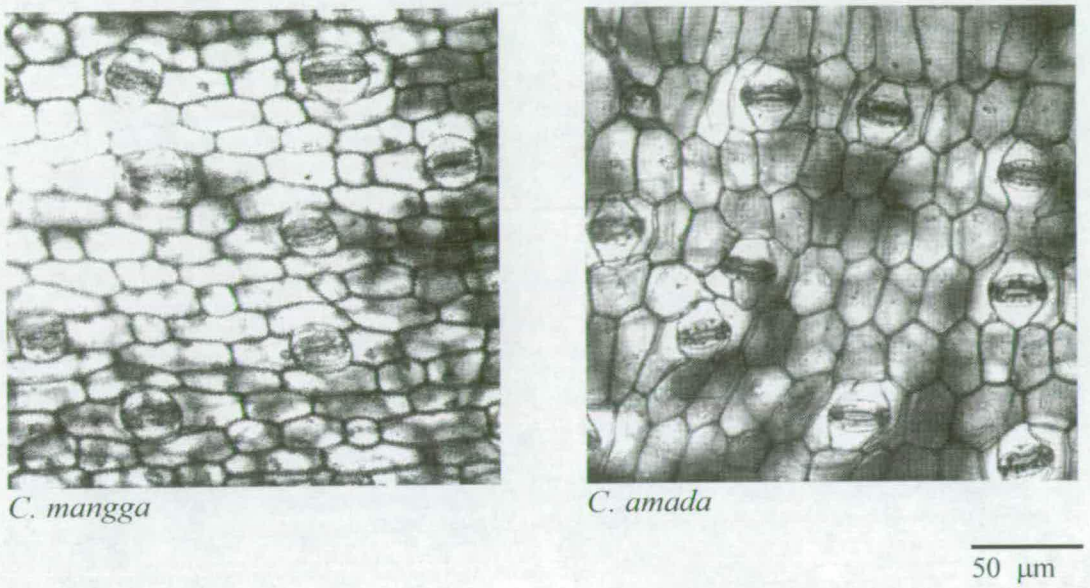
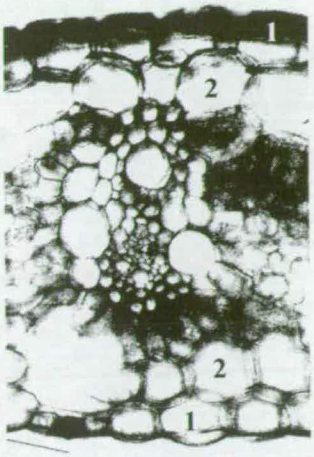
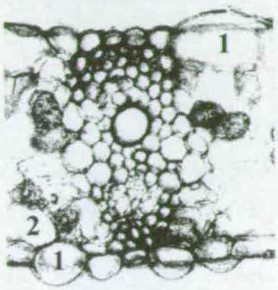


Figure 3.22 Abaxial epidermis of *C. mangga* and *C. amada*.

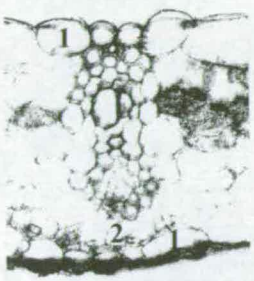
C. heyneana



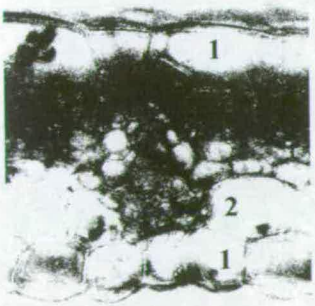
C. heyneana



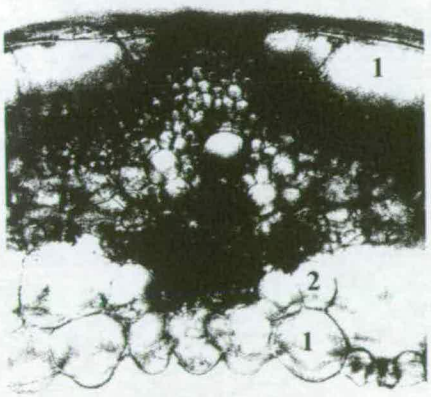
C. longa



C. longa



C. roscoeana



C. roscoeana

Figure 3.23 Leaf transverse section.

1= Epidermis; 2= Hypodermis ——— = 50 μm.

3.4.2.3 Seeds (Figure 3.24)

Only four samples were examined for the SEM of the seeds, they are *C. aurantiaca, C. petiolata, C. aromatica,* and *C. australasica.* Their surfaces show similarity between each other. Under 500x magnification, the surface of *C. australasica* seems to be different from the others at first examination. Bars were seen between the ridges. However, under higher magnification (2000x), these bars are not very clear, and they turn to look like the rest of the species. The seeds of *C. petiolata*, which were taken from herbarium specimens, did not seem to dry up gradually during preservation. Hence their surfaces are artifactual and looked rather different from the rest of the species.

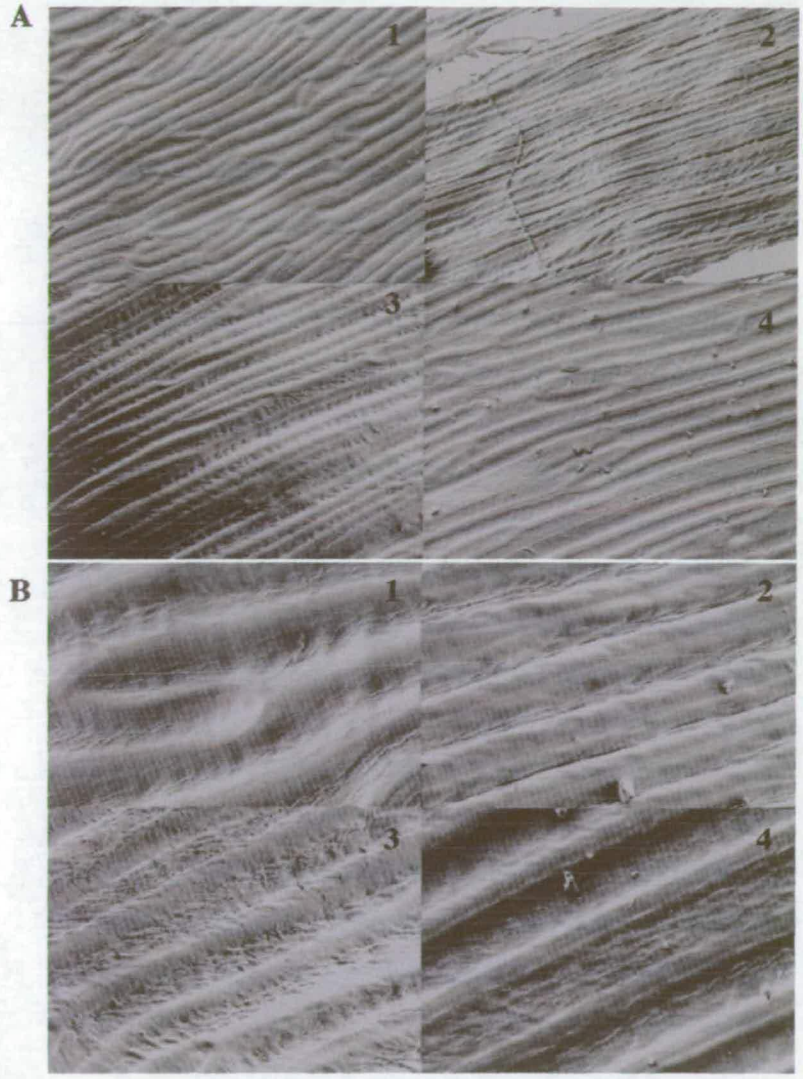


Figure 3.24 SEM of seed surface.
A. Under 500x magnification. **B**. Under 2000x magnification.
1. *C. aurantiaca*, **2**. *C. petiolata*, **3**. *C. cf. australasica*, **4**. *C. aromatica*

3.4.3 MORPHOMETRIC OF EPIDERMIS

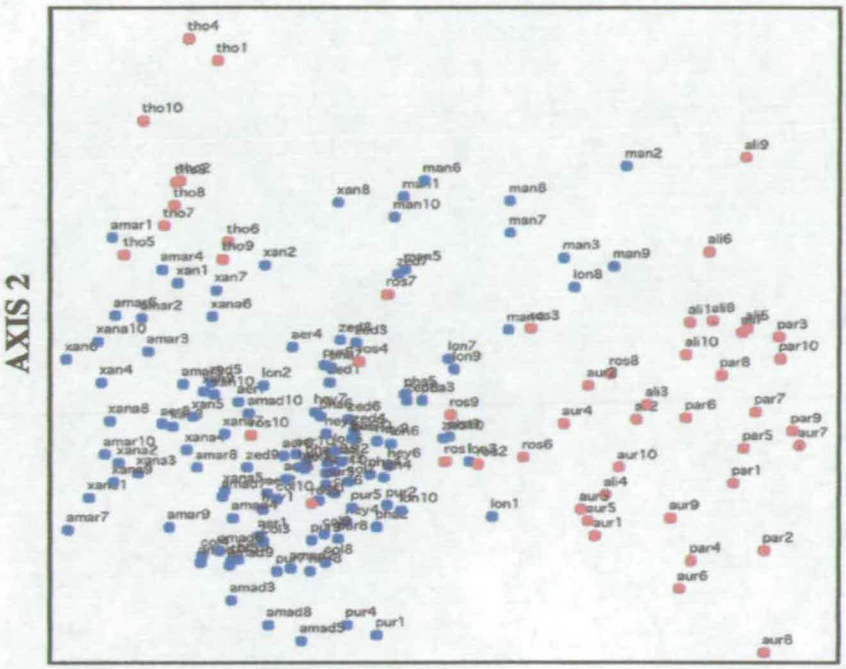
The size of epidermal cells (of adaxial or abaxial) of *Curcuma* subgenus *Hitcheniopsis* is slightly larger than those of *Curcuma* from subgenus. Measurements of epidermal and stomatal cells can be seen in **Appendix 4**. To investigate whether this is significant, morphometric of epidermal cells and stomatal size was carried out (**Table 3.4**). The result shows that no groupings are found in the PCA though one cluster, which consists of *Hitcheniopsis* species, seems quite separate from the rest. *C. roscoeana* and *C. thorelii,* however, form a cluster with species from subgenus *Curcuma* though rather at peripheral (**Figure 3.25**).

The first three components (axes) explained 66.94% of the total variance (**Table 3.4**). The first component, which explains 32.113% of total variance shows strong negative correlation with width and height of adaxial and abaxial stomata, and strong positive correlation with width of adaxial and abaxial epidermis (**Figure 3.25**). This means that species with larger eigenvectors has narrower and shorter stomata and wider epidermis. The second component explains 17.807% of total variance, and is strongly positively correlated with width of abaxial epidermis and strongly negatively correlated with length of abaxial epidermis (**Figure 3.25**). This means that species with larger eigenvectors has wider and shorter abaxial epidermis. The third component explains 17.020% of total variance, and is strongly positively correlated with width of adaxial epidermis and negatively correlated with width of abaxial epidermis and width of adaxial and abaxial stomata (**Figure 3.25**). This means that species with larger eigenvectors has wider adaxial epidermis, narrower abaxial epidermis, and narrower stomata. Subgenus *Hitcheniopsis* generally has wider epidermis and smaller stomata.

The epidermal characters, therefore, are quite uniform within *Curcuma* spp. This is probably related to the function of the epidermis, such as the stomatal for gas exchange during respiration or photosynthesis, which is the same for all *Curcuma*.

Table 3.4 Variance extracted, first 8 axes
(of morphometric of epidermal characters)

AXIS	Eigenvalue	% of Variance	Cum. % Var.	Broken-stick Eigenvalue
1	2.569	32.113	32.113	2.718
2	1.425	17.807	49.920	1.718
3	1.362	17.020	66.940	1.218
4	0.995	12.436	79.377	0.885
5	0.554	6.921	86.298	0.635
6	0.485	6.059	92.356	0.435
7	0.388	4.847	97.203	0.268
8	0.224	2.797	100.00	0.125



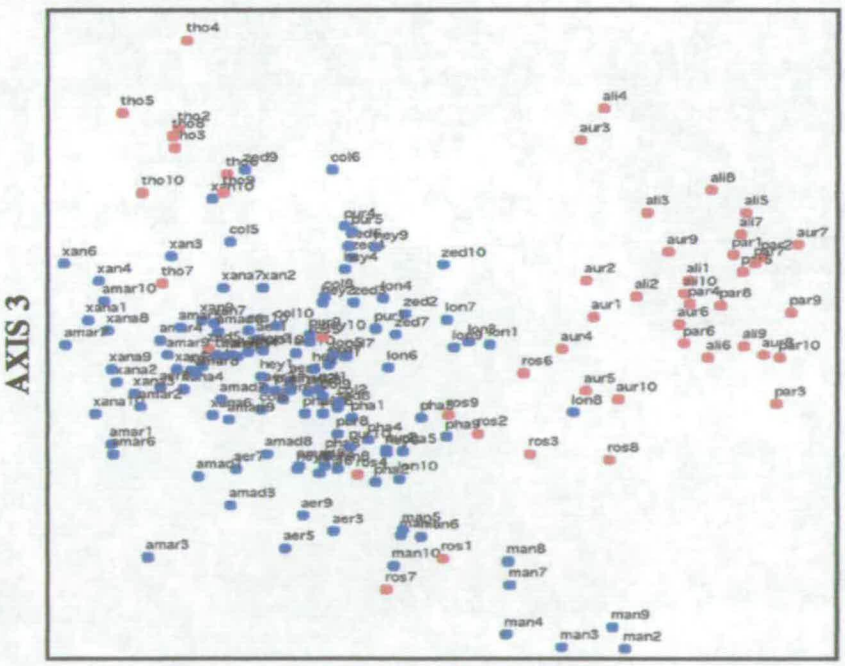


Figure 3.25 Principal component plot of *Curcuma*.
Red dot: subgenus *Hitcheniopsis*, blue dot: subgenus *Curcuma*.

3.4.4 MAPPING MORPHOLOGICAL CHARACTERS OF SOME SPECIES ONTO MOLECULAR TREE (Fig. 3.26-3.28)

3.4.4.1 Habit

The mapping of habit characters onto the molecular tree required only one evolutionary step (**Figure 3.26**). Tall habit, which is possessed by subgenus *Curcuma,* seems to have evolved from small habit, subgenus *Hitcheniopsis,* during the course of evolution. The evolution of the habit is probably interfered by the evolution of chromosome numbers. All of tall species have a polyploid chromosome number.

3.4.4.2 Rhizome structure

The mapping of the rhizome structure onto the molecular tree required one evolutionary step (**Figure 3.26**). Rhizome structure largely showed congruence with combined ITS and *trn*L-F data. Except *C. cf. australasica* and *C. petiolata,* all other species in subgenus *Hitcheniopsis* possess hardly developed rhizome. Well-developed rhizome is also probably related to polyploidy, that leads to development of rhizomes.

3.4.4.3 Colour of internal rhizome

The mapping of colour of internal rhizome onto molecular tree required eight evolutionary steps (**Figure 3.26**). Colour of internal rhizome has evolved several times independently on the tree. The ancestor has whitish rhizome, which evolve to coloured rhizomes. Except *C. cf. australasica,* all species from subgenus *Hitcheniopsis* have whitish rhizome. The colour of rhizomes has some value in systematics. This is an important character to recognize *C. longa* Group.

3.4.4.4 Leaf shape

The mapping of leaf shape onto molecular tree required two steps. Elliptic to elliptic oblong leaf shape dominates the genus. Very lanceolate or rather linear leaf shape is only possessed by *C. alismatifolia* which resembles the outgroup. Therefore, it is an autapomorphy (not illustrated).

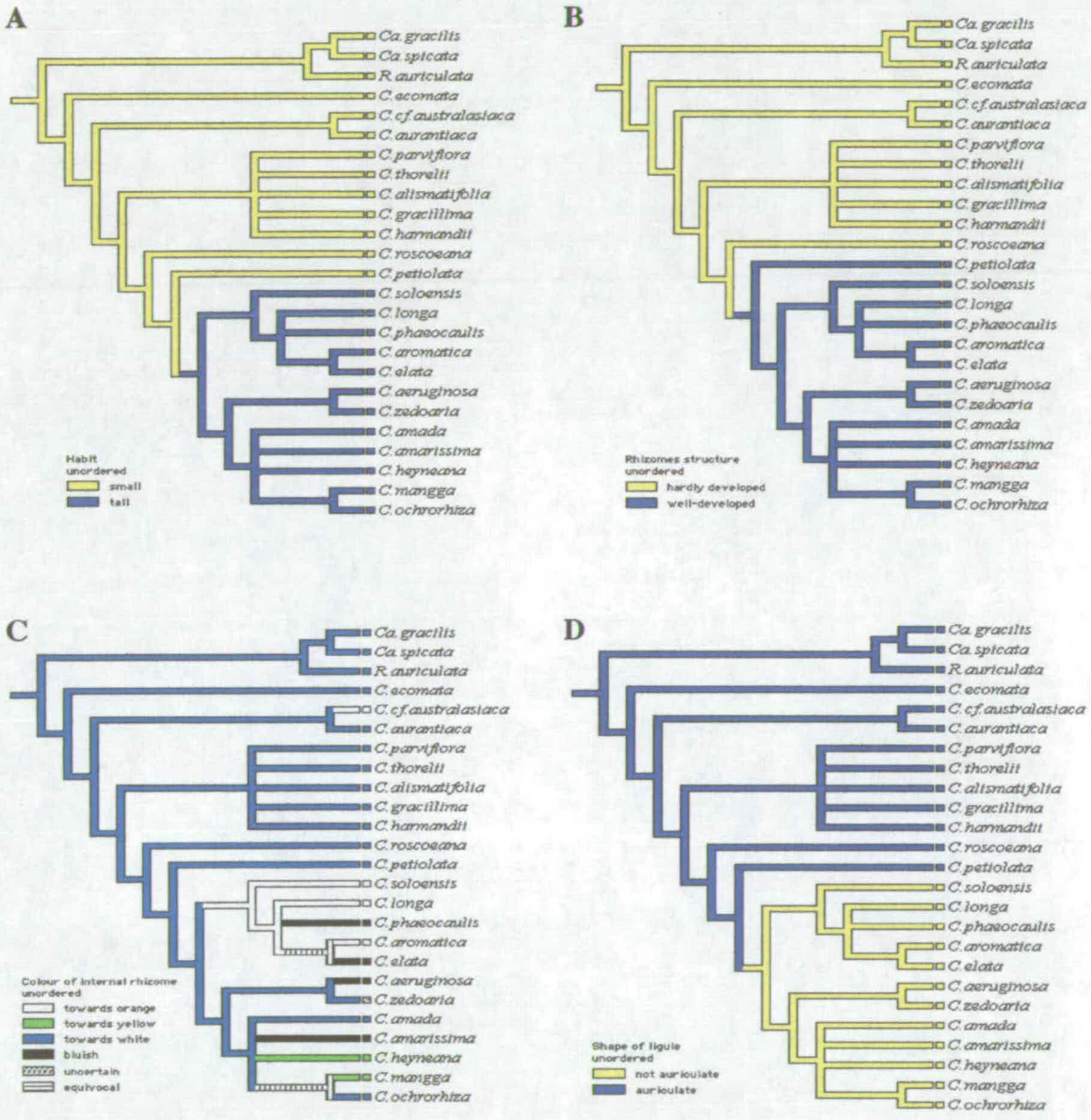


Figure 3.26 Mapping the habit (**A**), rhizome structure (**B**), colour of internal rhizome (**C**), and shape of ligule (**D**) onto molecular tree.

3.4.4.5 Shape of ligule

The mapping of the shape of ligule onto molecular tree required only one step (**Figure 3.26**). This character is nicely fit the subgeneric division. Subgenus *Hitcheniopsis* possess auriculate ligule, while subgenus *Curcuma* does not show this shape. Auriculate ligule has been lost during the course of evolution. Straight ligule replaced the auriculate one, which perhaps suit the habit of subgenus *Curcuma.*

3.4.4.6 Colour of midrib

The mapping of colour of midrib onto molecular tree required only one step. It is of little value, as green midrib dominates the whole taxa, except the only brownish midrib in *C. alismatifolia.* Brownish midrib is also possessed by *C. zanthorrhiza.*

3.4.4.7 Colour of sheaths

The mapping of colour of sheath onto molecular tree required three steps. The mapping is almost the same as that of the colour of the midrib. Green sheath dominates the whole taxa including the outgroup taxa. *C. elata* and *C. phaeocaulis* possess brownish sheath. In some way, this character will easily distinguish for example *C. phaeocaulis* from *C. aeruginosa.*

3.4.4.8 Purple flush on leaves

The mapping of purple flush on leaves onto molecular tree required three evolutionary steps. All the purple flush arises in some species from subgenus *Curcuma.*

3.4.4.9 Position of inflorescence

The mapping of position of inflorescence onto molecular tree required four evolutionary steps (**Figure 3.27**). Position of inflorescence evolved several times independently during the course of evolution. This result proves that character of inflorescence position is homoplastic. Therefore, classification on the basis of this character can be misleading.

3.4.4.10 Shape of bracts

The mapping of the shape of the bracts onto molecular tree required two evolutionary steps. The majority of shape of bract in the genus is elliptic to lanceolate. Only *C. harmandii* has a linear bract. The rest has elliptic or lanceolate bract. The common ancestor probably has linear bract.

3.4.4.11 Length of coma bracts compared to length of fertile bracts

The mapping of the length of coma bracts compared to the length of fertile bracts onto molecular tree required four evolutionary steps. All species from subgenus *Curcuma* have coma bracts that are longer than the fertile bracts. Subgenus *Hitcheniopsis* consists of species which has coma bracts that are longer than the fertile bracts, and also some species which has coma bracts that are shorter than or the same length as the fertile bracts. The short coma bracts appeared to have evolved several times independently.

3.4.4.12 Colour of coma and fertile bracts

The mapping of the colour of coma and fertile bracts onto molecular tree required three evolutionary steps. Stable uniform colour of coma bracts is only possessed by three species, *C. ecomata, C. harmandii,* and *C. roscoeana. C. longa* has two types of colour, ie. uniform colour and different colour between, coma and fertile bracts. Except *C. longa,* all other species from subgenus *Curcuma* have different colour between coma and fertile bracts.

3.4.4.13 Flower structure

The mapping of flower structure onto molecular tree required three evolutionary steps. All species from subgenus *Curcuma* have open big complex type of flower (simple type). Subgenus *Hitcheniopsis* possesses several type of flower, varied from simple, complex to small type. The common ancestor appeared to have complex type. Then it was switched to simple type in *C. australasica.* It was switched to small type in

C. thorelii, C. parviflora, and *C. gracillima.* During the course of evolution, it appeared that the complex type lost, and a simple type is attained in species from subgenus *Curcuma.* This phenomenon is most likely related to the pollination system of the taxa (see Chapter Four).

3.4.4.14 Shape of petals

The mapping of the shape of petals onto molecular tree required one evolutionary step. Shape of petals is elliptic in the majority of species. The exception is *C. ecomata,* which has lanceolate petal.

3.4.4.15 Colour of petals

The mapping of colour of petals onto molecular tree required five evolutionary steps. The majority of the species have yellowish or oranges petal. If we re-map flower structure onto colour of petals, we can see that except *C. ecomata* and *C. roscoeana,* all complex and small flower have whitish or greenish petal. Simple type, in general, has yellowish or reddish petal.

3.4.4.16 Cucullate on dorsal petal

The mapping of cucullate on dorsal petal onto molecular tree required three evolutionary steps. Cucullate on dorsal petal is prevalent in subgenus *Curcuma*, except the two species, *C. cf. australasica* and *C. petiolata.* The common ancestor appeared to have dorsal petal without cucullate.

3.4.4.17 Shape of labellum

The mapping of shape of labellum onto molecular tree required three evolutionary steps. The complex and small group, except *C. roscoeana,* have elongate labellum. On the other hand, the complex types have roundish labellum. The common ancestor appeared to have elongate labellum, which re-appear in complex and small type clade (*C. thorelii, C. gracillima, C. alismatifolia,* and *C. harmandii*).

3.4.4.18 Colour of labellum

The mapping of colour of labellum onto molecular tree required two evolutionary steps. It is almost congruence with the mapping of the shape of labellum. The exception is *C. ecomata.* Species with elongate labellum have purple or white and purple colour, while the simple types have yellow or orange labellum. In the majority of species with simple type, their colours of labellum are yellow or orange. The majority of complex or small species have purple or white with purple labellum.

3.4.4.19 Hair on labellum blade

The mapping of hair on labellum blade onto molecular tree required one evolutionary step. The majority of species have no hair on the labellum blade. *C. ecomata* is the only species which has hair on the labellum blade.

3.4.4.20 Hair on the middle band of the labellum

The mapping of hair on labellum blade onto molecular tree required one evolutionary step. Hair on labellum band (**Figure 3.27**) is only possessed by four species in the study, *C. thorelii, C. parviflora, C. gracillima* (the small type), and *C. harmandii.* Those four species have no hair on the labellum blade.

3.4.4.21 Shape of lateral staminodes

The mapping of this character onto molecular tree required one evolutionary step. The majority of species in the study have obovate to oblanceolate lateral staminodes. Linear odd lateral staminode is only possessed by *C. harmandii.*

3.4.4.22 Arrangement of lateral staminodes

The mapping of arrangement of lateral staminodes onto molecular tree required two evolutionary steps (**Figure 3.27**). The complex and small clade (*C. thorelii, C. gracillima, C. alismatifolia,* and *C. harmandii*) has free lateral staminodes, not like the rest of the species, which have clasping lateral staminodes. The flower of species on the

clade have little opening, while those of simple type, which have clasping lateral staminodes, have wide opening.

3.4.4.23 Colour of lateral staminodes

The mapping of the colour of lateral staminodes onto molecular tree required three evolutionary steps. The complex and small clade has whitish lateral staminodes (*C. thorelii, C. gracillima, C. alismatifolia*) and greenish colour (*C. harmandii*). The rest of the taxa except the outgroup have yellowish or orangish colour, which means the same as the rest of flower parts, such as petals and labellum.

3.4.4.24 Groove on lateral staminodes

The mapping of groove on lateral staminodes onto molecular tree required only two evolutionary steps (**Figure 3.27**). This character is congruence with the division of the genus at subgeneric level. The outgroups have lateral staminodes without groove except in *R. auriculata*. The groove that I observed is perhaps not homolog in *R. auriculata*. The common ancestor appeared to have evolved from lateral staminodes without groove. Subgenus *Curcuma* clade seemed to attain groove on the lateral staminodes during the course of evolution.

3.4.4.25 Patch of granules at apex of lateral staminodes

The mapping of patch of granules at the apex of lateral staminodes onto molecular tree required two evolutionary steps. Granule patch, which supposedly indicates pigment cells, appeared to be in majority of the species in subgenus *Curcuma*. Subgenus *Hictheniopsis* lacks these pigment cells, except in two species *(C. cf. australasica* and *C. petiolata*). If we re-map flower type onto this character, it appeared that complex species have these pigment cells, except in *C. aurantiaca*. The cells are probably required as signals by the pollinator. Further more data are required to answer this phenomenon.

3.4.4.26 Length of anther

The anther is short in all taxa except one species, *C. ecomata.* The mapping of this character onto molecular tree required one evolutionary step. The common ancestor appeared to have evolved from long anther. During the course of evolution, the long anther was replaced by short anther that fits the structure of flower.

3.4.4.27 Spur on anther

Spur on anther is not completely congruence with the division at subgeneric level (**Figure 3.28**). The mapping of this character onto molecular tree required three evolutionary steps. The anomaly is *C. ecomata, C. cf. australasica* and *C. petiolata* that have spur on the anther.

3.4.4.28 Crest on anther

The mapping of crest on anther onto molecular tree required three evolutionary steps (**Figure 3.28**). All subgenus *Curcuma* have no crest on the anther, while all species from subgenus *Hitcheniopsis* except *C. petiolata* and *C. cf. australasica* have crest on the anther. The outgroup has crest on the anther. The reappearance of the anther crest is odd and interesting. Perhaps the common ancestor has a crest on the anther, and then this was lost during the course of evolution.

3.4.4.29 Anther dehiscence

The mapping of anther dehiscence onto molecular tree required one evolutionary step. Anther dehiscence that stretches out along locules up to the base is only possessed by *C. aurantiaca.*

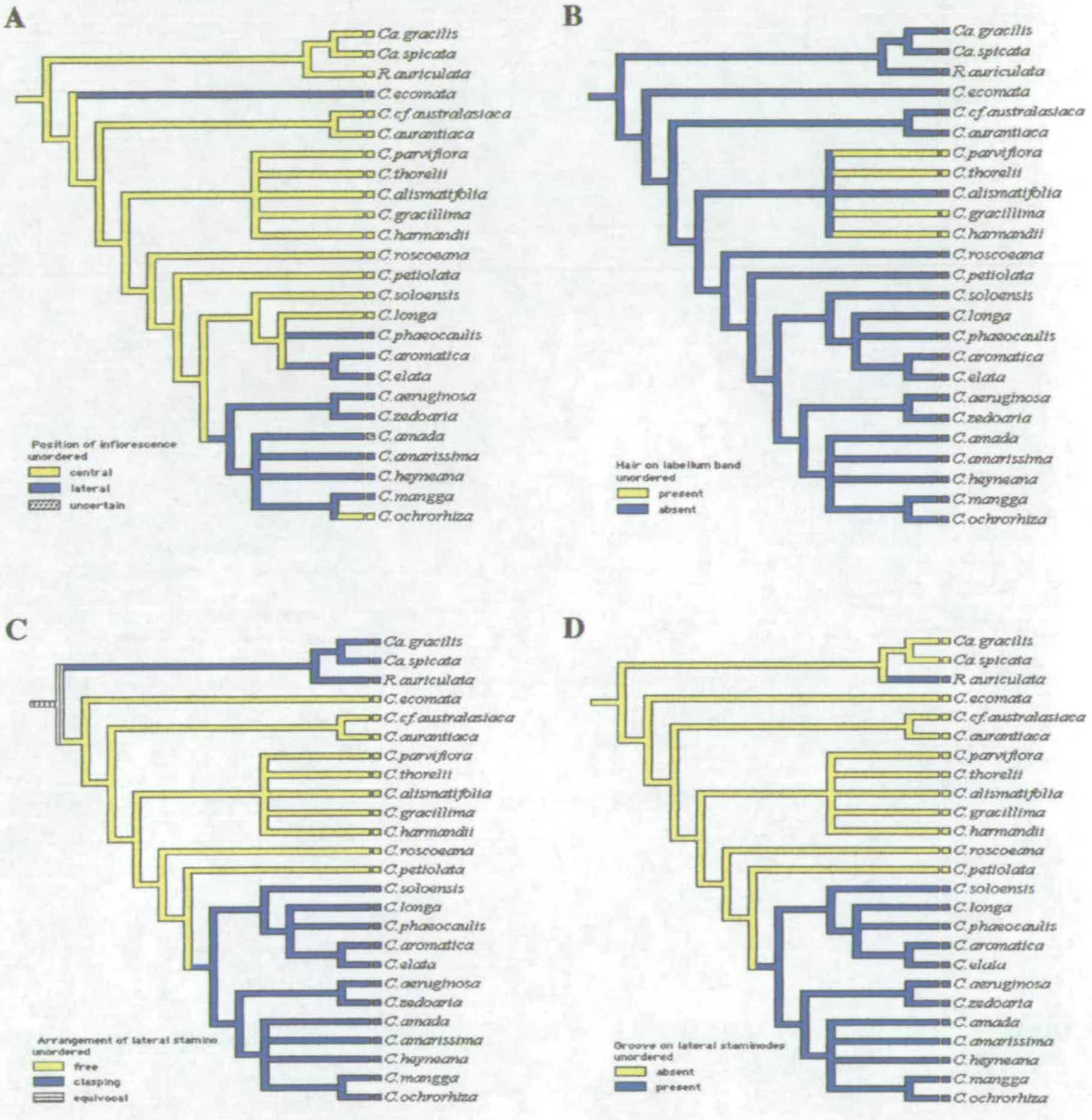


Figure 3.27 Mapping position of inflorescence (**A**), hair on the midddle band of the labellum (**B**), arrangment of lateral staminodes (**C**) onto molecular tree, and groove on lateral staminodes (**D**).

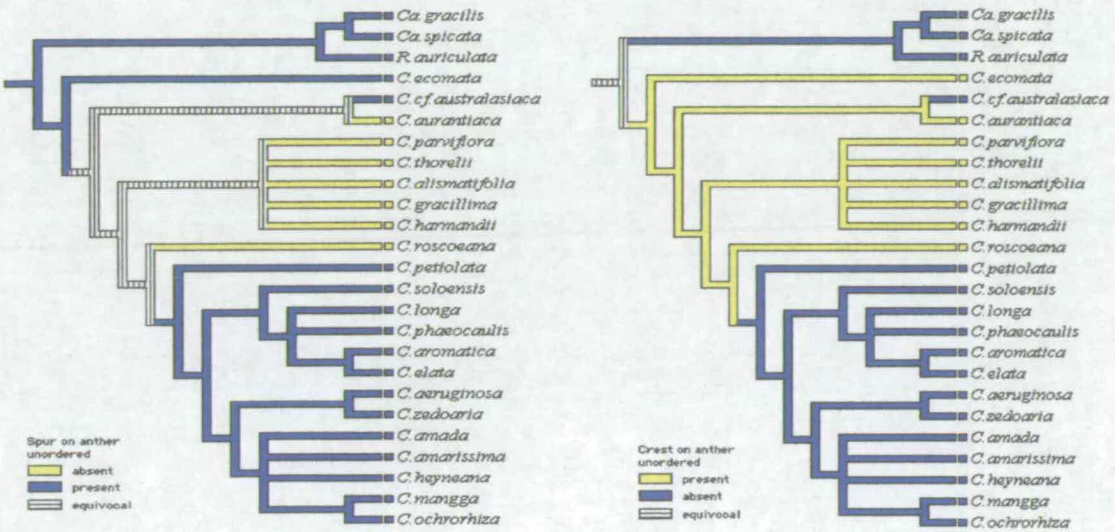


Figure 3.28 Mapping spur on anther (**A**) and crest on anther (**B**) onto molecular tree.

3.4.4.30 Stigma type

The mapping of stigma type onto molecular tree required three evolutionary steps. All species from subgenus *Hitcheniopsis,* except *C. aurantiaca* and *C. petiolata,* have non-inflated stigma. On the other hand, subgenus *Curcuma* has inflated stigma. The common ancestor appeared to have evolved from funnel-shaped stigma. During the course of evolution, inflated stigma was gained in subgenus *Curcuma.*

3.4.4.31 Stylar growth

The mapping of stylar growth onto molecular tree required only one evolutionary step. Stylar has been lost in the complex and small flower clade (*C. thorelii, C. gracillima, C. alismatifolia,* and *C. harmandii*).

3.4.4.32 Ring of hair on corolla tube

The mapping of ring of hair on corolla tube onto molecular tree required two steps. Ring of hair on corolla tube is congruence with the simple flower type. The subgenus *Curcuma* clade with *C. petiolata* and a clade of *C. cf. australasica* and *C. aurantiaca* possess the ring. The ring present in a constriction part along the throat of the flower.

3.5 DISCUSSION

3.5.1 PHYLOGENETIC RELATIONSHIPS WITHIN CURCUMA

3.5.1.1 C. ecomata

C. ecomata, are nested at the basal next to the outgroups. This is supported by the result of the analysis from molecular data. Five characters namely lateral inflorescence, coma bracts as long as fertile bracts, hairy dorsal petal at the tip, hairy lebellum blade, are autapomorphies of the species. The anther which is long is very similar to that of the outgroups. As explained in Chapter Two (p.72), Gagnepain (1908) placed *C. ecomata* in subgenus *Curcuma*. All species in subgenus *Hitcheniopsis* have no spur and have central position of inflorescence. The spurred anther and the lateral position of inflorescence are the characters that possibly encouraged him to place the species in the subgenus *Curcuma*. Yet the result from morphological data does not support his grouping either.

3.5.1.2 C. aurantiaca *and* C. cf. australasica

Analysis of the morphological data shows that *C. aurantiaca* and *C. cf. australasica* do not form a clade. This result is in contrast with that of the ITS data which showed strong support for the *C. aurantiaca* and *C. cf. australasica* clade. The different colour of coma and fertile bracts and the presence of hairy ring inside the corolla tube are synapomorphies of *C. aurantiaca, C. cf. australasica, C. petiolata,* and species in subgenus *Curcuma*. Anther dehiscence which is along the locules up to the base is the autapomorphy of *C. aurantiaca*.

Analysis of the morphological data shows that *C. cf. australasica* is more closely related to subgenus *Curcuma*. Several synapomorphies unite the species with *C. petiolata* and subgenus *Curcuma*. These characters are the simple flower type, the presence of hair on the ovary, the presence of hair on the dorsal petal, the presence of cucullate on the dorsal petal, the presence of granules patch (at apex) on the lateral staminodes, the spurred anther. The stigma which is inflated is the autapomorphy of *C. cf. australasica*.

C. aurantiaca and *C. cf. australasica* are placed in subgenus *Hitcheniopsis* by Valeton (1918). This is supported by the result of the analysis of morphological data. The two species are nested between the other species of subgenus *Hitcheniopsis, C. roscoeana* and *C. petiolata*. As it has been explained in Chapter Two (p.73), Schumann (1904) placed *C. australasica* within subgenus *Curcuma* on the basis of the rhizomes character. Both types of rhizome characters possibly occur. More samples of *C. australasica* are needed to verify this.

The close relationship between *C. cf. australasica* and the species in subgenus *Curcuma* hypothesize that the subgenus *Curcuma*, which are triploid sterile, possibly were derived from *C. cf. australasica*, which is diploid fertile, as one of the parentage. However, analysis of the ITS region shows that *C. cf. australasica* is more closely related to subgenus *Hitcheniopsis*. More molecular data is needed to verify the relationship between *C. cf. australasica* and the subgenus *Curcuma*.

3.5.1.3 The C. parviflora *clade*

The clade, which contains five species namely *C. parviflora, C. thorelii, C. alismatifolia, C. gracillima,* and *C. harmandii,* is not well supported by the analysis of morphological data. However, it is strongly supported by molecular analysis of the ITS region. Six characters unite the clade, i.e. the complex and small types of flower, whitish petals (greenish in *C. harmandii*), purple or white with purple labellum, presence of hair on the labellum band (except in *C. alismatifolia*), whitish lateral staminodes (greenish in *C. harmandii*), and the absence of the stylar growth. As it has been explained in Chapter Two (p.74), Valeton (1918) excluded these species from the genus

Curcuma. The exclusion of the species from the genus will make *Curcuma* a paraphyletic group, as the *C. parviflora* clade is nested between *C. ecomata* and *C. roscoeana*. Therefore, I suggest that the species in the *C. parviflora* clade are not transferred to any other genera. More sampling of *Curcuma* will possibly be able to verify this.

3.5.1.4 *The subgenus* Curcuma *clade*

The subgenus *Curcuma* clade is not only strongly supported by the molecular analysis but also is supported by the analysis of morphological data. However, the two subclades which exist from the analysis of molecular data are not found in the tree resulted from the morphological analysis. One subclade which contains *C. elata, C. amarissima, C. zanthorrhiza, C. aeruginosa,* and *C. phaeocaulis* is found from the morphological analysis.

The clade share common characters of the large habit, the non-auriculate ligule, the clasped and the grooved lateral staminodes. As it has been explained in Chapter Two (p.75), the species in this subgenus are not good species. The result of morphological analysis, therefore, also reveals the natural grouping of Baker's (1894) sections *Exantha/Mesantha*, and Schumann's (1904) and Valeton's (1918) subgenus *Curcuma*.

The characters that unite the subclade *C. elata* are the bluish colour of the internal rhizome (except in *C. zanthorrhiza*), the brownish leaf sheath (except in *C. aeruginosa* and *C. zanthorrhiza*), the presence of the purple flush on leaves (except in *C. elata*), and the yellowish petals (except in *C. zanthorrhiza, C. aeruginosa,* and *C. phaeocaulis*).

The sectional level in the all existing classification of *Curcuma* is not supported by morphological analysis. *Exantha* and *Mesantha* (sections of Baker) are scattered in the tree. Sectional level should be abandoned. This is supported by molecular analysis also.

3.5.1.5 C. roscoeana *and* C. petiolata

The result of the analysis from the morphological data shows that the close relationship of both species found in the analysis of the molecular data is separated by the insertion of *C. aurantiaca* and *C. cf. australasica.* However, *C. roscoeana* is more closely related to the subgenus *Hitcheniopsis* and *C. petiolata* is more closely related to the subgenus *Curcuma.* Therefore, the result of both the analyses differ.

The position of *C. roscoeana* in the tree resulted from morphological data supports the placement of the species in Baker's (1894) section *Hitcheniopsis* and Schumann's (1904) subgenus *Hitcheniopsis.* The morphological result, supported by molecular data, showed that the exclusion of the species by Valeton (1918) is not supported. The position of *C. petiolata* in the tree resulted from morphological data supports the placement of the species in Baker's (1894) section *Hitcheniopsis*, Schumann's (1904) and Valeton's (1918) subgenus *Hitcheniopsis.* As mentioned in Chapter Two (p.76), the species has intermediate characters between the two subgenera.

CHAPTER 4: EVOLUTION OF FLORAL DIVERSITY IN *CURCUMA*

4.1 INTRODUCTION

Floral morphology is one of the most important aspects of plant-pollinator interactions. It affects pollinator accessibility to nectar, efficiency of pollen deposition on the pollinator body, and efficiency of pollen acquisition by the stigma from the pollen vectors (Sakai *et al.* 1999). Variation in the site of pollen placement on the pollinator body can promote coexistence of plant species sharing common pollinators (Armbruster *et al.* 1994 in Sakai *et al.* 1999). Pollination syndromes is discussed earlier in the thesis (p. 81).

Sakai *et al.* 1999 show that among 29 species studied, which represent 11 genera of Zingiberaceae in rain forest in Borneo, eight were pollinated by spiderhunters (Nectariinidae), 11 by medium-sized *Amegilla* bees (Anthophoridae), and ten by small halictid bees. One single genus could have more than one pollinator, for example in *Amomum,* three pollination guilds (Spiderhunter, *Amegilla,* and small halictid bees) pollinated the genus.

Alpinia glabra Ridl., *Globba brachyanthera* K. Schum., *Amomum calyptratum* Nagam. & S. Sakai, *A. gyrolophos* R.M. Sm., *A. oliganthum* K. Schum., *Elettariopsis* sp., *Plagiostachys* sp., *P. crocydocalyx* (K. Schum.) B.L. Burtt & R.M. Sm., *Zingiber longipedunculatum* Ridl. were pollinated by Amegilla bees (Sakai et al. 1999). Prana (1977) observed *Amegilla* probably *A. buruensis* and *A. elegans,* visiting the flowers of *Curcuma* quite frequently and regularly. *Amegilla* are solitary bees that make nests underground. They were observed all year-round at the study site of Sakai *et al.* 1999. Amegilla-pollinated flowers had wider lips than other ginger species, which function as a platform for the pollinators (Sakai *et al.* 1999).

Most of *Boesenbergia,* such as *B. grandifolia* (Valeton) Merr., *B. gracilipes* (K. Schum.) R.M. Sm., *B.* aff. *variegata* R.M. Sm., *Elettaria* sp., *E. longituba* (Ridl.) Holt., *Amomum coriaceum* R.M. Sm., *A. durum* Nagam. & S. Sakai, *A. polycarpum* (K.

Schum.) R.M. Sm., *A. somniculosum* S. Sakai & Nagam, *Elettariopsis sp.*, *E.* aff. *kerbyi* R.M. Sm. were pollinated by small halictid bees (Sakai *et al.* 1999). The small whitish or greenish flowers in *Curcuma,* such as *C. parviflora, C. thorellii, C. gracillima, C. harmandii,* maybe pollinated by small halictid bees as well. The construction of the flowers of those *Curcuma* spp., such as the length of the labellum, the width of the labellum, the length of the anther, the length of the filament, the width of the filament and the width of the stigma, almost overlaps with that of the flowers pollinated by small halictid bees in Sakai's study.

Etlingera, Hornstedtia, Amomum roseisquamosum Nagam & S. Sakai, *Plagiostachys strobilifera* (Baker) Ridl. were pollinated by spiderhunters. The pollination guilds found in gingers in Sarawak are comparable to those of neotropical Zingiberales, namely hummingbird-, and euglossine-bee-pollinated guilds (Sakai *et al.* 1999).

Multivariate analysis has been applied in several taxonomical and pollination studies in plants and animals (Ortiz *et al.* 1999, Sakai *et al.* 1999). In pollination studies, floral morphology has usually been described qualitatively. Morphological similarity of the flowers in the same or different pollination guilds has rarely been quantified (Sakai *et al.* 1999). The correlation between flower morphology and pollination systems has been quantitatively studied (Sakai *et al.* 1999). An attempt to classify flower types that are likely to be related to pollination syndrome has also been made (Harrison *et al.* 1999).

There are relatively few studies on the pollination ecology of *Curcuma. Amegilla* was observed to be the pollinator of Javanese *Curcuma* (Prana 1977). The flowers in *Curcuma* spp. seem to have some different patterns. To check that those flowers have certain types, a multivariate Principal Component Analysis study was carried out.

4.2 MATERIALS

Information on the taxa used is tabulated in **Table 4.1**.

4.3 METHODS

4.3.1 Morphometric procedure

Morphometric analysis in the form of an ordination has been carried out in an attempt to classify *Curcuma* flowers on the basis of their quantitative variation. Twenty-eight measurements (**Table 4.2**) from single flowers of 22 species of *Curcuma* were taken (**Appendix 5**). Specimens were from fieldwork in Java (M. Ardiyani), a field expedition to Thailand (M. Newman & C. Ngamriabsakul), and species cultivated at the Royal Botanic Garden Edinburgh, UK. The drawing can be seen in **Figure 4.1**. Measurement was carried out under the light microscope, then converted to mm by calibration. Data were finally analysed with PC-Ord Program Version 3.18 (McCune & Mefford 1995) with principal component analysis option. Due to limited accessions, only five flowers of different *C. longa* were measured to check intraspecific variability.

4.3.2 Phylogenetic analysis and mapping

Mapping of floral characters onto a molecular phylogeny can help elucidate the evolutionary origins of floral variation within *Curcuma*. Floral characters were mapped onto a molecular tree based on internal transcribed spacer (ITS) sequence data (Ardiyani *et al.*, unpubl. res. see Chapter Two). Some species included in the morphometric analysis were omitted from the phylogeny because of unavailability of sequence data. Analysis of character-state transitions was performed in MacClade Version 3.08a (Maddison & Maddison 1999). Where flower material was unavailable in those accessions used in phylogenetic construction, different accessions were used for the morphometric analysis, namely *C. aromatica, C. cf. australasica, C. petiolata,* and *C. purpurascens* (see **Table 4.1**).

Table 4.1 *Curcuma* taxa used in this study

Species	Authority	Abbreviation	Accession number/Source	Distribution
C. aeruginosa	Roxb.	AER	RBGE 19780186	Burma, cultivated
C. alismatifolia	Gagnep.	ALI	M944 (E)	China, Thailand
C. amada	Roxb.	AMAD	RBGE 19810001	India, Thailand
C. amarissima	Roscoe	AMAR	RBGE 19871252	India, Thailand
C. aromatica	Salisb.	ARO	G&H1066 (E)	India, Thailand
			DNA: R.C. Joshi 4/1982 (E)	
C. aurantiaca	Zijp	AUR	35MA (BO)	Thailand, Java, cultivated
C. cf. australasica	Hook.f.	AUS	Woods 115 (E)	Australia, New Guinea
			DNA: K.M. Nagata 2312 (E)	
C. colorata	Valeton	COL	RBGE 19771290	Java
C. ecomata	Craib	ECO	CNG38 (E)	Thailand
C. euchroma	Valeton	EUC	49MA (E, BO)	Java
C. gracillima	Gagnep.	GRA	CNG60 (E)	China, Thailand
C. harmandii	Gagnep.	HAR	CNG46 (E)	China, Thailand
C. heyneana	Valeton & Zijp	HEY	RBGE 19780189	Java
C. longa (1)	L.	LON1	RBGE 19931919	cultivated throughout the tropics
C. longa (2)	L.	LON2	RBGE 19721701	cultivated throughout the tropics
C. longa (3)	L.	LON3	RBGE 19720174	cultivated throughout the tropics
C. longa (4)	L.	LON4	RBGE 19782126	cultivated throughout the tropics
C. longa (5)	L.	LON5	RBGE 19711837	cultivated throughout the tropics
C. mangga	Valeton & Zijp	MAN	RBGE 19780191	Java
C. parviflora	Wall.	PAR	RBGE 19851661	Burma, Thailand, Malay Peninsula
C. petiolata	Roxb.	PET	RBGE 19771293	Burma, Thailand, Malay
			DNA: K.M. Nagata 3871 (E)	Peninsula, Java
C. purpurascens	Blume	PUR	RBGE 19780193	Java
			DNA: K.M. Nagata 3886 (E)	
C. roscoeana	Wall.	ROS	RBGE 19973658	Burma, Thailand

Table 4.1 (continued) *Curcuma* taxa used in this study

Species	Authority	Abbreviation	Accession number/Source	Distribution
C. soloensis	Valeton	SOL	47MA (BO)	India, Java
C. thorelii	Gagnep.	THO	M945 (E)	Indochina, Thailand
C. zanthorrhiza	Roxb.	ZAN	RBGE 19740965	China, Thailand, Ambon

Note: E is Edinburgh herbarium; BO is Herbarium Bogoriense; MA= Marlina Ardiyani; M= Mark Newman; CNG: Chatchai Ngamriabsakul; RBGE: Royal Botanic Garden Edinburgh.

Table 4.2 Characters scored for morphometric analysis.

Character Number	Measurement (mm)
1	Height of ovary
2	Length of calyx
3	Length of corolla tube
4	Circumference of corolla tube at the base
5	Circumference of corolla tube at the opening
6	Length of dorsal corolla lobe
7	Width of dorsal corolla lobe at the widest point
8	Length of lateral corolla lobe at the shortest point from the base
9	Length of lateral corolla lobe at the longest point from the base
10	Width of lateral corolla lobe at the widest point
11	Length of lateral staminode at the shortest point from the base when flattened
12	Length of lateral staminode at the longest point from the base when flattened
13	Width of lateral staminode at the base
14	Width of lateral staminode at the widest point
15	Length of labellum when flattened
16	Width of labellum at the base
17	Width of labellum at the widest point
18	Length of the whole flower when flattened
19	Length of anther (only thecae)
20	Width of anther
21	Thickness of anther at the narrowest point
22	Thickness of anther at the widest point
23	Length of anther crest
24	Height of filament from the insertion of lateral staminode to corolla tube
25	Width of filament at the base
26	Width of filament at the narrowest point
27	Length of stigma
28	Width of stigma

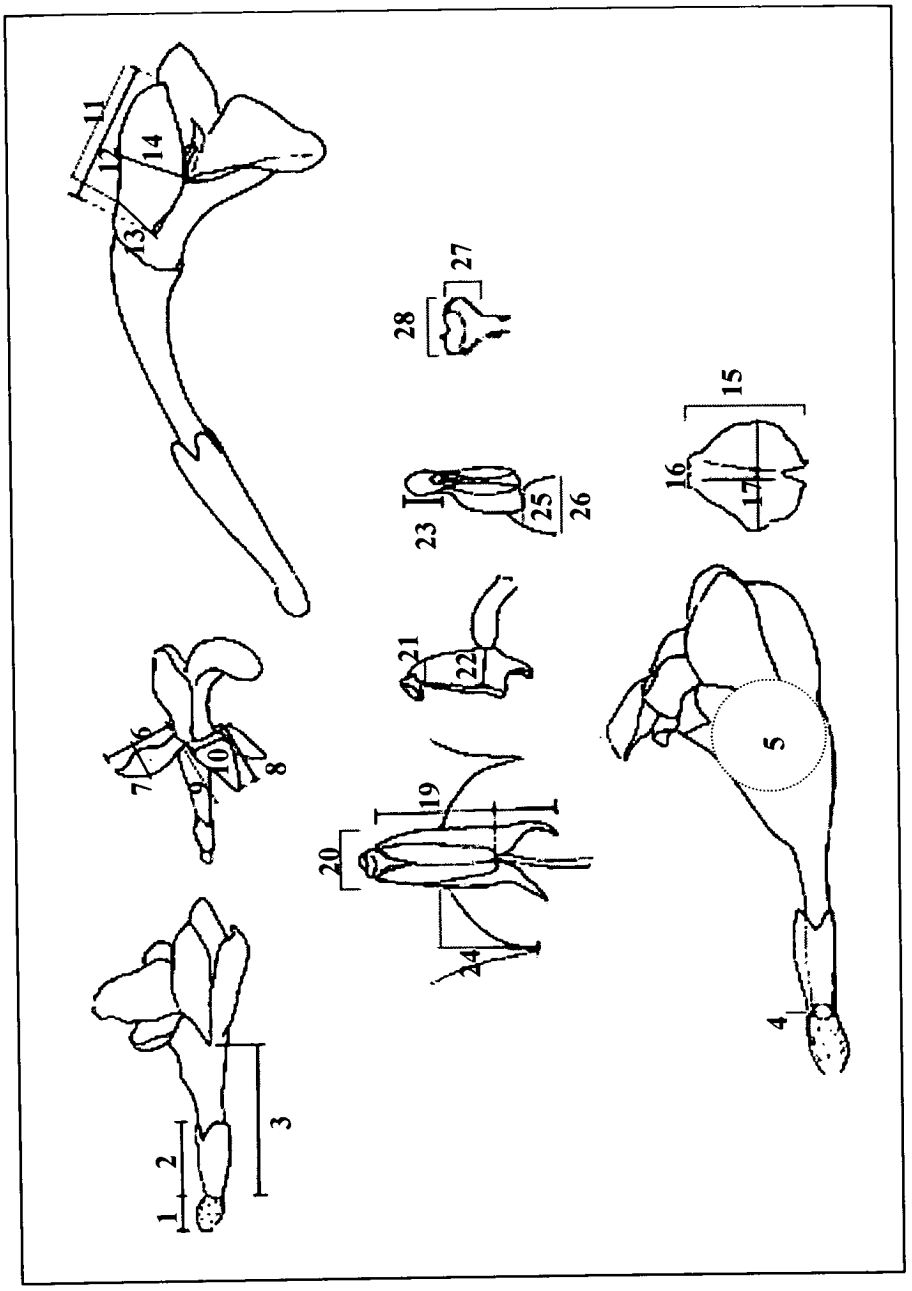


Figure 4.1 Measurement of flowers for morphometric analysis. (1 to 28, except 18, indicates characters scored in **Table 4.2**)

4.4 RESULTS AND DISCUSSION

4.4.1 AXES: CHARACTERS DETERMINED THE GROUPINGS

The eigenvalues for the first and the second axes are 17.440 and 3.592 respectively. The broken-stick eigenvalues for the first and the second axes are 3.927 and 2.927. Therefore, both the broken-stick eigenvalues are less than the eigenvalues. If the broken-stick eigenvalue is less than the actual eigenvalue for an axis, then the axis contains more information than expected by chance and should be considered for interpretation (McCune & Mefford 1995). The third axis contains a broken-stick eigenvalue which is higher than the eigenvalue.

The first two components (axes) explained 75.11% of the total variance. The first component, which explains 62.28% of total variance (**Table 4.3**), shows strong negative correlation with the overall size of the flower (**Figure 4.2**). This means that flowers with larger eigenvectors have smaller overall size of flower. The largest flower is that of *C. zanthorrhiza,* while the smallest flower is that of *C. gracillima.* Flower of three species (*C. gracillima, C. parviflora,* and *C. thorelii*) make up a group of species with small flowers. The rest of the species of *Curcuma* have larger flowers.

The second component explains 12.83% of total variance (**Table 4.3**), and is positively correlated with length of the anther crest, length of the lateral staminodes at the shortest point, length of the calyx, and length of the anther, and is negatively correlated with width of stigma (**Figure 4.2**). This means that flowers with larger eigenvectors have longer anther crests, longer lateral staminodes, longer calyces, longer anthers, and narrower stigmas. The larger eigenvector for the second axis makes the grouping of five species, *C. roscoeana, C. alismatifolia, C. ecomata, C. harmandii,* and *C. aurantiaca.* The type of flowers of those species is called complex. The flowers of species which have smaller eigenvectors of the first and the second axes make a group called simple flowers. The simple flower, therefore, has larger size, shorter anther crest, shorter lateral staminode, shorter calyx, shorter anther, and wider stigma.

Figure 4.2 Eigenvectors of the first and the second axes for the 28 floral characteristics

4.4.2 FLOWER GROUPINGS

All three axes quite clearly define three groups of flower. As explained previously (p. 146), the three groups are the simple, the complex and the small (**Figure 4.3**). The simple flower contains all the species from subgenus *Curcuma* studied, and two species from subgenus *Hitcheniopsis, C. cf. australasica* and *C. petiolata* (**Figure 4.4-4.6**). The species from the subgenus *Curcuma* are *C. mangga, C. euchroma, C. amada, C. longa, C. amarissima, C. zanthorrhiza, C. purpurascens, C. aeruginosa, C. heyneana, C. colorata, C. soloensis,* and *C. aromatica. C. australasica* and *C. petiolata* have flowers which are similar to those of subgenus *Curcuma*. Records shows that *Amegilla* bees pollinate the flowers of *Curcuma* subgenus *Curcuma* (Prana 1977). The simple flower has a very short anther crest, short and clasping lateral staminodes, shorter anther (but quite long spur), and wider stigma. The *Amegilla* bees push the spur of the anther when they go into the simple flower. This will rotate the anther so that it touches the back of the bee.

The small flower contains three species namely *C. thorelii, C. parviflora,* and *C. gracillima* (**Figure 4.4-4.6**). They belong to subgenus *Hitcheniopsis.* They may be pollinated by small halictid bees. The grouping of this flower type is in accordance with the colour of the flowers which is whitish. This is in contrast with that of the simple type which has a bright yellow flower.

The complex type contains *C. roscoeana, C. alismatifolia, C. ecomata, C. harmandii,* and *C. aurantiaca* (**Figure 4.4-4.6**). They belong to subgenus *Hitcheniopsis.* The complex flower has a long anther crest, long lateral staminodes, long anther (without spur), and narrower stigma. Further research to study the pollination of the genus is needed to observe the pollinator of these species.

The position of *C. petiolata, C. purpurascens, C. aromatica,* and *C. australasica* are quite isolated (**Figure 4.4**). Their factor loadings on axis 1 (2.4, 3.3, 4.5 and 5.2 respectively) are between the simple and the small type. The shape is the same as those of simple type, but the size is slightly different.

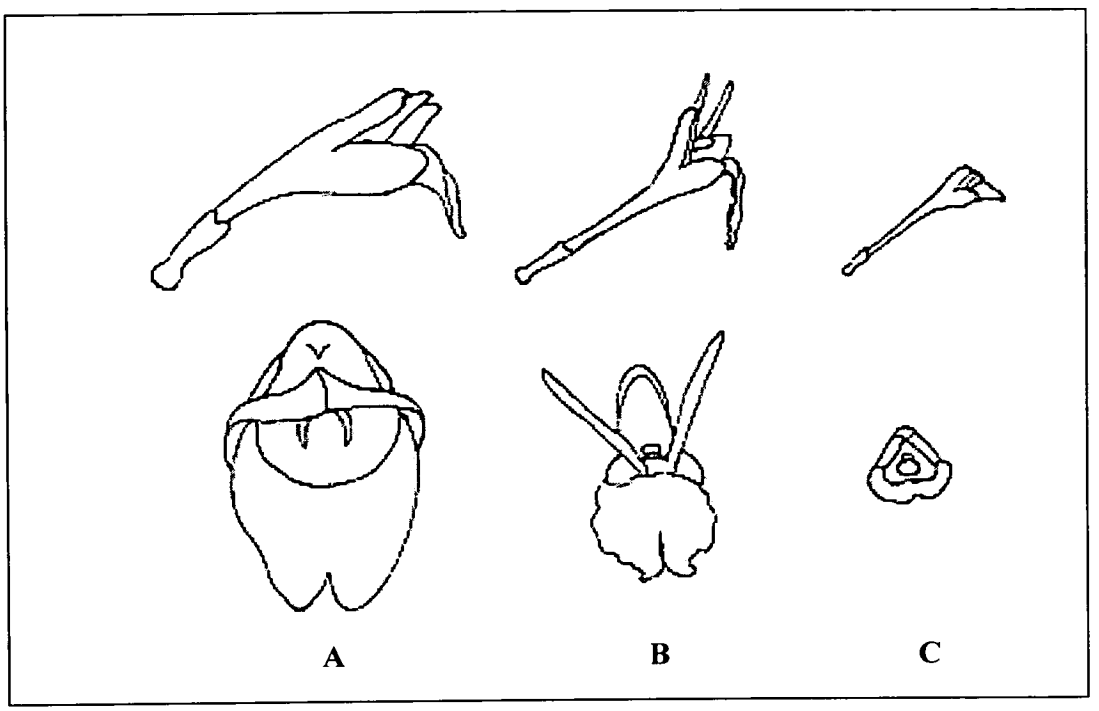


Figure 4.3 Floral types of *Curcuma*
A. Simple type; B. Complex type; C. Small type
(First row, lifesize. Second row, twice lifesize.)

Table 4.3 Variance extracted, first 10 axes.

AXIS	Eigenvalue	% of Variance	Cum.% of Var.	Broken-stick Eigenvalue
1	17.440	62.284	62.284	3.927
2	3.592	12.830	75.114	2.927
3	1.885	6.732	81.846	2.427
4	1.267	4.526	86.373	2.094
5	.829	2.961	89.333	1.844
6	.692	2.473	91.806	1.644
7	.503	1.798	93.604	1.477
8	.388	1.387	94.991	1.334
9	.327	1.169	96.161	1.209
10	.247	.882	97.043	1.098

Note: Cum.= cumulative; Var.=variance

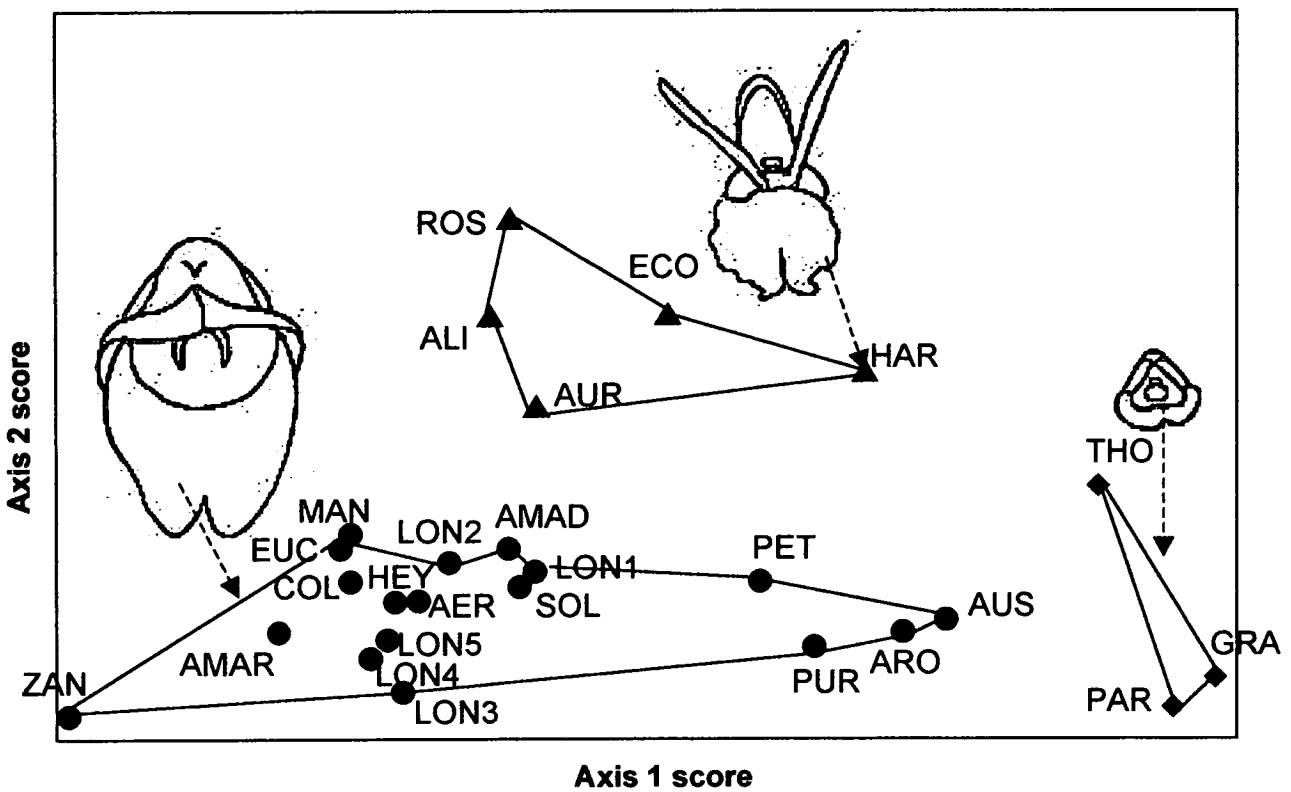


Figure 4.4 Principal component plot of *Curcuma*. The floral types shown in **Figure 4.3** are distinguished: the top group are complex type (▲), followed by large type ( ) and the small type (◆). Size and shape extremes are illustrated by the outline drawings. Axes 1 and 2 accounted for 62.28% (Eigenvalue 17.44) and 12.83% (Eigenvalue 3.60), respectively, of the variation.

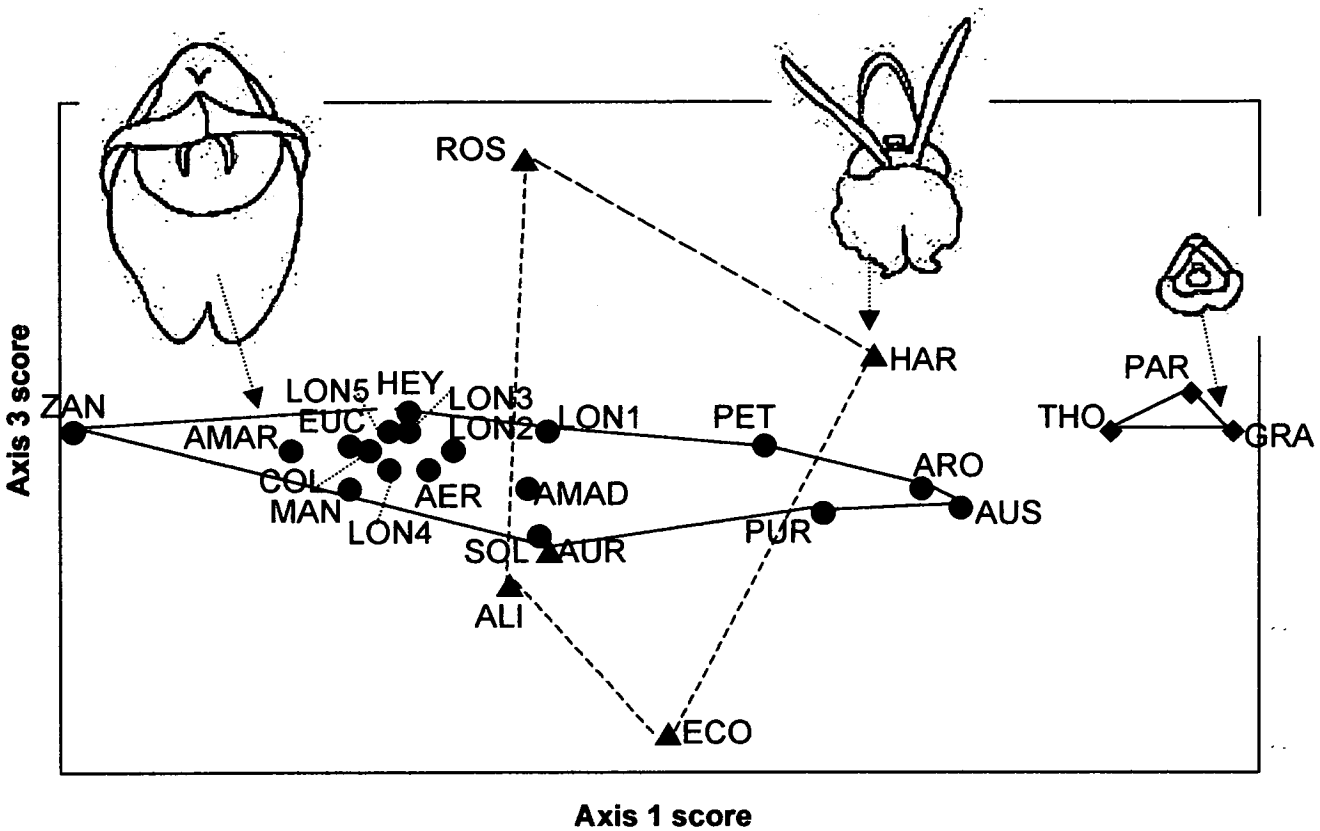


Figure 4.5 Principal component plot of *Curcuma.* The floral types shown in **Figure 4.3** are distinguished: the top group are complex type (▲), followed by large type (●) and the small type (◆). Size and shape extremes are illustrated by the outline drawings. Axes 1 and 3 accounted for 62.28% (Eigenvalue 17.44) and 6.73% (Eigenvalue 1.89), respectively, of the variation.

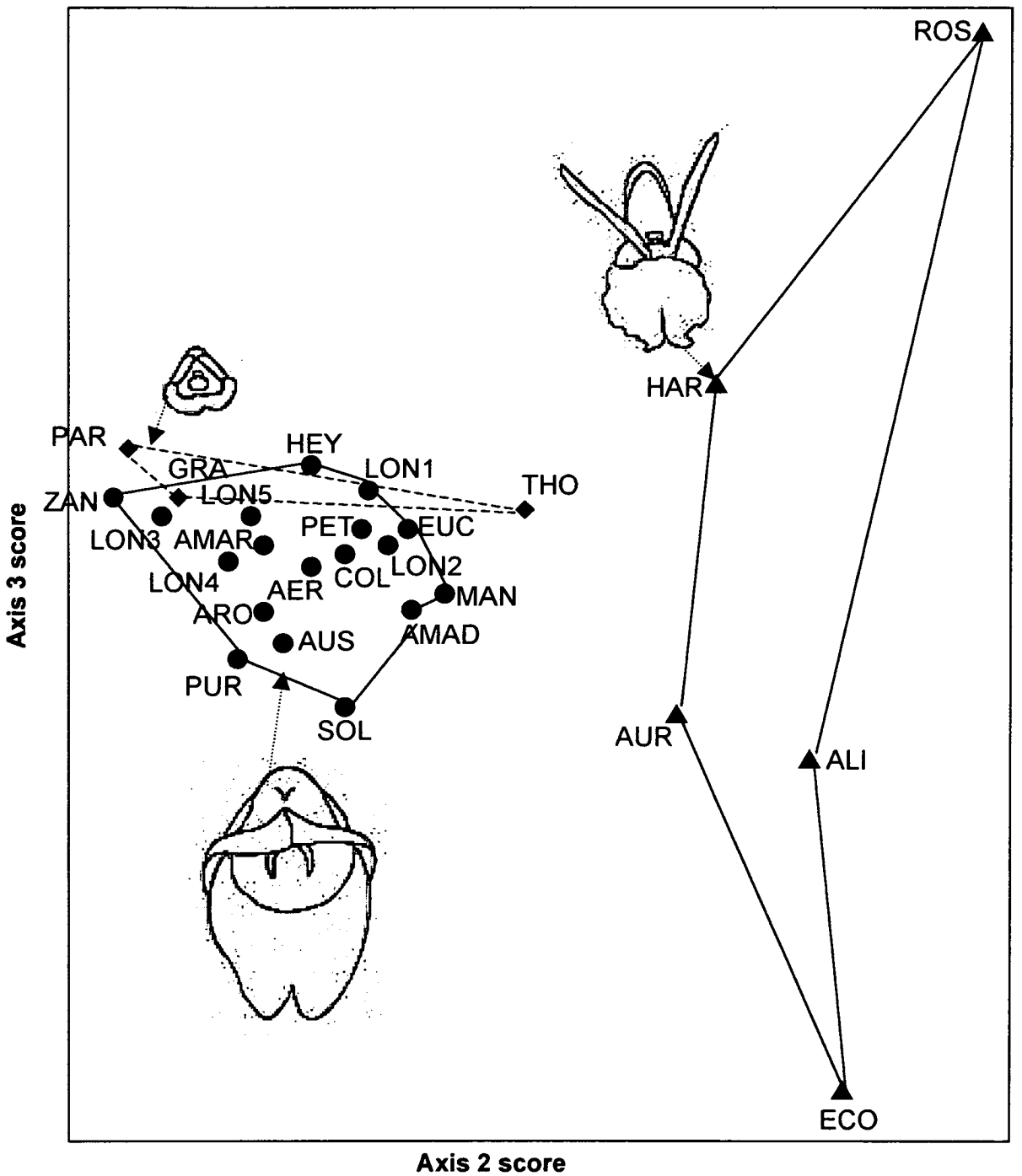


Figure 4.6 Principal component plot of *Curcuma.* The floral types shown in **Figure 4.3** are distinguished: complex type (▲), followed by large type (●) and the small type (◆). Size and shape extremes are illustrated by the outline drawings. Axes 2 and 3 accounted for 12.83% (Eigenvalue 3.60) and and 6.73% (Eigenvalue 1.89), respectively, of the variation.

4.4.3 MAPPING FLOWER STRUCTURE ONTO THE MOLECULAR TREE

The mapping of flower structure onto the molecular tree based on the ITS sequence data required three evolutionary steps (**Figure 4.7**). All species from the subgenus *Curcuma* have a simple type of flower. Subgenus *Hitcheniopsis* has all types of flower, the simple, the complex, and the small type. The common ancestor appeared to have the complex type. During the course of evolution, this type seems to have derived to the simple type. The clade that contains *C. aurantiaca* and *C. cf. australasica* consists of two different types of flower (complex type in *C. aurantiaca* and simple type in *C. cf. australasica*). However, *C. aurantiaca* is placed quite closely to the simple type cluster in the ordination. The clade that contains *C. parviflora, C. thorelii, C, gracillima, C. harmandii,* and *C. alismatifolia* also consists of two different types of flower, the complex type (*C. harmandii* and *C. alismatifolia*) and the small type (*C. thorelii, C. parviflora,* and *C. gracillima*). The small type has possibly been derived from the complex one. The clade that contains subgenus *Curcuma* with *C. petiolata* have a simple flower type. This type of flower has evolved twice independently. Flower type is probably too complex to map it on to molecular tree. A character should be treated usefully in the unitary character way of mapping it on to a molecular tree.

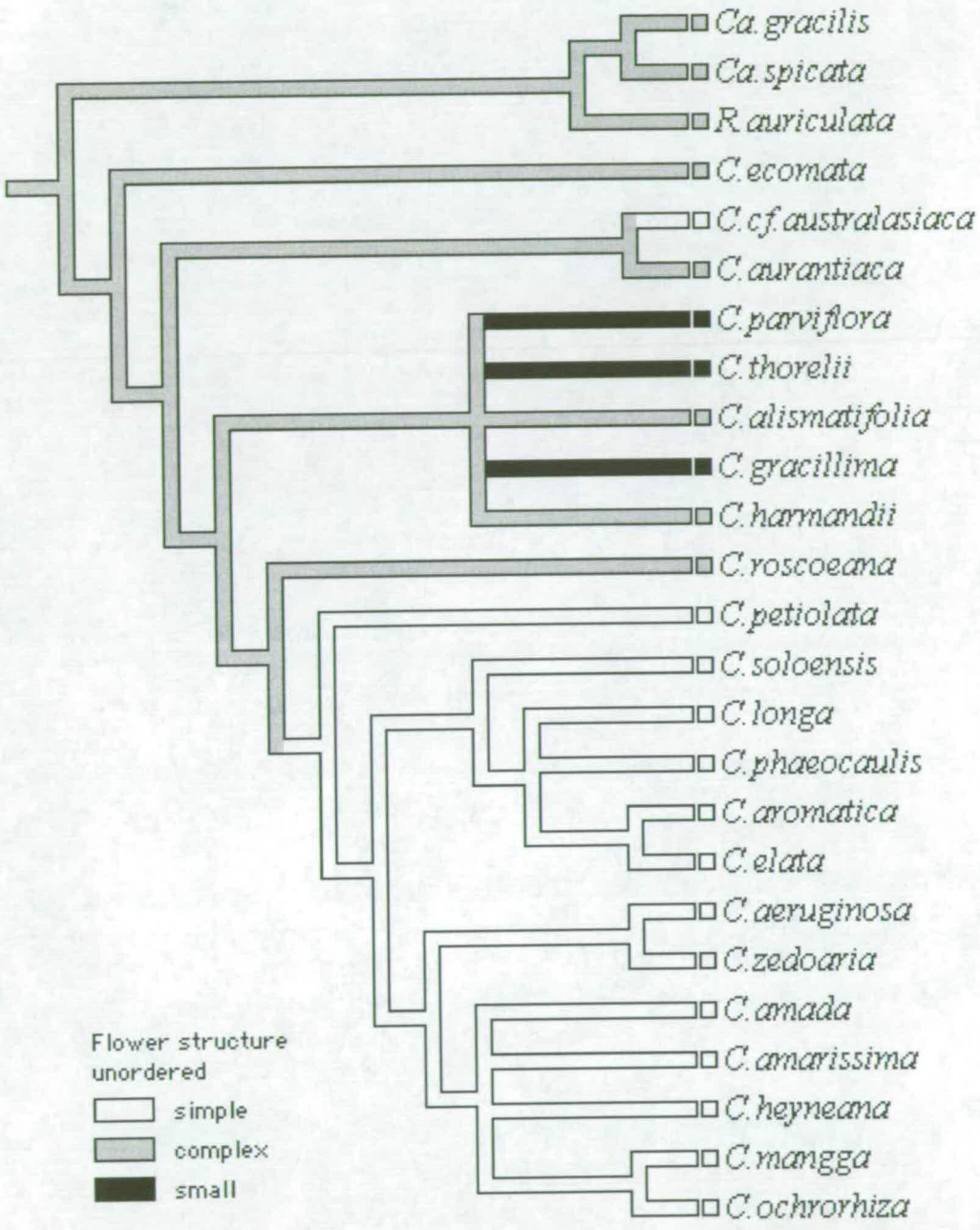


Figure 4.7 The mapping of flower type onto molecular tree.

CHAPTER 5: CHROMOSOME STUDY

5.1 INTRODUCTION

Before the study on meiosis by Ramachandran (1961) was carried out, it was hypothesized that the base numbers of *Curcuma* were 7 and 8. This was because the first chromosome count in *Curcuma* (Sugiura 1931) was 2n=64, and then Sato (1938) reported 2n=32. It was assumed that Sugiura's plant was octoploid and Satos's was tetraploid suggesting a base number of 8. In 1948, Raghavan & Venkatasuban reported 2n=42 in *C. aromatica,* so their species was hexaploid if the base number was 7. Therefore, basic numbers of 7 and 8 were suggested for the genus. However, Ramachandran (1961) observed regular formation of bivalents in metaphase I. This apparent diploid (2n=42) implied that the base number is 21, and not 7 or 8.

Chromosome numbers of some Thai species (2n=32 in *C. alismatifolia,* 2n= 24 in *C. gracillima,* 2n=20 in *C. harmandii,* and 2n=34 and 36 in *C. thorelii*), assuming they are diploid, suggest a base number that has deviated significantly. Further study is required to understand more about chromosome number variation in *Curcuma.*

Existing studies of chromosome numbers in *Curcuma* suggest that the genus contains diploid, triploid, and tetraploid cytotypes (**Table 5.2**). Cytological study of most Javanese *Curcuma* species carried out by Prana (1977) reported widespread triploidy in Javanese *Curcuma.* Only three species were reported to be diploid, i.e. *C. mangga, C. aurantiaca,* and *C. petiolata.* Severe sterility in Javanese species might indicate polyploidy. However, before any conclusions are drawn, further study is needed to check this hypothesis.

Chromosome number is one of the most widely used cytological characters in taxonomy. This is due to the fact that in vascular plants, there is great diversity of chromosome numbers. Chromosome number also frequently correlates with taxonomic groupings. It often demonstrates general stability and constancy within populations, species, and genera. This study aims to check the chromosome numbers in some species of *Curcuma* from mitotic and meiotic preparations.

5.2 MATERIALS

Materials used in this study are from living collections in the Research Glass House, The Royal Botanic Garden Edinburgh. A list of plants with their accession number and source of origin is given in **Table 5.1** below.

Table 5.1 List of materials used in the study.

Taxon	Accession number	Source of origin
C. aeruginosa	19981844	Java, Indonesia
C. alismatifolia	19973657	Thailand
C. colorata	19780188	Java, Indonesia
C. heyneana	19780189	Java, Indonesia
C. longa	19931919	not known
C. mangga	19780191	Java, Indonesia
C. parviflora	19851661	Sukhothai, Thailand
C. phaeocaulis	19771293	Java, Indonesia
C. roscoeana	19973658	Thailand
C. thorelii	19973859	Thailand
C. zanthorrhiza	19740965	not known
C. zanthorrhiza	19780187	Java, Indonesia
C. zedoaria	19771296	East Java, Indonesia

5.3 METHOD

The method used in this study follows Jong (1997). A flowchart of the method is shown in **Figure 5.1** below.

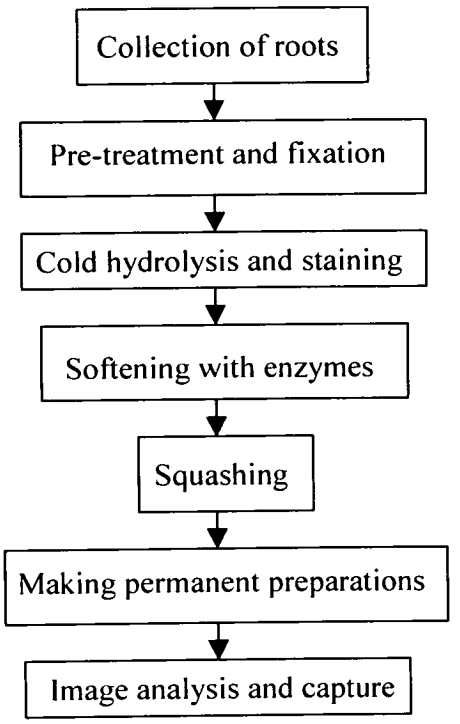


Figure 5.1 Flowchart of chromosome study.

5.3.1 Collection of roots

To induce formation of new roots, young plants were repotted. The plants were then watered regularly in the morning and late afternoon and were maintained in the glass house at a minimum temperature of 20°C and a maximum of 30 °C. The soil was monitored to see that it was moist enough and not too wet to suit the growth of new roots. After one to two weeks new fresh roots were observed.

At about mid-day, healthy growing fleshy white active roots with translucent caps were selected. They were rinsed with tap water at room temperature to clean off any compost particles. Ten to 15 of these roots were cut with a sharp scalpel, then were removed into a petri dish filled with distilled water. Several rinses were needed to clean them again. A fine paintbrush helped to remove compost particles that adhered to the roots. After that, the roots were placed on Whatman paper to blot off excess water. As soon as possible they were immersed simultaneously into pre-treatment (or fixative) in a sealed bottle for the required time. The bottle was given a good shake.

5.3.2 Pre-treatment and fixation

A pre-treatment chemical is used to increase the proportion of metaphases in the root tip meristem by inhibiting the formation of the spindle (Dyer 1979). Two pre-treatment agents were tried in this study, i.e. alpha-Bromonaphthalene (αBr) and Hydro-oxyquinolene (OQ). The roots were kept in the pre-treatment in the fridge at about 10 to 13 °C from to six and a half hours for OQ. αBr was only tried for two hours. To allow respiration by the roots, the bottle was shaken during this pre-treatment step to increase oxygen in the solution. This occasional agitation also to ensured uniform penetration of fixing fluid.

After pre-treatment, the roots were washed in two to three changes of distilled water. Excess water was blotted off, then they were placed in fixative (Farmer's solution) which was made from three parts of absolute alcohol (96%) and one part of glacial acetic acid. The fixing fluid should be freshly prepared just before use. Material fixed in an alcoholic fixative (3:1) may be stored in it or in 70% alcohol and kept in a refrigerator at about 5°C for up to 4 weeks or in deep-freeze at -20°C for prolonged storage.

5.3.3 Cold hydrolisis and staining

The roots were removed from the fixative and washed in two to three changes of distilled water. After that, they were hydrolized in 5N HCl for 15 to 35 minutes at room temperature (approximately 20°C). They were washed again in distilled water to remove the acid. Then, excess water was blotted off.

Feulgen staining was tried for three hours. It is a specific test for deoxyribonucleic acid (DNA). The chemical reaction involves hydrolysis of the DNA by hydrochloric or nitric acid and the development of a colour reaction in combination with colourless basic fuchsin. It gives a deep magenta colour while the other cell components remain unstained (Jong 1996). Next, the roots were transferred to tap water. The ions in the tap water help to fix and intensify the stain. Staining with haematoxylin for up to 45 minutes was also tried.

5.3.4 Softening with enzymes and squashing

A mixture of 4% cellulase and 4% pectinase can help soften the tissue. Cellulase will help to break the cell wall while pectinase helps to break the pectin that occurs between cells. The roots were exposed to these enzymes for 15 to 30 minutes. A softened root was placed onto a clean slide. The root tip, which is the most densely stained part, was cut off from the distal part and from the cap. These unnecessary parts were removed from the slide. A few drops of 45% acetic acid were put on the root tip. Using a blunt needle, the root tip was macerated to form fine particles. A clean coverslip was put on top of this macerated tissue. Squashing was done by placing the thumb gently on the coverslip and pressing firmly.

5.3.5 Quick-freeze method for making squash preparations permanent

This technique is fast and satisfactory for making squash preparations permanent with the aid of dry-ice or liquid nitrogen. First, a block of metal was frozen in liquid nitrogen with a pair of tongs. This was then removed and placed in a polystyrene holder. The squash preparation was placed on top of this frozen metal block with the coverslip directly over the block for freezing. After about 30 seconds, the slide was removed. The coverslip was then levered off.

As soon as possible, the coverslip was placed in a coplin jar, and the slide in another coplin jar, each containing 95% or 100% ethanol. They were left for two minutes. Both the slide and the coverslip were transferred to a second jar of alcohol and left for two to five minutes. Next, the slide was lifted from the alcohol and was drained on a piece of filter paper. One drop of euparal was placed near the top of material. The coverslip was lifted and gently pushed so that the euparal was drawn towards the material. Finally, the coverslip was lowered to cover all the material. The slide was left to dry for a few days in a 40^{o}C oven or on a slide warming plate.

5.3.6 Image analysis and capture

Observation of chromosomes was carried out by Axiophot Zeiss light microscope. Images were captured using Optimas 6.2.

5.4 RESULTS AND DISCUSSION

The chromosomes observed were very small and very numerous, so in counting was not easy. However, it was still possible to count the chromosomes in reasonably good preparations. The chromosome numbers observed can be seen in **Table 5.1**. The size of the chromosomes was not carefully measured, but the range is approximately 0.5-1.5 μm. Ramachandran's (1961) measurement was 0.6-1.7 μm. The shape of the chromosomes could not be observed due to difficulties in examining them. To date, there is no publication of the karyotype of the chromosomes of *Curcuma*. Previous workers encountered chromosomal chimaeras (see **Table 5.2**).

Except in *C. thorelii* (2n=38), the chromosome numbers from the successful preparations were all 2n=63. The chromosome count of *C. mangga* was 2n=63. This result was different from that of Prana (1977) which is 2n=42. The determination of the material (*C. mangga*), maintained in the glasshouse of the Royal Botanic Garden, Edinburgh, was not doubtful. Moreover, the plant was collected and identified previously by Prana himself. Therefore, *C. mangga* has diploid and triploid forms. Tetraploidy was only reported in *C. aromatica* with 2n=86 (Ramachandran 1961). In Java, triploidy is widespread in the genus except the diploid *C. aurantiaca, C. petiolata,* and *C. mangga* (Prana 1977). *C. aurantiaca* (2n=42) was found to set seeds (Valeton 1918, Prana 1977). I observed this in the field. Many herbarium specimens of *C. aurantiaca* have record on the seeds, however this is not the case with *C. petiolata.* This is probably due to the limited member of specimens observed. *C. mangga* (2n=42,63) was

Table 5.2 Results of chromosome counts in this study.

Taxon	Accession number	Chromosome counts (2n)
C. heyneana	19780189	63
C. phaeocaulis	19771293	not successful
C. parviflora	19851661	not successful
C. mangga	19780191	**63***
C. zanthorrhiza	19740965	63
C. roscoeana	19973658	not successful
C. longa	19931919	not successful
C. zedoaria	19771296	63
C. thorelii	19973859	38?
C. alismatifolia	19973657	not successful
C. aeruginosa	19981844	63
C. zanthorrhiza	19780187	not successful
C. colorata	19780188	not successful

Note: number in bold with *= the counting is different from Prana's result (1977) where the chromosome number of *C. mangga* was 42 (2n= 42).

Table 5.3 Chromosome counts from this study and from literature.

Taxon	Chromosome counts (2n)	Literature
C. aeruginosa	63	Prana (1977), Ardiyani (unpubl.)
C. alismatifolia	**32**	
C. amada	**42**	Chakravorti 1948, Sharma & Bhattacaryya 1959, Ramachandran 1961
C. angustifolia	**42**	Chakravorti 1948, Sharma & Bhattacaryya 1959
C. aromatica	**42**	Raghavan & Venkatasuban 1943, Chakravorti 1952
	63, 86	Ramachandran 1961
C. aurantiaca	**42**	Prana (1977)
C. colorata	62,63	Prana (1977)
C. decipiens	**42**	Ramachandran 1961
C. gracillima	**24**	
C. harmandii	**20**	
C. heyneana	63	Prana (1977), Ardiyani (unpubl.)
C. longa	**32**	Sato 1948
	62	Ragghavan & Venkatasuban 1943
	62, 93	Sharma & Bhattacaryya 1943
	63	Ramachandran 1961, Prana (1977)
	62,63,64	Chakravorti 1948
	64	Sugiura 1931
C. mangga	**42**	Prana (1977)
	63	Ardiyani (unpubl.)
C. neilgherrensis	**42**	Chakravorti 1948
C. parviflora		
C. petiolata	**42**	Prana (1977)
	64	Venkatasuban 1946
C. phaeocaulis	62,63,64	Prana (1977)
C. purpurascens	63	Prana (1977)
C. roscoeana	**42**	
C. soloensis	63	Prana (1977)
C. thorelii	**34,36**	
	38?	Ardiyani (unpubl.)
C. zanthorrhiza	63,64,70	Prana (1977)
	63	Ardiyani (unpubl.)
C. zedoaria	63,64,66	Prana (1977)
	63	Ramachandran 1961, Ardiyani (unpubl.)
	63, 64	Chakravorti 1948
	64	Venkatasuban 1946

Note: number in bold indicate diploidy

reported to set seeds by Valeton (1918), but neither Prana nor I observed seeds in the field or on herbarium specimens.

Based on the study on pollen stainability and germination tests, Prana (1977) found the diploid *C. aurantiaca* was fertile. *C. mangga* and *C. petiolata* were also fertile, but the fertility was very low. He hypothesized that sterility in these species is due to genetic rather than chromosomal causes. Further study is needed to verify this.

Triploidy can occur from diploid parents. When failure of reduction in meiosis occurs, reduced and unreduced gametes (eggs or pollen) may unite. Triploidy can also occur through hybridization between a diploid and a tetraploid. Tetraploidy in *Curcuma*, however, was only recorded in *C. aromatica.* Nothing is known of the origin of triploid *Curcuma*, whether they are formed through auto- or allopolyploidy. One specimens collected from Java showed polymorphism in its ITS sequence (see Chapter Seven) with one sequence resembling that of *C. aurantiaca* while the other was not similar to it (data not displayed). Thus, allopolyploidy could have happened in the species involving *C. aurantiaca* as one of the parents. This is in accordance with Prana's prediction that Javanese triploid species could be hybrid forms in which *C. aurantiaca* and *C. mangga* might have been involved. Further study focusing on those species might be able to give insight into evolutionary history in *Curcuma.*

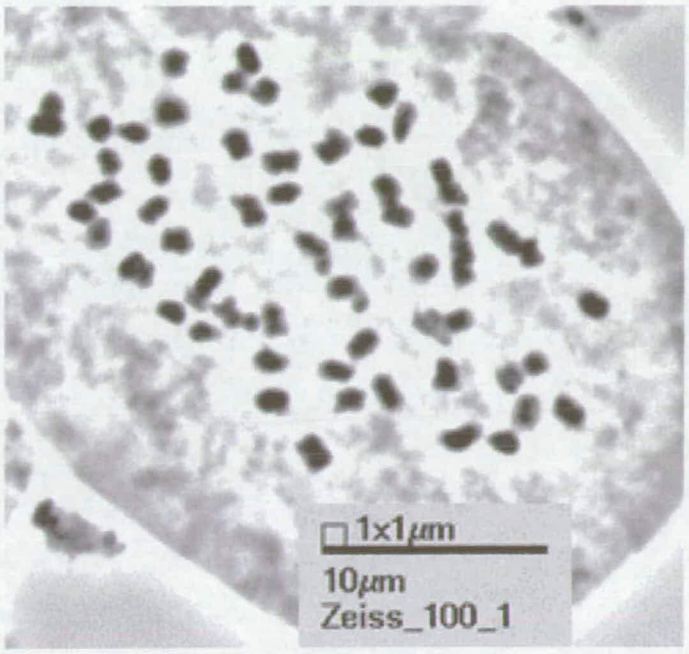


Figure 5.2 Chromosomes of *C. aeruginosa.*

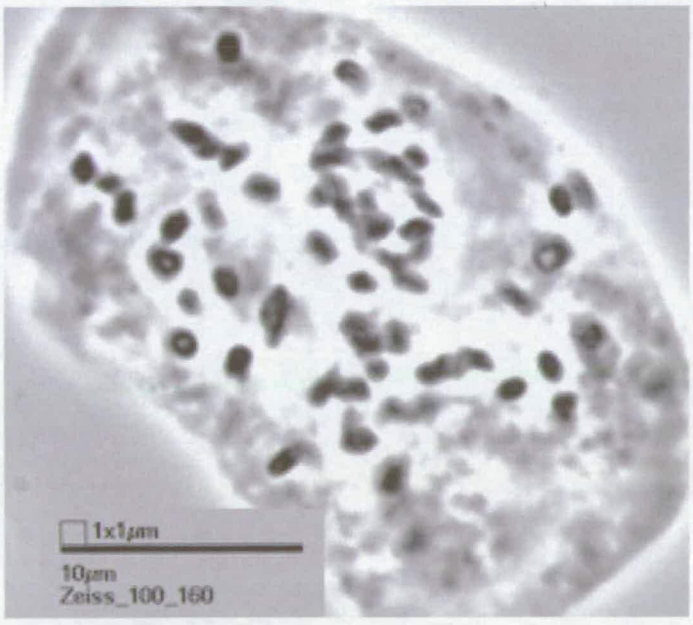


Figure 5.3 Chromosomes of *C. heyneana.*

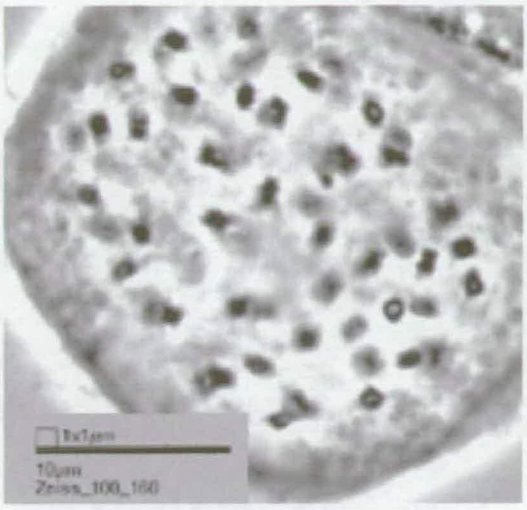


Figure 5.4 Chromosomes of *C. mangga*.

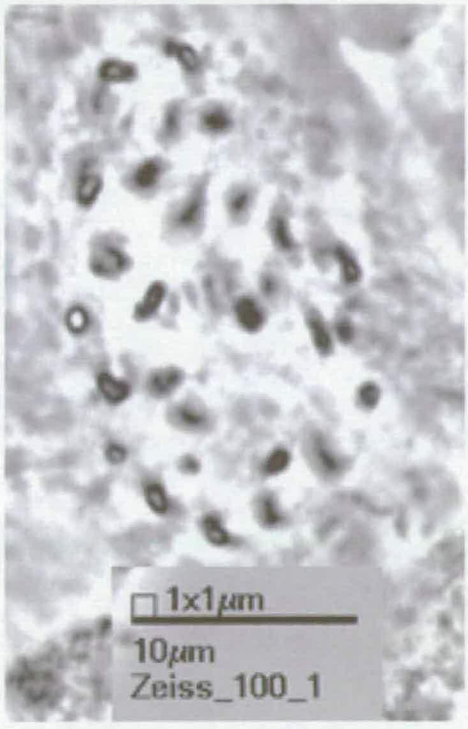


Figure 5.5 Chromosomes of *C. thorelii*.

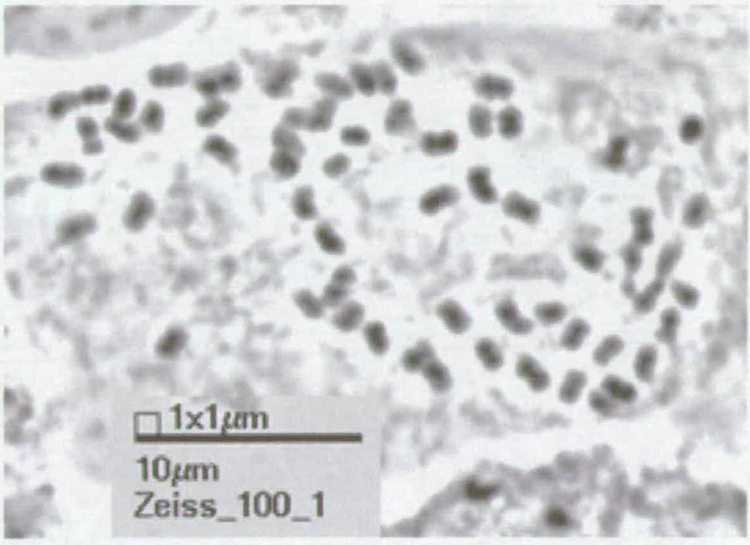


Figure 5.6 Chromosomes of *C. zanthorrhiza.*

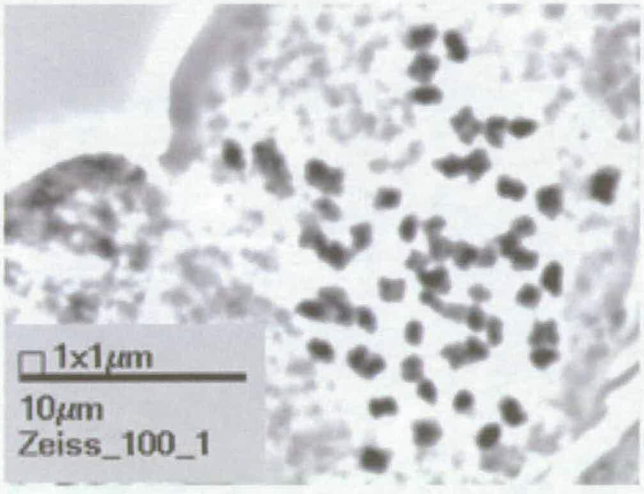


Figure 5.7 Chromosomes of *C. zedoaria.*

CHAPTER 6: ISOZYME VARIATION IN SOME *CURCUMA* SPECIES

6.1 INTRODUCTION

6.1.1 Isozyme electrophoresis

Isozymes are defined as multiple molecular forms of an enzyme encoded for by the same or by different loci. Allozymes are multiple molecular forms of an enzyme encoded for at the same locus. Therefore, allozymes are allelic forms of isozymes (Hollingsworth 1998). Diagrammatic representation of isozyme loci and allozymes is shown in **Figure 6.1**.

Isozyme electrophoresis involves running a crude tissue homogenate on a gel that has an electric current applied to it. The homogenate is first absorbed onto small pieces of filter paper (wicks) before loading it onto the gel. When an electric current is applied, thousands of different proteins present in the crude tissue homogenate will migrate across the gel at different speeds based on their net charge. The migration rates are also affected by the gel type. After the gel run is completed, it is then stained. Staining, by soaking the gel in the substrate for the enzyme of choice, allows visualization of a single type of enzyme among those present in the homogenate. The bands resulting from the staining (known as a zymogram) indicate the position that the enzyme has migrated to.

Enzymes are types of protein. Proteins are built from amino acids which connect together to form polypeptide chains. The order of the amino acids in these polypeptide chains is genetically controlled. This is known as the primary structure of the enzyme. The polypeptide chains can consist of a single chain (known as monomer), two chains (dimer), three chains (trimer), four chains (tetramer), etc. Disregarding the tissue or the organism of the enzymes, the numbers of poplypeptide chains that make up enzymes tend to be conserved. For instance, malate dehydrogenase (MDH) is a dimer, phosphoglucomutase (PGM) is a monomer. Knowledge of the number of polypeptide

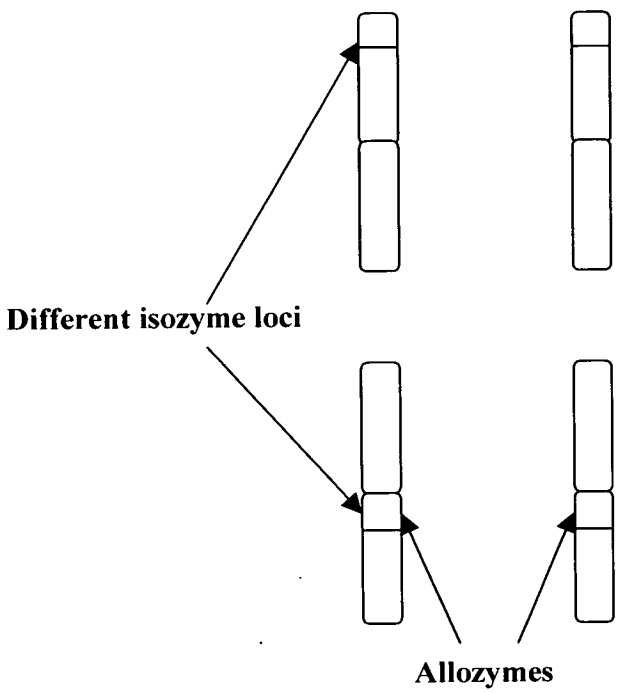


Figure 6.1 Diagrammatic representation of isozyme loci and allozymes.

The diagram represents an organism with four chromosomes, two paternally inherited and the other two maternally inherited. There are two isozyme loci, and at each locus there is the potential for two allozymes (alleles), one inherited maternally and one paternally (Hollingsworth 1998).

chains that makes up an enzyme is required to interpret the type of pattern produced on a gel.

The amino acids that build up enzymes have characteristic side arms that can be positively or negatively charged, or can be uncharged or neutral. Hence, the overall net charge of a protein will depend on the composition of amino acids that form the polypeptide chain. Mutations in DNA sequence in genes coding for the production of particular proteins can lead to substitutions in the amino acids that make up a polypeptide chain. Although the molecules are still functional, the overall net charge can be different. Amino acid substitutions may not only cause differences in the charge, but may also alter the shape or the size of a protein. Thus, DNA sequence variation may result in the forms of a protein that differ in net charge, size or shape. These differences can be detected using the technique of electrophoresis.

The discovery of starch gel electrophoresis of isozymes led to studies of animals (Harris & Hopkinson 1976), fungi (Micales 1986), plants (Soltis & Soltis 1989) and bacteria (Selander *et al.* 1986). It started when Smithies (1955) described gel electrophoresis and Hunter & Markert (1957) reported histochemical staining of enzyme gels. Harris (1966) and Lewontin & Hubby (1966) thereafter demonstrated the simple co-dominant Mendelian inheritance of allelic forms of isozymes, and the extent of polymorphisms in natural populations.

6.1.2 Advantages and disadvantages of the technique

The isozyme technique is a cheap, technically straightforward, and fast method to analyse large numbers of individuals to gain allelic data. According to Klaas (1998) when several taxa, accessions and individuals are to be compared, isozymes are very useful, as the assumption of homology is more accurate than with some DNA markers. Ample studies have demonstrated the simple Mendelian inheritance of a considerable number of isozymes (Hollingsworth 1998).

Epistatic interactions where one gene effects the expression of another, and pleiotropic interactions where one gene controls several apparently unrelated characters, may not be selectively neutral. Comparatively few studies have reported problems of

the nature suggested above (Wendel & Weeden 1989). Koehn & Hilbish (1987) and Watt (1983) reported the fact of selection that can occur for specific isoforms. Ryan *et al.* 1991 also reported that non-genetic variation in banding patterns could occur. This puts doubt in the use of isozymes as population genetic and biosystematic markers. However, careful experimental design may reduce or eliminate the potential pitfalls of the isozyme technique. It also suggested that, when absolute measures of variation are required, the isozyme technique may lead to under-estimates of the level of genetic diversity. Electrophoretically detectable variation will be an underestimate of total variation. However, the problem can be eliminated if comparative measures of variation are required (Hollingsworth 1998).

6.1.3 Application of isozyme

Isozymes have been used in investigations into interspecific hybridization (Crawford 1989, Hollingsworth *et al.* 1995, Raybould *etal.* 1991, Hollingsworth *et al.*1999); intergeneric hybridization (Soltis & Soltis 1986); identification of cultivars and lines; species delimitation and conservation (Chamberlain 1998); assessment of genetic variability in species and populations (Eanes & Koehn 1978, Ellstrand & Roose 1987); assessment of gene flow; evolutionary origin of polyploid species (Soltis & Rieseberg 1986; Ness *et al.* 1989, Ranker & Haufler 1989, Wolf *et al.* 1990, Soltis *et al.* 1991, Ashton & Abbot 1992, Raybould *etal.* 1991a, Raybould *etal.* 1991b, Roose & Gottlieb 1976); phylogeny (Patton & Avise 1983); variation in wild and cultivated species (Lange & Schifino-Wittmann 2000); and taxonomic delimitation and characterization of the germplasm (Chamberlain 1998; Lange & Schifino-Wittmann 2000).

Isozyme studies have been carried out in *Curcuma* to check the variation in some species of *Curcuma* (Ibrahim 1996) and to identify some early flowering *Curcuma* (Apavatjrut *et al.* 1999). The widespread polyploids *Curcuma* could use isozyme technique to investigate allo- or autoployploid origin. The formation of alloploids via interspecific hybridization and subsequent chromosome doubling is a very important mode of speciation in higher plants (Lewis 1980; Stace 1987). To understand the

evolutionary success of polyploid species, study of the formation and establishment of newly formed polyploids under natural conditions is required (Raybould *et al.* 1991).

6.2 MATERIALS AND METHOD

The material used in this study can be seen in **Table 6.1**. Some of the materials used in this study consisted of clonal lines collected in the field and subsequently maintained in pot culture in the glasshouse of the Royal Botanic Garden, Edinburgh.

Table 6. 1 *Curcuma* taxa (all are triploid) used in this study.

Species	Source	Locality sampled
C. aeruginosa Roxb. *(AE1)*	RBGE 19780186	Indonesia
C. aeruginosa (AE2)	RBGE 19771288	Java, Indonesia
C. aeruginosa (AE3)	RBGE 19981844	Java, Indonesia
C. aeruginosa (AE4)	RBGE 19981841	Java, Indonesia
C. colorata Valeton *(CO1)*	RBGE 19780188	Java, Indonesia
C. colorata (CO2)	RBGE 19771290	Java, Indonesia
C. colorata (CO3)	RBGE 19771294	Java, Indonesia
C. heyneana Valeton & Zijp *(HE1)*	RBGE 19780189	Java, Indonesia
C. heyneana (HE2)	RBGE 19981842	Java, Indonesia
C. heyneana (HE3)	RBGE 19940453	Java, Indonesia
C. longa L. *(LO1)*	RBGE 19931919	not known (cultivated)
C. longa (LO2)	RBGE 19782126	not known (cultivated)
C. longa (LO3)	RBGE 19720174	Java, Indonesia
C. longa (LO4)	RBGE 19711837	not known (cultivated)
C. longa (LO5)	RBGE 19730708	Java, Indonesia
C. longa (LO6)	RBGE 19721701	not known (cultivated)
C. longa (LO7)	RBGE 19720175	Java, Indonesia
C. longa (LO8)	RBGE 19990607	West Java, Indonesia (cultivated)
C. mangga Valeton & Zijp *(MA1)*	RBGE 19780191	Java, Indonesia
C. mangga (MA2)	RBGE 19710260	not known
C. mangga (MA3)	RBGE 19710261	not known
C. zanthorrhiza Roxb. *(ZA1)*	RBGE 19740965	not known
C. zanthorrhiza (ZA2)	RBGE 19780194	Indonesia
C. zanthorrhiza (ZA3)	RBGE 19771295	West Java, Indonesia
C. zanthorrhiza (ZA4)	RBGE 19780187	Java, Indonesia
C. zedoaria (Christm.) Roscoe *(ZE1)*	RBGE 19771296	East Java, Indonesia
C. zedoaria (ZE2)	RBGE 19730871	Sri Lanka
C. zedoaria (ZE3)	RBGE 19990612	West Java, Indonesia

A flowchart of the general protocol of protein-electrophoresis can be seen in **Figure 6.2**.

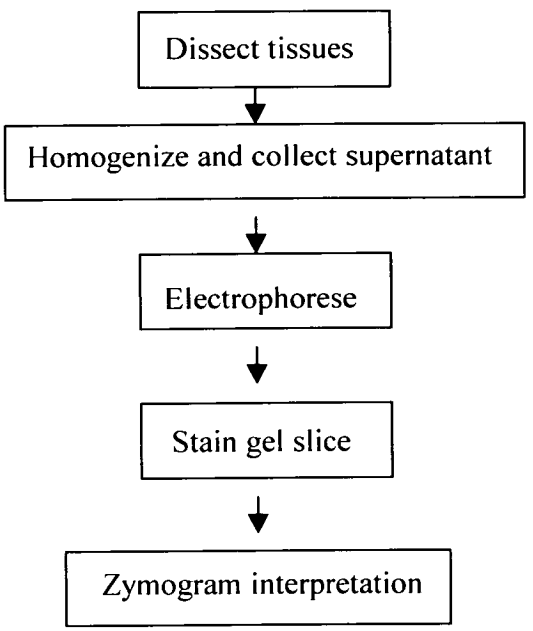


Figure 6.2 General protocol for protein-electrophoretic surveys (modified from Avise 1994)

6.2.1 Gel preparation

A 12% gel was\prepared by weighting out 42 g of starch and dissolving it into 350 ml gel buffer (for 20-30 samples) in a Buchner flask. The solution was agitated for 30 seconds to make the starch go into suspension. The flask was then put in a microwave and was heated on high power. After a few minutes, the solution was swirled again, and then heated again. After the solution had boiled, it was removed. From this stage onwards, it was essential to work as quickly as possible as the starch cooled and set.

A vacuum hose was attached to the flask side arm, and a rubber bung was placed on top of the flask. The flask was degassed until the small bubbles had disappeared and large bubbles were spread evenly throughout the solution. The vacuum pump was switched off and the rubber bung was slid slowly off the top of the flask. Then the vacuum hose was detached.

Prior to preparing the starch gel, a gel former was placed on a level surface on top of a paper towel. The gel was then poured into the gel former in a horizontal zigzag pattern. The gel was allowed to cool for 30 minutes. After it had set, a cling film was wrapped over the gel before keeping it in a fridge to allow cooling to 4°C.

6.2.2 Sample preparation

A washing up bowl was prepared by filling it with ice (up to 2/3 volume). A glass beaker was filled with water and was placed in the ice bucket. A ceramic grinding plate was put on ice. When the plate was cold (any condensation must be wiped away), a five mm square portion (or a punched tube) of fresh and healthy leaf tissue was placed in one of the wells. Two drops of extraction buffer were added. After that the tissue was homogenized using a flared glass rod. As soon as the tissue was homogenized, a filter paper wick was placed in the homogenate and was left to absorb the extract. The above steps were repeated for the number of the samples required. A drop of food dye was placed in an empty well and then a filter paper wick was added.

The extraction or grinding buffer was made up from $LiBO_3$ gel buffer (50ml), KCl (37mg), $MgCl_2$.$6H_20$ (10mg), 19mg of EDTA tetrasodium salt, PVPP (25mg), Triton x100 (0.5ml) and 2-mercaptoethanol (1.25ml).

6.2.3 Electrode tank preparation

The electrode tank and sponges were washed thoroughly and dried. A sponge was placed in each electrode tank. The blue plug was connected to the negative (cathode), and the orange plug to the positive (anode). The tank was filled with electrode buffer to within about one cm of the top.

6.2.4 Gel cutting and loading

The gel was removed from the refrigerator. A spatula was run around the margins of the gel to trim any excess from the edge of the gel former. The surface of the gel was then covered with cling film to prevent any creases or air bubbles. On the side of the gel former, an eight-cm running zone was marked out allowing at least five-cm from either end of the former. The wicks are usually placed close to the cathodal edge of the gel. The cling film was peeled back to the beginning of the running zone. Using a ruler and the flat end of a spatula, the gel was cut through the base along the start of the running zone. One side of the gel was pulled back gently to make a gap open up (**Figure 6.3**).

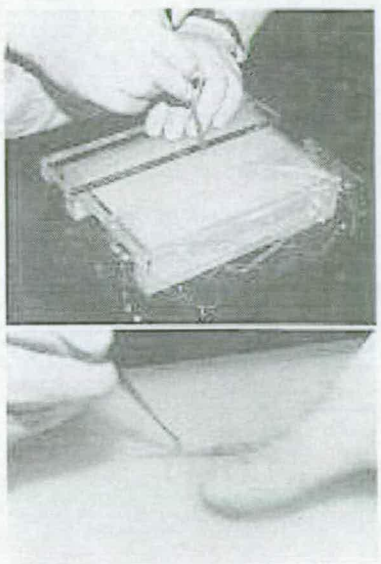


Figure 6.3 Cutting and loading the gel (Murphy *et al.* 1996).

Using a fine forceps, a filter paper wick was picked up. Any excess sample extract was dabbed off onto a paper towel. After that it was placed in the gap at the start of the running zone. This step was repeated for the remaining samples and also for the food dye marker. At least three-mm was allowed between wicks. After all the wicks were in place, a wide gauge drinking straw was inserted between the short side of the gel and the gel former end wall. The straw keeps the two parts of the gel in close contact maximising conductivity.

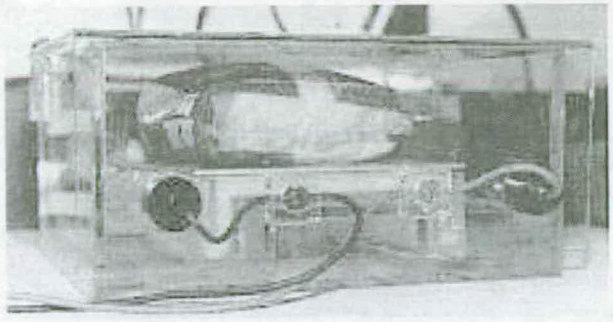


Figure 6.4 Horizontal starch gel apparatus during electrophoresis (Murphy *et al.* 1996).

The compressing force of the straw can squeeze out excess extract along the line of wells. This was dabbed using a paper towel. The cling film was pulled back over the wicks leaving about four cm of gel to exposed. A similar area at the opposite end was also exposed. The gel and the gel former were placed on the electrode rig. The sponges of each tank were placed overlapping with the exposed gel surface. The sponges must be flat and evenly placed on the gel surface. Any excess cling film was placed at either end of the gel on top of the sponge-gel overlap. Two A4 plastic bags were put on top of the gel. After that, an ice pack was placed on top of the bags (**Figure 6.4**).

The whole rig was then placed in the fridge, connected to a power pack and run for approximately four hours. For $LiBO_3$ buffers it was run at a constant voltage of

240V which should give a current of approximately 70mA. This will decrease to approximately 25mA after four hours. For MC8 buffers, it was run at a constant current of 40mA, which should give a voltage of approximately 150V. After ten minutes running, the dye should be checked to ensure that it was migrating in the correct direction.

6.2.5 Stain preparation

PGI or GPI (glucose 6 phosphase isomerase), 6PGD (6 phosphogluconate dehydrogenase), PGM (phosphoglucomutase), G-6-PDH (glucose 6 phosphase isomerase), IDH (isocitrate dehydrogenase), MDH (malate dehydrogenase), GOT or AAT (aspartic aminotransferase), and SKDH (shikimate dehydrogenase) enzymes were tried in the experiment. The recipe for each enzyme is written below. The chemicals according to instructions in the recipe were weighed out or measured. Most of the reagents used in the staining recipes are very hazardous. Therefore, treat with appropriate care should be taken, for example handling the reagents in a fume hood, wearing gloves.

PGI in $LiBO_3$ was made up from Tris 0.1M pH 8.0 (50ml), fructose-6-phosphate (40mg); NADP (7mg), MTT (12mg), PMS (3ng) in 0.5ml of 10% $MgCl_2$; glucose-6-phosphate dehydrogenase (35µl). PGI in $MC_8$was also tested.

PGD in MC_8 was composed from Tris 0.1M pH 8.0 (50ml), 6-phosphogluconic acid (50mg); NADP (10mg), MTT (15mg), PMS (3mg) in 0.5ml of 10% $MgCl_2$; $MgCl_2$ (50mg).

PGM in $LiBO_3$ was made up from Tris 0.1M pH 7.5 (50ml), glucose-1-phosphate (100mg); NADP (10mg), MTT (15mg), PMS (3mg) in 0.5ml of 10% $MgCl_2$; ATP (20mg), and glucose-6-phosphate dehydrogenase (15µl).

G-6-PDH in MC_8 was made up from Tris 0.1M pH 7.5 (50ml), glucose-6-phosphate sodium salt (50mg), NADP (10mg), MTT (15mg), PMS (3mg) in 0.5ml of 10% $MgCl_2$.

IDH in MC_8 was made up from Tris 0.1M pH 8.0 (50ml), isocitric acid (100mg); NADP (10mg), MTT (15mg), PMS (3mg) in 0.5ml of 10% $MgCl_2$; $MgCl_2$ (100mg).

MDH in MC_8 was made up from Tris 0.1M pH 8.5 (50ml), 750mg of malic acid; (Na salt); NAD (10mg), MTT (10mg), PMS (3mg) in 1ml dH_20.

GOT in $LiBO_3$ was composed of GOT substrate (50ml), fast blue BB salt (200mg), and pyridoxal-5-phosphate (1mg). Fast blue and pyridoxal were to be weighed dry and to be added to buffer later. GOT substrate was composed of Tris 0.1M pH 8.5 (50ml), α-ketoglutaric acid (18mg), aspartic acid (65mg), PVP 40T (250mg), 25mg of EDTA (Na_2 salt), and disodiumhydrogen phosphate (710mg).

SKDH in $LiBO_3$ was composed of Tris 0.1M pH 8.0 (50ml), shikimic acid (60mg); NADP (10mg), MTT (15mg), PMS (3mg) in 0.5ml of 10% $MgCl_2$.

Tris buffers were made up from 0.1M of Tris HCl, 12.11g of Tris Base per litre H_20. Ph was adjusted with HCl to required value.

The morpholine citrate (MC_8) electrode buffer (1L) was made up from citric acid (8.4g), 900ml of dH_20, 17ml of N-(3-aminopropyl)-morpholine at pH 8.0. To make 350ml gel buffer of morpholine citrate (MC_8, 14ml of the electrode buffer was diluted in 336ml of distilled water.

The lithium borate ($LiBO_3$) electrode buffer (1L) was made up from boric acid (11.9g) and lithium hydroxide (1.2g). The solution was adjusted to pH approximately 8.3. The gel buffer (1L) was composed of Tris base (5.45g), citric acid (1.28g) and electrode buffer (100ml).

6.2.6 Gel slicing and staining

The power pack was turned off, and left until the voltage dropped to zero. The gel and the gel former were removed from the electrode rig. Using a spatula the gel at the end of the running zone (around 8 cm from the wicks) was cut. The portion of gel in front of the running zone was discarded. A notch in the top right hand corner of the gel

was cut to facilitate orientation. The running zone area of the gel was carefully lifted up supporting it underneath and keeping it as flat as possible. After that, the running zone portion was placed on a paper towel and the upper and lower surface was dabbed gently. Any wicks that were still attached to the gel were removed. The gel was lifted up, and the top surface was placed up on the gel slicer. From underneath, between the gel and the slicer, presence of air bubbles must be checked and removed by tapping the gel down gently. After that, a glass plate was carefully placed on top of the gel. Next, the fishing line was held tight across the furthest end of the gel slicer from the worker. The line was pulled back along the gel (**Figure 6.5**).

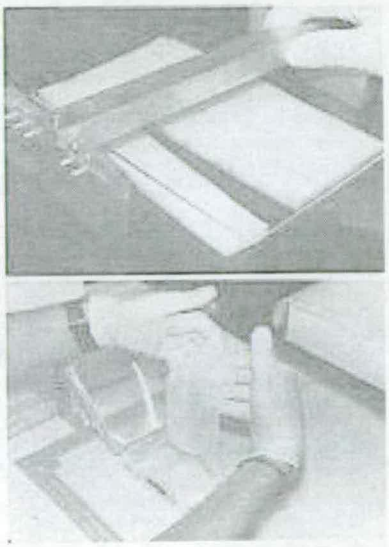


Figure 6.5 Slicing the gel for staining (Murphy *et al.* 1996).

The slicer, the glass plate, and the gel were inverted. Using a spatula, the thin slice was lifted up, then was placed in the relevant staining tray. The recently exposed surface of the remainder of the gel was dabbed with a paper towel. After that, it was placed on the gel slicer. The previous steps were repeated for the remaining slices to get some more gel slices.

Reagents were added to gels in staining trays. The trays were covered with lids, and they were put in an incubator in the dark at 38 ^{o}C until the bands developed. After the bands had developed, the gel was removed to a glass plate. Finally, it was photographed using a SLR camera.

6.3 RESULT AND DISCUSSION

Detectable isozymes can occur from three different genetic and biochemical phenomena: 1. multiple allelism at a single locus; 2. multiple loci coding for a single enzyme; 3. post-translational processing and the formation of secondary isozymes. All of these situations must be carefully considered when trying to interpret electrophoretic data (Micales *et al.* 1986). Interpretation of the banding patterns was also based on knowledge of sub-unit structure and subcellular compartmentalization (Weeden & Wendel 1989).

From eight enzyme systems that were tested, only three enzyme systems (ie. PGI, PGM, and MDH) were resolved. The rest, ie. 6PGD, G-6-PDH, IDH, GOT or AAT, and SKDH were unresolved.

PGI is a functional dimeric enzyme, occuring in the cytosol and/or plastids and related to the reversible reaction of fructose-6-phosphate and glucose-6-phosphate isomerization (Weeden & Wendel 1990).

PGI was the best resolved and the most stable of all enzyme systems. However, the one- to three-banded phenotype produced by PGI, did not prove to be readily interpretable in terms of number of loci (**Figure 6.6**). Except for the band in *C. parviflora*, the rest of the bands show quite clear homo- and heterozygote patterns. Considering the size of the bands, they could have been the result from more than one locus with two alleles (A and A'). Type A could represents homozygote AAAA, while type B could be heterozygote AAAA' with proportion 9:6:1. Type C could be heterozygote AAA'A' with proportion 1:2:1. Type D could be heterozygote AA'A'A' with proportion 1:6:9.

PGI of *C. aeruginosa, C. amada, C. amarissima, C. mangga, C. phaeocaulis, C. zedoaria, C. parviflora,* and *C. roscoeana* did not show any polymorphisms. Further

study using more samples is needed to verify this. On the other hand, PGI of the rest of the species (i.e. *C. colorata, C. heyneana, C. longa, C. zanthorrhiza*) showed variable patterns.

PGI of Hey1 showed band types A and B. PGI of Lon1 also produced band type A, while that of Lon2, Lon3, Lon5, Lon 6, and Lon7 produced three bands (band type C). PGI of Zan1, Zan2, Zan3, and Zan4 produced three-banded phenotype of type B while Zan2 produced slightly different band phenotype (type D). The middle and the lowest bands in Zan2 are wider than the top band, while on the rest of *C. zanthorrhiza* species the top and the middle bands are bigger than the lowest one. PGI of Col3 produced a three-banded phenotype (type F) with possible proportions of 1:2:1. PGI of Col 1 and Col2 produced a three-banded phenotype (type B).

The patterns in PGM were also consistent with one locus with no variation, except for Zan3 in which a two-banded phenotype was found. Therefore, *C. zanthorrhiza* showed homo- and heterozygosity.

MDH is a dimeric enzyme in most plants and is involved in the oxidation of malate to oxalacetate (Weeden & Wendel 1990). The patterns in MDH showed quite clearly two putative loci. The fastest bands or MDH1 were faint and not consistently scorable, so they were omitted from the analysis. The slower bands or MDH2 produced a one- to three-banded phenotype. This, however, also proved not to be readily interpretable in terms of number of loci. Variation within taxa was only found in *C. heyneana* that produced one very wide single band, and a three-banded phenotype for the rest of the samples.

Figure 6.6 Isozyme phenotypes for PGI, PGM, and MDH. Letters of the alphabet designate individual phenotypes for each isozyme. Aer= *Curcuma aeruginosa*, Amd= *C. amada*, Amr= *C. amarissima*, Col= *C. colorata*, Hey= *C. heyneana*, Lon= *C. longa*, Man= *C. mangga*, Par= *C. parviflora*, Pha= *C. phaeocaulis*, Ros= *C. roscoeana*, Zan= *C. zanthorrhiza*, Zed= *C. zedoaria*.

6.3.1 Variation among taxa

Bands specific taxa are encountered in *C. parviflora,* and *C. roscoeana,* which are diploid species, namely in the PGI and MDH systems (**Figure 6.6**). However, only a single sample for each species was available. More samples are needed to check the genetic variation of these two diploid species. The rest of the band phenotype did not hint for any specific species.

6.3.2 Variation within taxa

Some taxa show variation in the phenotype bands, ie. *C. heyneana, C. longa, C. colorata, C. zanthorrhiza* in PGI; *C. zanthorrhiza* in PGM; and *C. heyneana* in MDH.

Four multi-enzyme phenotypes were detected in *C. heyneana* based on variation in PGI and MDH. Populations ranged from being monomorphic to dimorphic. Two multi-enzyme phenotypes were found in *C. longa* or *C. colorata* based on variation in PGI. Populations were found to be mono- and dimorphic. Four multi-enzyme phenotypes were found in *C. zanthorrhiza* based on variation in PGI and PGM. Populations also ranged from being monomorphic to dimorphic. All the species are widely cultivated, and it is often difficult to know their origin.

Most *Curcuma* species studied, except *C. parviflora* and *C. roscoeana*, are widely cultivated, polyploid (triploid), and rarely set fruit. These polyploid plants, which propagate via vegetative reproduction, are highly morphologically similar, hardly distinguishable from herbarium specimens. The isozyme data observed here invite a question of how variation within species could happen.

The rhizome of Curcuma is widely used as spice or medicine. Intensive propagation by means of rhizome cuttings may have caused generative reproduction to switch off. The fact that some *Curcuma* show genetic variation (*C. longa, C. heyneana, C. colorata, C. zanthorrhiza*) is supported by polyploidy and sterile phenomenon, invite a hypothesis of hybridization long before the species establish themselves.

Populations of *C. colorata, C. heyneana, C. longa,* and *C. zanthorrhiza* show isozyme polymorphism. Though populations of the remaining species show no

polymorphism, other enzyme systems may produce polymorphic bands. Further investigation should be carried out to verify this.

CHAPTER 7: POLYMORPHISM IN ITS

7.1 INTRODUCTION

Problems with the use of nrDNA genes for molecular systematics include polymorphisms within the arrays. Homogenization of nrDNA is not instantaneous and individual plants may contain a mixture of older and more-derived alleles. Recombination can also result in individual allelles with multiple lineages (Buckler 1996).

Intraindividual polymorphism in ITS of most Javanese *Curcuma* is dealt with here. The same phenomenon has been discovered in Winteraceae (Suh *et al.* 1993), conifers (Karvonen & Savolainen 1993), peonies (Sang *et al.* 1995), *Zea* (Buckler & Holtsford 1996), *Amelanchier* (Campbell *et al.* 1997), *Cucurbita* (Jobst *et al.* 1998), *Gilia* (Morrell & Rieseberg 1998), *Castilleja* (Mathews & Lavin 1998), *Allium* (Mes *et al.* 1999), *Larix* and *Pseudotsuga* (Gernandt & Liston 1999), and *Aeschynanthus* (Denduangboripant & Cronk 2000). Polymorphisms in 18s rDNA and *trn*K of Japanese *Curcuma* is also present (Cao Hui personal communication).

7.2 MATERIALS AND METHOD

In Chapter Two, consensus sequences were given for polymorphic sequences. Here I am attempting to disentangle the two overlapping sequences by trying to find the base insertions and deletions (indel). The method involves tracking the bases starting from where the indel begins (**Figure 7.1**). Other techniques, such as DNA cloning, would, of course, be more powerful, but this has not been possible in the time available.

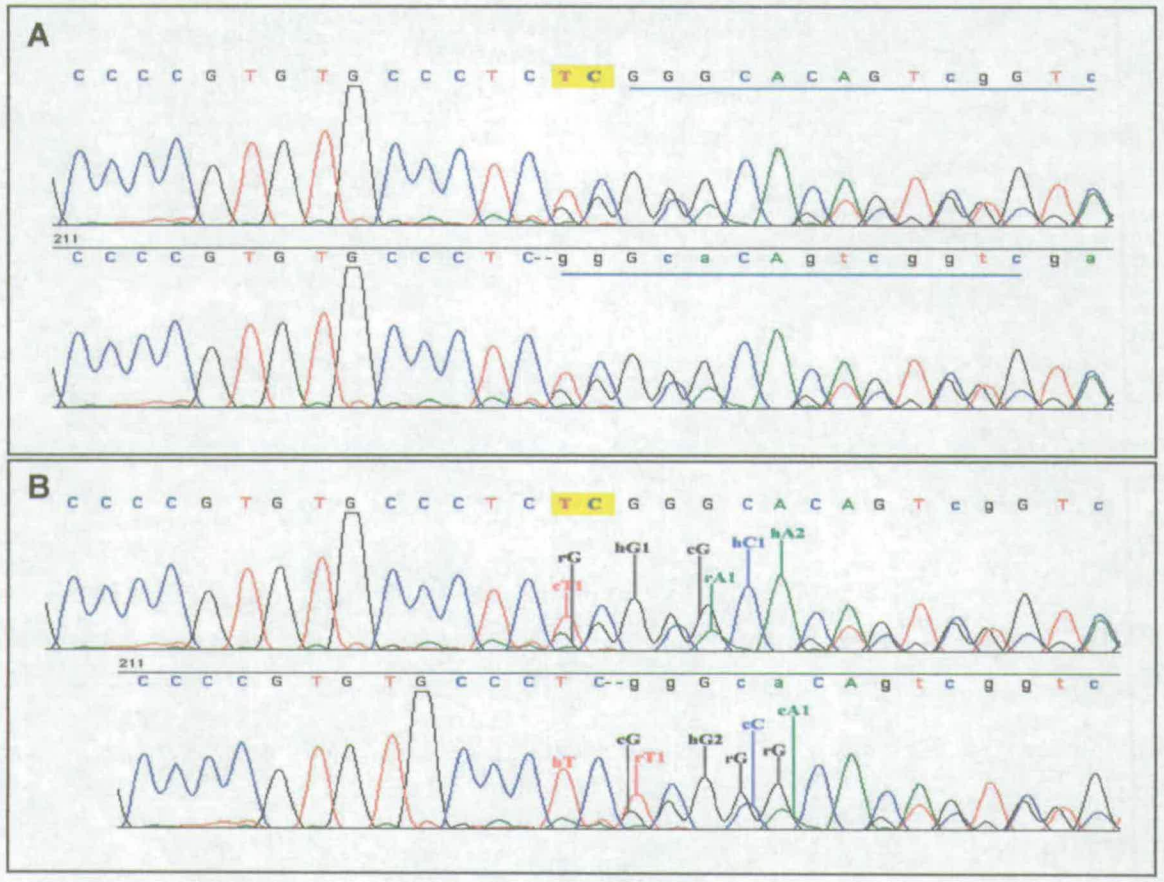


Figure 7.1 Electropherogram

A. Part of an ITS sequence which shows polymorphism. The two sequences are the same. The first line shows addition of TC after editing. The second line shows deletion of TC after editing.

B. Part of an ITS sequence which is similar with that at picture A. However, it was aligned so that the "TC" on the second line will be adjacent to the additive TC on the first line. h=helpful; c=confirmed; r=rejected.

7.3 RESULTS AND DISCUSSION

The result shows many indels in a single ITS sequence of Javanese *Curcuma*, except in three species namely *C. aurantiaca, C. petiolata,* and *C. ochrorhiza.* **Table 7.1** and **Table 7.2** shows the indel polymorphisms in Javanese *Curcuma.*

Indels in ITS1 are far more difficult to track down. This may indicate severe indels in the sequences. Only the very start and the very end of the sequence can be tracked for their indels. The very end indel (indel position II) sites are quite interesting as all traceable species from subgenus *Hitcheniopsis* have no insertion of TTCT except *C. aurantiaca.* Four traceable species from subgenus *Curcuma, C. aromatica, C. cf. longa, C. colorata,* and *C. elata,* show indel polymorphism of TTCT. This hints at hybridization of species having TTCT insertion and species having TTCT deletion. More samples are needed to verify this.

Indels in ITS2 are easier to track down. Eleven indel positions were recorded with the size of one to four bp. Insertion of TGC (indel position I) and TTTA (indel position XI) only occurs in *C. aurantiaca.* Four species, *C. longa 5, C. cf. longa, C. purpurascens,* and *C. colorata,* shows indel polymorphisms of those TGC and TTTA. Hence, those species could be derived by hybridization of *C. aurantiaca* and other species. A more powerful technique is indispensable to check this.

Insertion of AGCG (indel position II) occurs in *C. aurantiaca* and *C. aromatica.* Two other traceable sequences of *C. elata* and *C. cf. amada* show indel polymorphism of the bases. Indel polymorphism of TC (indel position III) occurs in *C. longa* and *C. soloensis.* Insertion of this TC is not found in all traceable species studied. Fourteen accessions show indel polymorphism of GT in indel position IV. Only *C. ochrorhiza* has the insertion the bases. Further study is needed to check if the insertion of the GT of *C. ochrorhiza* contributes to the indel polymorphism of GT in those fourteen accessions. Insertion of T (indel position V) not only occurs in Javanese *Curcuma,* but also in Thai *Curcuma* and the outgroups. Deletion of this T only occurs in *C. ecomata* and *Sm. Supraneanae.* Two accessions, *C. zedoaria 1* and *C. cf. zedoaria,* shows indel polymorphism of the bases. Indel polymorphism of G (indel position VII) only occurs in *C. amada* and *C. amarissima.* No species with deletion of the base is recorded yet.

Table 7.1 Indel polymorphism in ITS1 region

INDELS	I	II
Position (bp)	152	192-195
Ca. gracilis	-	----
Ca. spicata	-	----
R. auriculata	-	----
R. schneideriana	-	----
Stahlianthus sp.	-	----
Smithatris sp.	-	----
C. parviflora	-	----
C. thorelii	-	----
C. roscoeana	-	----
C. alismatifolia	-	----
C. gracillima	-	----
C. ecomata	-	----
C. harmandii	-	----
C. cf.australasica	-	----
C. petiolata	-	----
C. ochrorrhiza	C	----
C. aeruginosa	(-)(C)	----
C. amarissima	(-)(C)	----
C. aurantiaca	-	TTCT
C. heyneana	C	----
C. longa 1	-	----
C. longa 2	-	----
C. amada	(-)(C)	----
C. soloensis	(-)(C)	----
C. aromatica	-	(----)(TTCT)
C. longa cf.	?	(----)(TTCT)
C. colorata	?	(----)(TTCT)
C. elata	?	(----)(TTCT)

Table 7.2 Indel polymorphism in ITS2 region.

INDELS	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Position (bp)	3 - 5	42 - 45	70 - 71	157 - 158	170	173	174	189 - 192	208 - 211	240-241	249 - 252
Ca. gracilis	---	----	--	--	T	-	G	----	----	--	----
Ca. spicata	---	----	--	--	T	-	G	----	----	--	----
R. auriculata	---	----	--	--	T	-	G	----	----	--	----
R. schneideriana	---	----	--	--	T	-	G	----	----	--	----
St.involucratus	---	----	--	--	T	-	G	----	----	--	----
Sm.supraneanae.	---	----	--	--	-	C	G	----	----	--	----
C. parviflora	---	----	--	--	T	-	G	----	----	--	----
C. thorelii	---	----	--	--	T	-	G	----	----	--	----
C. roscoeana	---	----	--	--	T	-	G	----	----	--	----
C. alismatifolia	---	----	--	--	T	-	G	----	----	--	----
C. gracillima	---	----	--	--	T	-	G	----	----	--	----
C. ecomata	---	----	--	--	-	C	G	----	----	--	----
C. harmandii	---	----	--	--	T	-	G	----	----	--	----
C. cf.australasica	---	----	--	--	T	-	G	----	----	--	----
C. petiolata	---	----	--	--	T	-	G	G-AA	----	--	----
C. ochrorrhiza	---	----	--	GT	T	-	G	----	----	--	----
C. aeruginosa	---	----	--	(--)(GT)	T	-	G	----	----	--	----
C. phaeocaulis	---	----	--	(--)(GT)	T	-	G	----	----	--	----
C. amarissima	---	----	--	(--)(GT)	T	(-)(C)	(-)(G)	----	----	--	----
C. aurantiaca	TGC	AGCG	--	--	T	C	G	TCAA	-G-T	--	TTTA
C. heyneana	---	----	--	--	T	-	G	----	(----)(TGAT)	--	----
C. longa 1	---	----	(--)(TC)	--	T	-	G	(----)(TAAA)	(----)(TGAT)	--	----
C. longa 2	---	----	(--)(TC)	--	T	-	G	(----)(TCAA)	(----)(TGAT)	--	----

Table 7.2 (continued) Indel polymorphism in ITS2 region.

INDELS	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Position (bp)	3 - 5	42 - 45	70 - 71	157 - 158	170	173	174	189 - 192	208 - 211	240-241	249 - 252
C. amada	---	----	--	(--)(GT)	T	(-)(C)	(-)(G)	----	----	--	----
C. zedoaria 1	---	----	--	(--)(GT)	(-)(T)	(-)(C)	G	(----)(TCAA)	----	--	----
C. zedoaria 2	---	----	--	(--)(GT)	T	-	G	(----)(TCAA)	----	--	----
C. zedoaria 3	---	----	--	(--)(GT)	T	-	G	(----)(TCAA)	----	--	----
C. zedoaria cf.	---	----	--	(--)(GT)	(-)(T)	(-)(C)	G	(----)(TCAA)	----	--	----
C. zanthorrhiza 1	---	----	--	(--)(GT)	T	-	G	----	(----)(TGAT)	--	----
C. zanthorrhiza 2	---	----	--	(--)(GT)	T	-	G	----	(----)(TGAT)	--	----
C. soloensis	---	----	(--)(TC)	(--)(GT)	T	-	G	(----)(TAAA)	(----)(TGAT)	--	----
C. aromatica	---	AGCG	--	--	T	-	G	(----)(GCAA)	----	(--)(CG)	----
C. longa 3	---	----	(--)(TC)	--	?	?	?	?	----	--	----
C. longa 4	---	----	(--)(TC)	?	?	?	?	?	?	?	?
*C. longa 5**	(---)(TGC)	?	?	?	?	?	?	?	?	?	(----)(TTTA)
*C. longa cf.**	(---)(TGC)	?	?	?	?	?	?	?	?	?	(?)(TTTA)
*C. purpurascens**	(---)(TGC)	?	--	?	?	?	?	?	?	?	(----)(TTTA)
*C. colorata**	(---)(TGC)	?	?	?	?	?	?	?	?	?	(----)(TTTA)
C. elata	---	(----)(AGCG)	--	--	?	?	?	?	?	?	?
C. amada cf.	---	(----)(AGCG)	--	(--)(GT)	?	?	?	?	----	--	----
C. zanthorrhiza 3	---	----	--	(--)(GT)	?	?	?	?	(----)(TGAT)	--	----
C. zanthorrhiza 4	---	----	--	(--)(GT)	?	?	?	?	?	--	?

Notes: *: one copy of ITS2 sequences of these species is suspected to contain sequence that is identical to that of *C. aurantiaca.*

---: deletion

A/C/G/T: insertion

(-)(A/C/G/T): indel polymorphic

?: sequences are unreadable

Indel positions VIII is complex as different type of base insertion are encountered. Eight Javanese species show this indel polymorphism. *C. aurantiaca* and *C. petiolata* might have contributed the insertion of the bases.

Analysis of each single sequence (by tracking down the indel polymorphisms in this Chapter) shows similar result from the analysis of the consensus sequence in Chapter Two. The tree resulted from both analysis is congruent. Further study focusing on the indel polymorphisms of Javanese *Curcuma* would give more insight into the evolutionary history of the genus. Therefore, cloning the DNA to get an isolated single sequence is indispensable. Parsimony analyses were carried out. Different type of analysis resulted in slightly different phylogenetic signal (**Table 7.3**). However, the tree topology resulted are congruent (**Figure 7.2-7.4**).

Unlike other genera in Zingiberaceae such as in *Alpinia* (Rangsiruji *et al.* 2000), *Roscoea* and *Cautleya* (Ngamriabsakul *et al.* 2000), ITS sequence in *Curcuma* are found to be polymorphic. The ITS in *Curcuma* shows two different modes of evolution. First, highly homogenized copies were resulted from concerted evolution. Molecular drive seems to work well in some species, ie. *C.parviflora, C.thorelii, C.roscoeana, C.alismatifolia, C.gracillima, C.ecomata, C.harmandii, C.petiolata, C. aurantiaca* and *C.australasica.* Except *C. australasica,* which only occur in Northern Australia and New Guinea, the rest of those species occur in the Asian continent. *C. petiolata* and *C. aurantiaca* distribute both in the continent and the Malesian archipelago. Those homogenized-ITS species are fertile (the seeds are set). They have diploid chromosomes. Secondly, molecular drive has been failure in the process of concerted evolution. Length polymorphisms with indel events were found in most of Javanese and also Indian polyploid species (*C.ochrorhiza, C.aeruginosa, C.phaeocaulis, C.amarissima, C.heyneana, C.longa, C.amada, C.zedoaria, C.zanthorrhiza, C.soloensis, C.aromatica* and *C. elata*). This polymorphism could have been the result of incomplete homogenization or hybridization process. Further study is needed to check this.

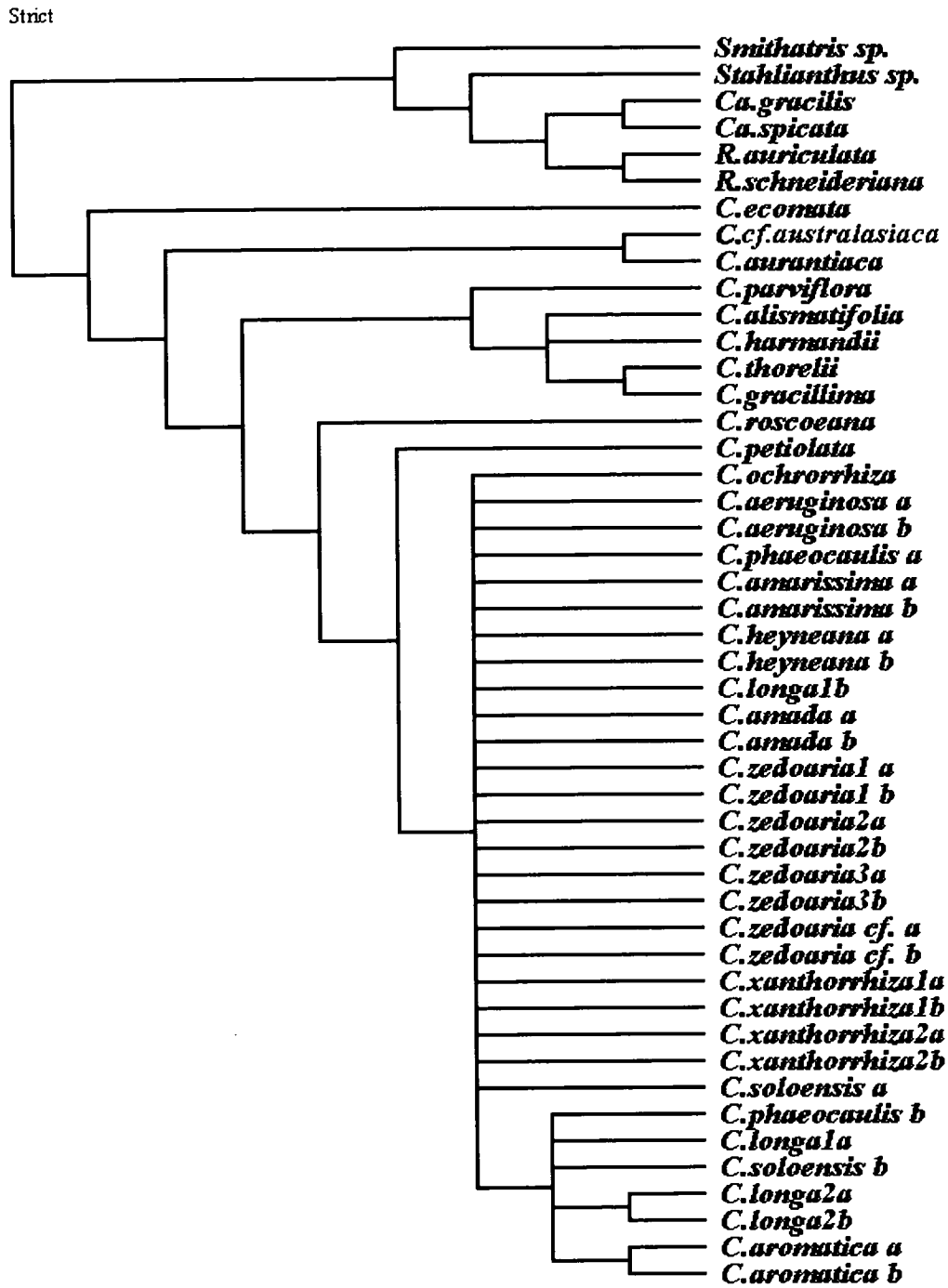


Figure 7.2 Strict consensus tree obtained from 1000 equally most parsimonious trees of length 167 steps resulting from equally weighted parsimony analysis of ITS2 data of non- and polymorphic *Curcuma* and the outgroups, with all sites analysed (CI=0.677; RI=0.830; RC=0.562).

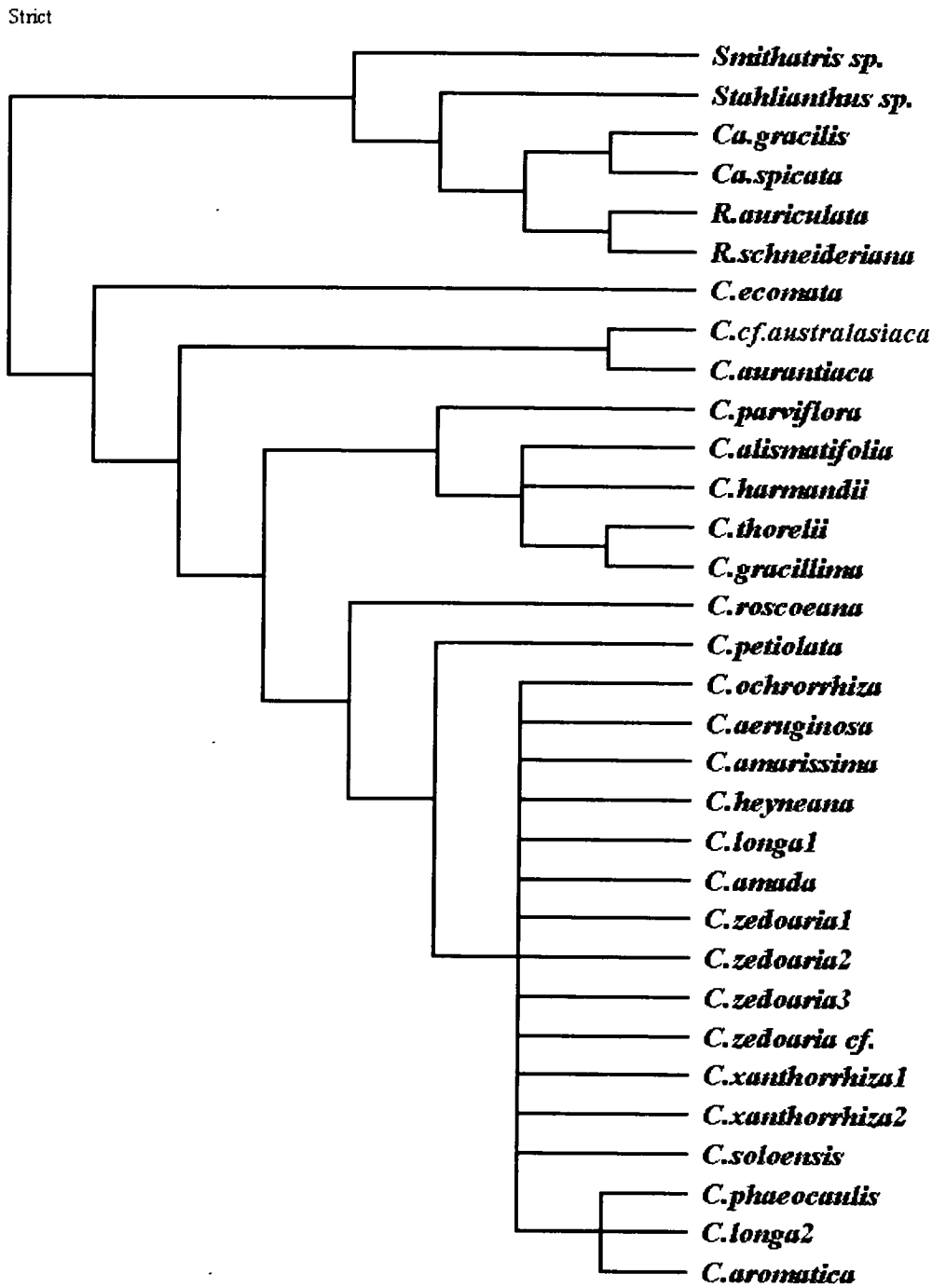


Figure 7.3 Strict consensus tree obtained from 112 equally most parsimonious trees of length 147 steps resulting from equally weighted parsimony analysis of ITS2 data of non- and polymorphic *Curcuma* and the outgroups, with indel polymorphic sites excluded (CI=0.701; RI=0.835; RC=0.585).

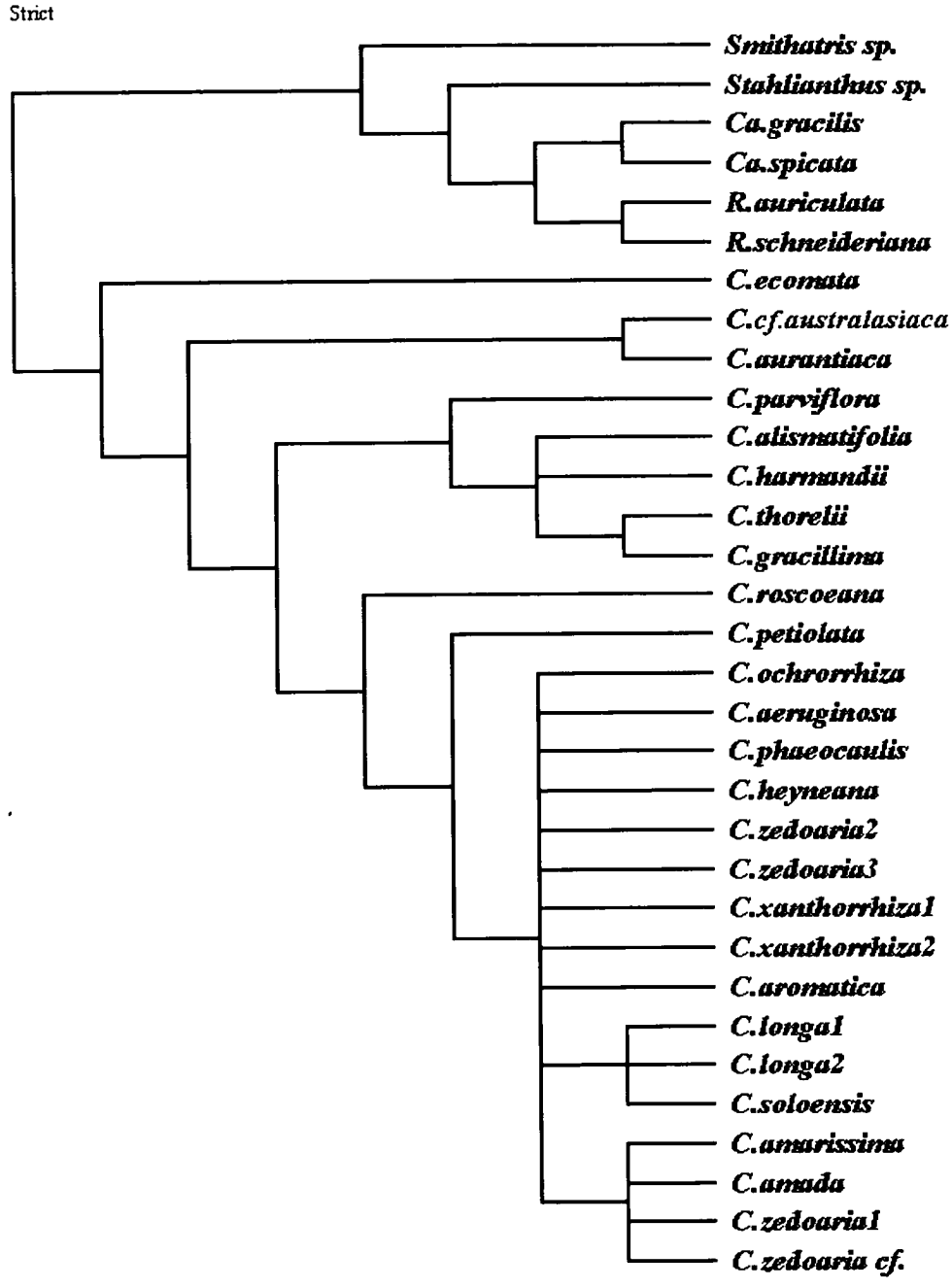


Figure 7.4 Strict consensus tree obtained from 1000 equally most parsimonious trees of length 163 steps resulting from equally weighted parsimony analysis of ITS2 data of non- and polymorphic *Curcuma* and the outgroups, with indel polymorphic sites coded as present or /and absent (CI=0.693; RI=0.828; RC=0.574).

Table 7.3 Descriptive statistics reflecting the amount of phylogenetic signal under different conditions of parsimony analysis for data on **Appendix 6.**

Descriptive Statictics	*analysis 1*	*analysis 2*	*analysis 3*	*analysis 4*
Tree length	167	147	163	171
No. of trees	1000	112	112	112
CI	0.677	0.701	0.693	0.702
CI excl. uninf.	0.620	0.639	0.630	0.630
HI	0.323	0.299	0.307	0.298
HI excl. uninf.	0.380	0.361	0.370	0.370
RI	0.830	0.835	0.828	0.826
RC	0.562	0.585	0.574	0.580

Notes: No. is number; CI: Consistency Index; HI: Homoplasy Index; RI: Retention Index; RC: Rescaled Consistency index; excl. is excluding; uninf. is uninformative. In analysis 1, all sites were analyzed. In analysis 2, copy sequences from polymorphic species were combined with indel polymorphic sites excluded. In analysis 3, similar to analysis 2 but with indel polymorphic sites coded gaps as present or absent. In analysis 4, similar to analysis 3 but alignment gaps were coded as present or absent.

Table 8.1 Taxonomic treatment of Javanese *Curcuma*.

Valeton (1918)	Proposed new classification
Subgenus *Curcuma* (Baker) K. Schum.	Subgenus *Curcuma* (Baker) K. Schum.
Section *Mesantha* Horan.	*C. longa* L.
C. longa L.	
C. purpurascens Blume	*C. longa* L. var. *aeruginosa* (Roxb.) Ardiyani
C. viridiflora Roxb.	*C. longa* L. var. *phaeocaulis* (Valeton) Ardiyani
C. colorata Valeton	*C. longa* L. var. *zedoaria* (Christm.) Ardiyani
C. euchroma Valeton	*C. longa* L. var. *mangga* (Valeton & Zijp) Ardiyani
C. soloensis Valeton	*C. longa* L. var. *ochrorhiza* (Valeton) Ardiyani
C. brog Valeton	*C. longa* L. var. *viridiflora* (Roxb.) Ardiyani
C. ochrorhiza Valeton	*C. longa* L. var. *heyneana* (Valeton & Zijp) Ardiyani
	C. longa L. var. *brog* (Valeton) Ardiyani
Section *Exantha* Horan.	*C. longa* L. var. *soloensis* (Valeton) Ardiyani
C. zedoaria (Christm.) Roscoe	*C. longa* L. var. *zanthorrhiza* (Roxb.) Ardiyani
C. zanthorrhiza Roxb.	*C. longa* L. var. *purpurascens* (Blume) Ardiyani
C. phaeocaulis Valeton	*C. longa* L. var. *euchroma* (Valeton) Ardiyani
C. aeruginosa Roxb.	*C. longa* L. var. *colorata* (Valeton) Ardiyani
C. mangga Valeton & Zijp	
C. heyneana Valeton & Zijp	
Subgenus *Paracurcuma* (Baker) K. Schum.	Subgenus *Hitcheniopsis* (Baker) K. Schum.
C. petiolata Roxb.	*C. petiolata* Roxb.
C. aurantiaca Zijp	*C. aurantiaca* Zijp

beginning, Burtt proposed to conserve *Curcuma* Roxburgh with *C. angustifolia* Roxb. as the type. However, later, after investigating the nomenclature he found out that Manjella-Kua of Rheede could be chosen as a lectotype of *C. longa*. However, to choose this as a lectotype for the genus would end up with the name *Curcuma* L. quoad lecto. excl. descr. since the description of Linnaeus does not describe *C. longa*. Burtt in 1981 proposed again *Curcuma* Roxburgh with *C. longa* as a type. But this is rejected by the Committee and *Curcuma* Linnaeus is at the end conserved for the generic name of the commercial turmeric. The history is summarized and tabulated in **Table 8.3**.

Table 8.2 The original description of Linnaeus Species Plantarum 1753

rotunda	1. CURCUMA foliis lanceolato-ovatis; nervis lateralibus rarissimis. Curcuma foliis ovatis utrinque acuminatis; nervis lateralibus paucissimis. *Roy. lugdb. 12. Fl. zeyl. 7* Manja-kua. *Rheed. mal. II. p. 19. t. 10.* *Habitat in* India.
longa	2. CURCUMA foliis lanceolatis; nervis lateralibus numerosissimis. Curcuma foliis lanceolatis utrinque acuminatis: nervis lateralibus numerosissimis. *Roy. ludg. 12. Fl. zeyl.7. Mat. med. 5.* Curcuma radice longa. *Herm. ludg. 208. t. 209.* *Habitat in* India.

8.2 MATERIALS AND METHOD

Materials are herbarium as well as living specimens. Herbarium specimens (**Appendix 8**) are from Royal Botanic Garden Herbarium Edinburgh (E), and loans from Herbarium Bogoriense (BO), Leiden Herbarium (L), and Kew Botanic Gardens Herbarium (K).

Data on species name, authority, collector, collection number, date of collection, collection site, latitude, longitude, etc are stored in Pandora Data Base in the Royal Botanic Garden Edinburgh. Descriptions were written by hand instead of with the Delta program. Generating description by Delta was once tried (Ardiyani 1997).

Table 8.3 Summary of the history of typification of *Curcuma*

Year	Summary of history of typification of *Curcuma*
1737	Linnaeus in Musa Cliffortiana included only *Curcuma rotunda* in genus *Curcuma.*
1753	Linnaeus in Genera Plantarum added *C. longa* without modifying the description of the genus, so the description was only based on *C. rotunda.*
?	*Curcuma rotunda* was transferred to other genus *Boesenbergia,* therefore *C. longa* is the only species which can be chosen as lectotype of *Curcuma* so long as the identity is clear .
1821	Steudel chose *C. longa* as a lectotype of the genus.
1918	Valeton in his study of Zingiberaceae of Java and Malaya rejected *C. longa* as *nomen dubium* on the basis of that the original description and illustration of Hermann referred to *C. aromatica* Salisb. He proposed *C. domestica* Valeton as the correct name for *C. longa* L. This was followed by, for example Trimen (1931), Holttum (1950), Backer & Bakhuizen f. 1968).
1923	Britton & Wilson chose *C. longa* as a lectotype of the genus.
1929	Hitchcock & Green chose *C. longa* as a lectotype of the genus.
1935	Merrill chose Hermann's *C. longa* as a lectotype of the genus.
1959	Mansfeld pointed out if the typification of *C. longa* proposed by Merrill is accepted, *C. longa* can be taken as lectotype of the name *Curcuma.*
1972	Burtt & Smith proposed to conserve *Curcuma* Roxburgh (1810) non Linnaeus (1753) with *C. angustifolia* Roxb. as its type species. Their reason was that there was no support of the choice from Britton & Wilson (1923) and Hitchcock & Green (1929). However, if the identity of *C. longa* L. as valid species can be established, it does not preclude the re-adoption of *C. longa.*
1974	The Committee rejected Burtt & Smith's proposal to conserve *Curcuma* Roxb. They agreed with Mansfeld (1959) so regarded *C. longa* as a lectotype.
1977	Burtt reinvestigated *C. longa* and found out Manjella Kua of Rheede can be chosen as lectotype of *Curcuma.*
1981	Burtt revised the proposal and proposed again to conserve *Curcuma* Roxb. on the basis of that Roxburgh gives a description of *C. longa* while there is no description in Linnaeus (1753) which can cause the genus only be cited *Curcuma* L. quoad lecto. excl. descr.
1984	The Committee agrees with Burtt in saying that the lectotypification of *C. longa* is in conflict with the protologue. However, committee voted 10-1 (one abstention) that the conserved name should date from 1753 and be attributed to Linnaeus. *Curcuma* Linnaeus is conserved with a new type *C. longa* Linnaeus.

8.3 RESULTS

8.3.1 Generic re-description

Curcuma L.

Curcuma , C. von Linnaeus, father, Sp.pl. 1 (1753) 2 — Syntype: *Curcuma longa* L. (BM)
Erndlia
Hitcheniopsis , H.N. Ridley, Fl.Malay Penins. 4 (1924) 252

Plant of about 30-155 cm height. *Rhizome* white, bright yellow, yellow orange, deep orange internally, pungent or young mango-like fragrant, 3.0-11.0 by 1.5-2.5 cm. *Leaf-sheath* green, glabrescent or pubescent, edge sometimes hairy, c. 5.0 to more than 40.0 cm long. *Leafless sheath* green or reddish brown, broad linear, round, mucronate at apex, glabrescent to pubescent, sometimes hairy on its edge, the outer one smaller than the inner one, c. 4.0-35.0 cm long. *Petiole* green, c. 0-22.0 cm long, glabrous or glabrescent. *Ligule* c. 1.0-3.0 mm by 1.2 to 3.0 cm, ciliate, auriculate or not. *Blade* green with or without purplish brown flush on distal half of the leaves on upper or on both surface, lanceolate to broadly lanceolate, acuminate at apex, rounded, slightly acuminate, acute to obtuse decurrent at base, glabrous or glabrous and hairy on edge at tip on upper surface, glabrous or glabrous and hairy on edge at apex on one side on lower surface, c. 16.5-85.0 by 5.0-23.0 cm. *Midrib* green, dark red brown on upper surface, green on lower surface. *Inflorescence* central or lateral. *Scape* green, c. 6.5-50.0 cm long, slightly glabrescent or pubescent or puberulous, covered with 3-6 leafless sheaths. *Spike* c. 10.0-20.0 by 4.0-9.0 cm. *Leafless sheath* on scape c. 4.0-24.0 cm long, glabrescent or pubescent to densely pubescent. *Spike* c. 9.0-19.0 by 3.5-9.0 cm. *Fertile bract* green tipped with purplish, c. 8-20, oblong, orbicular, obovate, broad ovate, broad elliptic to rounded, rather acute or obtuse to slightly rounded, glabrescent or pubescent to densely pubescent on outer surface, pubescent on inner surface, c. 3.5-6.5 by 1.6-5.2 cm. *Coma bract* purple at tips, dark green getting dull white towards base, c. 4-9, lanceolate to broad lanceolate to broad elliptic, acute to slightly obtuse or obtuse to rounded, slightly mucronate or mucronate or not so, pubescent to densely pubescent on outer surface, c. 3.5-8.4 by 0.9-4.5 cm. *Bracteole* boat-shaped, c. 1.6-3.5 by 0.9-2.6 cm, sparsely shortly hairy with dense hair at apex, white pelucid or white with pink top. *Calyx* c. 1.0-1.4 cm long and c. 1.35 cm wide, three-toothed, with one the deepest among others, hairy especially at tips, apex of tooth truncate slightly cleft, white pelucid and pinkish at tips. *Corolla tube* c. 1.6-3.2 cm long, white, pale yellow, pink, yellowish white at the base, pinkish at apex. *Corolla lobes* pink, pale pink, reddish or brownish. *Dorsal corolla-lobe* elliptic-rounded, ovate, hooded with cucullate apex, c. 1.0-2.0 by 0.6-1.4 cm. *Lateral corolla-lobes* broad oblong, elliptic, apex rounded, c. 1.0-1.5 by 0.7-1.1 cm. *Labellum* almost orbicular with blunt or pointed apex, shallowly cleft at apex, c. 1.4-2.0 by 1.2-1.8 cm, and c. 1.0-1.2 cm wide at base, citrine, yellow, light orange, orange, median darker with purple spot in the centre. *Lateral staminodes* unequal elliptic or oblong with slightly rounded apex, broad

obovate, c. 1.0-1.6 cm long and c. 6.0-9.0 mm wide at base, c. 8.0-18 mm at the widest part, light yellow, light orange, orange. *Anther* slim, c. 3.5-4.0 mm long, c. 2.0-2.5 mm thick, thecae c. 1.5-2.0 mm wide, protruded on posterior side, spur almost as long as theca, c. 2.0-4.0 mm long, curved. *Filament* c. 3.5-8.0 by 4 mm. *Style* thin and slender. *Stigma* bilabiate with saccate of two-lobes on dorsal side. *Stylodes* 5.0-7.0 mm long.

Ecology: teak forests, dry grassy lands, cultivated, waste ground and abandoned cultivation.
Altitude range: 0 - 2500 m

8.3.2 Key to subgenera *(partly after Valeton, 1918)*

Rhizome developed; leaves narrowed at the base; spurs are almost as long or half as long as the anther; lateral staminodes folded; ligule not auriculate..... .....**subgenus *Curcuma***
Rhizome hardly developed; leaves rounded at the base; no spurs or very short spurs on anther; lateral staminodes not folded; ligule auriculate .....**subgenus *Hitcheniopsis***

8.3.3 Key to varieties in Javanese Curcuma longa

The key leads involve poor characters such as colours and smells. They are best applied to living material.

1. Rhizomes bitter, white with blue tinge; leaves with purple flush 2
   Rhizomes light yellow, yellow, dark yellow or orange; leaves green or with purple flush..... 3

2. Leaf sheath green..... ***aeruginosa***
   Leaf sheath purplish..... ***phaeocaulis***

3. Rhizome light yellow, mango smell or not 4
   Rhizome yellow, dark yellow or orange.....6

4. Rhizome bitter, not mango smell; leaves with intense purple streak along the midrib.. ..... ***zedoaria***
   Rhizome slightly bitter, mango smell; leaves green.....5

5. Rhizome with clear mango smell, very slightly bitter; inflorescence lateral..... ..... ***mangga***
   Rhizome very slightly manggo smell, bitter; inflorescence central..... ..... ***ochrorhiza***

6. Leaves green without purple streak.....**7**
   Leaves with purple streak or with purple midrib.....**11**

7. Inflorescence all green or sometimes coma bracts pinkish; inflorescence central.....
   .....**8**
   Coma bracts pink or purplish, flower bracts greenish; inflorescence lateral or central..**9**

8. Inflorescence all green or coma bracts pinkish; rhizome dark orange, turmeric smell...
   ..... ***longa***
   Inflorescence all greens; rhizome orange..... ***viridiflora***

9. Rhizome yellow, unique smell; inflorescence lateral..... ***heyneana***
   Rhizome citrine or orange yellow; inflorescence central.....**10**

10. Rhizome citrine; flowers light yellow..... ***brog***
    Rhizome orange-yellow..... ***soloensis***

11. Leaves with purplish midrib; inflorescence central.....**12**
    Leaves with purple streak not along the midrib; inflorescence lateral.....
    ..... ***zanthorrhiza***

12. Rhizome dark orange or yellow-orange; coma bracts pinkish, flower bracts greenish
    .....**13**
    Rhizome dark yellow; bracts all green..... ***purpurascens***

13. Rhizome yellow-orange..... ***euchroma***
    Rhizome dark orange..... ***colorata***

***Curcuma longa* L.** Sp. Pl. 1. p.3. 1753

Finding a type citation or citing a type for *Curcuma longa* was beyond the scope of the thesis.

Amomum curcuma Jacq.
Curcuma domestica Valeton , T. Valeton, Bull.Jard.Bot. Buitenzorg 27 (1918) 31

Rhizome deep orange (21A or 22A) externally and internally, the young tips white. *Blade* green. *Inflorescence* central. *Flower bract* green. *Coma bract* white or white with purple towards. *Flower* pale yellow. *Corolla tube* white. *Corolla lobes* white. *Lateral corolla-lobes* ovate, rounded at apex. *Labellum* creamy white with yellow median band. *Lateral staminodes* creamy white.

Vernacular. Kunyit

Altitude range:
250 - 1281 m

***Curcuma longa* L. var. *aeruginosa* (Roxb.) Ardiyani**, W. Roxburgh, Asiat.Res. 11 (1810) 335

Rhizome cylindric, bluish inside, bitter. *Blade* green with purplish brown flush on distal half of the leaves on upper surface (on both surface, Holttum), *midrib* green. *Inflorescence* lateral. *Flower bract* green tipped with purplish. *Coma bract* purple at tips, dark green getting dull white towards base. *Calyx* transparent and pinkish at tips. *Corolla tube* yellowish white (dark pink red, Valeton; deep-crimson pink, Holttum). *Corolla lobes* reddish or brownish (pale red, Baker). *Lateral staminodes* light yellow.

Vernacular. Koneng hideung, Kunyir hideung (Sunda), Temu ireng (Jawa), Temo ereng (Madura).

Note. *aeruginosa* is meant for the aeruginous colour of the rhizomes. The specimen cited in the protologue comes from Pegu. Roxburgh reduces Rumph's "temu itam" (which is *C. aeruginosa* Roxb.) to a Bengalese species called *C. caesia* Roxb.

***Curcuma longa* L. var. *phaeocaulis* (Valeton) Ardiyani** , T. Valeton, Bull.Jard.Bot. Buitenzorg 27 (1918) 69

Rhizome yellow (20C) in the centre, orangish (25D) and bluish in the middle, and white or cream at peripheral; bitter. *Leafless-sheath* reddish brown. *Leaf-sheath* reddish brown with green at apex. *Petiole* green. *Blade* green with purple streak along the midrib; *midrib* green. *Inflorescence* lateral. *Flower bract* elliptic, acute, green tipped with purplish. *Coma bract* red purple at top and white at the lower half. *Corolla lobes* red. *Labellum* deep yellow .

Vernacular. Temu itam; Temu santen (Sumedang, West Java).

Note. The description on inflorescence is taken from Valeton 1918. In daily use, the rhizomes of this species is mixed up with those of *C. aeruginosa*.

Altitude range:
177 - 301 m

***Curcuma longa* L. var. *zedoaria* (Christm.) Ardiyani** Monandr. Pl. Scitam. 1828: 41

Roscoea lutea (Blanco) Hassk.
Roscoea nigrociliata Hassk.
Curcuma zedoaria (Bergius) Roscoe
Costus luteus Blanco
Amomum zedoaria Christm. , G.F. Christmann, G.W.F. Panzer, Vollst.Pflanzensyst. 5 (1779) 12

Rhizome cylindric, light yellow internally (20C). *Leafless-sheath* green with brown flush, mucronate at apex. *Leaf-sheath* green. *Petiole* green. *Blade* green with reddish brown flush along the midrib on both surface, *midrib* purplish green on upper surface, green on lower surface. *Inflorescence* lateral. *Scape* green. *Flower bract* elliptic (broad obovate, Horaninow), green tipped with purplish. *Coma bract* dark pink at tip getting dull white towards the base. *Calyx* yellowish white with pinkish at tip. *Corolla tube* yellowish white. *Corolla lobes* white. *Labellum* yellow, median darker with purple spot in the centre. *Lateral staminodes* light yellow.

Vernacular. Temu kuning.

Altitude range:
50 - 626 m

***Curcuma longa* L. var. *mangga* (Valeton & Zijp) Ardiyani** in Bull. Jard. Bot. Buitenz. XXVI. 1918: 50

Rhizome young mango-like fragrant, light yellow inside (3C when young; 20B on peripheral and 25B in thecentre when old) (citrine, Valeton). *Leafless-sheath* green. *Leaf-sheath* green (sometimes slightly purple, Holttum). *Petiole* green. *Blade* green, *midrib* green. *Inflorescence* lateral. *Scape* green. *Flower bract* oblong to elliptic, obtuse to rather acute, green tipped with purplish. *Coma bract* dark pink at tips getting dull white towards base. *Calyx* pelucid. *Corolla tube* yellowish white at the base, pinkish at apex. *Corolla lobes* pinkish white (flower pure white, Valeton, Holttum). *Lateral staminodes* light yellow.

Vernacular. Temu mangga; Tema, Tema poh (Madura, East Java and Yogyakarta); Temu bajangan (local name in Bojonegoro), Temu lalab (Jakarta).

Note. mangga means mango. The rhizome has young mango-like odour.

Altitude range:
250 - 259 m

***Curcuma longa* L. var. *ochrorhiza* (Valeton) Ardiyani** , T. Valeton, Bull.Jard.Bot. Buitenzorg 27 (1918) 45

Rhizome light-yellow (1B) when old and yellowish white (3C) when young. *Blade* green. *Inflorescence* central. *Flower bract* broad oblong ovate, obtuse, light green. *Coma bract* obovate, obtuse to slightly acute, white with rose apex. *Corolla tube* pale yellow. *Corolla lobes* pale rose.

Vernacular. Temu lawak which is for *C. zanthorrhiza* (Valeton 1918).

Note. Specimen cited in the protologue i.e. Heyne 705 from Randublatung. *ochrorhiza* is named from the externally and internally white, in the centre greenish-lemon tinged rhizome (Valeton 1918).

Altitude range:
259 - 260 m

***Curcuma longa* L. var. *viridiflora* (Roxb.) Ardiyani** , W. Roxburgh, Asiat.Res. 11 (1810) 341

Rhizome nearly pure yellow mixed with brown tinged (deep yellow, Roxburgh) internally. *Root tuber* ovate, very light ash-coloured with gold yellow endodermis. *Blade* green, *midrib* very faintly purplish on upper surface (Holttum). *Inflorescence* central. *Flower bract* narrowly ovate, obtuse, light green. *Coma bract* snow white with partly light brown dots, sometimes with sporadic light brown dots at apex. *Corolla lobes* faintly pink, the rest of the flower light cream.

Vernacular. Temu lati, Lati putih, Temu kebo, Temu prit (Jawa).

Note. A native of Sumatra, and other eastern islands. Roxburgh described the species from a specimen from Bencoolen (Bengkulu) which was sent by Dr. Charles Campbell (Roxburgh 1820). Specimen cited in the protologue is a specimen of Dr. Charles Campbell from Bencoolen (Bengkulu). *viridiflora* probably means green flower or inflorescence. The description above is from Valeton (1918).
Altitude range:
138 - 600 m

***Curcuma longa* L. var. *heyneana* (Valeton & Zijp) Ardiyani** in Bull. Jard. Bot. Buitenz. XXVI. 1918: 54;

Rhizome yellow (7A) inside (pure bright yellow, Valeton; whitish sometimes yellowish in the centre, Backer & Bakhuizen). *Leaf-sheath* green. *Petiole* green. *Blade* green, *midrib* green. *Inflorescence* lateral. *Scape* green. *Flower bract* green tipped with purplish. *Coma bract* dark pink at tip getting dull white towards base. *Calyx* transparent and pinkish at tip. *Corolla tube* yellowish white at the base and pinkish at apex. *Corolla lobes* pinkish white.

Vernacular. Temu giring (Central Java), Jaha (West Java), Temu giring, Tema licin, Tema koneng (East Java), Tema lateng (local name in Mt. Yang).

Altitude range:
177 - 900 m

***Curcuma longa* L. var. *brog* (Valeton) Ardiyani** , T. Valeton, Bull.Jard.Bot. Buitenzorg 27 (1918) 48 p.24

Rhizome pure lemon yelow internally, yellowish white externally. *Root tuber* very pale yellowish internally. *Inflorescence* central. *Scape* pubescent. *Flower bract* pale yellow green on lower bracts, pale green with violet stripes on upper bracts, pubescent. *Coma bract* almost white at the base, red violet upperhalf towards the apex, pubescent. *Corolla lobes* very light pink.

Note. Specimen cited in the protologue i.e. specimens from Randublatung. The description above is based on the herbarium specimen from Randublatung with Valeton's handwriting. The data on underground parts is adopted from Valeton's protologue.

***Curcuma longa* L. var. *soloensis* (Valeton) Ardiyani** , T. Valeton, Bull.Jard.Bot. Buitenzorg 27 (1918) 46

Rhizome orange internally (25A), bitter and minty fragrance. *Root tuber* ellipsoidal, light grey with yellow or lemon yellow endodermis. *Blade* green. *Inflorescence* central. *Flower bract* very light pure green on lower bracts, with spotted violet at apex on upper bracts (Valeton). *Coma bract* white with pink towards the apex, dark violet at apex. *Corolla lobes* very light.

Vernacular. Gelenye, Belenye.

Note. Specimen cited in the protologue, i.e. Heyne 50 from Solo. *soloensis* from the word Solo, a town in Central Java.

Altitude range:
50 - 610 m

***Curcuma longa* L. var. *zanthorrhiza* (Roxb.) Ardiyani**, Fl.ind. I (1820) 25

Rhizome orange inside (21A or 22A). *Root tuber* orange (10E) when young, orange (17A or B) when old. *Leafless-sheath* green with brown flush. *Leaf-sheath* green. *Petiole* green. *Blade* green with narrow purplish brown flush along the midrib on both surface, purple flush remain on down the middle in old leaves, *midrib* purplish green on upper surface, green on lower surface. *Inflorescence* lateral. *Scape* green. *Flower bract* broadly ovate, rather acute or pointed, green tipped with purplish. *Coma bract* dark pink at tip getting dull white towards base. *Calyx* transparent or colourless (toothlets light red, Valeton). *Corolla tube* yellowish white. *Corolla lobes* pinkish white. *Lateral staminodes* light yellow with pinkish spot at the back or whitish according to Holttum.

Vernacular. Temu lawak (Central/East Java), Koneng gede (Sunda), Temu labak (Madura).

Note. The species that was described by Roxburgh came from Amboyna (Ambon) which was brought to the Botanic Garden at Calcutta in 1798. However, this plant flowered for the first time in April and May 1810 (Roxburgh 1820).

Altitude range:
177 - 400 m

***Curcuma longa* L. var. *purpurascens* (Blume) Ardiyani**, C.L. von Blume, Enum.pl.Javae (1827) 46

Rhizome yellow (17C). *Root tuber* elliptical, orange yellow in the inner cortex, grey pleroma. *Blade* green, *midrib* dark red brown on upper surface. *Inflorescence* central. *Flower bract* light green. *Coma bract* white at the base, light green to nearly white towards the apex, light brown spotted at apex. *Bracteole* pellucid white. *Corolla lobes* snow white, the rest of the flower very pale cream yellow. *Lateral staminodes* elliptic, falcate, obtuse.

Vernacular. Tis, Pinggang, Tinggang, Gelenye or Belenye (Valeton 1918), Kunyir santen, Koneng santen (Sunda) (Koorders 1911).

Note. Specimen cited in the protologue is from province of Bantam (Banten), West Java. The word *purpurascens* is probably for the purple flush on the leaves. The description of flower is based on the herbarium specimen.

Altitude range:
260 - 850 m

***Curcuma longa* L. var. *euchroma* (Valeton) Ardiyani** , T. Valeton, Bull.Jard.Bot. Buitenzorg 27 (1918) 42 p.26

Rhizome bright orange (25A or B) internally, the young one bright orange yellow internally. *Root tuber* orange yellow. *Blade* green, *midrib* reddish on upper surface, almost disappear on mature plant. *Inflorescence* central. *Flower bract* broad ovate, green with red purple tip. *Bracteole* pelucid and pink at apex. *Coma bract* light green at base red purple at apex. *Flower* diluted ochraceous. *Corolla lobes* pink (56A). *Corolla tube* yellow. *Lateral staminodes* light orange. *Labellum* light orange with darker (orange) median band. *Filament* orangish. *Anther* and spur white.
Vernacular. Kunir batok, Temu prit, Temu lati.

Note. Specimen cited in the protologue, ie. Heyne 449 from Mojokerto; Heyne 52 from Kediri; Heyne s.n. from Soemenep (Sumenep) Madura. *euchroma* means well-coloured.

Altitude range:
50 - 308 m

***Curcuma longa* L. var. *colorata* (Valeton) Ardiyani**, T. Valeton, Bull.Jard.Bot. Buitenzorg 27 (1918) 40 p.5, p.25

Rhizome deep orange (25B) internally, smell pleasant, taste mild or carrot-like (Holttum). *Root tuber* orange in the centre, grey on edge. *Blade* green, *midrib* dark red brown on both surfaces. *Inflorescence* central. *Flower bract* green on lower bracts, green with violet stripes and pink at top on upper bracts, pubescent. *Coma bract* white or light green, dark purple at apex, pubescent. *Corolla lobes* pale pink (Valeton).

Vernacular. There is no well-established native name. Tis or Tinggang, Temu ketek.

Note. Specimen cited in the protologue, ie. Heyne 35; Backer 11348 from Mount Willis; K.1645 from Randublatung. *colorata* is probably meant for the colour of the coma which is dark purple, and the flower which is orange (Valeton 1918). The tallest flowers of any *Curcuma* of Java. It has some resemblance to *C. petiolata* (Valeton 1918).

Altitude range:
177 - 600 m

8.3.4 Key to species of subgenus Hitcheniopsis

No spur on the anther ..... ***C. aurantiaca* Zijp**
Spur on the anther is short..... ***C. petiolata* Roxb.**

Curcuma aurantiaca Zijp , C. van Zijp, Recueil Trav.Bot.Néerl. 12 (1915) 345 —

Rhizome hardly developed, orange (21A or 22A) internally. *Blade* green. *Inflorescence* central. *Flower bract* green. *Coma bract* pink (55B or C). *Flower* pale yellow. *Corolla tube* orange (28B). *Corolla lobes* orange (28B). *Lateral corolla-lobes* ovate, rounded at apex. *Labellum* orange (28B). *Lateral staminodes* orange (28B). *Anther* whitish with no spur.

Altitude range:
50 - 550 m

Curcuma petiolata Roxb. , Fl.ind. 1 (1820) 36

Rhizome hardly developed, very pale sulphurous internally. *Blade* green. *Inflorescence* central. *Flower bract* green, dark purple-brown at tip. *Coma bract* dark purple-brown. *Flower* very light orange. *Corolla tube* white. *Corolla lobes* white with a yellow or pink top. *Lateral corolla-lobes* rotundate-ovate-oblong. *Labellum* light orange with yellow median band. *Lateral staminodes* light orange. *Anther* with short spur. (Valeton 1918)

Vernacular. Temu putri (Jakarta)

Note. petiolata means long petiole. In Java, the species is cultivated in Batavia and Buitenzorg and seems to be rare (Valeton 1918). The species is native from Pegu and Martaban. It was first discovered by Mr. F. Carey which then sent to the Calcutta Botanic Gardens. Then, it was described by Roxburgh (Hooker f. 1870). The species is closely related to the turmeric (*C. longa*) and *C. australasica* Hook. f. The two latter species, however, have narrow leaf at the base, longer spikes, and not too deep pouches of its bracts (Hooker f. 1870).

Altitude: 100 m

Green leaf sheath of subgenus *Hitcheniopsis*

Green leaf sheath of subgenus *Curcuma*

Brown leaf sheath of subgenus *Curcuma*

Ligule auriculate

Ligule not auriculate

Leaf entirely green without purple flush

Leaf green, midrib brown, without purple flush

Leaf green with purple flush along the midrib

Leaf green with purple flush on distal half of the leaves

Rhizome hardly developed

Rhizome well-developed

Rhizome section white with blue tinged

Rhizome section light yellow

Rhizome section yellow

Rhizome section dark yellow

Rhizome section orange – colour chart designed

Rhizome section orange-yellow

Rhizome section dark orange

Figure 8.1 Index of picture to use in Figure 8.2

Inflorescence lateral; coma bracts pinkish, flower bracts greenish

Inflorescence central; coma bracts greenish, flower bracts greenish

Coma bracts pinkish, flower bracts greenish

Living material not seen

L Lateral inflorescence

C Central inflorescence

Figure 8.1 (continued) Index of picture to use in Figure 8.2

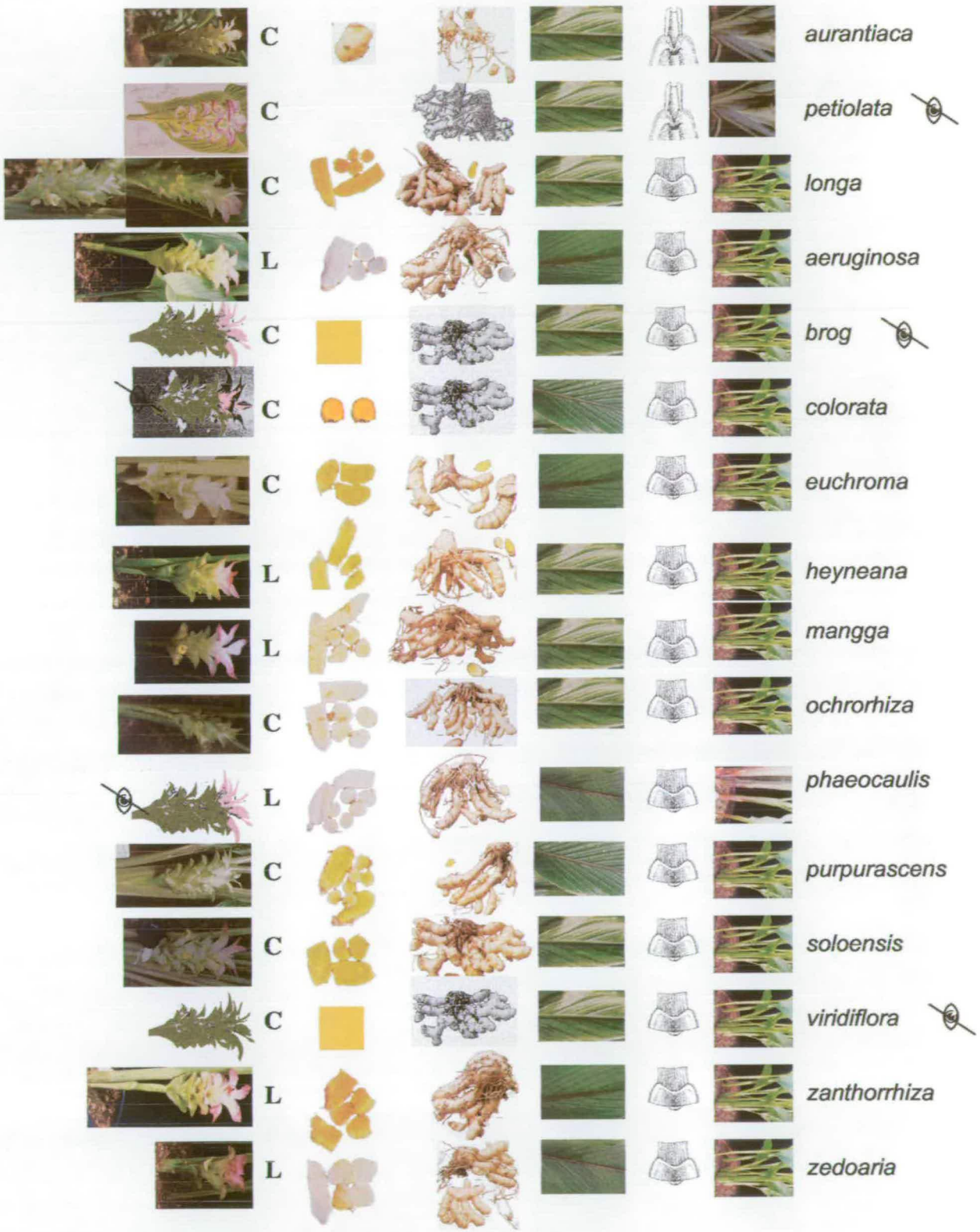


Figure 8.2 Illustration of Javanese *Curcuma*.
From the left to the right: inflorescence, rhizome section, rhizome structure, leaf, ligule, and leaf sheath. An eye with a slash indicates the material is not seen;.C: central; L: lateral.

CHAPTER 9: GENERAL DISCUSSION AND CONCLUSIONS

9.1 PHYLOGENETIC STUDY BASED ON COMBINED MORPHOLOGICAL AND MOLECULAR DATA

The tree resulting from the combined molecular and morphological data can be seen in **Figure 9.1**. It is less resolved than those resulting from the molecular or the morphological data. The subgenus *Curcuma* clade is nested terminal in the tree. This is supported by the analysis of the molecular or the morphological data. Therefore, the present classification of the genus into the subgenus *Curcuma* is confirmed. Two subclades within the subgenus *Curcuma* clade are similar with those in the molecular tree. However, this does not reflect the two sections in the subgenus. The sectional level classification is not supported, and should be abandoned. As mentioned in Chapter Two, there are no morphological data which support the grouping into the two subclades. *C. petiolata* is nested next to subgenus *Curcuma* clade. This is supported by the analysis of the molecular or the morphological data. Hence, the species is closely related to the subgenus *Curcuma* having an intermediate character between the two subgenera in *Curcuma*. The *C. aurantiaca* and *C. cf. australasica* clade, the *C. parviflora* clade, *C. roscoeana,* and *C. ecomata* are nested in a polytomy. Nevertheless, the data show the close relationship among species of the subgenus *Hitcheniopsis*. Therefore, subgenera *Curcuma* and *Hitcheniopsis* are phylogenetically distinct. The *C. parviflora* clade is well resolved. *C. harmandii,* which has a complex floral type, is nested at the base of the clade. *C. thorelii* and *C. gracillima,* which have a small floral type, are nested at the terminal end of the clade. It appears that the floral type has evolved from the complex to the small type.

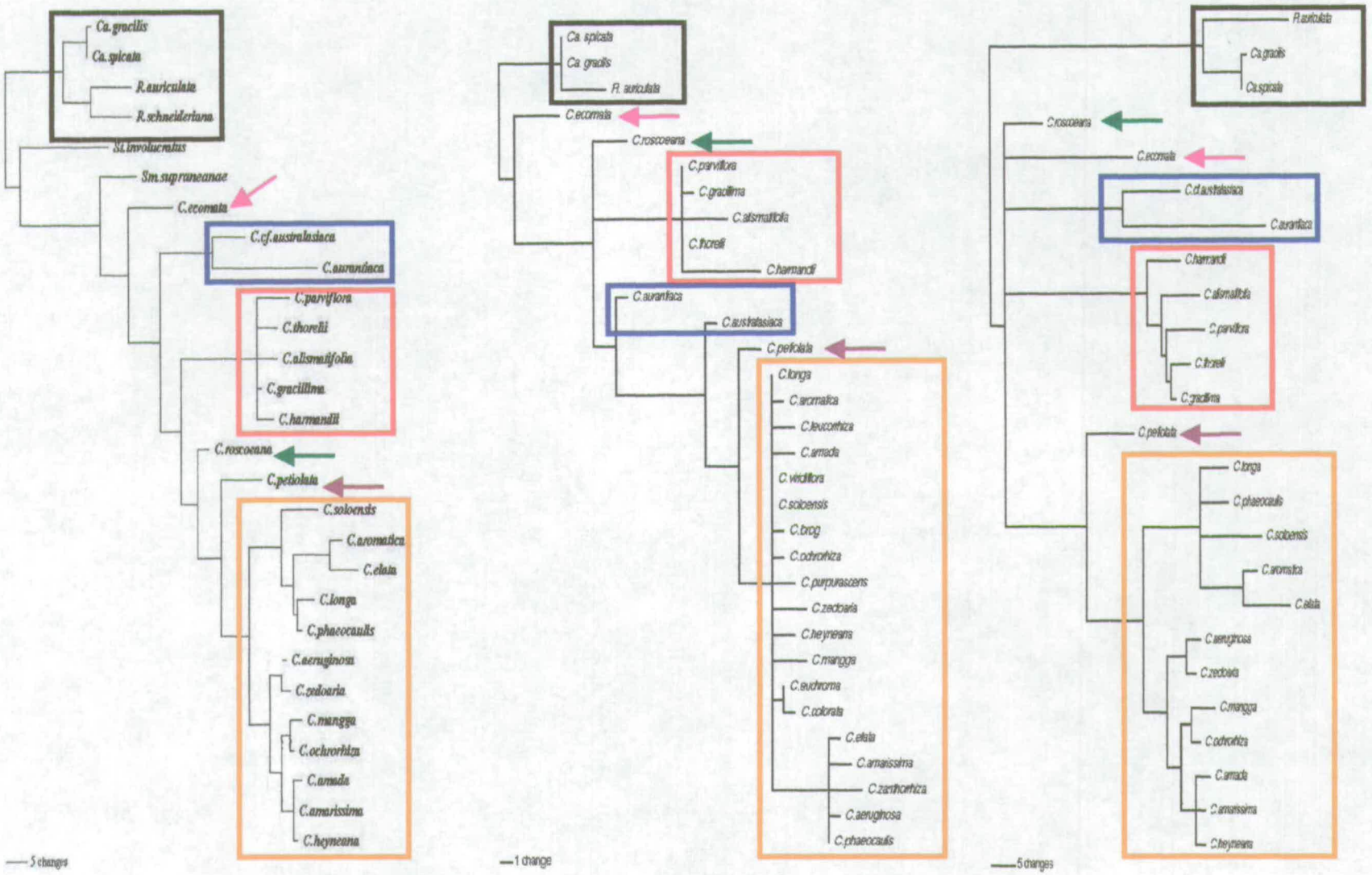


Figure 9.1 Trees constructed from molecular data (the left), morphological data (the middle), and combined both molecular and morphological data (the right)

9.2 EVALUATION OF THE CHARACTERS USED IN THE EXISTING CLASSIFICATION OF CURCUMA

9.2.1 The rhizomes

The well-developed rhizome character is uniformly distributed within subgenus *Curcuma*. In contrast, the hardly developed rhizome is prevalent in subgenus *Hitcheniopsis*. One species from this subgenus, *C. petiolata*, has an intermediate character having very short rhizomes. Almost none of the subgenus *Curcuma* species with well-developed rhizomes set seeds. This sterility may result from constant vegetative reproduction using the rhizomes which have been intensively used as traditional medicines. During the course of evolution, the rhizomes of *Curcuma* may have evolved from none or very short to well developed rhizomes. The process had been possibly affected by human influence or through domestication.

9.2.2 The leaves

The leaves of the species in subgenus *Hitcheniopsis* are rounded at the base according to Valeton (1918) in the delimitation of the subgeneric level. *C. alismatifolia* from the subgenus *Hitcheniopsis*, however, has narrow leaves acuminate at the base. This character, therefore, is not consistent with the division of the two subgenera. The ligules are auriculate for all species from subgenus *Hitcheniopsis*. They are not auriculate in subgenus *Curcuma*. So far, this ligule character is consistent with the division of the subgenera.

9.2.3 The bracts

The bracts are mostly not adnate above the middle in subgenus *Curcuma* according to Valeton (1918). They are connected at least partly beyond the middle in subgenus *Hitcheniopsis* (Valeton 1918). This character of the adnation of the bracts is not consistent. In subgenus *Hitcheniopsis*, *C. harmandii* has bracts that are adnate for much less than half their length. Hence, the character of the adnation of the bracts is not a good for separating the two subgenera. The coma bracts in subgenus *Hitcheniopsis* are

shorter than the fertile bracts (Valeton). *C. alismatifolia* has shorter fertile bracts than coma bracts. This character of the length of the coma bracts is therefore inconsistent with the division of the subgenera.

9.2.4 The lateral staminodes

The lateral staminodes are longitudinally grooved and folded or clasped under the dorsal corolla lobe in subgenus *Curcuma* (Valeton 1918). These characters are congruent with the division of the subgenera. Free (not folded) and non-grooved lateral staminodes occur in all species from subgenus *Hitcheniopsis*.

9.2.5 The anthers

All species from subgenus *Curcuma* have spurs on the anther. Three species from subgenus *Hitcheniopsis* studied, *C. ecomata, C. cf. australasica,* and *C. petiolata,* have spurred anthers. The rest of the species from subgenus *Hitcheniopsis* studied have spurless anthers. Therefore, the character of the spurred anther is not specific to subgenus *Curcuma* alone.

Spurs are assumed to help in the process of pollination. When a pollinator tries to enter the flower, it has to push the spurs. This in turn will rotate the anther, since the anther is versatile, and will position it so that it touches the back of the pollinator. The pollen then will be trapped on the pollinator's back and will be carried to other flowers.

The connectives of the anther cells lengthen at the apex to form a crest which encloses the stigma entirely or only its base. This anther crest occurs in all species from the subgenus *Hitcheniopsis* being a constant character for the subgenus.

9.3 EVALUATION OF THE CLASSIFICATION OF CURCUMA FROM PHYLOGENETIC INSIGHT

9.3.1 The existing classification of Curcuma

The exclusion of several species from the genus *Curcuma* by Valeton (1918) does not result in a natural grouping. Therefore, the delimitation of Valeton's subgenus

Paracurcuma does not accommodate the rest of the species from the subgenus *Hitcheniopsis*. Some characters used in the delimitation of the subgenera are inaccurate. They are the shape of the base of the leaves, the adnation of the bracts, the length of the coma bracts in comparison with that of the fertile bracts, and the spurs on the anther (p.221-222). Some other characters are good for the delimitation of the subgenera, i.e. the shape of the ligules, the groove on the lateral staminodes, the clasping lateral staminodes, and the crest on the anther.

The characters used in the delimitation of Baker's (1894) sectional level and Schumann's (1908) subgeneric level classifications are also inaccurate. The position of the inflorescence has been proved homoplasious. The shape of the apex of the bracts in subgenus *Hitcheniopsis* is not always obtuse. It is acuminate in *C. harmandii*. The adnation of the bracts, as explained earlier, is not a good character for separating the two subgenera. Although the delimitation of the subgeneric taxa is not really appropriate, the groupings of the species into the subgenera are accurate.

The sections of Schumann (1908), Baker (1894), and Valeton (1918), which are based on the position of the inflorescence, have to be rejected as discussed previously (p.75; p.145).

The classification of *Curcuma* by Velayudhan *et al.* (1996) is mainly based on the rhizome characters. Due to limited samples, the classification can not be completely checked. In the field, *C. longa* (which is in the section *Tuberosa* subsection two) was found to produce flower spikes from the tip of the sessile tuber, and from the tip of the primary mother rhizome in another clone. Therefore, the division into subsections in section *Tuberosa* does not seem natural. Further study is needed to verify this.

9.3.2 *Proposed new classification of* Curcuma

Subgenus Curcuma (Baker) K. Schum.

- ***Diagnostic description (partly after Valeton 1918)***

Rhizomes well developed, forming lateral branches. Ligules not forming an auricle on both sides of the base of the petiole. Lateral staminodes longitudinally grooved, folded under the dorsal lobe. The connective of the anther cells not lengthened towards the top, not forming a crest.

C. longa L., *C. longa* var. *aeruginosa*, *C. longa* var. *zedoaria*, *C. longa* var. *amada*, *C. longa* var. *heyneana*, *C. longa* var. *ochrorhiza*, *C. longa* var. *soloensis*, *C. longa* var. *aromatica*, *C. longa* var. *elata*, *C. longa* var. *amarissima*, *C. longa* var. *zanthorrhiza*, *C. longa* var. *brog*, *C. longa* var. *euchroma*, *C. longa* var. *colorata*, *C. longa* var. *mangga*, *C. longa* var. *ochrorhiza*, *C. longa* var. *purpurascens*, *C. longa* var. *viridiflora*, and *C. longa* var. *phaeocaulis*.

Subgenus Hitcheniopsis (Baker) K. Schum.

Diagnostic description (partly after Valeton 1918)

Rhizomes lacking or short, not forming lateral branches. Ligules forming an auricle on both sides of the base of the petiole. Lateral staminodes not longitudinally grooved, not folded under the dorsal lobe. The connective of the anther cells lengthen towards the top forming a crest.

C. ecomata, *C. australasiaca*, *C. aurantiaca*, *C. thorelii*, *C. gracillima*, *C. alismatifolia*, *C. harmandii*, *C. roscoeana*, and *C. petiolata*.

Further study that includes more samples of *Curcuma* sp. is needed to check if the diagnostic characters are able to accommodate them.

APPENDIX 1. The chemicals used in the molecular approach.

- 2xCTAB extraction buffer (2% CTAB: cetyltrimethylammonium bromide): 1.4 M NaCl, 20 mM EDTA (Ethylenediaminetetraacetic acid) disodium salt, 100 mM Tris-HCl pH 8, 1% PVPP (polyvinyl pyrrolidone) with added 1% beta-mercaptoethanol prior to use.
- TE (10 mM Tris-HCl and 1 mM EDTA).
- 10xTBE buffer stock (89mM Tris-HCl, 89 mM boric acid, 2mM EDTA pH 8.0).
- agarose (Promega, Madison, WI, USA).
- 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 Hyperladder (Promega, USA).
- 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, Espoo, Finland.
- dNTP mix (Sigma Chemicals, Poole, Dorset, UK).
- primers (Oswel DNA Service, Southampton, UK).
- Dynazyme™ II thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland).
- QIAquick™ PCR Purification Kit (Qiagen Ltd, Dorking, Surrey, UK).

APPENDIX 2. Sequence data matrix (displayed from 5' to 3') of aligned ITS1 and ITS2 regions of 28 taxa of Zingiberaceae. Numbers in bold italic (1 to 35) indicate the number and position of alignment gaps. Uncertain nucleotide states are coded based on PAUP conventions (Swofford 1993) as follows: K=G/T, M=A/C, R=A/G, S=C/G, W=A/T, Y=C/T, N=A/T/G/C. Square brackets at the end of sequences show the real spacer length of ITS1 plus ITS2 regions.

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                             10        20        30        40        50        60        70        80        90
                    ITS1      .         .         .         .         .         .         .         .     1    .
                              .     123 4         .         .         .         .    5    .6 789    .     0    .
Ca.gracilis         TTGTTGAGAGAGCTTA-----GAATGATGGATGGTTGTGAATGTGTAAATGTGCCCCTTTCCTTTCCCCA-------TGTTGG----TGG
Ca.spicata          TTGTTGAGAGAGCATA-----GAATGATGGATGGTTGTGAATGTGTAAATGTGCCCCTTTCCTTTCCCCA-------TGTTGG----TGG
R.auriculata        TTGTTGAGAGAGCATA-----GAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT-CCCCA-------TCTCGG----TGG
R.schneideriana     TTGTTGAGAGAGCATA-----GAATGATGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT-CCCCA-------TATCGG----TGG
St.involucratus     TTGTTGAGAGAGTATA-----GAATGATGGATGATTGTGAATGTGTGAGCGTGCTCCTTTCCTTGCCCAA-------TGTTGG----TGG
Sm.supraneanae      TTGTTGAGAGAGCATA-----GAATGATGGATGATTGTGAACGTGTGAACGTGACCCTTTCGTTAGCCCA-------CGTTGG----TGG
C.parviflora        TTGTTGAGAGAGCATA---TAGAATGACGGATGAATGTGAATGTGTGAACGTGACCCTTTCTTTAGCCCA-------TGTTGG----TGG
C.thorelii          TTGTTGAGAGAGCATA---TAGAATGATGGACGAATGTGAATGTGTGAACGTGACCCTTTCTTTAGCCCA-------TGTTGG----TGG
C.roscoeana         TTGTTGAGAGAGCATA-----GAATGATGGATGATTGTGAATGTGTGAACGTGACCCTTTCGTTAGCCCA-------TGTTGGAACATGG
C.alismatifolia     TTGTTGAGAGAGCATA---TAGAATGACGGATGAATGTGAACGTGTGAACGTGACCCTTTCTTTAGCCCA-------TGTTGG----TGG
C.gracillima        TTGTTGAGAGAGCATA---TAGAATGACGGATGAATGTGAATGTGTGAACGTGACCCTTTCTTTAGCCCA-------TGTTGG----TGG
C.ecomata           TTGTTGAGAGAGCATA-----GAATGATGGATGATTGTGAATGTGTGAACGCGACCCTTTCGTTAGCCCA-------CGTTGG----TGG
C.harmandii         TTGTTGAGAGAGCATA---TAGAATGATGGATGAATGTGAATGTGTGAACGTGACCCTTTCTTTAGCCCA-------TGTTGG----TGG
C.cf.australasica   TTGTTGAGAGAGCATA-----GAATGATGGATGATTGTGAATGCGTGAACGTGACCCTTTCGTTAGCCCA-------TGTTGG----TGG
C.petiolata         TTGTTGAGAGAGCATA-----GAATGATGGATGATTGTGAATGTGTGAACGTGACCCTTTCGTCCATCGG---CCCATGTTGG----TGG
C.aurantiaca        TTGTTGAGAGAGCATAGCATAGAATGATGGATGATTGTGAATGTGTGAACGTGACCCTTTCGTTAGCCCA---TCCATGTTGG----TGG
C.aeruginosa        TTGTTGAGAGAGCATA-TACAGAATGATGGATGATTGTGAACGTGTGAACGYGACCCTTTCGTCAGCCCA----TCAAKTTGG----TGG
C.amada             TTGTTGAGAGAGCATAGCATAGARTGATGGATGATTGCKAWCGTGTGAACGTGACCCTTTCGTCRGCCCA--KCCCATGTTGG----TGG
C.amarissima        TTGTTGAGAGAGCATAGCATARAATGATGGATGATTGCKAWCGTGTGAACGTGACCCTTTCGTCRGCCCA--TCCCRTGTTGG----TGG
C.aromatica         TTGTTGAGAGAGCAT--CATAGAATGATGGATGATTGTGAACGTGTGAACGTGACCCTTTCKTCAGCMGCCCGCCCACGTTGG----TGG
C.elata             TTGTTGAGAGAGCAT--CATAGAATGATGGATGATTGTGAACGTGTGAACGTGACCCTTTCGTCAGCCCG---CCCACGTTGG----TGG
C.heyneana          TTGTTGAGAGAGCATAGCATAAAATGATGGATGATTGCTAACGTGTGAACGTGACCCTTTCRTCGGCCCA--TCCCATGTTGG----TGG
C.longa             TTGTTGAGAGAGCAT--CATAGAATGATGGATGATTGTGAACGTGTGAACGTGACCCTTTCGTCATCAGCCCGCCCATGTTGG----TGG
C.mangga            TTGTTGAGAGAGCATAGCACAGAATGAAGGATGAWTGCGAACGTGTGAACGTGACCCTTTTGTCGGCCCA-------TGTTGG----TGG
C.ochrorhiza        TTGTTGAGAGAGCATAGCACAGAATGATGGATGATTGCGAACGTGTGAACGTGACCCTTTCGTCGGCCCA-------TGTTGG----TGG
C.phaeocaulis       TTGTTGAGAGAGCAT--CACAGAATGATGGATGATTGTGAACGTGTGAACGTGACCCTTTCGTCATCCGCCCGCCCGTGTTGG----TGG
C.soloensis         TTGTTGAGAGAGCAT--CATAGAATGATGGATGATTGTGAACGTGTGAACGTGACCCTTTCGTCGTCAGCCCGCCCATGTTGG----TGG
C.zedoaria          TTGTTGAGAGAGCATA-TATAGAATGATGGATGATTGTGAACGTGTGAACGCGACCCTTTCGTCGGCCCA----TCAAKTTGG----TGG
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                      100       110       120       130       140       150       160       170       180
                    1  .1 1 1    .         .         .         .         .     1    .      11.         .
                    1  .2 3 4    .         .         .         .         .     5    .      67.         .
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R.auriculata         GCGATT-GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTGGCGTGCCC--GGGGAGCCCAA
R.schneideriana      GCGATT-GACCGTAGCTCAGTGCGATCGGCATTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTCGCGTGCGCGGGGGGAGCCCAA
St.involucratus      GCGATT-GAACGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTCGAAAGCAGAGGGTCCC-TTGGCGTGCCC--GGGGAGCCAAA
Sm.supraneanae       GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TCGCCGTGAGC--GGGGAGCACAA
C.parviflora         GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTGAAAGCAGAGGGCCCC-TTGGCGTGAGC--GGGGAGCACAA
C.thorelii           GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTGAAAGCAGAGGGCCCC-TTGGCGTGAGC--GGGGAGCACAA
C.roscoeana          GCGATTTGACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTGGAAGCATAGGGCCCC-TTAGCGTAAGC--GGGGAGCACAA
C.alismatifolia      GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTGAAAGCAGAGGGCCCC-TTGGCGTGAGC--GGGGAGCACAA
C.gracillima         GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTGAAAGCAGAGGGCCCC-TTGGCGTGAGC--GGGGAGCACAA
C.ecomata            GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTGCTGTGAGC--GGGGAGCCCAA
C.harmandii          GCGATT-GAC--TA---CGGTGCGATCGGCACTAAGGAACAATGAATTTGAAAGCARAGGGCCCC-TTGGCGTGAGC--GGGGAGCACAA
C.cf.australasica    GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTAGAAGCAGAGGGCCCC-TTGCCATGAGC--GGGGAGCACAA
C.petiolata          GCGATT-GACTG-AGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTGGAAGCAGAGGGCCCC-TTAGCGTGAGC--GGGGAGCACAA
C.aurantiaca         GCGATT-GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTTGGAAGCAGAGGGCCCC-TTGCCGTGAGC--GGGGAGCACAA
C.aeruginosa         GCGATT-GACTG-AGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTAGAAGCAGAAGGCCCCCTTAGCGTGAGC--GGGGAGCCCAA
C.amada              GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAACGAACTTGGAAGCAGAGGGCCCCCTTAGCGTGAGC--GGGGAGCCCAA
C.amarissima         GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAACGAAMTTGGAAGCAGAGGGCCCCCTTAGCGTGAGC--GGGGAGCCCAA
C.aromatica          GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAARGAACAACGAAMTTGGAAGCAGAGGGCCCCCTTAGCGTGAGC--GGGGAGCCCAA
C.elata              GCGATT-GACCGTASCTCGSTGCGATCGGCACTAAGGAACAACGAACTTGGAAGCAGAGGGCCCC-TTAGCGTGAGC--GGGGAGCCCAA
C.heyneana           GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAACGAACTTRGAAGCAGAGGGCCCC-TCAGCGTGAGC--GGGGAGCCCAA
C.longa              GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAACGAACTTGGAAGCAGAGGGCCCCCTTAGCGTGAGC--GGGGAGCCCAA
C.mangga             GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAACGAACTTGGAAGCAGAGGGCCCC-TCAGCGTGAGC--GGGGAGCCCAA
C.ochrorhiza         GCGAAT-GACCGTAGCTCGGKGCGATCGGCACTAAGGAACAACGAACTTGGAAGCAGAGGACCCCCTTAGCGTGAGC--GGGGAGCCCAA
C.phaeocaulis        GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAACGAACTTGGAAGCAGAGGACCCCCTTAGCGTGAGC--GGGGAGCCCAA
C.soloensis          GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAACGAACTTGGAAGCAGAGGGCCCC-TCAGCGTGAGC--GGGGAGCCCAA
C.zedoaria           GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAAGAACAACGAACTTGGAAGCAGAGGGCCCC-TCAGCGTGAGC--GGGGAGCCCAA
                     GCGATT-GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAACGAACTTGGAAGCAGAAGGCCCCCTTAGCGTGAGC--GGGGAGCCCAA
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                          190       200       210       220       230       240       250       260      270
                                               ITS2
                           .    1 .       .1    2  .         .         .         .   22    .        .
                           .    8 .       .9    0  .         .         .         .   12    .        .
Ca.gracilis          TGCGTCGGAGATTTTT----CGAAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCGTTATTGGCATCGAGCG----CGGAAATTGGC
Ca.spicata           TGCGTCGGAGATTTTT----CGAAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCGTTATTGGCATCGAGCG----CGGAAATTGGC
R.auriculata         TGCGTCGGAGATTTCT----CGAAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCATTGCTGGTGTCGAGCG----CGGAAATTGGC
R.schneideriana      TGCGTAAGAGATTTCT----CGAAATCAAA-TGAAT---CGTCGCTTTAGCTCCATGCGTTGCTGGTGTCAAGCG----CGGAAATTGGC
St.involucratus      AGCATCGTAGATTCCT----CGGAATCAAAATGAAT---CGTCGCTTTTGCTCCATGCTTTGCTGGCGTCGATCG----CGGAAATTGGC
Sm.supraneanae       TGCGTCGAAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTTTTGGCATTGAGCG----CGGAAATTGGC
C.parviflora         TGCGTCGAAGATTCTT----CGGAATCAAA-TGAAT---TGTCGCTTATGCTTCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGC
C.thorelii           TGCGTCGAAGATTCTT----CGGAATCAAA-TGAAT---TGTCGCTTATGCTCCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGC
C.roscoeana          TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---TGTCGCTTTTGCTCCATGCTTTGTTGGCATTGAGCG----CGGAAATTGGC
C.alismatifolia      TGCGTCGAAGATTCTT----CGGAATCAAA-TGAAT---TGTCGCTTATGCCCCATGCTTTGTTGGCATCGAGTG----CGGAAATTGGC
C.gracillima         TGCRTCGAAGATTCTT----CGGAATCAAA-TGAAT---TGTCGCTTATGCTCCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGC
C.ecomata            TGCATCGAAGATTCCT----CGGAATCAAA-TGAAT---TGTCGCTTTTGCTCCATGCTTCGTYAGCATTGAGCG----CGGAAATTGGC
C.harmandii          TGCGTCGAAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTATGCTCCATGCTTCGTTGGCATTGAGTG----CGGAAATTGGC
C.cf.australasica    TGCGTCGGAGATTCTT----CAGAATCAAA-TGAAT---TGTCGCTTTTGCTCCATGCTTTATTAGCATTGAGCG----CGGAAATTGGC
C.petiolata          TGCGTCGAAGATTCTT----CGGAATCAAA-TGAAT---TGTCGCTTTTGCTCCATGCTCTGTTGGCATTGAGCG----CGGAAGTTGGC
C.aurantiaca         TGCGTCGGAGATTCTTTCTTCGGAATCAAA-TGAATTGCCGCCGCTTTTGCTCCATGCTTTATTAGCATTGAGC-AGCGCGAAAATTGGC
C.aeruginosa         TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGC
C.amada              TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAARTTGGC
C.amarissima         TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGC
C.aromatica          TGCGTCGGAGATTCTTTCTTCGGAATCAAA-TGAGT---TGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGTGAGCGCGGAAGTTGGC
C.elata              TGCGTCGGAGATTCTTTCTTCGGAATCAAA-TGA-T---TGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGTGAGCGCGKAAGTTGGC
C.heyneana           TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGC
C.longa              TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGCG----CGGAAGTTGGC
C.mangga             TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGC
C.ochrorhiza         TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGC
C.phaeocaulis        TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---MGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGCG----CGGAAGTTGGC
C.soloensis          TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGC
C.zedoaria           TGCGTCGGAGATTCTT----CGGAATCAAA-TGAAT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGC
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                        280       290       300       310       320       330       340       350       360
                          .  2      .         .         2         .         .         .         .         .
                          .  3      .         .         4         .         .         .         .         .
Ca.gracilis             CTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGAGTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAAC
Ca.spicata              CTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGAGTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAAC
R.auriculata            CTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGAGTGGG-TAGTCCGAAGTCGTCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAGAAC
R.schneideriana         CTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGAGTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAAC
St.involucratus         CTCGTGTGCCCTC--AGGCACAGTCGGTTGAAGAGTGGG-TAGTCGGCAGCCGTCGGGCATGATGGGTGTTGGTCGCCGTTAGCGGGAAC
Sm.supraneanae          CTCGTGTGCCCTC--GAGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAGTCGTCGAGCACGACGGATGTTGGTCGCCATGAGCGGGAAC
C.parviflora            CCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGAGTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAAC
C.thorelii              CCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGAGTGGG-CACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAAC
C.roscoeana             CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.alismatifolia         CCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGAGTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAAC
C.gracillima            CCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGAGTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAAC
C.ecomata               CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTATTCGTCGAGCACGATGGATGTTGGTCGTCGCGAACGGGAAC
C.harmandii             CCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGAGTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAAC
C.cf.australasica       CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGGGTATCCGGTAGTCGTCGAGCACGATGGATGTTGGTCGTCACGAGCGAGAAC
C.petiolata             CCCGTGTGCCCTC--GAGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCACGAGCGAGAAC
C.aurantiaca            CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGGGTAGCCGGTAGTCGTCGAGCACGATGGATGTTGGTCGTCACGAGCGAGAAC
C.aeruginosa            CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.amada                 CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.amarissima            CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGAYGGACGTTGGTCGTCGCGAGCGAGAAC
C.aromatica             CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGCGGG-TAGTCGGCAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.elata                 CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGYGGG-TAGTCRGCAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.heyneana              CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.longa                 CCCGTGTGCCCTCTCGGGCACAGTCGGTCGAAGAGCGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.mangga                CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.ochrorhiza            CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGACGGACGTTGGTCGTCGCGAGCGAGAAC
C.phaeocaulis           CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGCGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
C.soloensis             CCCGTGTGCCCTCTCGGGCACAGTCGGTCGAAGAGCGTG-TAGTCGGTAATCGTCGAGCGCGATGGACGTTCGTCGTCGCGAGAGAGAAC
C.zedoaria              CCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGAGTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAAC
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                    370        380        390        400        410        420        430        440        450
                    .2         . 2 2      .          .  2       .          .  2    3 3.          .          .
                    .5         . 6 7      .          .  8       .          .  9    0 1.          .          .
Ca.gracilis         AGAACGTCGT--CCCCGTCGT---TTTGGGAAT-GTCCTCAA----GAGACCCTGTGTGAT-------TGTGATGTCGTGTGAAAGTGCC
Ca.spicata          AGAACGTCGT--CCCCGTCGT---TTTGGGAAT-GTCCTCAA----GAGACCCTGTGTGAT-------TGTGATGTCGTGTGAAAGTGCC
R.auriculata        AGAACGTCGT--CCCCGTCGT---TTTAGGATT--TCCTCAA----GAGACCCCGTGTGAT-------TGTGATGCGGTGTGAAAGCCCC
R.schneideriana     AGAACGTCGT--CCCCGTCGC---TTTAGGATT-GTCCTCAA----GAGACCCCGTGTGAT-------TGTGATGTCGTGCGAAAGTGCC
St.involucratus     TGAACGTTGT--CCCCGTCGT---GCTGGGATGAGACCTCAA----GAGACCCTGTGTGAT------TTGCGGAGTCGGGTGAAAGTGCC
Sm.supraneanae      TGAACGTCGT--CCTCGTCGT---TTCGGAACGAGTCCTCAA----GAGACCCTGTGTGAT------TTGCGGAGTCGGACGAAAGTGCC
C.parviflora        TGAACGTCGT--CCTCGTCAT---TTTGGGATGAGTCCTCAA----GAGACCCTATGTGAT-------TGTGATGTCGTGTGAAAGCGAT
C.thorelii          TGAACGTCGT--CCTCGTCAT---TTTGGGATGAGTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGACGAAAGCGCT
C.roscoeana         TGAACGTCGT--CCTCGTCGT---TTTGGGATGAGTCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
C.alismatifolia     TGAACGTCGT--CCTCGTCAT---TTTGGGATGAGTCCTCAA----GAGACCCTACGTGAT-------TGCAGAGTCGGATGAAAGCGCT
C.gracillima        TGAACGTCGT--CCTCGTCAT---TTTGGGATGAGTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGACGAAAGCGCT
C.ecomata           TGAACGTCGT--CCTCGTCGT---TTCGGGATGAGTCCTCAA----GAGACCCTGTGTGAT-------TGCGGAGTCGGTTGAAAGTGCC
C.harmandii         TGAACGTCGT--CCTCGTCAT---TTTGGGATGAGTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGATGAAAGCGCT
C.cf.australasica   TGAGCATCGT--CCTTGTCGC---TTTGGAACGAATCCTCAA----GAGACCCAATGTGAT----GATTGTGGAGTCGGATGAATGTTCC
C.petiolata         TGAACGTCGT--CCTCGTCGT---TTTGGGATGAGTCCTCAAGAA-GAGACCCTGTCTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
C.aurantiaca        TGAACATCGT--CCTTGTCGTCGTTTCGGAACGAACCCTCAATCAAGAGACCCTACTATGT----GATTGCGGAGTCGGATGAATGTTCC
C.aeruginosa        TGAACGTCGT--CCTCGTCGT---TTTGGGATGAGTCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
C.amada             TGAACGTCGTGTCCTCGTCGT--TTTCGGGATGAGTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
C.amarissima        TGAACGTCGTGTCCTCGTCGT---TTTGGGATGAGTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
C.aromatica         TGAACGTCGT--CCTCGTCGT---TTTGGGATGAGCCCTCAA----GAGACCCTGCGTGAT----GATTGCGGAGCCGCGCGAAAGCGCC
C.elata             TGAACGTCGT--CCTCGTCGT---TTTGGGATGAGCCCTCAAGAAAGAGACCCTGTGTGAT----GATTGCGCACCCGCGCGAGAGCGCC
C.heyneana          TGAACGTCGTGTCCTCGTCGT---TTTGGGATGAGTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
C.longa             TGAACGTCGT--CCTCGTCGT---TTTGGGATGAGCCCTCAAKAAAGAGACCCTGTGTGATTGATGATTGCGGACCCGCGCGAACGCGCC
C.mangga            TGAACGTCGTGTCCTCGTCGT---TTTGGGATGAGTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
C.ochrorhiza        TGAACGTCGTGTCCTCGTCGT---TTTGGGATGAGTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
C.phaeocaulis       TGAACGTCGT--CCTCGTCGT---TTTGGGATGAGCCCTCAA----GAGACCCTGTGTGATWGATGATTGCRGACCCGCGCGAAAGCCCC
C.soloensis         TGAACGTCGT--CCTCGTCGT---TTTGGGATGAGYCCTCAA----GAGACCCTGTGTGAT----TGATGCAGAGTCGCGTGAAAGCGCT
C.zedoaria          TGAACGTCGTGTCCTCGTCGT--TTTCGGGATGAGTCCTCMA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCC
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	460 470	
	3 . 33 3 .	
	2 . 34 5 .	
Ca.gracilis	GTG--TCCATCA---AATTGT	[415]
Ca.spicata	GTG--TCCATCA---AATTGT	[415]
R.auriculata	GTG--TCCATCA---AATTGT	[412]
R.schneideriana	GTG--TCCATCA---AATTGT	[415]
St.involucratus	GTG--TCCATCA---AATTGT	[417]
Sm.supraneanae	GTG--TCAATCA----TTTGT	[415]
C.parviflora	GTG--TCAATCA-TCATTTGC	[419]
C.thorelii	GTG--TCAATCA-TCATTTGC	[420]
C.roscoeana	GTG--TCAATCA----TTTGC	[421]
C.alismatifolia	GTG--TCAATCA-TCATTCGC	[419]
C.gracillima	GTG--TCAATCA-TCATTTGC	[419]
C.ecomata	GTG--TCAATCA----TTTGT	[409]
C.harmandii	GTG--TCAATCA-TCATTTGC	[419]
C.cf.australasica	GTG--TCAATCA----TTTGC	[417]
C.petiolata	GCG--TCAATCA----TTTGC	[424]
C.aurantiaca	GTG--CCAATCATTTATTTGC	[447]
C.aeruginosa	GCG--TCAATCA----TTTGC	[425]
C.amada	GCG--TCAATCA----TTTGC	[431]
C.amarissima	GCG--TCAATCA----TTTGC	[430]
C.aromatica	GCGCGTCAATCA----TTTGC	[437]
C.elata	GCGCGTCAATCA----TTTGC	[437]
C.heyneana	GCG--TCAATCA----TTTGC	[430]
C.longa	GCG--TCAATCA----TTTGC	[437]
C.mangga	GCG--TCAATCA----TTTGC	[425]
C.ochrorhiza	GCG--TCAATCA----TTTGC	[425]
C.phaeocaulis	GCG--TCAATCA----TTTGC	[431]
C.soloensis	GCG--TCAATCA----TTTGC	[429]
C.zedoaria	GCG--TCAATCA----TTTGC	[428]

APPENDIX 3. Sequence data matrix (displayed from 5' to 3') of aligned ITS1 and ITS2 regions of 28 taxa of Zingiberaceae. Numbers in bold italic (1 to 35) indicate the number and position of alignment gaps. Uncertain nucleotide states are coded based on PAUP conventions (Swofford 1993) as follows: K=G/T, M=A/C, R=A/G, S=C/G, W=A/T, Y=C/T, N=A/T/G/C. Square brackets at the end of sequences show the real spacer length of ITS1 plus ITS2 regions.

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                    10        20        30        40        50        60        70        80        90
                     .         .         .         .         .         .        1.         .         .
                     .         .         .         .         .         .         .         .         .
Ca.spicata          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAAACTAAGGTTTATCAAA
R.humeana           TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTT--------------TATCAAA
Sm.supraneanae      TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.thorelii          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.roscoeana         TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.alismatifolia     TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.gracillima        TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTGAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.ecomata           TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTT--------------TATCAAA
C.harmandii         TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTT--------------TATCAAA
C.cf.australasica   TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.petiolata         TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.ochrorhiza        TGGTAAcTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.aeruginosa        TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.phaeocaulis       TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.aurantiaca        TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.heyneana          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.longa             TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.amada             TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.zedoaria          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.zanthorrhiza      TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.soloensis         TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.aromatica         TGGTAAATTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.purpurascens      TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.elata             TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
C.colorata          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA
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                    100       110       120       130       140       150       160       170       180
                     .         .         .         .         .         .         .         .         .
                   2 .   345   .         .         .         .         .         .         .         .
Ca.spicata          CTAGAATAAAAAAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTTGGTAGTTGGAATC
R.humeana           CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTTGGTAGTTGGAATC
Sm.supraneanae      CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.thorelii          CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.roscoeana         CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.alismatifolia     CTAGAATAAAAAAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.gracillima        CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.ecomata           CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.harmandii         CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.cf.australasica   CTAGAATAAAAAAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.petiolata         CTAGAATAAAAAAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.ochrorhiza        CTAGAA-AAAAAAA---GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.aeruginosa        CTAGAA-AAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.phaeocaulis       CTAGAA-AAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.aurantiaca        CTAGAATAAAAAAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.heyneana          CTAGAA-AAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.longa             CTAGAA-AAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.amada             CTAGAA-AAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.zedoaria          CTAGAA-AAAAAAA---GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.zanthorrhiza      CTAGAA-AAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.soloensis         CTAGAA-AAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.aromatica         CTAGAA-AAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.purpurascens      CTAGAA-AAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.elata             CTAGAA-AAAAAAA---GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
C.colorata          CTAGAA-AAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCGTCGGTAGTTGGAATC
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                    190       200       210       220       230       240       250       260       270
                     .         .         .         .         .         .         .         .         .
                     .         .         .         .         .         .         .         .         .
Ca.spicata          CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
R.humeana           CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
Sm.supraneanae      CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.thorelii          CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.roscoeana         CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.alismatifolia     CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.gracillima        CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.ecomata           CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.harmandii         CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.cf.australasica   CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.petiolata         CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.ochrorhiza        CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.aeruginosa        CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.phaeocaulis       CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.aurantiaca        CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.heyneana          CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.longa             CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.amada             CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.zedoaria          CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.zanthorrhiza      CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.soloensis         CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.aromatica         CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.purpurascens      CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.elata             CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
C.colorata          CGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGATTAATCATGACT
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                    280       290       300       310       320       330       340       350       360
                     .         .         .         .         .         .         .         .         .
                     .         .         .         .         .         .         .         .         .
Ca.spicata          CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAATCCAATGGAAGTCGAAAGAAGA
R.humeana           CGAATCCATTATATTATATGGATAATTATAATATTAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCAATGGAAGTTGAAAGAAGA
Sm.supraneanae      CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.thorelii          CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.roscoeana         CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.alismatifolia     CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.gracillima        CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.ecomata           CGAATCCATTATATTATATGGATAATTCTAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.harmandii         CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTMAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.cf.australasica   CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.petiolata         CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
C.ochrorhiza        CGAATCCATTATATTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGA
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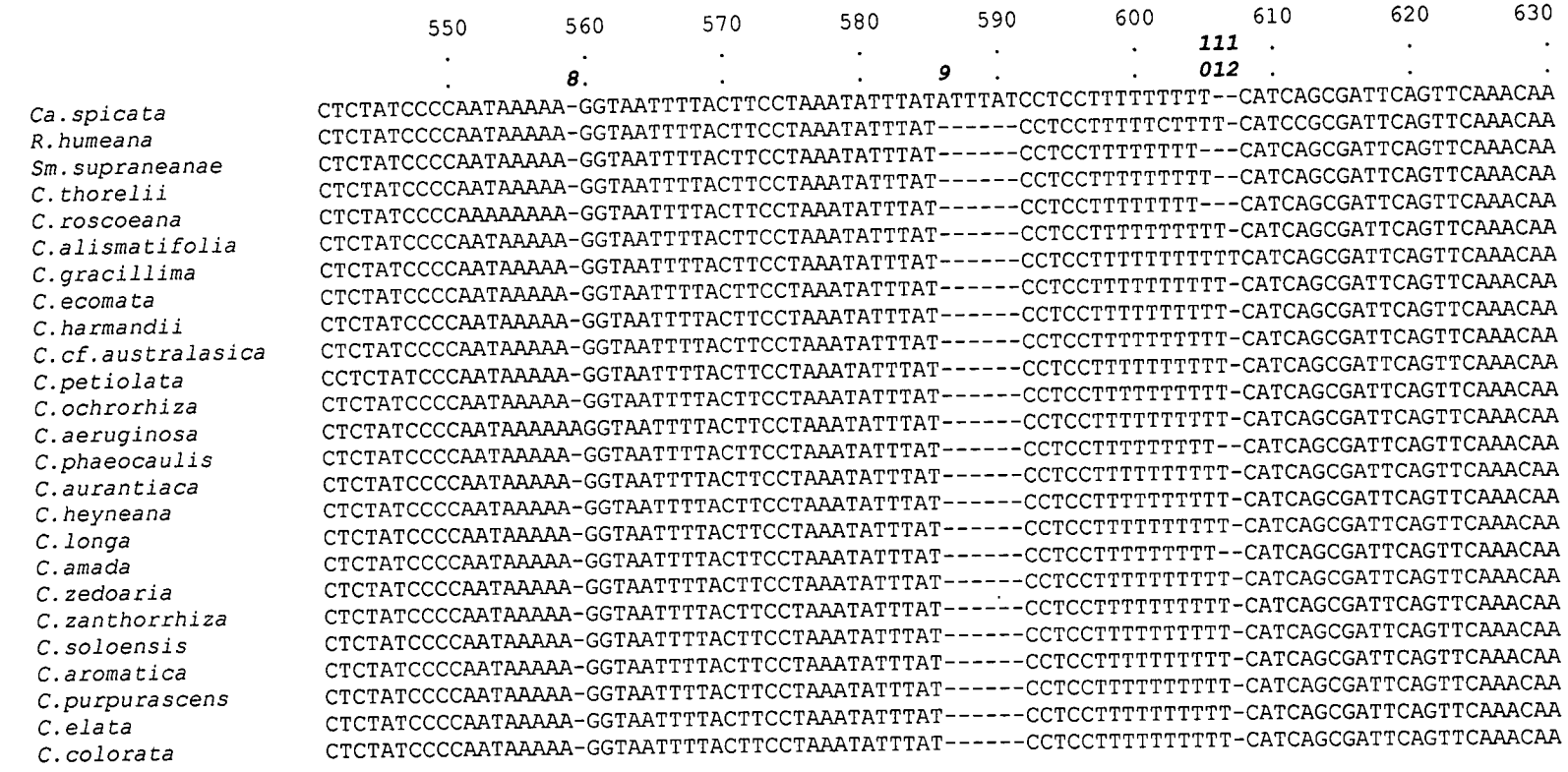
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R.humeana CTCTATCCCCAATAAAAA-GGTAATTTTACTTCCTAAATATTTAT------CCTCCTTTTTCTTTT-CATCCGCGATTCAGTTCAAACAA
Sm.supraneanae CTCTATCCCCAATAAAAA-GGTAATTTTACTTCCTAAATATTTAT------CCTCCTTTTTTTT---CATCAGCGATTCAGTTCAAACAA
C.thorelii CTCTATCCCCAATAAAAA-GGTAATTTTACTTCCTAAATATTTAT------CCTCCTTTTTTTTT--CATCAGCGATTCAGTTCAAACAA
C.roscoeana CTCTATCCCCAAAAAAAA-GGTAATTTTACTTCCTAAATATTTAT------CCTCCTTTTTTTT---CATCAGCGATTCAGTTCAAACAA
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Sm.supraneanae  AATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGAT
C.thorelii      AATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGAT
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C.cf.australasica AATTCACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGAT
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C.heyneana          TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.longa             TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.amada             TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.zedoaria          TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.zanthorrhiza      TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.soloensis         TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.aromatica         TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.purpurascens      TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.elata             TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
C.colorata          TAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA
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910

Ca.spicata	GTTGGTAGAGC [908]
R.humeana	GTTGGTAGAGC [881]
Sm.supraneanae	GTTGGTAGAGC [900]
C.thorelii	GTTGGTAGAGC [901]
C.roscoeana	GTTGGTAGAGC [900]
C.alismatifolia	GTTGGTAGAGC [903]
C.gracillima	GTTGGTAGAGC [903]
C.ecomata	GTTGGTAGAGC [887]
C.harmandii	GTTGGTAGAGC [886]
C.cf.australasica	GTTGGTAGAGC [903]
C.petiolata	GTTGGTAGAGC [903]
C.ochrorhiza	GTTGGTAGAGC [899]
C.aeruginosa	GTTGGTAGAGC [902]
C.phaeocaulis	GTTGGTAGAGC [900]
C.aurantiaca	GTTGGTAGAGC [903]
C.heyneana	GTTGGTAGAGC [901]
C.longa	GTTGGTAGAGC [900]
C.amada	GTTGGTAGAGC [900]
C.zedoaria	GTTGGTAGAGC [899]
C.zanthorrhiza	GTTGGTAGAGC [901]
C.soloensis	GTTGGTAGAGC [900]
C.aromatica	GTTGGTAGAGC [901]
C.purpurascens	GTTGGTAGAGC [900]
C.elata	GTTGGTAGAGC [899]
C.colorata	GTTGGTAGAGC [900]

APPENDIX 4. Measurements (μm) of epidermal and stomata cells.

Preparation	LE1	WE1	LE2	WE2	HS1	HS2	WS1	WS2
zed1	101.00	35.00	38.00	37.00	49.00	42.00	63.00	43.00
zed2	72.00	30.00	65.00	47.00	45.00	47.00	43.00	47.00
zed3	57.00	35.00	58.00	40.00	47.00	48.00	50.00	48.00
zed4	70.00	39.00	65.00	29.00	48.00	45.00	55.00	45.00
zed5	82.00	23.00	51.00	33.00	49.00	49.00	63.00	49.00
zed6	75.00	36.00	65.00	33.00	45.00	50.00	43.00	50.00
zed7	90.00	37.00	42.00	61.00	47.00	47.00	50.00	47.00
zed8	88.00	24.00	43.00	51.00	48.00	45.00	55.00	45.00
zed9	72.00	41.00	76.00	18.00	49.00	49.00	63.00	49.00
zed10	60.00	44.00	62.00	25.00	45.00	44.00	43.00	44.00
pha1	87.00	28.00	48.00	41.00	39.00	46.00	53.00	54.00
pha2	92.00	19.00	55.00	44.00	39.00	41.00	46.00	59.00
pha3	85.00	37.00	46.00	52.00	42.00	40.00	59.00	52.00
pha4	100.00	18.00	54.00	48.00	43.00	43.00	49.00	49.00
pha5	72.00	32.00	35.00	33.00	41.00	43.00	52.00	46.00
pha6	65.00	20.00	56.00	50.00	45.00	41.00	63.00	55.00
pha7	72.00	38.00	47.00	40.00	44.00	45.00	62.00	53.00
pha8	100.00	28.00	41.00	39.00	38.00	44.00	63.00	54.00
pha9	66.00	38.00	52.00	45.00	40.00	38.00	64.00	50.00
pha19	75.00	25.00	58.00	38.00	46.00	41.00	63.00	53.00
Zan1	84.00	27.00	35.00	38.00	53.00	48.00	73.00	47.00
Zan2	85.00	43.00	40.00	46.00	49.00	49.00	68.00	51.00
Zan3	109.00	26.00	56.00	28.00	50.00	52.00	60.00	49.00
Zan4	104.00	22.00	55.00	35.00	58.00	48.00	67.00	54.00
Zan5	69.00	22.00	49.00	19.00	49.00	48.00	58.00	52.00
Zan6	94.00	29.00	53.00	32.00	53.00	52.00	73.00	61.00
Zan7	61.00	39.00	39.00	29.00	49.00	49.00	68.00	53.00
Zan8	69.00	28.00	25.00	60.00	50.00	44.00	60.00	47.00
Zan9	99.00	33.00	45.00	32.00	58.00	40.00	67.00	52.00
Zan10	77.00	42.00	61.00	24.00	49.00	51.00	58.00	57.00
aer1	75.00	24.00	75.00	28.00	44.00	45.00	66.00	53.00
aer2	65.00	29.00	60.00	26.00	41.00	45.00	60.00	62.00
aer3	77.00	22.00	43.00	42.00	38.00	41.00	53.00	65.00
aer4	81.00	28.00	52.00	55.00	44.00	48.00	62.00	56.00
aer5	63.00	22.00	39.00	27.00	42.00	38.00	60.00	63.00
aer6	39.00	26.00	66.00	29.00	44.00	44.00	58.00	60.00
aer7	46.00	20.00	48.00	31.00	45.00	45.00	52.00	65.00
aer8	66.00	18.00	55.00	27.00	45.00	51.00	57.00	62.00
aer9	79.00	27.00	31.00	24.00	41.00	40.00	54.00	61.00
aer10	70.00	33.00	60.00	33.00	43.00	45.00	60.00	58.00
hey1	105.00	31.00	58.00	38.00	44.00	41.00	77.00	55.00
hey2	103.00	37.00	57.00	43.00	45.00	45.00	65.00	49.00
hey3	99.00	25.00	38.00	33.00	42.00	42.00	62.00	53.00
hey4	88.00	41.00	72.00	34.00	43.00	43.00	57.00	58.00

APPENDIX 4(continued) Measurements (μm) of epidermal and stomata cells.

Preparation	LE1	WE1	LE2	WE2	HS1	HS2	WS1	WS2
hey5	107.00	25.00	61.00	26.00	49.00	40.00	60.00	57.00
hey6	71.00	25.00	51.00	35.00	42.00	41.00	54.00	48.00
hey7	85.00	35.00	49.00	39.00	44.00	44.00	64.00	53.00
hey8	105.00	21.00	62.00	30.00	42.00	43.00	55.00	52.00
hey9	80.00	44.00	65.00	37.00	49.00	42.00	47.00	55.00
hey10	78.00	35.00	54.00	21.00	48.00	41.00	60.00	46.00
man1	85.00	21.00	22.00	78.00	41.00	48.00	58.00	45.00
man2	75.00	24.00	17.00	102.00	39.00	38.00	51.00	39.00
man3	78.00	20.00	18.00	80.00	43.00	35.00	48.00	40.00
man4	69.00	22.00	21.00	60.00	39.00	37.00	51.00	45.00
man5	62.00	25.00	26.00	54.00	37.00	48.00	58.00	45.00
man6	48.00	25.00	18.00	56.00	45.00	45.00	55.00	38.00
man7	67.00	20.00	25.00	75.00	40.00	43.00	51.00	39.00
man8	61.00	27.00	19.00	68.00	46.00	39.00	55.00	35.00
man9	66.00	28.00	18.00	73.00	36.00	38.00	46.00	40.00
man10	65.00	20.00	21.00	64.00	44.00	44.00	62.00	41.00
lon1	91.00	45.00	68.00	49.00	35.00	40.00	69.00	47.00
lon2	98.00	38.00	37.00	31.00	49.00	43.00	68.00	50.00
lon3	95.00	47.00	57.00	49.00	41.00	38.00	65.00	48.00
lon4	88.00	45.00	61.00	36.00	42.00	42.00	63.00	52.00
lon5	70.00	29.00	68.00	45.00	48.00	41.00	55.00	57.00
lon6	45.00	35.00	71.00	40.00	48.00	37.00	68.00	48.00
lon7	103.00	43.00	48.00	58.00	47.00	41.00	55.00	45.00
lon8	78.00	53.00	35.00	70.00	41.00	37.00	55.00	49.00
lon9	99.00	48.00	45.00	57.00	48.00	36.00	64.00	47.00
lon10	89.00	32.00	39.00	26.00	35.00	41.00	63.00	47.00
pur1	101.00	35.00	76.00	33.00	45.00	35.00	48.00	64.00
pur2	66.00	19.00	69.00	47.00	40.00	42.00	49.00	57.00
pur3	104.00	31.00	40.00	39.00	41.00	39.00	65.00	58.00
pur4	71.00	43.00	96.00	24.00	45.00	38.00	73.00	53.00
pur5	112.00	41.00	71.00	45.00	44.00	45.00	46.00	62.00
pur6	82.00	33.00	50.00	57.00	43.00	43.00	75.00	54.00
pur7	89.00	30.00	71.00	40.00	38.00	42.00	67.00	70.00
pur8	125.00	34.00	42.00	36.00	39.00	39.00	68.00	55.00
pur9	98.00	33.00	72.00	44.00	38.00	45.00	64.00	63.00
pur10	103.00	29.00	45.00	49.00	39.00	43.00	74.00	47.00
col1	96.00	33.00	59.00	44.00	40.00	42.00	66.00	61.00
col2	88.00	36.00	45.00	32.00	41.00	42.00	62.00	54.00
col3	100.00	24.00	62.00	41.00	42.00	42.00	58.00	68.00
col4	103.00	21.00	75.00	46.00	41.00	46.00	65.00	71.00
col5	98.00	31.00	78.00	25.00	48.00	45.00	60.00	57.00
col6	65.00	50.00	87.00	35.00	45.00	45.00	55.00	65.00
col7	88.00	40.00	48.00	27.00	41.00	43.00	51.00	59.00

APPENDIX 4 (continued) Measurements (μm) of epidermal and stomata cells.

Preparation	LE1	WE1	LE2	WE2	HS1	HS2	WS1	WS2
col8	61.00	31.00	86.00	27.00	42.00	44.00	63.00	53.00
col9	68.00	24.00	75.00	43.00	39.00	44.00	49.00	69.00
col10	90.00	31.00	75.00	50.00	47.00	41.00	63.00	66.00
Zana1	101.00	23.00	67.00	30.00	46.00	49.00	88.00	58.00
Zana2	63.00	25.00	67.00	30.00	46.00	45.00	94.00	60.00
Zana3	95.00	19.00	60.00	36.00	50.00	43.00	85.00	55.00
Zana4	80.00	24.00	61.00	38.00	43.00	47.00	82.00	57.00
Zana5	86.00	33.00	64.00	33.00	42.00	44.00	93.00	51.00
Zana6	94.00	27.00	38.00	47.00	45.00	48.00	95.00	40.00
Zana7	80.00	39.00	66.00	38.00	45.00	46.00	82.00	57.00
Zana8	95.00	28.00	48.00	25.00	50.00	48.00	71.00	61.00
Zana9	85.00	23.00	70.00	40.00	49.00	43.00	91.00	63.00
Zana10	81.00	22.00	42.00	37.00	48.00	49.00	89.00	55.00
tho1	85.00	35.00	31.00	90.00	54.00	51.00	73.00	60.00
tho2	77.00	63.00	40.00	40.00	55.00	52.00	70.00	62.00
tho3	83.00	40.00	63.00	66.00	60.00	53.00	67.00	55.00
tho4	71.00	68.00	55.00	73.00	59.00	55.00	88.00	56.00
tho5	93.00	53.00	62.00	53.00	53.00	54.00	81.00	65.00
tho6	97.00	55.00	46.00	51.00	51.00	51.00	71.00	58.00
tho7	69.00	35.00	48.00	50.00	50.00	54.00	73.00	57.00
tho8	85.00	51.00	54.00	52.00	52.00	56.00	75.00	56.00
tho9	88.00	49.00	53.00	51.00	51.00	51.00	74.00	55.00
tho10	84.00	55.00	32.00	53.00	53.00	55.00	78.00	61.00
ros1	66.00	30.00	33.00	31.00	30.00	42.00	65.00	46.00
ros2	93.00	36.00	57.00	57.00	31.00	43.00	80.00	40.00
ros3	85.00	47.00	44.00	77.00	37.00	37.00	84.00	43.00
ros4	85.00	28.00	43.00	59.00	43.00	41.00	73.00	49.00
ros5	95.00	33.00	60.00	34.00	41.00	45.00	60.00	56.00
ros6	83.00	38.00	57.00	45.00	40.00	40.00	55.00	37.00
ros7	65.00	25.00	25.00	62.00	37.00	43.00	62.00	57.00
ros8	76.00	45.00	45.00	67.00	35.00	37.00	61.00	42.00
ros9	89.00	33.00	56.00	59.00	36.00	44.00	74.00	40.00
ros10	74.00	36.00	58.00	32.00	49.00	42.00	68.00	58.00
ali1	85.00	44.50	62.50	75.00	40.00	45.00	35.00	27.50
ali2	84.00	48.00	55.00	45.00	40.00	41.50	37.50	30.00
ali3	90.00	49.00	70.00	57.50	45.00	41.50	35.00	29.00
ali4	99.00	50.00	85.00	42.50	45.00	45.00	29.00	30.00
ali5	100.00	55.00	65.00	80.00	45.00	40.00	30.00	27.50
ali6	90.00	42.50	45.00	85.00	45.00	40.00	30.00	29.00
ali7	52.50	62.50	55.00	45.00	42.50	40.00	30.00	25.00
ali8	77.50	57.50	70.00	72.50	45.00	41.50	30.00	32.50
ali9	77.50	52.50	35.00	90.00	42.50	42.50	30.00	30.00
ali10	70.00	48.00	60.00	60.00	40.00	42.50	35.00	29.00

APPENDIX 4 (continued) Measurements (μm) of epidermal and stomata cells.

Preparation	LE1	WE1	LE2	WE2	HS1	HS2	WS1	WS2
par1	90.00	42.50	77.50	57.50	37.50	42.50	26.00	25.00
par2	90.00	47.50	80.00	50.00	37.50	37.50	25.00	28.00
par3	70.00	40.00	50.00	70.00	37.50	40.00	25.00	25.00
par4	87.50	40.00	70.00	35.00	40.00	37.50	24.50	27.00
par5	90.00	45.00	70.00	60.00	40.00	40.00	26.50	25.00
par6	90.00	35.00	60.00	60.00	42.50	40.00	26.00	26.00
par7	85.00	47.50	70.00	65.00	44.00	37.50	26.50	25.00
par8	75.00	55.00	42.50	40.00	40.00	40.00	27.00	25.00
par9	87.50	50.00	60.00	60.00	40.00	36.00	27.00	24.00
par10	80.00	42.50	58.00	75.00	38.00	40.00	25.00	27.00
aur1	110.00	30.00	67.50	45.00	40.00	42.50	30.00	30.00
aur2	110.00	35.00	57.50	60.00	45.00	45.00	31.00	27.50
aur3	120.00	50.00	70.00	35.00	45.00	44.00	31.00	28.00
aur4	75.00	30.00	60.00	45.00	47.50	40.00	32.50	30.00
aur5	105.00	30.00	50.00	35.00	40.00	40.00	30.00	30.00
aur6	75.00	37.50	75.00	30.00	35.00	40.00	30.00	27.00
aur7	75.00	55.00	73.00	57.50	35.00	41.00	27.50	26.00
aur8	90.00	38.00	80.00	40.00	32.50	35.00	28.00	27.50
aur9	90.00	45.00	75.00	45.00	40.00	40.00	31.00	30.00
aur10	65.00	30.00	58.00	38.00	37.50	42.50	30.00	29.50
amar1	104.00	28.00	27.00	66.00	52.00	45.00	85.00	68.00
amar2	82.00	33.00	37.00	45.00	54.00	41.00	79.00	67.00
amar3	105.00	18.00	32.00	65.00	52.00	36.00	88.00	68.00
amar4	64.00	33.00	43.00	41.00	54.00	47.00	70.00	60.00
amar5	112.00	34.00	45.00	45.00	53.00	43.00	68.00	63.00
amar6	67.00	31.00	28.00	32.00	52.00	42.00	78.00	68.00
amar7	101.00	30.00	60.00	24.00	48.00	43.00	85.00	72.00
amar8	95.00	27.00	61.00	42.00	53.00	38.00	80.00	58.00
amar9	111.00	35.00	52.00	26.00	50.00	38.00	77.00	65.00
amar10	87.00	32.00	58.00	23.00	53.00	44.00	77.00	63.00
amad1	95.00	23.00	52.00	27.00	42.00	39.00	78.00	64.00
amad2	94.00	33.00	65.00	33.00	38.00	41.00	79.00	58.00
amad3	95.00	19.00	56.00	29.00	40.00	38.00	73.00	65.00

APPENDIX 4 (continued) Measurements (μm) of epidermal and stomata cells.

Preparation	LE1	WE1	LE2	WE2	HS1	HS2	WS1	WS2
amad4	93.00	31.00	63.00	32.00	41.00	45.00	75.00	59.00
amad5	91.00	31.00	58.00	20.00	42.00	32.00	73.00	62.00
amad6	113.00	31.00	64.00	32.00	44.00	43.00	76.00	58.00
amad7	118.00	29.00	45.00	35.00	41.00	45.00	68.00	62.00
amad8	95.00	18.00	65.00	26.00	40.00	40.00	65.00	59.00
amad9	92.00	20.00	64.00	29.00	39.00	45.00	71.00	58.00
amad10	81.00	37.00	54.00	41.00	45.00	44.00	70.00	63.00

Notes:
LE1= Length of adaxial epidermis; WE1= Width of adaxial epidermis; LE2= Length of abaxial epidermis; WE2= Width of abaxial epidermis; HS1= Height of stomatal on adaxial; WS1= Width of stomatal on adaxial; HS2= Height of stomatal on abaxial; WS2= Width of stomatal on abaxial.
zed: *C. zedoaria;* pha: *C. phaeocaulis;* Zan: *C. zanthorrhiza 1;* aer: *C. aeruginosa;* hey: *C. heyneana;* man: *C. mangga;* lon: *C. longa;* pur: *C. purpurascens;* col: *C. colorata;* Zana: *C. zanthorrhiza 2;* tho: *C. thorelii;* ros: *C. roscoeana;* ali: *C. alismatifolia;* par: *C. parviflora;* aur: *C. aurantiaca;* amar: *C. amarissima;* amad: *C. amada.*

APPENDIX 5 Measurements of flower in the morphometric analysis.

SPECIES	1	2	3	4	5	6	7	8	9	10	11
C.aeruginosa	3.000	12.375	24.313	7.261	25.750	13.750	12.250	11.838	12.125	9.500	10.125
C.alismatifolia	2.625	9.875	19.500	6.673	18.375	14.875	9.375	16.250	17.375	5.875	13.750
C.amada	3.500	9.750	26.250	6.280	21.063	14.500	10.250	10.875	12.000	7.875	10.500
C.amarissima	4.000	11.625	27.625	9.813	28.500	15.250	11.375	13.625	14.375	11.063	10.750
C.aromatica	2.250	6.313	16.750	4.514	13.688	5.875	7.875	8.438	9.375	4.688	7.875
C.aurantiaca	2.625	12.500	22.000	6.084	20.188	14.750	8.000	17.188	19.375	8.125	11.375
C.cf.australasica	2.000	6.125	14.375	4.710	12.125	10.375	7.500	8.438	9.375	5.813	6.813
C.colorata	2.625	9.875	32.875	8.243	28.625	12.875	13.000	12.625	14.250	10.625	11.000
C.ecomata	2.125	13.000	21.500	4.318	12.000	12.500	7.875	18.750	18.750	4.625	8.125
C.euchroma	3.000	10.438	34.500	7.261	24.750	12.000	11.375	12.250	14.500	9.375	11.500
C.gracillima	1.250	5.125	15.500	2.944	7.313	4.500	3.188	3.750	3.875	2.438	5.563
C.harmandii	1.875	11.250	23.250	3.925	8.750	8.875	5.250	8.750	9.625	4.625	12.625
C.heyneana	2.875	17.125	32.750	9.028	28.250	12.500	11.250	12.000	12.500	10.125	9.000
C.longa 1	2.625	10.750	34.500	6.084	22.500	10.375	10.125	9.125	10.625	6.625	8.500
C.longa 2	3.063	12.250	26.000	8.635	24.625	12.875	10.000	11.375	13.750	8.500	9.875
C.longa 3	2.750	10.375	31.250	6.673	30.313	11.875	12.750	11.688	12.750	10.500	8.125
C.longa 4	3.750	11.188	30.500	7.261	29.625	12.750	12.188	12.250	12.750	9.750	8.500
C.longa 5	3.000	10.625	26.938	7.850	29.375	13.125	11.938	11.125	11.500	9.063	9.125
C.mangga	2.750	11.125	27.000	7.850	27.313	15.000	11.000	13.500	13.813	9.750	13.375
C.parviflora	1.813	6.125	11.688	4.121	7.750	5.313	3.875	4.863	5.125	3.438	5.625
C.petiolata	2.250	11.188	23.875	5.888	20.125	9.125	8.375	9.000	9.125	2.750	8.625
C.purpurascens	2.500	8.500	18.125	5.495	16.125	9.125	8.250	9.875	10.000	6.250	6.750
C.roscoeana	2.625	22.000	36.500	5.495	12.313	12.250	6.625	11.625	11.875	7.188	13.250
C.soloensis	3.125	8.250	16.875	7.065	24.250	10.250	10.125	12.500	14.688	6.438	10.500
C.thorelii	1.875	5.938	15.000	1.963	5.563	6.688	4.563	6.188	6.875	3.750	10.000
C.zanthorrhiza	5.000	8.250	39.625	10.205	36.375	15.000	16.438	15.000	16.000	12.500	10.375

APPENDIX 5 (continued) Measurements of flower in the morphometric analysis.

SPECIES	12	13	14	15	16	17	18	19	20	21	22
C.aeruginosa	13.063	5.750	7.688	15.500	10.250	16.500	40.375	4.500	3.000	2.125	2.625
C.alismatifolia	16.250	3.750	3.125	22.500	5.875	12.375	38.375	6.250	2.313	1.438	1.875
C.amada	13.500	5.125	7.375	17.000	7.500	13.125	43.250	4.688	2.500	1.750	2.375
C.amarissima	15.125	6.375	9.000	15.200	11.375	19.500	46.750	4.125	3.000	2.625	2.750
C.aromatica	9.625	3.063	5.125	10.625	5.438	8.875	28.625	2.500	1.938	1.500	1.750
C.aurantiaca	14.125	5.625	7.500	16.500	6.125	17.000	38.750	6.000	2.250	1.000	2.125
C.cf.australasica	8.063	2.625	4.250	8.750	3.875	10.000	24.438	3.063	1.875	0.875	1.375
C.colorata	15.000	7.125	9.125	18.500	10.375	16.500	50.500	5.000	2.500	1.750	2.500
C.ecomata	8.375	2.250	6.875	15.000	5.000	10.750	32.000	8.750	2.125	1.000	1.875
C.euchroma	14.700	5.500	8.438	18.000	10.500	19.000	52.200	4.875	3.188	2.500	2.500
C.gracillima	5.438	1.813	1.938	5.250	2.250	3.875	22.188	1.563	1.188	0.750	1.063
C.harmandii	13.500	0.938	1.188	16.500	4.125	8.750	38.625	3.875	1.875	1.000	1.500
C.heyneana	9.625	6.125	7.125	16.500	11.625	15.500	45.250	4.375	2.500	2.125	2.625
C.longa 1	10.750	5.000	5.750	13.375	8.875	12.875	47.875	4.563	2.500	1.625	2.875
C.longa 2	14.000	5.500	8.750	15.500	9.688	16.500	43.063	4.188	2.625	1.625	2.625
C.longa 3	10.750	6.500	6.875	14.500	12.625	17.500	44.750	4.250	2.875	2.125	2.750
C.longa 4	11.250	6.375	8.125	14.500	12.625	18.000	45.500	4.438	2.500	2.125	2.875
C.longa 5	12.000	5.875	9.063	18.000	13.313	20.000	41.938	4.250	2.500	2.125	3.063
C.mangga	13.750	5.938	9.750	16.500	10.688	16.500	43.500	4.375	2.625	2.250	2.500
C.parviflora	7.000	1.688	2.000	7.625	2.563	4.188	20.500	1.500	1.375	0.750	1.250
C.petiolata	10.500	4.500	6.500	10.875	8.000	12.000	36.625	3.250	1.750	1.000	1.875
C.purpurascens	8.375	3.750	4.875	10.000	6.500	9.750	29.000	3.500	2.250	1.625	2.000
C.roscoeana	15.125	2.125	10.875	18.000	4.500	19.500	54.250	3.938	2.125	1.125	2.875
C.soloensis	12.750	5.188	7.125	16.500	9.750	14.000	32.750	4.625	2.875	1.875	2.625
C.thorelii	10.625	1.188	3.563	12.000	1.875	7.500	27.500	2.188	1.188	0.563	1.063
C.zanthorrhiza	14.125	8.250	9.875	17.500	14.063	17.000	58.750	4.375	3.375	2.750	2.875

APPENDIX 5 (continued) Measurements of flower in the morphometric analysis.

SPECIES	23	24	25	26	27	28
C.aeruginosa	0.063	2.625	4.000	2.250	31.438	0.667
C.alismatifolia	1.125	3.938	5.000	2.500	29.688	0.348
C.amada	0.250	3.750	3.313	1.250	34.688	0.909
C.amarissima	0.063	3.000	4.375	2.000	34.750	0.879
C.aromatica	0.188	2.313	2.125	1.000	21.563	0.606
C.aurantiaca	0.688	1.875	2.813	1.250	29.875	0.758
C.cf.australasica	0.375	1.125	3.000	0.938	18.563	0.606
C.colorata	0.188	3.500	4.000	1.688	41.375	1.212
C.ecomata	1.125	6.000	2.500	1.000	36.250	0.545
C.euchroma	0.125	3.750	3.250	1.750	43.125	0.788
C.gracillima	1.188	2.313	1.438	0.938	19.375	1.273
C.harmandii	2.000	1.625	2.750	1.500	28.750	0.606
C.heyneana	0.125	2.500	4.375	1.875	39.625	0.636
C.longa 1	0.313	3.625	3.625	1.875	42.688	0.364
C.longa 2	0.500	3.750	3.938	1.688	33.938	0.818
C.longa 3	0.250	2.625	4.688	2.063	38.125	1.061
C.longa 4	0.250	3.875	4.250	1.938	38.813	0.818
C.longa 5	0.375	3.375	4.313	2.063	34.563	0.909
C.mangga	0.250	4.375	4.750	2.125	35.750	0.667
C.parviflora	1.688	1.125	1.813	1.188	14.313	1.970
C.petiolata	0.313	3.000	3.125	1.250	30.125	0.636
C.purpurascens	0.125	2.063	2.125	1.063	23.688	0.606
C.roscoeana	3.813	2.500	3.563	1.813	42.938	0.365
C.soloensis	0.375	3.125	4.125	2.000	24.625	0.758
C.thorelii	1.000	1.438	1.313	0.813	18.625	0.303
C.zanthorrhiza	0.188	3.250	5.813	3.188	47.250	1.061

Note: Number 1-28 refers to the characters listed in **Table 4.2**

APPENDIX 6 Sequence data matrix (displayed from 5' to 3') of aligned ITS2 region of 32 accessions representing 27 taxa of Zingiberaceae.

Taxon	**ITS2** (positions 10, 20, 30, 40, 50, 60, 70, 80, 90; indels ***1***, ***2***, ***3***)
Ca.gracilis	AT---CGTCGCTTTTGCTCCATGCGTTATTGGCATCGAGCG----CGGAAATTGGCCTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGA
Ca.spicata	AT---CGTCGCTTTTGCTCCATGCGTTATTGGCATCGAGCG----CGGAAATTGGCCTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGA
R.auriculata	AT---CGTCGCTTTTGCTCCATGCATTGCTGGTGTCGAGCG------GAAATTGGCCTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGA
R.schneideriana	AT---CGTCGCTTTAGCTCCATGCGTTGCTGGTGTCAAGCG----CG-AAATTGGCCTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGA
St.involucratus	AT---CGTCGCTTTTGCTCCATGCTTTGCTGGCGTCGATCG----CGGAAATTGGCCTCGTGTGCCCTC--AGGCACAGTCGGTTGAAGA
Sm.supraneanae	AT---CGTCGCTTTTGCTCCATGCTTTTTTGGCATTGAGCG----CGGAAATTGGCCTCGTGTGCCCTC--GAGCACAGTCGGTCGAAGA
C.parviflora	AT---TGTCGCTTATGCTTCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.thorelii	AT---TGTCGCTTATGCTCCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.roscoeana	AT---TGTCGCTTTTGCTCCATGCTTTGTTGGCATTGAGCG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.alismatifolia	AT---TGTCGCTTATGCCCCATGCTTTGTTGGCATCGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.gracillima	AT---TGTCGCTTATGCTCCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.ecomata	AT---TGTCGCTTTTGCTCCATGCTTCGTTAGCATTGAGCG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.harmandii	AT---CGTCGCTTATGCTCCATGCTTCGTTGGCATTGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.cf.australasica	AT---TGTCGCTTTTGCTCCATGCTTTATTAGCATTGAGCG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.petiolata	AT---TGTCGCTTTTGCTCCATGCTCTGTTGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GAGCACAGTCGGTCGAAGA
C.ochrorrhiza	AT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCYTC--GGGCACAGTCGGTCGAAGA
C.aeruginosa a	AT---YGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.aeruginosa b	AT---YGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.phaeocaulis a	AT---YGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.phaeocaulis b	AT---YGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.amarissima a	AT---CGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.amarissima b	AT---CGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.aurantiaca	ATTGCCGCCGCTTTTGCTCCATGCTTTATTAGCATTGAGC-AGCGCGAAAATTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.heyneana a	AT---CGTCGCTTTTGCTCCATGCTTYGYCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.heyneana b	AT---CGTCGCTTTTGCTCCATGCTTYGYCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.longa1a	AT---CGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC**TC**GGGCACAGTCGGTCGAAGA
C.longa1b	AT---CGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.longa2a	AT---YGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC**TC**GGGCACAGTCGGTCGAAGA
C.longa2b	AT---YGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.amada a	AT---CGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----YGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.amada b	AT---CGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----YGGAARTTGGCCCCGTGTGCCCTC--NGGCACAGTCGGTCGAAGA

Taxon	ITS2	10	20	30	40	50	60	70	80	90
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C.zedoaria1 a		AT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.zedoaria1 b		AT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.zedoaria2a		AT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.zedoaria2b		AT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.zedoaria3a		AT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.zedoaria3b		AT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTRCCCTC--GGGCACAGTCGRTCGAAGA								
C.zedoaria cf. a		AT---CGTCGCTTTTRCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTRCCCTC--GGGCACAGTCGGTCGAAGA								
C.zedoaria cf. b		AT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.zanthorrhiza1a		AT---YGTSGCTTTWGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.zanthorrhiza1b		AT---YGTSGCTTTWGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTRCCCTC--GGGCACAGTCGRTCGAAGA								
C.zanthorrhiza2a		AT---CGTCGCTTTTRCTCCATGCTTYGTCGRCATTGAGCG----CGGAAGTTGGCCCCGTGTRCCCTC--GGGCACAGTCGRTCGAAGA								
C.zanthorrhiza2b		AT---CGTCGCTTTTRCTCCATGCTTYGTCGRCATTGAGCG----CGGAAGTTGGCCCCGTGTRCCCTC--GGGCACAGTCGRTCGAAGA								
C.soloensis a		AT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC**TC**GGGCACAGTCGGTCGAAGA								
C.soloensis b		AT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.aromatica a		GT---TGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGTGAGCGCGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								
C.aromatica b		GT---TGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGTGAGCGCGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA								

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Ca.gracilis         GTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT--TTT-GGGAAT-
Ca.spicata          GTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT--TTT-GGGAAT-
R.auriculata        GTGGG-TAGTCCGAAGTCGTCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT--TTT-AGGAT--
R.schneideriana     GTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGT-----CCCCGTCGC--TTT-AGGATT-
St.involucratus     GTGGG-TAGTCGGCAGCCGTCGGGCATGATGGGTGTTGGTCGCCGTTAGCGGGAACTGAACGTTGT--CCCCGTCGT--GCT-GGGATGA
Sm.supraneanae      GTGGG-TAGTCGGTAGTCGTCGAGCACGACGGATGTTGGTCGCCATGAGCGGGAACTGAACGTCGT--CCTCGTCGT---TTCGGAACGA
C.parviflora        GTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.thorelii          GTGGG-CACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.roscoeana         GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.alismatifolia     GTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.gracillima        GTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.ecomata           GTGGG-TAGTCGGTATTCGTCGAGCACGATGGATGTTGGTCGTCGCGAACGGGAACTGAACGTCGT--CCTCGTCGT---TTCGGGATGA
C.harmandii         GTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.cf.australasica   GTGGGGTATCCGGTAGTCGTCGAGCACGATGGATGTTGGTCGTCACGAGCGAGAACTGAGCATCGT--CCTTGTCGC--TTT-GGAACGA
C.petiolata         GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCACGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.ochrorrhiza       GTGGG-TAGTCGGTAATCGTCGAGCACGACGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCYTCGTCGT--TTT-GGGATGA
C.aeruginosa a      GYGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.aeruginosa b      GYGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.phaeocaulis a     GYGGG-TAGTYGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.phaeocaulis b     GYGGG-TAGTYGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGW
C.amarissima a      GTGGG-TAGTCGRTAATCGTCGAGCACGAYGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.amarissima b      GTGGG-TAGTCGRTAATCGTCGAGCACGAYGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTTC-GGATGA
C.aurantiaca        GTGGGGTAGCCGGTAGTCGTCGAGCACGATGGATGTTGGTCGTCACGAGCGAGAACTGAACATCGT--CCTTGTCGTCGTTTCGGAACGA
C.heyneana a        GYGGG-TAGTCGGTAACCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.heyneana b        GYGGG-TAGTCGGTAACCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.longa1 a          GCGGG-TAGTCGGTAATCGTTGARCAAGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.longa1 b          GKGGG-TAGTCSGTAATCGTTGARCAAGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.longa2 a          GCGGG-TAGTCGGTAATCGTCGAGCACGATAGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.longa2 b          GCGGG-TAGTCGGTAATCGTCGAGCACGATAGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.amada a           GTGGG-TAGTCGGTAAYCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.amada b           GTGGG-TAGTCGNTAAYCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTTC-GGATGA
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C.zedoaria1 a     GTGGG-TAGTCGGYAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.zedoaria1 b     GTGGG-TAGTCGGYAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT---TTCGGGATGA
C.zedoaria2a      GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.zedoaria2b      GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.zedoaria3a      GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.zedoaria3b      GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.zedoaria cf. a  GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT---TTCGGGATGA
C.zedoaria cf. b  GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.zanthorrhiza1a  GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.zanthorrhiza1b  GTGGG-TAGTCGGYAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.zanthorrhiza2a  GYGGG-TAGTCGGYAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.zanthorrhiza2b  GYGGG-TAGTCGGYAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.soloensis a     GCGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.soloensis b     GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.aromatica a     GCGGG-TAGTCGGCAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTKAACGTCGT--CCTCGTYGT--TTT-GGGATGA
C.aromatica b     GCGGG-TAGTCGGCAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTKAACGTCGT--CCTCGTYGT--TTT-GGGATGA
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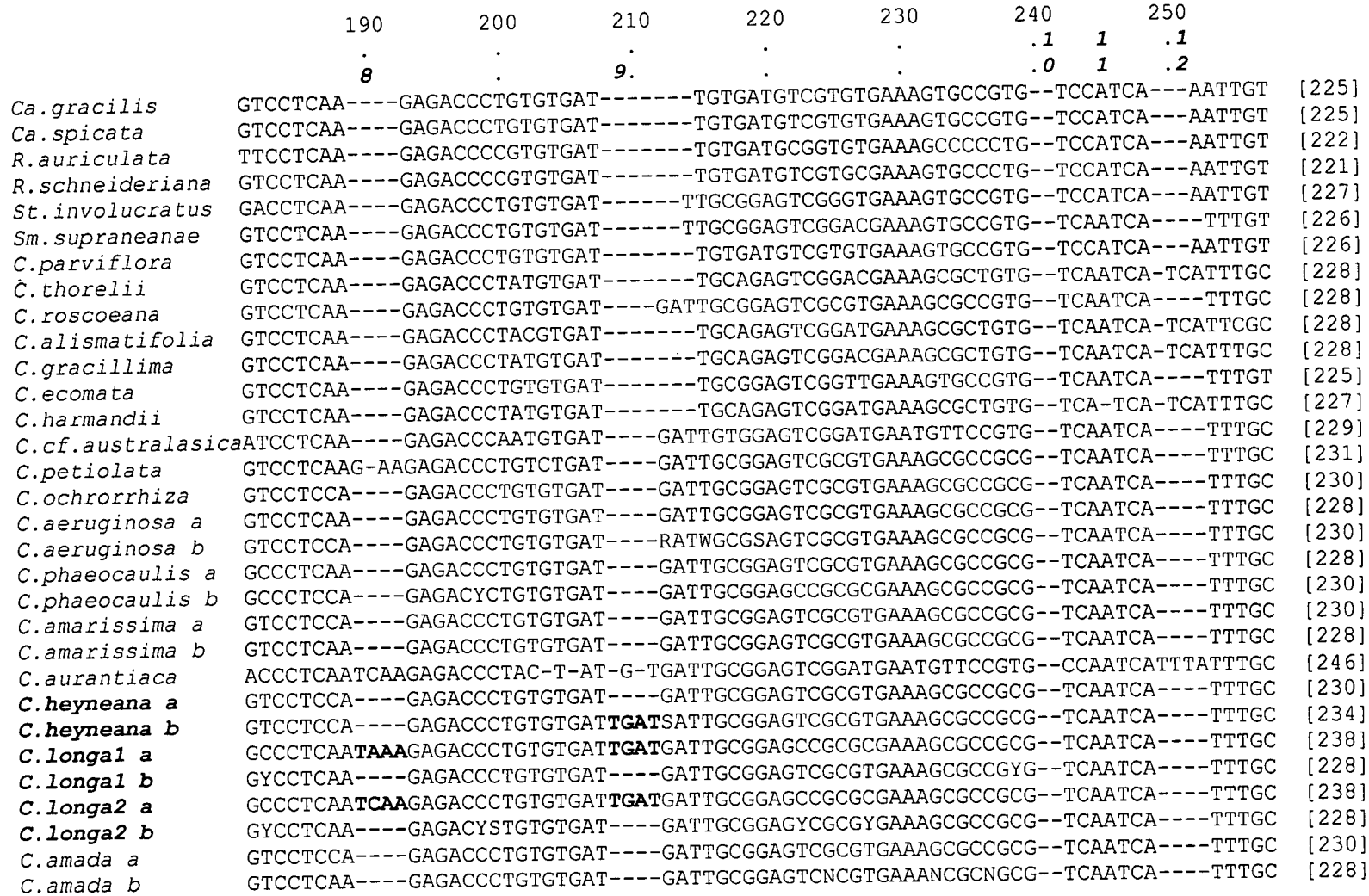
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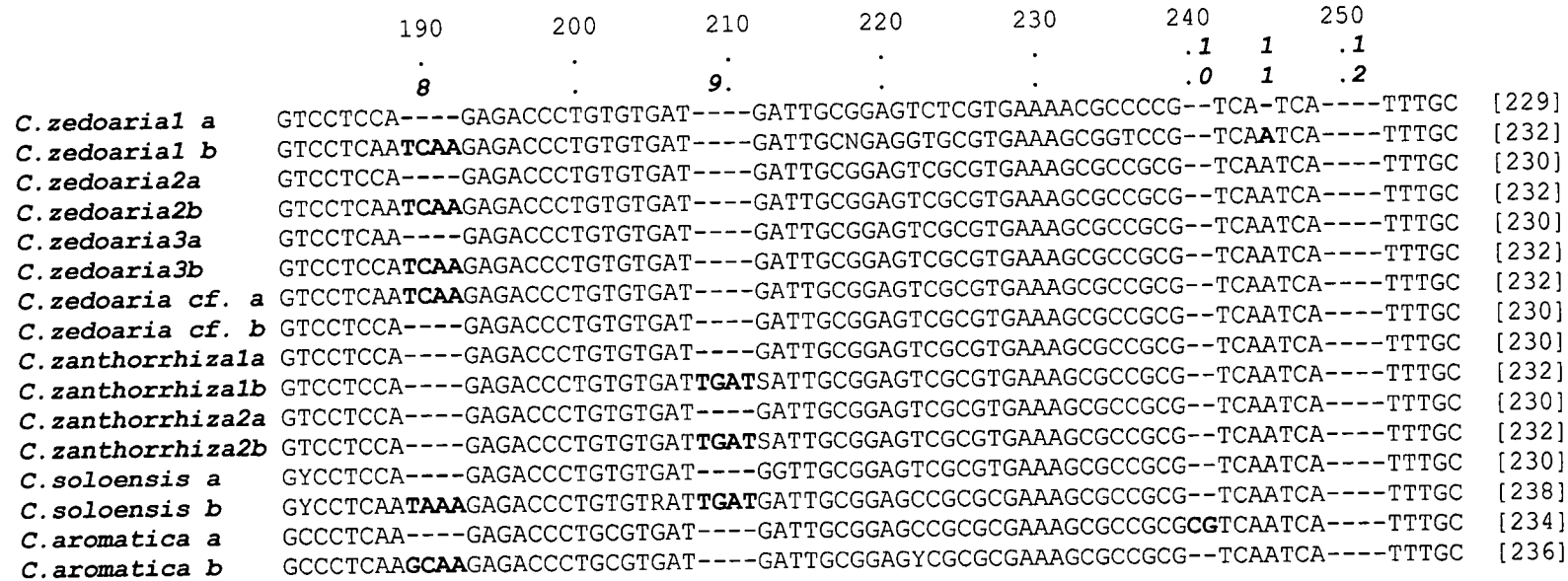
Ca.spicata GTCCTCAA----GAGACCCTGTGTGAT-------TGTGATGTCGTGTGAAAGTGCCGTG--TCCATCA---AATTGT [225]
R.auriculata TTCCTCAA----GAGACCCCGTGTGAT-------TGTGATGCGGTGTGAAAGCCCCCTG--TCCATCA---AATTGT [222]
R.schneideriana GTCCTCAA----GAGACCCCGTGTGAT-------TGTGATGTCGTGCGAAAGTGCCCTG--TCCATCA---AATTGT [221]
St.involucratus GACCTCAA----GAGACCCTGTGTGAT------TTGCGGAGTCGGGTGAAAGTGCCGTG--TCCATCA---AATTGT [227]
Sm.supraneanae GTCCTCAA----GAGACCCTGTGTGAT------TTGCGGAGTCGGACGAAAGTGCCGTG--TCAATCA----TTTGT [226]
C.parviflora GTCCTCAA----GAGACCCTGTGTGAT-------TGTGATGTCGTGTGAAAGTGCCGTG--TCCATCA---AATTGT [226]
C.thorelii GTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGACGAAAGCGCTGTG--TCAATCA-TCATTTGC [228]
C.roscoeana GTCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGTG--TCAATCA----TTTGC [228]
C.alismatifolia GTCCTCAA----GAGACCCTACGTGAT-------TGCAGAGTCGGATGAAAGCGCTGTG--TCAATCA-TCATTCGC [228]
C.gracillima GTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGACGAAAGCGCTGTG--TCAATCA-TCATTTGC [228]
C.ecomata GTCCTCAA----GAGACCCTGTGTGAT-------TGCGGAGTCGGTTGAAAGTGCCGTG--TCAATCA----TTTGT [225]
C.harmandii GTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGATGAAAGCGCTGTG--TCA-TCA-TCATTTGC [227]
C.cf.australasica ATCCTCAA----GAGACCCAATGTGAT----GATTGTGGAGTCGGATGAATGTTCCGTG--TCAATCA----TTTGC [229]
C.petiolata GTCCTCAAG-AAGAGACCCTGTCTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [231]
C.ochrorrhiza GTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [230]
C.aeruginosa a GTCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [228]
C.aeruginosa b GTCCTCCA----GAGACCCTGTGTGAT----RATWGCGSAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [230]
C.phaeocaulis a GCCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [228]
C.phaeocaulis b GCCCTCCA----GAGACYCTGTGTGAT----GATTGCGGAGCCGCGCGAAAGCGCCGCG--TCAATCA----TTTGC [230]
C.amarissima a GTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [230]
C.amarissima b GTCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [228]
C.aurantiaca ACCCTCAATCAAGAGACCCTAC-T-AT-G-TGATTGCGGAGTCGGATGAATGTTCCGTG--CCAATCATTTATTTGC [246]
C.heyneana a GTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [230]
C.heyneana b GTCCTCCA----GAGACCCTGTGTGATTGATSATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [234]
C.longa1 a GCCCTCAATAAAGAGACCCTGTGTGATTGATGATTGCGGAGCCGCGCGAAAGCGCCGCG--TCAATCA----TTTGC [238]
C.longa1 b GYCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGYG--TCAATCA----TTTGC [228]
C.longa2 a GCCCTCAATCAAGAGACCCTGTGTGATTGATGATTGCGGAGCCGCGCGAAAGCGCCGCG--TCAATCA----TTTGC [238]
C.longa2 b GYCCTCAA----GAGACYSTGTGTGAT----GATTGCGGAGYCGCGYGAAAGCGCCGCG--TCAATCA----TTTGC [228]
C.amada a GTCCTCCA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC [230]
C.amada b GTCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCNCGTGAAANCGCNGCG--TCAATCA----TTTGC [228]



Notes:
Numbers in bold italic indicate number and position of indel polymorphisms. A/T/G/C in bold shows insertion polymorphisms within one individu correspond with species name in bold font. Hypens indicate alignment gaps, while hypens in bold shows deletion polymorphisms within one individu correspond with species name in bold font. Uncertain nucleotide states are coded based on PAUP conventions (Swofford 1993) as follows: K=G/T, M=A/C, R=A/G, S=C/G, W=A/T, Y=C/T, N=A/T/G/C. a and b after species name indicate sequence copies a and b which were obtained after inspecting and editing the electropherogram and were not the results of cloning. Square brackets at the end of sequences show the real spacer length of ITS2 region.

APPENDIX 7 Sequence data matrix (displayed from 5' to 3') of aligned ITS2 region of 32 accessions representing 27 taxa of Zingiberaceae after polymorphic sequences combined.

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                          10        20        30        40        50        60        70        80        90
Taxon          ITS2       .         .         .         .         .         .         .         .         .
               1          .         .         .        .2 3  4 5  .         .         **        .         .
Ca.gracilis    AT---CGTCGCTTTTGCTCCATGCGTTATTGGCATCGAGCG----CGGAAATTGGCCTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGA
Ca.spicata     AT---CGTCGCTTTTGCTCCATGCGTTATTGGCATCGAGCG----CGGAAATTGGCCTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGA
R.auriculata   AT---CGTCGCTTTTGCTCCATGCATTGCTGGTGTCGAGCG------GAAATTGGCCTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGA
R.schneideriana AT---CGTCGCTTTAGCTCCATGCGTTGCTGGTGTCAAGCG----CG-AAATTGGCCTCGTGTGTCCTC--GGGCACAGTCGGTTGAAGA
St.involucratus AT---CGTCGCTTTTGCTCCATGCTTTGCTGGCGTCGATCG----CGGAAATTGGCCTCGTGTGCCCTC--AGGCACAGTCGGTTGAAGA
Sm.supraneanae AT---CGTCGCTTTTGCTCCATGCTTTTTTGGCATTGAGCG----CGGAAATTGGCCTCGTGTGCCCTC--GAGCACAGTCGGTCGAAGA
C.parviflora   AT---TGTCGCTTATGCTTCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.thorelii     AT---TGTCGCTTATGCTCCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.roscoeana    AT---TGTCGCTTTTGCTCCATGCTTTGTTGGCATTGAGCG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.alismatifolia AT---TGTCGCTTATGCCCCATGCTTTGTTGGCATCGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.gracillima   AT---TGTCGCTTATGCTCCATGCTTTGTTGGCATTGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.ecomata      AT---TGTCGCTTTTGCTCCATGCTTCGTTAGCATTGAGCG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.harmandii    AT---CGTCGCTTATGCTCCATGCTTCGTTGGCATTGAGTG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCATAGTCGGTCGAAGA
C.cf.australasica AT---TGTCGCTTTTGCTCCATGCTTTATTAGCATTGAGCG----CGGAAATTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.petiolata    AT---TGTCGCTTTTGCTCCATGCTCTGTTGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GAGCACAGTCGGTCGAAGA
C.ochrorrhiza  AT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCYTC--GGGCACAGTCGGTCGAAGA
C.aeruginosa   AT---YGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.phaeocaulis  AT---YGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.amarissima   AT---CGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.aurantiaca   ATTGCCGCCGCTTTTGCTCCATGCTTTATTAGCATTGAGC-AGCGCGAAAATTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.heyneana     AT---CGTCGCTTTTGCTCCATGCTTYGYCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.longa1       AT---CGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.longa2       AT---YGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----CGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.amada        AT---CGTCGCTTTTGCTCCATGCTYYGTYGGCATTGAGCG----YGGAARTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.zedoaria1    AT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.zedoaria2    AT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.zedoaria3    AT---CGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.zedoaria cf. AT---CGTCGCTTTTRCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTRCCCTC--GGGCACAGTCGRTCGAAGA
C.zanthorrhiza1 AT---YGTSGCTTTWGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.zanthorrhiza2 AT---CGTCGCTTTTRCTCCATGCTTYGTCGRCATTGAGCG----CGGAAGTTGGCCCCGTGTRCCCTC--GGGCACAGTCGRTCGAAGA
C.soloensis    AT---CGTCGCTTTTGCTCCATGCTTYGTCGGCATTGAGCG----CGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
C.aromatica    GT---TGTCGCTTTTGCTCCATGCTTTGTCGGCATTGAGTGAGCGCGGAAGTTGGCCCCGTGTGCCCTC--GGGCACAGTCGGTCGAAGA
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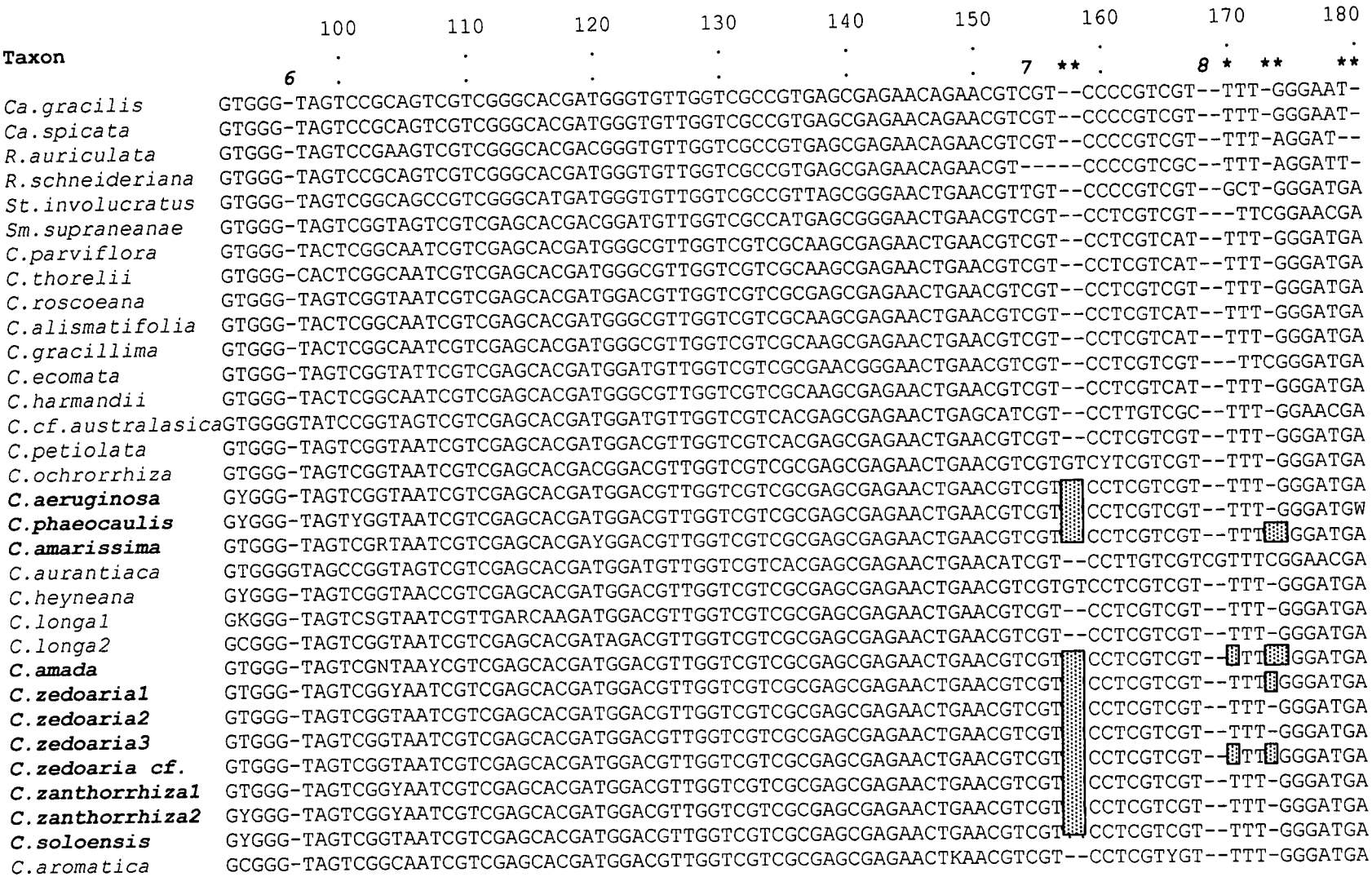
Taxon
100 110 120 130 140 150 160 170 180
6
7
8
Ca.gracilis GTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT--TTT-GGGAAT-
Ca.spicata GTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT--TTT-GGGAAT-
R.auriculata GTGGG-TAGTCCGAAGTCGTCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT--TTT-AGGAT--
R.schneideriana GTGGG-TAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGT-----CCCCGTCGC--TTT-AGGATT-
St.involucratus GTGGG-TAGTCGGCAGCCGTCGGGCATGATGGGTGTTGGTCGCCGTTAGCGGGAACTGAACGTTGT--CCCCGTCGT--GCT-GGGATGA
Sm.supraneanae GTGGG-TAGTCGGTAGTCGTCGAGCACGACGGATGTTGGTCGCCATGAGCGGGAACTGAACGTCGT--CCTCGTCGT---TTCGGAACGA
C.parviflora GTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.thorelii GTGGG-CACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.roscoeana GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.alismatifolia GTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.gracillima GTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.ecomata GTGGG-TAGTCGGTATTCGTCGAGCACGATGGATGTTGGTCGTCGCGAACGGGAACTGAACGTCGT--CCTCGTCGT---TTCGGGATGA
C.harmandii GTGGG-TACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT--TTT-GGGATGA
C.cf.australasica GTGGGGTATCCGGTAGTCGTCGAGCACGATGGATGTTGGTCGTCACGAGCGAGAACTGAGCATCGT--CCTTGTCGC--TTT-GGAACGA
C.petiolata GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCACGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.ochrorrhiza GTGGG-TAGTCGGTAATCGTCGAGCACGACGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCYTCGTCGT--TTT-GGGATGA
C.aeruginosa GYGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT-GGGATGA
C.phaeocaulis GYGGG-TAGTYGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT-GGGATGW
C.amarissima GTGGG-TAGTCGRTAATCGTCGAGCACGAYGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT GGATGA
C.aurantiaca GTGGGGTAGCCGGTAGTCGTCGAGCACGATGGATGTTGGTCGTCACGAGCGAGAACTGAACATCGT--CCTTGTCGTCGTTTCGGAACGA
C.heyneana GYGGG-TAGTCGGTAACCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT--TTT-GGGATGA
C.longa1 GKGGG-TAGTCSGTAATCGTTGARCAAGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.longa2 GCGGG-TAGTCGGTAATCGTCGAGCACGATAGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT--CCTCGTCGT--TTT-GGGATGA
C.amada GTGGG-TAGTCGNTAAYCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT GGATGA
C.zedoaria1 GTGGG-TAGTCGGYAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT GGGATGA
C.zedoaria2 GTGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT-GGGATGA
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C.zanthorrhiza1 GTGGG-TAGTCGGYAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT-GGGATGA
C.zanthorrhiza2 GYGGG-TAGTCGGYAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT-GGGATGA
C.soloensis GYGGG-TAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGT CCTCGTCGT--TTT-GGGATGA
C.aromatica GCGGG-TAGTCGGCAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTKAACGTCGT--CCTCGTYGT--TTT-GGGATGA

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                          190       200       210       220       230       240       250
Taxon                      .         .    1    .         .         .         .         .
                          ****       .  9 0 *******     .         .        **   *   ****
Ca.gracilis       GTCCTCAA----GAGACCCTGTGTGAT-------TGTGATGTCGTGTGAAAGTGCCGTG--TCCATCA---AATTGT
Ca.spicata        GTCCTCAA----GAGACCCTGTGTGAT-------TGTGATGTCGTGTGAAAGTGCCGTG--TCCATCA---AATTGT
R.auriculata      TTCCTCAA----GAGACCCCGTGTGAT-------TGTGATGCGGTGTGAAAGCCCCCTG--TCCATCA---AATTGT
R.schneideriana   GTCCTCAA----GAGACCCCGTGTGAT-------TGTGATGTCGTGCGAAAGTGCCCTG--TCCATCA---AATTGT
St.involucratus   GACCTCAA----GAGACCCTGTGTGAT------TTGCGGAGTCGGGTGAAAGTGCCGTG--TCCATCA---AATTGT
Sm.supraneanae    GTCCTCAA----GAGACCCTGTGTGAT------TTGCGGAGTCGGACGAAAGTGCCGTG--TCAATCA----TTTGT
C.parviflora      GTCCTCAA----GAGACCCTGTGTGAT-------TGTGATGTCGTGTGAAAGTGCCGTG--TCCATCA---AATTGT
C.thorelii        GTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGACGAAAGCGCTGTG--TCAATCA-TCATTTGC
C.roscoeana       GTCCTCAA----GAGACCCTGTGTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGTG--TCAATCA----TTTGC
C.alismatifolia   GTCCTCAA----GAGACCCTACGTGAT-------TGCAGAGTCGGATGAAAGCGCTGTG--TCAATCA-TCATTCGC
C.gracillima      GTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGACGAAAGCGCTGTG--TCAATCA-TCATTTGC
C.ecomata         GTCCTCAA----GAGACCCTGTGTGAT-------TGCGGAGTCGGTTGAAAGTGCCGTG--TCAATCA----TTTGT
C.harmandii       GTCCTCAA----GAGACCCTATGTGAT-------TGCAGAGTCGGATGAAAGCGCTGTG--TCA-TCA-TCATTTGC
C.cf.australasicaATCCTCAA----GAGACCCAATGTGAT----GATTGTGGAGTCGGATGAATGTTCCGTG--TCAATCA----TTTGC
C.petiolata       GTCCTCAAG-AAGAGACCCTGTCTGAT----GATTGCGGAGTCGCGTGAAAGCGCCGCG--TCAATCA----TTTGC
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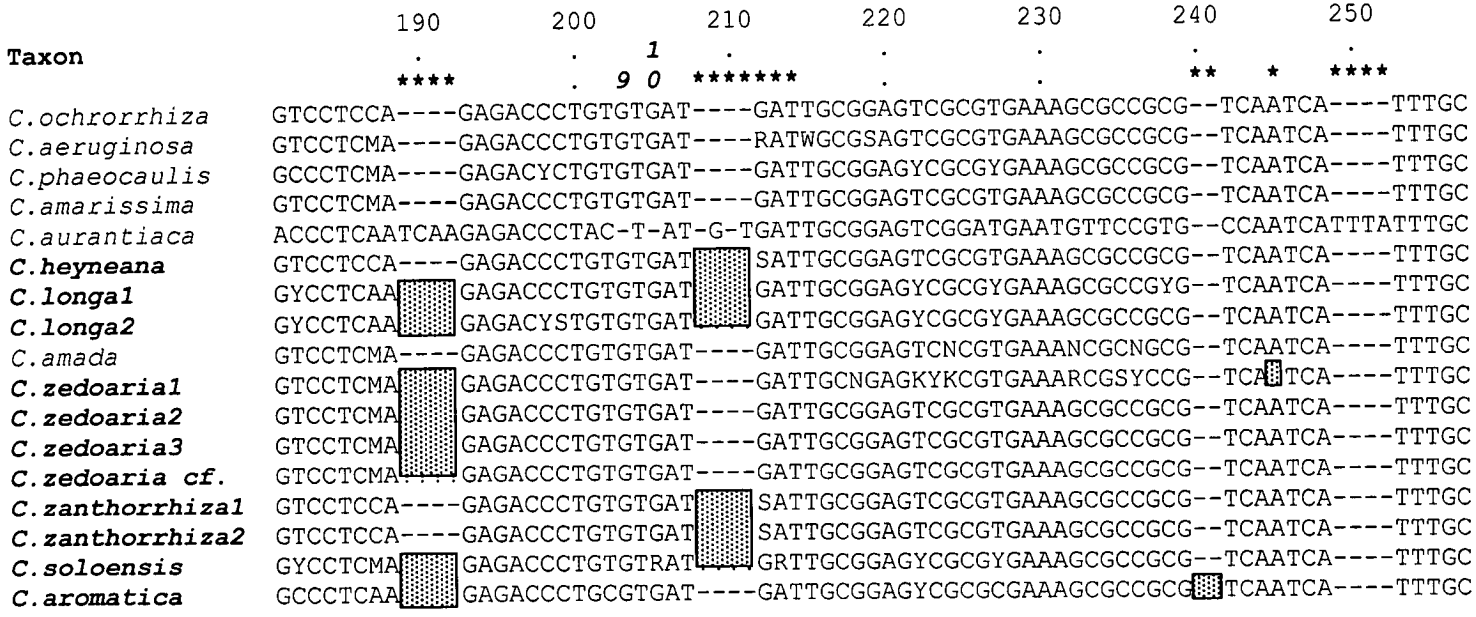

Numbers in bold italic indicate the number and position of alignment gaps. shows indel polymorphisms (correspond with species name in bold font). Hypens indicate alignment gaps. Uncertain nucleotide states are coded based on PAUP conventions (Swofford 1993) as follows: K=G/T, M=A/C, R=A/G, S=C/G, W=A/T, Y=C/T, N=A/T/G/C. * in bold indicates nucleotide sites which were excluded from part of phylogenetic analysis.

APPENDIX 8 Herbarium specimens examined

***Curcuma longa* L. var. *aeruginosa* (Roxb.) Ardiyani**

Exsiccatae:

Jawa: Banten, s.n. (L, L). Buitenzorg, #877 (BO, L). Purwokerto, Gunung Tugel, #C09021 (BO). Pradjekan, Pancur-Ijen Pancur-Ijen, Koorders #20751ß (BO). Sumedang, Marlina Ardiyani #25 (E). Karang Anyar, Kampung Sugat, Kelurahan Ngunut, Jumantono, Marlina Ardiyani #63MA (BO). *Kerala*: Trichur, Marlina Ardiyani #26 (E). Altitude range: 100 - 259 m

19771288

***Curcuma longa* L. var. *phaeocaulis* (Valeton) Ardiyani**

Exsiccatae:

Jawa: Tjibeber, Valeton #AcNo166880 (BO). Wonogiri, Kethu Forest, Marlina Ardiyani #43MA (E). Wonogiri, Desa Sukoharjo, Tirtomoyo, Marlina Ardiyani #51MA (BO). Wonogiri, Desa Blarak Sari, Kelurahan Sukoharjo, Tirtomoyo, Marlina Ardiyani #52MA (BO). Sukorejo, Kalipakis, Marlina Ardiyani #73MA (E). Altitude range:177 - 301 m

***Curcuma longa* L. var. *mangga* (Valeton & Zijp) Ardiyani**

Exsiccatae:

Jawa: Gunung Pandan, Kediri, Thorenaar #305 (BO). Karang Anyar, Kampung Sugat, Kelurahan Ngunut, Jumantono, Marlina Ardiyani #62MA (BO). Kediri, Ottens #705 (K, L). Buitenzorg, Botanic Garden, Heyne #85 (L). Bogor, Botanic Garden, Heyne #AcNo0012988 (K). Tjabak, Kalshoven #AcNo956017383 (L). Altitude range: 250 - 259 m

***Curcuma longa* L. var. *ochrorhiza* (Valeton) Ardiyani**

Exsiccatae:

Jawa: Karang Anyar, Kampung Sugat, Kelurahan Ngunut, Jumantono, Marlina Ardiyani #54MA (E). Karang Anyar, Dusun Talpitu, Desa Ngemplak, Kecamatan Karang Pandan, Marlina Ardiyani #57MA (BO). Kediri, Ottens #705 (BO). Randoeblatoeng, Kalshoven s.n. (BO). Altitude range: 259 - 260 m Heyne 705, iii 1917, Java: Bondowoso, Randublatung (BO).

***Curcuma longa* L.**

Exsiccatae:

Assam: Jashpur, Chota Nagpur, Wood #69 (K). Chota Nagpur, Wood #187 (K). Mungfoo Sealaha, Russel #20 (K). *Bangladesh*: Sylhet, Wallich #6605 (K). Chittagong Hill Tracts District, Wood #91 (K). *Bihar*: Parasnath, Chota Nagpur, Haranbagh, Clarke #33667a (K). Parasnath, Chota Nagpur, Haranbugh, Clarke #33768 (K).

Burma: Maymyo, Lace #5302 (K). Maymyo, Lace #5302 (E, E). Pegu, McLelland s.n. (K). *Haryana*: Saharampore, s.n. (E). *Hawaiian Is*: Kalaheo, Kauai, Nagata #3882

(E). *Jawa*: Buitenzorg, #AcNo0083973 (BO). Buitenzorg, #AcNo0083974 (BO). Wonogiri, Desa Sukoharjo, Tirtomoyo, Marlina Ardiyani #50MA (BO). Karang Anyar, Kampung Sugat, Kelurahan Ngunut, Jumantono, Marlina Ardiyani #60MA (BO). Karang Anyar, Kampung Sugat, Kelurahan Ngunut, Jumantono, Marlina Ardiyani #61MA (E). Buitenzorg, Bakhuizen van den Brink, Jr. #7202 (BO). Buitenzorg, Hallier #AcNo9083521305 (L). *Kalimantan*: Long Sungai Barang, Leaman #DL281 (E). *Karnataka*: Kanara, s.n. (E). *Natal*: Pietermaritzburg, Newman #799 (E, E). *New Caledonia*: Paita, McKee #36793 (E). *Papua New Guinea*: Masawara, Upper Markham, #8319 (E, E). Zenag, Kairo, Johnson #27977 (E). Vailala, Croft #LAE61234 (E). Kaiser Wilhelmsland, Hollrung #529 (K). Tanglide, Daru, Western province, Near Kunini village, Simaga #729 (K). *Philippines*: Mansalay, Mindoro, Merrill #908 (K). Island of Cuyo, Escritor #21378 (K). Palawan, Fenix #15536 (E). Coron Island, Ramos #41172 (K). *Sarawak*: Rumah Juing, Nanga Setusor, Sungai Mujok, Julau, Rantai Jawa #S67315 (E). Sarawak: Kampung Gumbang, Runi, Lai Shak Teck #S67408 (E). *Sri Lanka*: Colombo, s.n. (E). Colombo, Walker s.n. (E, E).
Tanzania: Korongwe, Lushoto, Archbold #3269 (K). *Thailand*: Bangkok, Marcan #2217 (K). *Uttar Pradesh*: Kumaon, Wallich #6605D (E). *Western Samoa*: Upolu, Reinecke #587 (E). *Zambia*: Mt. Makutu, Chilanga, Mwinilunga, Angus #3046 (K).
Altitude range: 250 - 1281 m

***Curcuma longa* L. var. *viridiflora* (Roxb.) Ardiyani**

Exsiccatae:
Bali: Bali, Nagata #3880 (E). *Jawa*: Rogodjampi, #768 (L). Tjikande, Blume #AcNo9033221921 (L). Tjikande, Blume #AcNo9033221926 (L). Bogor, Botanic Garden, Heyne #40 (L). Batavia, Valeton #40 (BO). Tomo, Jl. Raya Bandung-Sumedang c.10 km from Bandung, Marlina Ardiyani #37MA (BO). Tomo, Jl. Raya Bandung-Sumedang c. 10 km from Bandung, Marlina Ardiyani #40MA (BO). *Sumatera*: Muarabungo, Rahayu #331 (K). Altitude range: 138 - 600 m

***Curcuma longa* L. var. *heyneana* (Valeton & Zijp) Ardiyani**

Exsiccatae:
Jawa: Buitenzorg, Botanic Garden, s.n. (BO). Wonogiri, Kethu Forest, Marlina Ardiyani #42MA (BO). Tawangmangu, Harini #100 (L). Altitude range:177 - 900 m

***Curcuma longa* L. var. *brog* (Valeton) Ardiyani**

Exsiccatae:
Jawa: Getas, Randublatung, Kalshoven s.n. (BO). Getas, Randublatung, Kalshoven s.n. (L). Kalshoven s.n., 8 xii 1916, Java: Randublatung, Getas (BO, L).

***Curcuma longa* L. var. *soloensis* (Valeton) Ardiyani**

Exsiccatae:
Jawa: Buitenzorg, Botanic Garden (Heyne 691), s.n. (L). Buitenzorg, Botanic Garden (Heyne 683), s.n. (L). Solo, #50 (L, K). Wonogiri, Kethu Forest, Marlina Ardiyani

#41MA (BO). Wonogiri, Kethu Forest, Marlina Ardiyani #47MA (BO). Karang Anyar, Dusun Talpitu, Desa Ngemplak, Kecamatan Karang Pandan, Marlina Ardiyani #55MA (BO). Ngawi, Kampung Tambak Selo, Marlina Ardiyani #64MA (E). Ngawi, Kampung Tambak Selo, Marlina Ardiyani #65MA (BO). Ngawi, Kampung Tambak Selo, Marlina Ardiyani #66MA (E). Blora, Desa Getas, Marlina Ardiyani #70MA (BO). Randublatung, Kampung Banaran, Marlina Ardiyani #71MA (E). Tempoeran, Beumée #4989 (BO). Purworedjo, Heyne #AcNo166927 (BO). Wonogiri, Tukluk, Harini #86 (L). *Papua New Guinea*: Musgrave, along river, Nagata #3915 (E). Altitude range: 50 - 610 m *Specimen examined.* Heyne 691, s.d., Java: Bogor (L); Heyne 683, s.d., Java: Bogor (L); no collector 50, s.d., Java: Solo, Temu glenyeh (L);

***Curcuma longa* L. var. *zanthorrhiza* (Roxb.) Ardiyani**

Exsiccatae:

Jawa: Buitenzorg, #42 (L). Salatiga, #2421 (L). Wonogiri, Kethu Forest, Marlina Ardiyani #45MA (BO). Wonogiri, Kethu Forest, Marlina Ardiyani #46MA (E). Karang Anyar, Dusun Talpitu, Desa Ngemplak, Kecamatan Karang Pandan, Marlina Ardiyani #59MA (BO). Tjiemas, Backer #25561 (L). Buitenzorg, Botanic Garden Botanic Garden, Heyne #42 (L). Buitenzorg, Botanic Garden, Heyne #219 (L). Semarang, Leeuwen #365 (BO). Tjipetir, Koolhaas s.n. (L). Buitenzorg, Tjimahpar (Cimahpar) Tjimahpar (Cimahpar), Irsan s.n. (BO). *Peninsular Malaysia*: Kota Tinggi, Johore, 23.5 miles to Jamaluang Road, Sinclair #8074 (E, E). Kota Tinggi, Johore, 23.5 miles to Jemaluang Road, Sinclair #8079 (E). Kota Tinggi, Johore, 23.5 miles to Jemaluang Road, Sinclair #40295 (E). Altitude range:177 - 400 m

***Curcuma longa* L. var. *zedoaria* (Christm.) Ardiyani**

Exsiccatae:

Assam: Dalgaon, Chatterjee s.n. (E). *Bangladesh*:
Chimbuk, Newman, Rahman #989 (E). Kaptai, Between Kaptai and Chittagong, Soejarto, Rahman #5003 (K). *Hawaiian Is*: Oahu, Source unknown, Nagata #3640 (E).

Jawa: Tomo, s.n. (K). Tomo, #623 (L). Kedunghalang, Boerlage #405 (L). Tomo, J. Raya Bandung-Sumedang c. 10 km from Bandung, Marlina Ardiyani #38MA (BO). Karang Anyar, Dusun Talpitu, Desa Ngemplak, Kecamatan Karang Pandan, Marlina Ardiyani #56MA (BO). Jawa Timur, Jawa Timur, Marlina Ardiyani #72MA (E). Leuwiliang, Pasir Honje, Bakhuizen van den Brink, Jr. #7563 (L). Lengkong, Backer #17092 (L). Batavia, Kramat Sentiong, Backer #34373 (L). Garoetan, Mousset #1091 (BO). Grobogan, Vogel #79 (L). Bidaratjina, Meester Cornelis, Edeling #AcNo167036 (BO). *Kerala*: Trichur, s.n. (E). Anaimalai Hills, Karani, Fischer #33555 (K). Palai, Mangaly #10365 (E). Malabar, Lau s.n. (K). *New Caledonia*: Noumea, McKee #36123 (E). *Peninsular Malaysia*: Balek Palau, Ridley #7229 (K). Jalan Misjed, Penang, Sidek bin Kiah #254 (K). *Philippines*: Lucban, Tayabas Province, Elmer #7752 (E). Irosin, Mt. Bulusan, Sorsogon Province, Elmer #16738 (K). Lantouan, Mindoro Is., N. face of Mt. Halcon, Stone #820 (K). Novaliches, Rizal Province, Paradise farm, Mendoza #37422 (K). Mt. Mariveles, Lamao river, Whitford

#1267 (K). *Sarawak*: Rumah Gerasi, Nanga Ju, Sungai Mujok, near Julau, Rantai Jawa #S67309 (E). Sarawak: Kampung Silantek, 85th miles Simanggang Road, 2nd Division, Ilias bin Paie #S.42639 (E). Sarawak: Rumah Ubong, Sungai Balang, Nanga Gaat, Kapit, 7th Division, Lee, Awa #S.50038 (E). Sarawak: Rumah Ubong, Sungai Balang, Nanga Gaat, Kapit, 7th Division, Lee, Awa #S.50039 (E). Sarawak: Kampong Kuala Tellian, near Mukah, Kandau Jenang #S58115 (E). Sarawak: Kampung Gumbang, Runi, Lai Shak Teck #S67409 (E). *Singapore*: Bukit Timah, #11362 (K). *Sri Lanka*: Sinharaja, Burtt #6803 (E). Bopathella Falls, Marlina Ardiyani #28 (E). Galla, Dubuc s.n. (E).
Thailand: Hat Yai, Trang, Prince Songkla University, Newman #59 (E, E). Tripagodas, Burmese border, Bloembergen #48 (K). Mae Mawh, LAMPANG, Mae Mawh Lignite mine area, Maxwell #90-598 (E). Chiang Mai, summit of Doi Miang Awo, Maxwell #92-518 (E). Hin Dat, Put #39 (K). Muang Ngao, Lampang, Put #3998 (K). Aw Ong Kang, Geesink, Hattink, Phengklai #6624 (K).

***Curcuma longa* L. var. *purpurascens* (Blume) Ardiyani**

Exsiccatae:
Bali: Bali, Nagata #3886 (E). *Jawa*: Lebak, s.n. (L, L, K, K, L). Buitenzorg, Botanic Garden (Heyne 25) Botanic Garden (Heyne 25), s.n. (L). Tomo, Tomo Oost Preanger, #620 (K, L). Pulau Kangean, Beguin s.n. (K). Karang Anyar, Dusun Talpitu, Desa Ngemplak, Kecamatan Karang Pandan, Marlina Ardiyani #58MA (BO). Buitenzorg, Ottens #25 (K). Buitenzorg, Ottens #25 (BO, L). Tomo, Heyne #620 (BO). Sitoebondo, Clason-Laarman #987 (L). Dago, Bandung, Popta #702/65 (L). Altitude range: 260 - 850 m *Specimen examined.* Heyne 620, 8 iii 1916?, Java: Tomo, Koneng tinggang (BO)

***Curcuma longa* L. var. *euchroma* (Valeton) Ardiyani**

Exsiccatae:
Jawa: Soemenep, s.n. (L, BO). Tajoe, Ngarengan, Koorders #35742ß (BO). Wonogiri, Kampung Sulingi, Nguntoronadi, Marlina Ardiyani #48MA (BO). Wonogiri, Desa Tanjung Sari, Kecamatan Tirtomoyo, Marlina Ardiyani #49MA (E). Karang Anyar, Kampung Sugat, Kelurahan Ngunut, Jumantono, Marlina Ardiyani #53MA (E). Kediri, Ottens #703 (L, BO). Randoeblatoeng, Getas, Kalshoven #AcNo920182672 (L). Randoeblatoeng, Kalshoven #AcNo956017379 (L). Heyne 87, 1916, Madura: Sumenep, Temu lati (BO). Altitude range: 50 - 308 m

***Curcuma longa* L. var. *colorata* (Valeton) Ardiyani**

Exsiccatae:
Jawa: Buitenzorg, Botanic Garden (Heyne 100), Valeton s.n. (BO). Banjar, Marlina Ardiyani #34 (E). Wonogiri, Kethu Forest, Marlina Ardiyani #44MA (BO). Bondowoso, Heyne #592 (BO). Getas, Randublatung, Kalshoven #1645 (BO - Type of *Curcuma colorata* Valeton). *Thailand*: Ban Mae Pang, 30 km north of Mae Sariang, Larsen, Santisuk, Warncke #2338 (E, K). Altitude range:177 - 600 m

***Curcuma aurantiaca* Zijp**

Exsiccatae:

Jawa: Tomo, Tomo Oost Preanger, #619 (K, L, BO). Bogor, Botanic Garden, #862b (L, K). Mount Puger, Besuki, Buwalda #7298 (K, L). Randoeblatoeng, Koorders #42257ß (BO). Tjabak, Koorders #42524ß (BO). Tomo, Jl. Raya Bandung-Sumedang c. 15 km from Bandung, Marlina Ardiyani #35MA (BO). Tomo, Jl. Raya Bandung-Sumedang c.15 km from Bandung, Marlina Ardiyani #36MA (E). Ngawi, Kampung Tambak Selo, Marlina Ardiyani #67MA (E). Ngawi, Kampung Tambak Selo, Marlina Ardiyani #68MA (BO). Ngawi, Kampung Tambak Selo, Marlina Ardiyani #69MA (BO). Bogor, Botanic Garden Botanic Garden Botanic Garden, Alston #12624 (BO). Bodja, Beumée #3831 (BO). Saradan, Madiun, Wisse #156 (BO). Tjiandjur, Kiara Payung, Backer #23584 (L, BO). Djukongdjukong, Backer #27587 (BO). Djepara, Heyne #633 (BO). *Singapore*: Perlis, Beoih Hangat, Henderson #22869 (K). *Thailand*:

Kanchanadit, Surat, #13130 (K). Tung Song, Rabil Bunnag #165 (K). Altitude range: 50 - 550 m

***Curcuma petiolata* Roxb.**

Exsiccatae:

Hawaiian Is: Oahu, Origin unknown (Cultivated in Lyon Arboretum)., Nagata #3688 (E, E). *Jawa*: Buitenzorg, Botanic Garden, s.n. (L). Bogor, #47 (K, L). Bogor, Bogor Botanic Garden, #866 (K). Bodja, Beumée #3885 (BO, L). Buitenzorg, Botanic Garden, Heyne #47 (L, K). *Thailand*: Sai Yok, Marcan #2382 (K). Altitude:100 m

Curcuma indeterminate

Exsiccatae:

Jawa: Randoeblatoeng, Ottens #688 (K, L). Djukongdjukong, Backer #27587 (BO). Pulau Kangean, Backer #30009 (BO). Buitenzorg, Botanic Garden, Heyne #AcNo0082611 (BO). Gombong, Heyne #AcNo0082617 (BO). Mangkang, Leeuwen s.n. (L, BO); Randoeblatoeng, Koorders #42257ß (BO).

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