The Systematics of the Hedychieae (Zingiberaceae), with Emphasis on *Roscoea* Sm.

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I declare that this thesis has been composed by myself and the work contained within, unless otherwise stated, is my own.

ABSTRACT

The tribe Hedychieae (ginger lily) is the second largest in the ginger family, Zingiberaceae. I carried out a phylogenetic analysis of the Hedychieae using nuclear ribosomal DNA (ITS1, 5.8S and ITS2) and chloroplast DNA (*trn*L (UAA) 5′ exon to *trn*F (GAA)). The results of these two data sets are in accordance, though with differing levels of resolution. Hedychieae is confirmed to include Zingibereae, the true gingers, and is monophyletic. However, the genera *Boesenbergia* and *Curcuma* are not monophyletic. Two major clades are recognised in Hedychieae namely, the '*Curcuma* clade' and the '*Hedychium* clade'.

The '*Curcuma* clade' comprises *Camptandra*, *Pyrgophyllum*, *Stahlianthus* and a set of four morphologically very similar genera: *Curcuma*, *Hitchenia*, *Paracautleya* and *Smithatris*. In this clade, a subclade of *Camptandra/Pyrgophyllum* is the sister group to a very strongly supported '*Curcuma* complex': *Curcuma*, *Hitchenia*, *Paracautleya*, *Smithatris* and *Stahlianthus*. *Curcuma* is paraphyletic. Two subclades are found in the complex namely, *Stahlianthus/Curcuma* subg. *Hitcheniopsis*, and *Hitchenia/Paracautleya/Curcuma* subg. *Curcuma*. The dorsifixed versatile anther of the *Curcuma* complex has been lost independently in *Hitchenia* and *Stahlianthus*, while the basifixed versatile anther has arisen independently in *Camptandra* and *Cautleya/Roscoea*. Scanning electron micrographs of anther development in *Cautleya spicata* show that the appendages develop from the joint connective tissue and thus the anther with the appendages is versatile in mature plant. Observation of the appendages in *Curcuma* and *Paracautleya* reveals that the anther is dorsifixed and the appendages are derived from the thecae of the anther.

Within the '*Hedychium* clade', I recognise two main subclades: a clade of *Hedychium/Pommereschea/Rhynchanthus/Cautleya/Roscoea*, and a '*Boesenbergia* group' that has *Boesenbergia*, *Caulokaempferia*, *Cornukaempferia*, *Distichochlamys*, *Haniffia*, *Kaempferia*, *Scaphochlamys* and *Zingiber*. *Boesenbergia* is paraphyletic with respect to *Caulokaempferia*. *Zingiber* is sister to *Cornukaempferia* and the

large, narrow and curved anther crest found in these two genera is a morphological character also suggesting their close relationship. *Pommereschea* and *Rhynchanthus* have been traditionally placed in the tribe Alpinieae, but the lack of petaloid staminodes in these two genera can be seen as a derived character loss.

I carried out a detailed phylogenetic study of *Roscoea*, the only truly high altitude genus of an otherwise lowland tropical plant family using ITS. It is found along the Himalaya and on high mountains in Southwest China. The results show that *Roscoea* is monophyletic and *Cautleya* is the sister group. Furthermore, *Roscoea* is found to have two subclades, namely the 'Chinese clade' and the 'Himalayan clade', which show contrasting geographical distributions. These two groups are disjunct across the 'Brahmaputra gap', a region in which no *Roscoea* species has been recorded. Morphological data support these findings. These three lines of evidence: ITS, distribution and morphology are used to define a new species, *R. bhutanica*, from western populations of previously *R. tibetica*. A new identification key to *Roscoea* is presented. The morphological data used in the phylogenetic study of *Roscoea* are found to be limited as they contain more homoplastic characters than the ITS. Chromosome counts of *Roscoea alpina*, *R. auriculata*, *R. purpurea* and *Cautleya spicata* are presented. My counts of two species confirm the widely reported number of $2n = 24$. However, I found that both *R. alpina* and *C. spicata* have a chromosome number of $2n = 26$.

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CHAPTER ONE: INTRODUCTION

1.1 TAXONOMY AND SYSTEMATICS

Man classifies all that he sees and gives each thing a name in order to be able to remember it and communicate about it. This is a natural habit and always happens either consciously or subconsciously. In the natural world of which man is a part, there are so many living beings around that he needs some sort of a system to help him to take them all in efficiently. The very first categories of classification may be, for instance, usefulness (e.g. food, medicine, fuel) and harmfulness (poison).

Taxonomy is the term given to this discipline covering all three activities involved, namely **classification**, **nomenclature** and **identification**. The term was first coined by A.P. de Candolle (Davis and Heywood, 1963, p. 8) and the early study's aim was only to recognise the diversity of living organisms. In other words, each species should have a particular place in a system that can be used for data retrieval and communication. Cronquist (1968, 1988) gave a definition of taxonomy among various ones (Stace, 1989; Lincoln *et al.*, 1998) as "a study aimed at producing a system of classification of organisms which best reflects the totality of their similarities and differences". A classification system can be created for many different purposes using any sources of the data. However, stability, practicality and convenience are at the core of the system (Davis and Heywood, 1963).

Carolus Linnaeus is famously known as the father of modern taxonomy. His Species Plantarum (Linnaeus, 1753) which presented his classification of the plants of his time, set out the starting point of reference for nomenclature and a sample system of classification that is presently considered far from adequate. It was based on a few characters of the reproductive organs, such as the number of stamens and carpels, so it is often called the 'sexual system'. Nonetheless he succeeded in bringing the bewildering world of plant diversity into a sort of system. He is also frequently accredited with having first introduced the binomial system of

nomenclature, although Jean Bauhin founded the system a century earlier than Linnaeus (Lawrence, 1951, p. 17).

The advent of 'On the Origin of Species by Means of Natural Selection' (Darwin, 1859) with the evolutionary theories of C. Darwin and A.R. Wallace profoundly altered the world of biological studies, albeit it had had little impact in taxonomy until the present time of cladistics. The fundamental concept, as the title suggests, which was new at the time, is a truly luminous, revolutionary guide towards the better understanding of the biological world. It is now accepted that the diversity of life on earth is a result of evolution, the process by which the pool of variation in any species or population interacts with the surrounding environment or natural forces to produce change. As a result of cumulative change from generation to generation, differing characteristics arise in organisms and species are selected to survive and reproduce in those environments or go extinct. This process at the macro scale or above species level takes place very slowly over a long span of time, usually millions of years. The process is well captured by Darwin in the phrase 'descent with modification'.

A good classification system must reflect this pattern of branching or evolutionary relationships. The word 'good' here means **stable** and **predictable**. A classification that recognises the evolutionary history of the group is thought to have greater predictive value and can accommodate later findings from new sources of data with greater stability than ones that were not constructed by the recognition of the evolutionary pattern of the group. It can be said that such a classification is natural or phylogenetic. However, such a natural classification that is constructed from all available evidence, may or may not reflect phylogeny. Thus, a phylogenetic or evolutionary classification is preferable. Nonetheless, having this aim in mind, plant taxonomists often come up with different systems of classification (Cronquist, 1988; Thorne, 1992; Takhtajan, 1997). The differences are partly attributable to the characters taxonomists use to define their groupings. Another aspect that cannot be really justified is personal thought or belief deriving from the taxonomist's own experience. This renders the science of taxonomy **subjective**, and is always a topic of

debate in the community, besides attracting outside criticism. One attempt to produce an **objective** procedure of classification is termed cladistics to which I shall come back in a later part.

In the history of botanical nomenclature, three revolutionary initiations can be recognised (McNeill, 2000). The first is the introduction of the binomial system of plant naming by Linnaeus in 1753. The second is the agreement of the principle of priority. The third is the type method applied to plant names. The recent International Code of Botanical Nomenclature (Saint Louis Code) is also considered to be the best code published to date, though some aspects of the present code still need to be refined (McNeill, 2000). It can be considered here that taxonomy (in a strict sense i.e. classification) and nomenclature are two different activities and linked to each other only by the types of the plants.

Systematics is a more recent term than taxonomy. It is frequently used interchangeably with taxonomy, though to some taxonomists it has a wider meaning. As Stuessy's diagram illustrated (Stuessy, 1990, p. 8) besides taxonomy, it also includes the study of the process of evolution and the study of phylogeny. The term systematics is thus preferred here. It is accepted that the only theory that can unify all fields in biology is the theory of evolution. 'Nothing in biology makes sense except in the light of evolution' is an article title and famous quoted phrase of Dobzhansky (1973) that summarises it all. Similarly the only organising discipline in biology that can unify all others into one is systematics. All information from studies in biology can find its place in a theoretically single universal classification system that has the theory of evolution at its core. Such a classification is the ultimate goal that systematists should be aiming for. It is expected that it will not take long from now for the ideal system to be reached since the positions of all the major groups of flowering plants are already known (Bremer *et al.*, 1998; Soltis *et al.*, 1999; Soltis *et al.*, 2000). We are coming to an age that the systematists know their plants' phylogeny and this estimate, as more and more data are used to reconstruct plant evolutionary history, will truly reflect the natural, genealogical, phylogenetic or evolutionary relationship and open more opportunities for further research.

1.1.1 TYPOLOGICAL AND POPULATIONAL THINKING

Although it has been assumed that all species included in this study pass species rank recognition, or in other words, they are all well established morphologically as distinct species, the notion of typological and populational concepts is worth bearing in mind. While species exist in the real world, as we recognise, for examples, dogs, cats, roses and thistles, after Darwin, two points are made clear. Firstly species are not unchanging organisms as previously thought. Secondly species are not represented by types, but by a population or populations, a smallest unit of organism which natural selection plays upon. Variation within any population is the raw material for any changes in the course of evolution. The process that gives rise to all species on earth is a continuing one but classification is like a photograph in which a moment of evolution is frozen and portrayed.

We need, however, to have type concepts for taxonomic purposes (Cronquist, 1988), besides the fact that we all have type concepts for countless other things. A group or groups of individuals are best referred to species whose **multiple correlations of characters** are distinct from those of other species. The typological concept may not be totally correct but it is useful for study and communication. It is also biological rather than nomenclatural when we talk about it. Only names have types, not species (Davis and Heywood, 1963, p. 279). The width of one's typological concept of a particular species is variable depending on the variation of the species that one has seen or is aware of. This is of course a part of the taxonomist's expertise. Often knowledge pertaining to that species arises intuitively in one's mind. Different opinions are thus formed based on experience and concepts. At the species level and above the typological concept is mainly used and these studies lie in the area of phylogeny while studies at species level and below are considered to be in the field of population genetics. As we can see, both levels are the study of variation in the biological world or systematics.

1.2 CLADISTICS AND MOLECULAR SYSTEMATICS

Cladistics is the term given to a method of classification that relies solely on the recency of common ancestry for the classification. It was coined by a distinguished ornithologist E. Mayr (Mayr, 1969). Given a set of organisms and a set of characters in use for classification, this objective method will give ideally the same results by systematists. The characters that are used for grouping are shared derived ones or **synapomorphies**. This simple, yet very powerful method was first formalised by an entomologist named W. Hennig (Hennig, 1950, 1966). The principal concept is the **parsimony** of evolution or the requirement of minimum changes in the course of evolution. It means that the shortest hypothetical pathway of change that explains the present pattern of data used in the systematic study is considered to be the most likely evolutionary route.

Its philosophy stems from the notion that evolution is true. Species have evolved and shared common ancestors, giving rise to branching patterns of speciation. This gives credibility to the hierarchical system of classification that has been used since the time of Linnaeus. At present, the system has seven basic hierarchical levels, namely species, genus, family, order, class, division and kingdom, in order of the totality of similarities and differences among individuals or groups. It should be noted that above species level, the rank given to any taxon is largely arbitrary. These ranks should not be deduced as synonyms with the branching pattern of species in reality.

Rank recognition according to the Linnean system of nomenclature is probably the most subjective aspect of taxonomy which has been found to be problematic to use by modern cladists. Arguments as to whether to continue to use the existing traditional system of nomenclature with added modifications when needed or replace it with a new phylogenetic system have arisen in recent years and are unlikely to end in the near future. The PhyloCode (a phylogenetic code of biological nomenclature) that aims to give more stability to the species names and

reflect the phylogeny of the species studied has, however, been proposed recently to the community (Cantino and de Queiroz, 2000). McNeill (2000), however, stated that one may comprehend and find a better position in the dispute over the phylogeny and the names of plants if one understands that classification and nomenclature are two different activities.

There are terms of characters in the method that need to be clarified, i.e. apomorphy, autapomorphy, synapomorphy and plesiomorphy. Apomorphy is a derived character state. Synapomorphy is a shared derived character state. Autapomorphy is a character state that distinguishes a particular clade. Plesiomorphy is a primitive character state. The only useful character in cladistics is synapomorphy. These terms are relative in the group being studied. Upon finding the most parsimonious tree/s from the cladistic analysis, characters are then defined as plesiomorphic or apomorphic. Terminal species are also grouped as monophyletic, paraphyletic and polyphyletic. A monophyletic group is a branch that includes all terminal species arising from a common ancestor. This monophyletic branch is called a **clade**. Paraphyly occurs when not all terminal taxa are included in the branch and termed a **grade**. A polyphyletic group is a group that has more than one common ancestor or arises from more than one direct origin. It should be noted that not all synapomorphic characters indicate monophyly, but they also can indicate polyphyly due to parallelism or convergence (homoplasy).

One aspect that is paramount in the study of biology is homology. In order to understand the evolving nature of species, one must be able to differentiate homology from analogy. Unlike analogy where similarity is attributable to convergent evolution, homology is similarity of closely related species due to common descent. It ensures that like is being compared with like, i.e. the same characters, in the comparative study of biology. Its understanding aids in unravelling patterns of changes of the characters and the evolution of the plants as a whole. In cladistics, it is usually considered to be synonymous with synapomorphy. Normally we do not know beforehand which characters are plesiomorphic, synapomorphic or autapomorphic. Character identification is one of the results of cladistic analysis

when all the characters included interact in the process of finding the evolutionarily shortest trees. Each character is then identified in terms of both state (primitive or derived) and consistency (congruent or homoplastic), albeit tentatively or imperfectly because of its relative value.

Usually a few closely related species or outgroup species of the species being investigated (ingroup species) are included in the analysis, for the base of the study. Although it is not necessary to have more than one outgroup, the more complete the sampling of related taxa the greater the expectation of stability of future studies (Nixon and Carpenter, 1993). The outgroup species are normally selected based on their morphology or other similarities with the ingroup species. Then, a matrix of character states of all species in the study is constructed and used for the cladistic analysis. Not until the cladistic analysis has been completed are the outgroup species clearly identified and confirmed. In other words, the result of the analysis may suggest otherwise. If the outgroups are found to have a common direct ancestor with the ingroup species, it is then called the **sister** group (to the ingroup species).

Not all characters have the same value in systematics, a fact that is well known to the community. Good characters normally mean that they are useful in the process of identification and classification. Practitioners who know their plants well usually select only good characters for use and discard others or give different weight to the characters, a process that is called character weighting. There should be a biological explanation to any given differing values of the character states. In general, during the first cladistic analysis of any sources of data, each character is given the same value, one. Later analyses can be modified according to some statistical values observed from the resulting tree/s or can be tested according to some hypotheses.

There is also another method referred to as discrete one, apart from parsimony, for the cladistic analysis that should be mentioned. This method which was specially developed for use with molecular data is called **maximum likelihood** (Felsenstein, 1981). It seeks to find the trees that yield the maximum likelihood value

to an observed data set on the explicit model of evolution. In other words, it asks the model of evolution to find the shortest trees of the observed data set. This method is considered to be more versatile than parsimony to analysing the sequences because it evaluates all characters and can accommodate other assumptions, such as different rates and patterns of substitution. However it can give inconsistent results (Siddall, 1998) and is computationally demanding.

A problem in cladistics, known as NP (non-polynomial) - complete problems in mathematics, is criticised as a weakness in the method. It shows that as the number of species increases, the number of possible evolutionary trees quickly soars and even any imaginable increase in the speed of computers cannot evaluate them all to find the most parsimonious tree(s) in a life time (Pankhurst, 1991, 1995). This problem has been, in part, solved by the ever-increasing speed of computers and also by the introduction of new methodologies in cladistics. With the notion of inferred resultant phylogenetic trees as the estimates, or in other words, we do not know the true tree, the inferred trees are best used as a starting point of further investigation.

1.3 MORPHOLOGY AND MOLECULES IN SYSTEMATICS

The relative values of morphological and molecular data have been a topic of debate. Despite the fact that morphological data are cheap and readily obtained, plant systematists are often faced with plasticity within a species, e.g. morphological variation within or among populations. The plasticity of any plant species, apart from genetic variation among individuals, is largely attributable to various physical environments where the plant populations grow. Three main symptoms can be seen in plant plasticity. They are morphological, physiological and behavioural variation (Lincoln *et al.*, 1998). Moreover these highly affected morphological traits by different environments are not inheritable and there are also limited traits to be useful in the systematic study. There is sometimes a problem of the homology of morphological data, which unless firmly supported by its ontology (ontogenetic criterion) is arguable. So it is normally thought that molecular data are superior to

morphological data in that the environmental effect or selection is less active in molecules or DNA. Thus the phylogenetic information in molecules is more preserved and stable, hence more useful to reconstructing the life history of the group. Besides, by using a non-coding region, it also means that the result is less subject to environmental conditions which are sometimes found to play a very influential role in plant evolution.

DNA accumulates mutations over time. Its function is subject to how much it can tolerate before jeopardising its survival. The evolutionary variability of any molecule is a balance of mutationally neutral input and the constraints of structure and function. Its use as a source of phylogenetic information has two advantages over morphological data, first more data and, second generally easier interpretation of homology. There are hundreds or thousands of genes in any genomic set. Each part of the genome has its own properties, e.g. inheritance mode and rate of evolution. Thus, genes can be chosen for their suitability to a particular problem. For instance, genes of high rate of evolution can give rather well resolved trees in specific level studies, whereas, genes of slow rate of evolution are more suitable at generic level and above. Gene sequences also give the power of recognition of the frequency and evolutionary potential of hybridisation and introgression. Nevertheless, a study based on morphological data is still the only way to study relationships between living organisms and fossils.

Each nucleotide in any gene is one character whose states are four nucleotides, namely A, T, C and G. The use of nucleotide sequences in phylogenetic study involves fewer subjective decisions on the homology of character and character states. An 'A' at a particular site is an 'A', providing correctly identified homology and sequences alignment. This type of data is well suited to rigorous, algorithmic methods of analysis. The weighting step of molecular character is also easier than that of morphological character where the process is somewhat more of personal experience and again presents the field another debate. It also opens up the opportunity of studying distantly related species whose other comparative data, e.g. morphology are limited, if not prohibited at all. There are two types of origins of

genes. Orthologous genes are the ones that can be traced back to the speciation event, and will thus give a correct phylogenetic tree when used for evolutionary study. Paralogous genes are the ones that duplicate from one another in a species. This type of gene, as a result of duplication, when used in phylogenetic estimation, will give a gene tree rather than a species tree.

Molecular phylogenetics has twofold benefits. Firstly phylogenetic relationship or branching pattern is ascertained: monophyletic groups are defined and sister groups are identified. In addition, the relative timings of the speciation events that correspond to the lineage divergences are determined. Secondly, DNA-based phylogeny can then be compared to other traditional lines of evidence, e.g. anatomy and morphology (including data obtained by light microscope and electron microscope), palynology, chemistry, cytology and breeding system (Soltis and Soltis, 1995). It also has the power of predictability. Morphological data are commonly mapped on a molecular tree, therefore the evolutions of characters are studied. However, it should be noted that relationships are deduced on the basis of horizontal comparison (living organism) by using homology to refer to vertical (evolutionary) relationship.

Nonetheless, molecular data can occasionally be misleading depending on the history of the molecular data used (Doyle, 1992). Studies in *Gossypium* (Wendel *et al.*, 1995) and *Heuchera* (Soltis and Kuzoff, 1995) showed that cpDNA sequence-based phylogeny could not distinguish the true evolutionary interrelationships among the member species. This is because different sources of molecular data may have different histories, especially in cpDNA. Introgression or chloroplast capture is a factor among other biological phenomena, such as lineage sorting and mistaken orthology which may give rise to gene trees that are discordant with species trees (Doyle, 1992). Another source of error in molecular based phylogeny is known as long branch attraction (or Felsenstein Zone) (Felsenstein, 1978). It occurs when two or more species have disproportionately high rates of molecular evolution in respect to other species in the study. As a result, these two or more species are prone to group together due to their higher chance of nucleotide similarity (resulting from

convergent evolution). This might be considered to be only a problem of sampling that can be solved, in part, by sampling more taxa closely related to the problem taxa.

In plants, there are three different DNAs in a cell, namely nuclear DNA, chloroplast DNA and mitochondrial DNA. Their properties are shown in Table 1.1 (adapted from Judd *et al.*, 1999).

Table 1.1. The characteristics of plant genomes.

Source	Heredity	Genome Size (kbp)	Nature of Changes	Mutation Rate
Nuclear DNA (nrDNA)	Biparental	1.1×10^{6} to 1.1×10^{11}	Point mutation, Insertion & deletion	6X
Chloroplast DNA (cpDNA)	Uniparental (generally maternal)	135-160	Point mutation, Insertion & deletion	3X
Mitochondrial DNA (mtDNA)	Uniparental (generally maternal)	200-2500	Lots of relocation or shuffling of genes	X

Chloroplast DNA and nuclear DNA have been used as major sources of phylogenetic information since the early days of plant molecular phylogenetic study. A gene termed *rbc*L in the chloroplast DNA which encodes the large subunit of the most abundant protein in the world, ribulose-1, 5-bisphosphate carboxylase/oxygenase or Rubisco, has been extensively studied in the field, both for its restriction site variation and nucleotide substitution (Palmer *et al.*, 1988; Olmstead and Palmer, 1994). In nuclear DNA, ribosomal DNA that encodes for ribosomes, has played a significant role in plant phylogenetic study (Baldwin *et al.*, 1995; Soltis *et al.*, 1997). With the feasibility of direct DNA sequencing, *rbc*L gene sequences and ribosomal gene sequences have proved to be useful in plant phylogenetic estimates (Chase *et al.*, 1993; Soltis *et al.*, 1997). Unfortunately, plant mitochondrial DNA, unlike the other two kinds, is not suitable for plant phylogenetic reconstruction,

especially at lower level because of its frequent relocation or shuffling of member genes and slow rate of nucleotide substitution that are considered to be of limited value (Wolfe *et al.*, 1987; Palmer, 1992). However, there are some reports demonstrating that certain genes of plant mtDNA contain enough variation that when used in the species level phylogenetic estimation, give rather well resolved patterns (Duff and Nickrent, 1999; Bakker *et al.*, 2000).

Ideally both morphological and molecular data should be used in an evolutionary study because they have different rates of evolution which might yield insights into phylogeny at different hierarchical levels (Pennington, 1996). Whether to combine these two sources of data into one analysis or to analyse them separately for phylogenetic reconstruction is again subject to argument as to the optimal use of the data. Traditionally, molecular data are used to infer the group phylogeny first, then morphological data are mapped across the molecular tree. This practice has proved valuable for comparing taxa that are highly morphologically divergent, plesiomorphically simple or secondarily simplified by reduction (and hence have insufficient clearly homologous structures) and for elucidating cases of parallel evolution (Bateman, 1996). This practice is also considered, however, to be suboptimal in cases where there are good or discrete morphological data that can be used to infer a cladogram on their own (Bateman *et al.*, 1998). Seelanan *et al.* (1997) and Bateman (1999) have suggested similar ways of dealing with phylogenetic trees from different data. Originally they analyse each data set separately and, if there is no incongruency among the resultant trees topologically, then these data sets can be combined and analysed simultaneously to give 'total evidence' trees. If there is an incongruent clade, the data sets should not be combined and explanations must be sought for conflicting phylogenies. In the case of morphology alone, Bateman (1999) has suggested dividing soft or non-discrete states characters and hard or discrete states characters. Subsequently, only a set of hard characters is used in the phylogenetic analysis and the resultant trees are compared with trees from other sets of data. After reaching total evidence clades, soft characters are then mapped onto the trees.

1.4 THE PLANTS: ZINGIBERACEAE

Eighty-eight genera and about 2000 species (Kress, 1995), are accepted in the monophyletic order Zingiberales (Nakai, 1941; Tomlinson, 1962; Cronquist, 1981; Dahlgren *et al.*, 1985; Kress, 1990; Duvall *et al.*, 1993; Bremer *et al.*, 1998). They are grouped into eight families, namely Musaceae, Strelitziaceae, Lowiaceae, Heliconiaceae, Costaceae, Zingiberaceae, Cannaceae, and Marantaceae (see Table 1.2). Attempts have been made to work out the phylogenetic relationships of the families within the order (Tomlinson, 1962; Kress, 1990; Smith *et al.*, 1993; Kress, 1995) (see Figure 1.1). The most recent study of Kress (1995), based on molecular characters (*rbc*L) and 36 morphological characters, revealed that the clade of Marantaceae and Cannaceae is the sister clade of Zingiberaceae and Costaceae. The outgroup families were Haemodoraceae, Philydraceae, Pontederiaceae of Bromeliiflorae and Commelinaceae of Commeliniflorae (Dahlgren *et al.*, 1985), then all placed under Commelinales (Bremer *et al.*, 1998). The most recent studies using three molecular sources (18S rDNA, *rbc*L and *atp*B) (Chase *et al.*, 2000; Soltis *et al.*, 2000) confirmed that Zingiberales is monophyletic and with Commelinales as its sister group, though the relationships among the families are poorly resolved. Only Lowiaceae-Strelitziaceae is strongly supported. The family Zingiberaceae is also shown to be monophyletic with Alpinieae in basal position, then Globbeae, Hedychieae and Zingibereae, respectively.

There is no doubt that Costaceae is the sister family of Zingiberaceae. They used to be placed as a subfamily of the Zingiberaceae. Many characters are found to justify the family rank of both groups (Tomlinson, 1956). The Zingiberaceae are perennial herbs of the tropical forests, the greatest concentration of genera and species lying in Southeast Asia (Tomlinson, 1956; Dahlgren *et al.*, 1985). They are chiefly forest floor plants, growing in humus-rich shade or semi-shade habitats. All species have branched, fleshy rhizomes that may be above or under ground and many possess tuberous roots.

Figure 1.1. A 'rhizogram' of the Zingiberales (taken from Kress, 1990).

Zingiberaceae make up approximately half the total number of species in Zingiberales, 1000 species in 2000 and more than half the number of genera, 50 genera in 88 (Cronquist, 1981; Kress, 1995). The family has also been always considered a natural group (Kress, 1990; Kress, 1995) within the Zingiberales. The autapomorphic characters of the family are the fusion of the lateral staminodes of the inner staminal whorl into a labellum, the presence of two epigynous glands at the base of the style, and the occurrence of cells containing essential or ethereal oils (Kress, 1990). The latest classification divides the family into four tribes, namely Alpinieae, Globbeae, Hedychieae and Zingibereae (Burtt and Smith, 1972). The circumscriptions of the tribes according to one vegetative character and four floral characters (Smith, 1981) are tabulated (Newman, 1988) in Table 1.5 (see also Figure 1.2).

The name 'zingiber' probably originates from the Arabic word zanjabil and later the Sanskrit word singabera (meaning horn-root), which gave rise to the classical Greek name zingiberi and finally zingiber in Latin (Larsen *et al.*, 1999, p.1). Botanically, *Zingiber* is a genus name and gives the foundation to the family and

order names which the plant (*Zingiber officinale* Roscoe) belongs to.

People have used Zingiberaceae for various purposes, such as food, medicines and ornamentals. In daily cuisine, ginger (*Zingiber officinale* Roscoe or *khing* in Thai), turmeric (*Curcuma longa* L. or *khamin*) and galangal (*Alpinia galanga* (L.) Willd. or *kha*) are spices that are widely used in food. Another important species is *Elettaria cardamomum* (L.) Maton that gives cardamom (*krawan*). Many have beautiful showy inflorescences or flowers and are used as ornamentals. Among these are *Hedychium coronarium* Koenig (ginger lily), *Alpinia purpurata* (Vieill.) K. Schum., *Globba winitii* C.H. Wright and *Etlingera elatior* (Jack) K. Schum. Recently *Curcuma* species have been promoted as cut and pot flowers for export in Thailand, among them, *C. alismatifolia* Gagnep. (Siamese Tulip) and *C. roscoeana* Wall. *Roscoea* is probably the most well known genus in western horticulture. As the only genus that can stand the weather in summer outside, it is grown in many gardens. In spite of the many uses of gingers, we know little of the evolutionary relationships within the family and the genera.

Table 1.2. The families, genera, and species of Zingiberales showing geographical distribution (after Larsen *et al.*, 1998).

Family	Number of genera and species	Distribution
Musaceae Juss.	2 genera; 36-46 species *Musa* (30-40) and *Ensete* (6)	Old World Tropics and Subtropics
Heliconiaceae Nakai	1 genus (*Heliconia*); 200 species	Mainly New World Tropics
Lowiaceae Ridl.	1 genus (*Orchidantha*); 11 species	Southern China to Pacific Isles
Strelitziaceae Hutch.	3 genera; 6-7 species *Phenakospermum* (1) *Ravenala* (1) *Strelitzia* (4-5)	 Trop. S. America Madagascar Southern Africa
Marantaceae Petersen	31 genera; 450 species	Pantropical
Cannaceae Juss.	1 genus (*Canna*); 10-25 species	Tropical & subtropical Americas
Costaceae Nakai	4 genera; 108-113 species *Costus* (90) *Tapeinochilus* (15-20) *Dimerocostus* (2) & *Monocostus* (1)	 Pantropical, mainly in Americas S.E. Asia Tropical Americas
Zingiberaceae Lindl.	50 genera; 1300 species	Mainly Indo-Malayan; 3 endemic genera in Africa and Madagascar: *Aframomum* (50), *Aulotandra* (5), *Siphonochilus* (15), and *Renealmia* (75) mainly trop. S. America

Table 1.3. The systems of classification of the Zingiberales (modified from Kress, 1990).

Bentham and Hooker (1883) *Genera plantarum*	Petersen (Engler and Prantl edn. 1, 1889) *Nat. Pflanzenfamilien*	Schumann (Engler 1900, 1902, 1904)[1] *Pflanzenreich*	Hutchinson (1934, 1959) *Fam. Fl. Plants*	Nakai (1941)[2]
Family: Scitamineae	Order: Scitamineae	Order: Scitamineae	Order: Scitamineae (later Zingiberales)	Order: Zingiberales
Tribes: Museae (*Musa*, *Ravenala*, *Strelitzia*, *Heliconia*)	Families: Musaceae Tribes: Museae (*Musa*, *Ravenala*, *Strelitzia*) Heliconieae (*Heliconia*)	Families: Musaceae Subfamilies: Musoideae (*Musa*) Strelitzioideae Tribes: Strelitzieae (*Strelitzia*, *Ravenala*) Heliconieae (*Heliconia*) Lowioideae (*Orchidantha*)	Families: Musaceae (*Musa*) Strelitziaceae (*Strelitzia*, *Ravenala*, *Phenakospermum*, *Heliconia*) Lowiaceae (*Orchidantha*)	Families: Musaceae (*Musa*, *Ensete*) Strelitziaceae (*Strelitzia*, *Ravenala*, *Phenakospermum*) Heliconiaceae (*Heliconia*) Lowiaceae (*Orchidantha*)
Zingibereae	Zingiberaceae	Zingiberaceae Subfamilies: Zingiberoideae Tribes: Zingibereae Hedychieae Globbeae Costoideae Costeae	Zingiberaceae Tribes: Zingibereae Hedychieae Globbeae Costeae	Zingiberaceae Costaceae (*Costus*, *Tapeinochilus*, *Dimerocostus* *Monocostus*)
Maranteae	Marantaceae	Marantaceae	Marantaceae	Marantaceae
Canneae (*Canna*)	Cannaceae (*Canna*)	Cannaceae (*Canna*)	Cannaceae (*Canna*)	Cannaceae (*Canna*)

[1] Also included Loesener and Winkler (in Engler and Prantl, edn. 2, 1930).
[2] Also included Tomlinson (1962), Cronquist (1981), Dahlgren *et al.* (1985), Thorne (1992)

Table 1.4. The first 200 years of Zingiberaceae systematics.

Author	Year	Significant contributions
Linnaeus, C. (1707-1778)	1753	Five genera were recognised in Monandria Monogynia, **Species Plantarum**: *Amomum* (now, *Zingiber officinale*; *Z. zerumbet*; *Elettaria cardamomum*; *Aframomum* spp.) *Alpinia* (*Renealmia racemosa*), *Curcuma* (*C. longa*; *Boesenbergia rotunda*), *Kaempferia* (*K. galanga*; *K. rotunda*), *Costus* (*C. arabicus*)
König, J.G. (1728-1785)	1783	The first good botanical descriptions were made from living plants in Retzius's ***Observationes Botanicae*** (3:45-75). (Linnaeus's pupil who visited Thailand)
Retzius, A.J. (1742-1821)	1791	Koenig's notes and Retzius's own studies were further published in ***Observationes Botanicae*** (6:17-18).
Willdenow, C.L. (1765-1812)	1797	Some improvement on Retzius's classification was made in **Species Plantarum**.
Roscoe, W. (1753-1831)	1807	True Scitaminean plants (mainly members of present day defined Zingiberaceae) were separated from Jussieu's Cannae using the anther character *in* ***Transactions of the Linnean Society of London*** (8:330-357). Colour plates of Scitaminean plants were published during 1824-1829 (Monandria Plants of the order Scitaminae).
Roxburgh, W. (1751-1815)	1812	The plants may be separated into two groups, apart from by using the charater of the anther: (i) truly herbaceous (*Curcuma*, *Kaempferia*, *Zingiber* and *Globba*), and (ii) less herbaceous (*Canna*, *Phrynium*, *Amomum* and *Alpinia*), in Monandrous Plants of India, ***Asiatic Researches*** (11:318-362). The work was republished posthumously with additional taxa in Flora Indica (three editions, 1820, 1832 and 1874).
Blume, C.L. (1796-1862)	1827	The family was subdivided into natural groups that formed the basis of Meisner's tribes in **Enumeratio Plantarum Javae**.
Wallich, N. (1786-1854)	1829-1832	Excellent plates of several species were published in **Plantae Asiaticae Rariores**. Wallich's catalogue (A numerical list of dried specimens) was published in 1828.
Meisner C.D.F. (Meissner, C.D.F.) (1800-1874)	1842	A subdivision was proposed for the family in **Plantarum Vascularium Genera**. *Two of the four presently accepted tribes dated back from this subdivision (*Globbeae, *Zingibereae, Amomeae, Alpinieae and Costeae).

Horaninow, P.F. (1796-1865)	1862	The first monograph of the family was presented in **Prodomus Monographiae Scitaminearum.**
Baker, J.G. (1834-1920)	1890-1892	Zingiberaceae plants of India (including those known from the Malay Peninsula) were complied in Hooker's **Flora of British India** (6:198-264).
Petersen, O.G. (1847-1937)	1899	The monograph of the family was presented in Engler and Prantl's **Die Natürlichen Pflanzenfamilien** (edn.1).
Ridley, H.N. (1855-1956)	1899	Ridley's first account of the Scitamineae of the Malay Peninsula was published in ***Journal of the Straits Branch of the Royal Asiatic Society*** (32:85-184). He also published the family account in the Flora of the Malay Peninsula (1924,4:233-285).
Schumann, K.M. (1851-1904)	1904	A monograph of the family was published in Engler's **Das Pflanzenreich** (4:part 46).
Valeton, T.H. (1855-1929)	1904	Thorough study of the family in Java was first published in ***Bulletin de L' Institut Botanique de Buitenzorg*** (20). The work was continued through 1913, 1914 and 1918 (Bulletin du Jardin Botanique de Buitenzorg, 1918, 26).
Gagnepain, F. (1866-1952)	1908	Descriptions of new species, mainly from Indo-China, and an account of the family were written in **Flore Generale de L' Indo-Chine** (6:25-121) (H. Lecomte, ed.).
Loesener, L.E.T. (1865-1941)	1930	An account of Zingiberaceae was published following the work of Valeton in Engler and Prantl's **Die Natürlichen Pflanzenfamilien** (edn.2, part 15a).
Holttum, R.E. (1895-1990)	1950	The Zingiberaceae of the Malay Peninsula was publisehd in ***the Garden's Bulletin Singapore*** (13:part 1).

Table 1.5. The classification of tribes in Zingiberaceae (Burtt and Smith, 1972).

Tribe	**Lateral staminodes**	**Ovary**	**Filament**	**Anther crest**	**Plane of distichy of leaves compared with direction of growth of rhizome**
Zingibereae	Petaloid, fused with labellum	Trilocular. Placentation axile	Non-exserted	Wrapped around the style above the anther	Parallel
Globbeae	Petaloid, free from labellum	Unilocular. Placentation basal-axile or parietal	Exserted. Bow-like with style as bow-string	Lateral on the anther or absent	Parallel
Hedychieae	Petaloid, free from labellum	Trilocular. Placentation axile	Non-exserted or, if exserted, not bow-like	When present not wrapped around the style above the anther	Parallel
Alpinieae	Small, linear or tooth-like or absent	Trilocular. Placentation axile	Variable	When present, not wrapped around the style above the anther	Transverse

Figure 1.2. Floral parts of the four tribes in Zingiberaceae (after Smith, 1981).

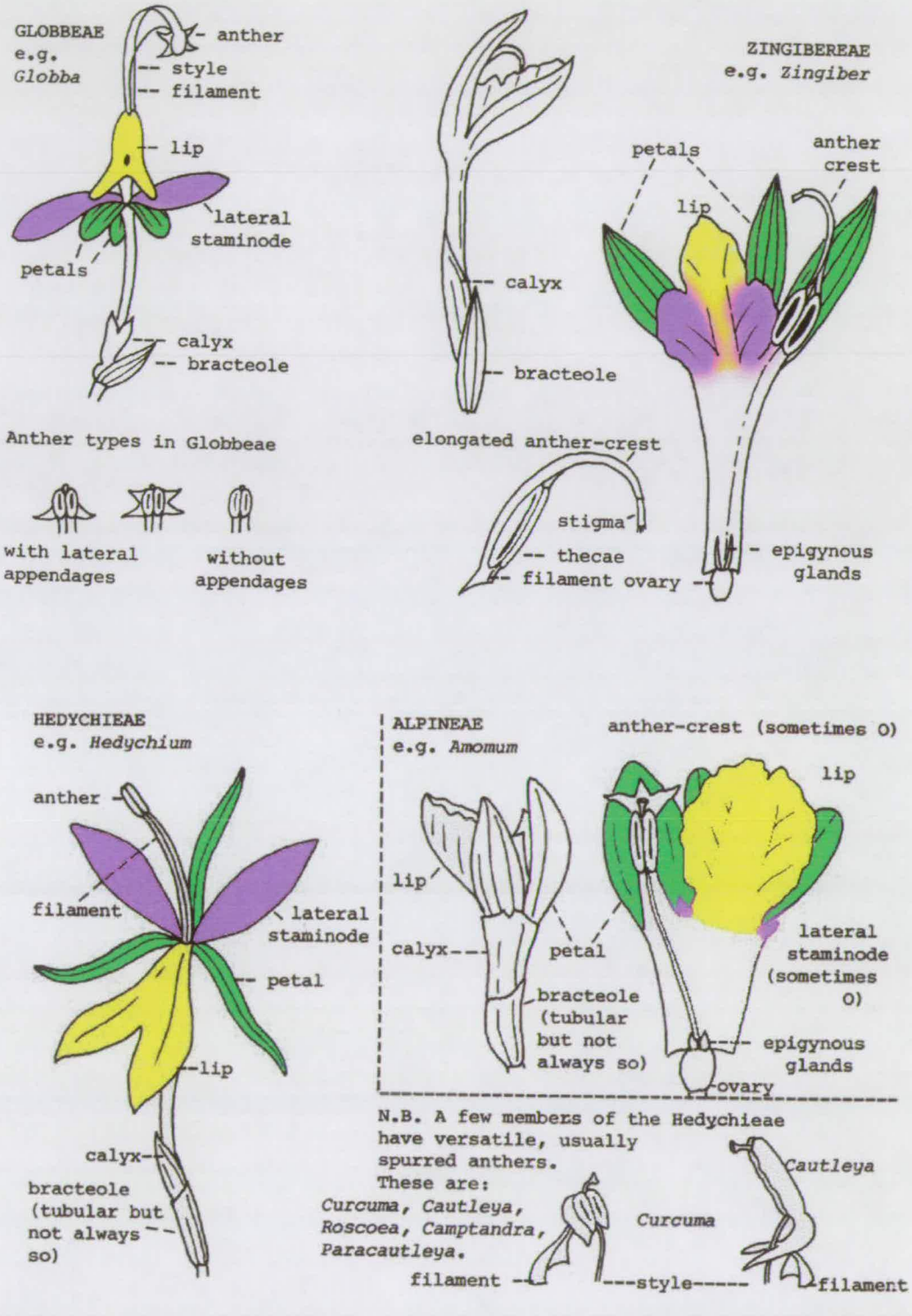


Table 1.6. Genera and numbers of species in the tribes of Zingiberaceae (after Larsen *et al.*, 1998).

Alpinieae A. Rich. (24 genera, ~800 spp.)	**Globbeae** Meisn. (4 genera, ~110 spp.)	**Hedychieae** Horan. (21 genera, ~303 spp.)	**Zingibereae** Meisn. (1 genus)
Aframomum K. Schum. (50)	*Gagnepainia* K. Schum. (3)	*Boesenbergia* Kuntze (60)	*Zingiber* Boehm. (100)
Alpinia Roxb. (227)	*Globba* L. (100)	*Camptandra* Ridl. (4)	
Amomum Roxb. (150)	*Hemiorchis* Kurz (3)	*Caulokaempferia* K. Larsen (10)	
Aulotandra Gagnep. (5)	*Mantisia* Sims (4)	*Cautleya* (Benth.) Hook. f. (2)	
Burbidgea Hook.f. (8)		*Cornukaempferia* J. Mood & K. Larsen	
Cyphostigma Benth. (1)		*Curcuma* L. (50)	
Elettaria Maton (7)		*Distichochlamys* M. F. Newman (1)	
Elettariopsis Baker (10)		*Haniffia* Holttum (2)	
Etlingera Giseke (70)		*Haplochorema* K. Schum. (3-4)	
Geocharis (K. Schum.) Ridl. (7)		*Hedychium* J. König (50)	
Geostachys (Baker) Ridl. (18)		*Hitchenia* Wall. (3)	
Hornstedtia Retz. (50)		*Kaempferia* L. (40)	
Leptosolena C. Presl (1)		*Paracautleya* R. M. Sm. (1)	
Nanochilus K. Schum. (1)		*Parakaempferia* A. S. Rao & D. M. Verma (1)	
Plagiostachys Ridl. (20)		*Pyrgophyllum* (Gagnep.) T. L. Wu & Z. Y. Chen (1)	
Pleuranthodium (K. Schum.) R.M.Sm. (25)		*Roscoea* Sm. (19)	
Pommereschea[A] Wittm. (2)		*Scaphochlamys* Baker (30)	
Renealmia L.f. (75)		*Siliquamomum*[C] Baill. (1)	
Rhynchanthus[A] Hook. f. (6)		*Siphonochilus*[D] J. M. Wood & Franks (15)	
Riedelia Oliv. (60)		*Smithatris*[E] W.J. Kress & K. Larsen (1)	
Siamanthus K. Larsen & J. Mood (1)		*Stahlianthus* Kuntze (6)	
Stadiochilus[B] R. M. Sm. (1)			
Tamijia S. Sakai & Nagam. (1)			
Vanoverbergia Merr. (1)			

[A] From new molecular cladistic analyses (Wood *et al.*, 2000; Kress, pers. comm.), it is placed within Hedychieae. *Pommereschea* and *Rhynchanthus* form a clade among other members of Hedychieae.
[B] Status is uncertain but *Stadiochilus* has an affinity with *Rhynchanthus*. In addition, there is a character, a groove bearing the filament in corolla tube, in common with many *Hedychium* spp. which is not found in any member of Alpnineae (Smith, 1980).
[C] From the molecular cladistic analysis (Kress, pers. comm.), it is placed within Alpinieae.
[D] From the molecular cladistic analysis, it is a sister taxon to all the rest of Zingiberaceae (Wilf *et al.*, 2000).
[E] It has not been published yet (Kress & Larsen, pers. comm.).

1.4.1 THE AUTHORITIES OF THE TRIBAL NAMES

Current classifications accept four tribes in Zingiberaceae namely Alpinieae A. Rich., Globbeae Meisn., Hedychieae Horan. and Zingibereae Meisn. (Burtt & Smith 1972; Smith, 1981; Dahlgren *et al.*, 1985; Larsen *et al.*, 1998). The delimitations of the tribes are now largely clear, though there are doubts about the placement of some genera in the classification (Smith, 1981; Larsen *et al.*, 1998). The following paragraph is a brief history of the correct authorities for the tribal names. Presently accepted tribal names are in bold.

The first person who divided the family into groups was C.L. Blume. Blume (1827) subdivided the family into five subdivisions or sections: Zingibera, Amomae, Alpiniae, Costae and Globbae. Unfortunately his ranks are not valid according to Article 4.1 of the International Code of Botanical Nomenclature (Greuter *et al.*, 2000). The first person who validly established the first ever tribe in Zingiberaceae: **Alpinieae** is A. Richard (1841). Then in the following year, C.D.F. Meisner (1842) published his subdivision of the family into five tribes: Alpinieae, Amomeae, **Globbeae**, **Zingibereae** and Costeae. Meisner's Amomeae was sunk into synonymy under Alpinieae (Burtt, 1972) while Costeae was raised to family rank (Nakai, 1941). Then in 1862, P.F. Horaninow established **Hedychieae**, Amomeae, Alpinieae and Costeae for Amomaceae, a synonym of Zingiberaceae. The seminal work of Schumann (1904) which has been a basis for later workers, however, used only three tribal names in his subfamily Zingiberoideae namely Hedychieae, Globbeae and Zingibereae (including the genera of Alpinieae). Schumann attributed all the tribes to Petersen who had written a monograph of the family (Petersen, 1899). Loesener (1930) followed the use of the three tribes and the authorities. In 1950, R.E. Holttum correctly pointed out that *Zingiber* is more closely related to Hedychieae than to *Amomum* and *Alpinia* (Alpinieae). He transferred *Zingiber* to Hedychieae. Zingibereae was taken up again at tribal rank (Burtt and Olatunji, 1972) after Holttum (1950) failed to rename his Hedychieae that included *Zingiber*, the type of the family, as Zingibereae in accordance with Article 19.4 of the Code.

1.4.2 BIOGEOGRAPHY

Biogeographical study of the Zingiberales has been advanced by findings of new fossilised remains. It has been found that Zingiberales already existed in the Late Cretaceous, about 83 million years before present (Friis, 1988; Herendeen and Crane, 1995; Bremer, 2000). Though *Spirematospermum* of the Santonian/Campanian of North America and the European Tertiary was thought at first to belong to the family Zingiberaceae, a new study (Rodriguez de la Rosa and Cevallos Ferriz, 1994) has suggested that it is better referred to the Musaceae. The very first fossils of Zingiberaceae have been found in Late Cretaceous to Early Eocene sediments of Western Interior North America (Hickey and Peterson, 1978). Three species of fossilised leaves of *Zingiberopsis* were calculated to date from c. 70 million years BP (Herendeen and Crane, 1995) and are morphologically similar to the extant genus *Alpinia*. The pattern of venation in these three species of *Zingiberopsis* also shows a clear trend toward loss of the wider parallel vein subsets over the approximately 20-million-year range of the genus (Hickey and Peterson, 1978). The fossil record is scant in the family, however, and cannot provide new or independent information because it needs to be typed with modern taxa.

The present distribution of Zingiberaceae can also be a guide to reconstructing its evolutionary history in relation and addition to the geological history of the earth (see Table 1.7). Out of 50 genera described so far in the family (Table 1.6), at least 21 genera belong to the tribe Hedychieae (Mood and Larsen, 1997; Larsen *et al.*, 1998; Larsen and Mood, 1998). The majority of these genera occur in continental Southeast Asia, i.e. Burma, Thailand, Laos, Cambodia, Vietnam and Peninsular Malaysia (see Table 1.7). Thailand in the centre of this area possesses the greatest number of genera in Zingiberaceae. The area is in fact a meeting point for elements concentrated in the west and those confined to the east (Ashton, 1990). In addition to the molecular phylogeny of the genera in Hedychieae, and the phylogeny of *Roscoea*, present distribution patterns of the members should provide further evidence to interpreting the evolutionary patterns of the groups.

Table 1.7 Distribution of Zingiberaceae in Asia.

(Colour scheme: green = Tribe Alpineae, pink = Tribe Globbeae, blue = Tribe Zingibereae; please see also Table 1.6 and Table 7.1).

Country/area	Number of genera	Number of species	The five largest genera	Source
Pakistan	3	4	*Curcuma* (2, cult.) *Roscoea* (1) *Zingiber* (1, cult.)	(Ghazanfar and Smith, 1982)
India	20 (2 monotypic genera: *Paracautleya*, SW and *Parakaempferia*, NE)	176	*Hedychium* (39) *Curcuma* (28) *Zingiber* (18) *Globba* (18) *Amomum* (16)	(Karthikeyan *et al.*, 1989; Jain and Prakash, 1995; Srivastava, 1998)
Sri Lanka	11 (1 monotypic genus: *Cyphostigma*)	34	*Amomum* (10) *Alpinia* (7) *Curcuma* (5) *Zingiber* (5) *Hedychium* (3)	(Burtt and Smith, 1983)
Nepal	11	36	*Hedychium* (12) *Roscoea* (6) *Amomum* (3) *Globba* (3) *Zingiber* (3)	(Press *et al.*, 2000)
Bangladesh	13	46	*Hedychium* (9) *Curcuma* (8) *Globba* (7) *Zingiber* (6) *Alpinia* (5) *Amomum* (5)	(Rahman and Yosaf, 1996; Rahman and Yosuf, 1997)

Bhutan	14	47	*Hedychium* (13) *Zingiber* (7) *Globba* (5) *Roscoea* (4) *Amomum* (4)	(Smith, 1994)
China	20 (2 monotypic genera: *Pyrgophyllum*, SW and *Siliquamomum*, S)	209	*Alpinia* (51) *Zingiber* (42) *Amomum* (39) *Hedychium* (28) *Roscoea* (13) *Curcuma* (12)	(Wu and Larsen, 2000)
Cambodia, Laos and Vietnam	At least 13 (2 monotypic genera: *Distichochlamys*, C Vietnam and *Siliquamomum*, N Vietnam)	150	*Globba* (22) *Amomum* (19) *Alpinia* (17) *Curcuma* (17) *Kaempferia* (13) *Zingiber* (13)	(Gagnepain, 1908; Newman, 1995; Larsen, 1996b)
Burma	21 (1 monotypic genus: *Stadiochilus*)	151	*Globba* (23) *Curcuma* (21) *Kaempferia* (17) *Zingiber* (16) *Alpinia* (14) *Amomum* (14)	(Smith, 1980; Kress, 2000)
Thailand	23 (2 monotypic genera: *Siamanthus*, S and *Smithatris*, C)	200	*Curcuma* (50) *Globba* (34) *Zingiber* (26) *Hedychium* (25) *Boesenbergia* (15) *Kaempferia* (15)	(Larsen, 1996a; Larsen and Mood, 1998; Sirirugsa, 1998; Theilade, 1999)
Malesian region	26	700	*Alpinia* (180) *Amomum* (100) *Etlingera* (50-60)	(Larsen, 1996b; Larsen, 1998)

			Riedelia (55) *Zingiber* (50) *Boesenbergia* (50)	
Peninsular Malaysia and Singapore	18	171	*Alpinia* (24) *Amomum* (19) *Scaphochlamys* (19) *Zingiber* (19) *Globba* (15)	(Larsen *et al.*, 1999)
Mount Kinabalu	11	56	*Etlingera* (11) *Alpinia* (10) *Amomum* (10) *Globba* (5) *Plagiostachys* (5)	(Beaman *et al.*, 1998)
Brunei	13	106	*Amomum* (22) *Boesenbergia* (18) *Alpinia* (10) *Etlingera* (9) *Plagiostachys* (9)	(Cowley, 2001)
Indonesia	18 (1 monotypic genus: *Nanochilus*, Sumatra)	366	*Alpinia* (92) *Amomum* (66) *Riedelia* (62) *Globba* (31) *Etlingera* (30) *Zingiber* (30)	(Riswan and Setyowati, 1996; Larsen *et al.*, 1998;)
Philippines	12 (2 monotypic genera: *Leptosolena* and *Vanoverberghia*)	99	*Alpinia* (40) *Amomum* (19) *Globba* (12) *Zingiber* (10) *Plagiostachys* (6)	(Madulid, 1996)
Australia	9	18	*Alpinia* (6) *Curcuma* (2)	(Smith, 1987)

			Hedychium (2) *Zingiber* (2) *Amomum* (2)	
Fiji Islands	6	17	*Alpinia* (9, 5 end.) *Hedychium* (2) *Zingiber* (2) *Etlingera* (2, 1 end.) *Curcuma* (1) *Elettaria* (1)	(Smith, 1979)

1.5 AIMS AND CHOICES OF PHYLOGENETIC INFORMATION

1.5.1 THE HEDYCHIEAE STUDY

This study is initiated to study evolutionary relationships in one of the four accepted tribes, Hedychieae in the family Zingiberaceae. The tribe has twenty-one genera described to date (see Table 1.6). To study the evolutionary or phylogenetic relationships among the genera of Hedychieae, two sources of molecular information are sampled. First, the internal transcribed spacers of ribosomal DNA (ITS) which are part of nuclear DNA were chosen (Baldwin, 1992; Rangsiruji, 1999). The second source of phylogenetic information comes from a region of circular-chloroplast DNA encoded *trn*L (UAA) 5′ exon – *trn*F (GAA) exon (referred to hereafter as *trn*L-F region) (Taberlet *et al.*, 1991; Gielly and Taberlet, 1994).

The ITS region is nested in the ribosomal DNA (rDNA). Ribosomal DNA is a set of many repetitive multicopies DNA sequences found in the nuclear genome that encodes for the synthesis of ribosomal RNA (rRNA). It is the best known example of a repetitive gene region that has undergone a process called concerted evolution (Zimmer *et al.*, 1980). The process homogenises all the mutations within

the multiple copies of the gene in a single individual and species. Thus it appears that all the copies of the gene evolve as if a single unit and are able to outstrip mutations that lead to speciation. The process is still inadequately understood but is thought to have occurred through mechanisms of molecular drive, for instance, biased gene conversion and unequal crossing over, among others (Li, 1997, p. 309-334). As a result of concerted evolution, ribosomal DNA possesses many advantages for DNA sequencing. For instance, it is easily detected because of the very high numbers of copies in the genome and the problem of homology in the comparison of the sequences from different species is eliminated. A representative sequence of an individual of a species can be safely used as such.

The ITS region is subdivided into the ITS1 region (<300 bp), which separates the 18S and 5.8S rDNA genes, and the ITS2 region (<300 bp), which is found between the 5.8S and 26S rDNA genes (Figure 1.3). The attributes of the ITS regions that simplify their PCR amplification, sequencing alignment and phylogenetic analysis are: small size, highly conserved flanks, high copy number, rapid concerted evolution and length conservation of angiosperm ITS sequences (Baldwin *et al.*, 1995). The most widely used regions of the ribosomal DNA, as sources of phylogenetic information at specific and generic level, are the internal transcribed spacers (ITS1 and ITS2) of the gene (Baldwin, 1992; Baldwin *et al.*, 1995; Downie and KatzDownie, 1996; Möller and Cronk, 1997a). Simultaneously, the 18S region has been used at familial level and above (Soltis *et al.*, 1997).

The disadvantage in using ITS sequences is that the small number of characters from these short spacers provides limited data for phylogenetic studies in angiosperms. Four-taxon simulations by Huelsenbeck & Hillis (1993) suggest that sequences of such short length are, under most conditions and types of analysis, less effective for accurate tree reconstruction than longer sequences. Useful variation must be more highly concentrated within a set of ITS1 and ITS2 sequences than in longer DNA regions in order to achieve the same level of phylogenetic resolution and support. Further constraints on the number of useful ITS characters can be imposed by the need to delete small indel regions from phylogenetic analysis

because of uncertain sequence alignment. Therefore, it is essential that data from other sources (*e.g.* chloroplast DNA) are used in conjunction or combined with ITS evidence to obtain enough characters for well-supported phylogenetic resolution (Kluge, 1989; Barrett *et al.*, 1991; Donoghue and Sanderson, 1992).

The noncoding *trn*L-F is then chosen in addition to the use of the ITS regions (Figure 1.4). The *trn*L-F region is found in a large single copy of chloroplast DNA (Hiratsuka *et al.*, 1989; Maier *et al.*, 1995). The region includes the 5′ *trn*L exon, the *trn*L intron, the 3′ *trn*L exon, the intergenic spacer and the *trn*F exon regions. The cpDNA *trn*L-F region has been used widely as a source of phylogenetic markers (Gielly *et al.*, 1996; Sang *et al.*, 1997; Kajita *et al.*, 1998; Bakker *et al.*, 1999). The region proves to be useful in reconstructing phylogeny at specific and generic level in these studies. Nonetheless, it was reported that the region in *Alpinia* spp. (Rangsiruji *et al.*, 2000) was about four times less variable than that of the ITS region of the genus, and thus yielded less resolved phylogenetic trees. It is then expected that the region may be more suitable at higher level i.e. generic level and above for reconstructing phylogeny in Zingiberaceae which is the aim of this study of the Hedychieae. In addition to phylogenetic studies, a study of versatile anther development using scanning electron microscope (SEM) in a group of five genera (*Camptandra*, *Cautleya*, *Curcuma*, *Paracautleya* and *Roscoea*) of the Hedychieae is also carried out to further test the monophyly of the group.

1.5.2 THE *ROSCOEA* STUDY

The study then takes a closer look into the phylogenetic relationships of one of the genera in Hedychieae, *Roscoea*, a peculiar genus in Zingiberaceae. Unlike most members of the family, *Roscoea* is mainly found in the north subtropical zone along the Himalaya, from Kashmir in the west to the east of Burma, and Southwest China (Cowley, 1982). This phenomenon prompts a question of how this originally tropical family has established a new home in a temperate region. In order to study the phylogenetic relationships and the biogeography of the genus, this part of the

study is not only based on cladistic analysis of molecular data (ITS sequences), but also incorporates the distribution pattern of all 19 species in the genus (Cowley, 1982; Cowley and Baker, 1996; Ngamriabsakul and Newman, 2000). A morphological cladistic analysis is also performed to find any congruence or discrepancy between the resultant evolutionary patterns from molecular and morphological analyses. Furthermore, five species of *Roscoea* and *Cautleya* are studied cytologically.

Figure 1.3. Repeat units of the nuclear ribosomal DNA and the organisation of the internal transcribed spaces (ITS) (adapted from Möller, pers. comm.). Arrows indicate orientation and approximate position of primer sites. Primer names and sequences are those of Möller & Cronk (1997a) and Rangsiruji (1999).

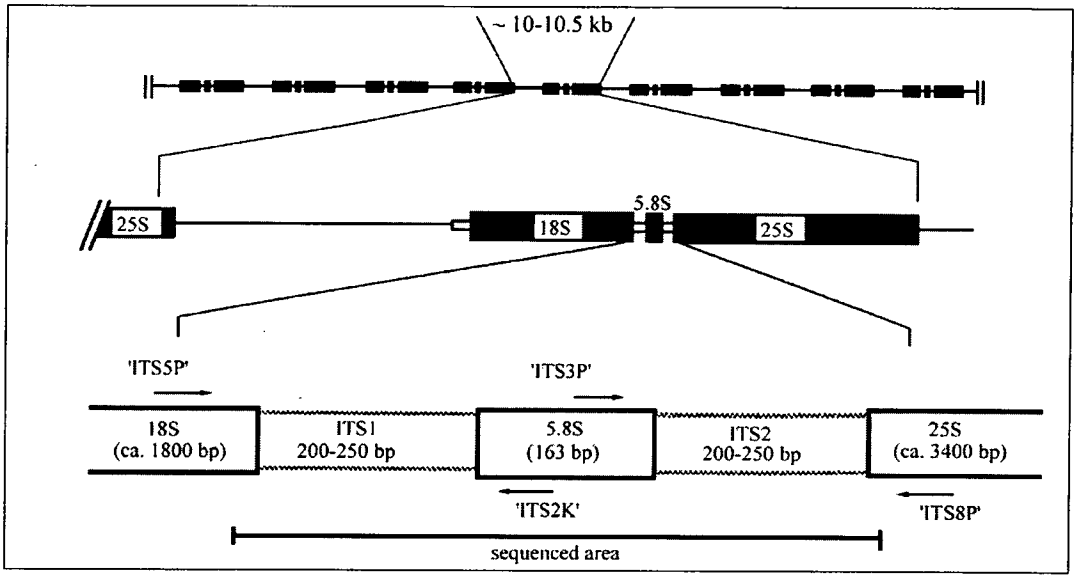
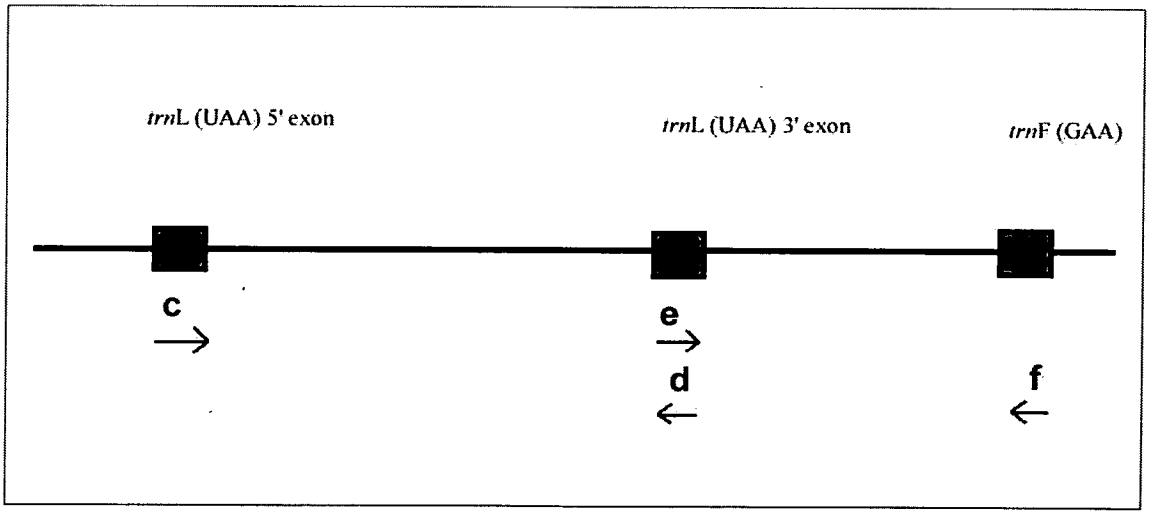


Figure 1.4. Approximate positions and directions of the primers used to amplify *trn*L-F region (Taberlet *et al.*, 1991).



CHAPTER TWO: PHYLOGENY OF THE HEDYCHIEAE BASED ON ITS (nrDNA) AND *trn*L-F (cpDNA)

2.1 ABSTRACT

A phylogenetic analysis of the tribe Hedychieae is performed using nuclear ribosomal DNA (ITS1, 5.8S and ITS2) and chloroplast DNA (*trn*L (UAA) 5′ exon to *trn*F (GAA)). The results of these two phylogenetic sources are in accordance, though with differing levels of resolution. Morphology, chromosome numbers and distribution ranges are discussed in the light of the molecular findings. Hedychieae is confirmed to include Zingibereae, the true gingers, and is monophyletic. However, the genera *Boesenbergia* and *Curcuma* are apparently not monophyletic. Two major subclades are recognised in Hedychieae, namely the '*Curcuma* clade' and the '*Hedychium* clade'.

The '*Curcuma* clade' comprises *Camptandra*, *Pyrgophyllum*, *Stahlianthus* and a set of four morphologically very similar genera: *Curcuma*, *Hitchenia*, *Paracautleya* and *Smithatris*. In this clade, a subclade of *Camptandra/Pyrgophyllum* is the sister group to a very strongly supported '*Curcuma* complex': *Curcuma*, *Hitchenia*, *Paracautleya*, *Smithatris* and *Stahlianthus*. *Smithatris* may be recognised as a distinct genus and sister group to the others in the complex. *Curcuma* is paraphyletic. Two subclades are found in the complex, namely *Stahlianthus /Curcuma* subgenus *Hitcheniopsis*, and *Hitchenia/Paracautleya/Curcuma* subgenus *Curcuma*. The dorsifixed versatile anther of the *Curcuma* complex has been lost independently in *Hitchenia* and *Stahlianthus*, while the basifixed versatile anther has arisen independently in *Camptandra* and *Cautleya/Roscoea*.

Within the '*Hedychium* clade', I recognise two main subclades: a clade of *Hedychium/Pommereschea/Rhynchanthus/Cautleya/Roscoea*, and a '*Boesenbergia* group' that has *Boesenbergia*, *Caulokaempferia*, *Cornukaempferia*, *Distichochlamys*,

Haniffia, *Kaempferia*, *Scaphochlamys* and *Zingiber*. *Pommereschea* and *Rhynchanthus* have been traditionally placed in the tribe Alpinieae, but the lack of petaloid staminodes in these two genera can be seen as a derived character loss. *Haniffia* is the sister group to the remaining genera of the '*Boesenbergia* group'. *Boesenbergia* is paraphyletic in respect to *Caulokaempferia*. *Zingiber* is sister to *Cornukaempferia* and the large, narrow and curved anther crest found in these two genera is a morphological character also suggesting their close relationship.

Under low stringency conditions, two bands of the *trn*L-*trn*F PCR product using either a set of primer 'c' and 'f', 'c' and 'd' or 'e' and 'f' are encountered in some species. The PCR amplification of the region needs stricter conditions than the ITS amplification of the same species, including a well-calibrated thermocycler. All the products used for sequencing in this study are obtained as a single band.

2.2 INTRODUCTION

Hedychieae, as the second largest tribe following Alpinieae, has twenty-one genera described to date (see Table 1.6). Almost all the genera in the tribe are confined to South and Southeast Asia. The only exception is *Siphonochilus* whose distribution lies in Africa and Madagascar. Two recent, preliminary molecular cladistic analyses based on the ITS (nuclear ribosomal DNA) (Searle and Hedderson, 2000, for Hedychieae) and with *mat*K (chloroplast DNA) (Kress, pers. comm., for the family) suggest that *Siphonochilus* is not a member of Hedychieae, but rather a taxon in Alpinieae as is *Siliquamomum*. On morphological grounds, the Hedychieae appears to form a monophyletic group. The synapomorphies of the tribe are free petaloid staminodes, trilocular ovary (infrequently incompletely trilocular or unilocular) with axile placentation and the plane of distichy of the leaves parallel with the direction of growth of the rhizome.

The internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA have proven to be useful in phylogenetic studies in many plant families, e.g.

Asteraceae (Baldwin, 1992), *Apiaceae* (Downie and KatzDownie, 1996), *Gesneriaceae* (Möller and Cronk, 1997a), and *Araliaceae* (Wen *et al.*, 1998). These regions have rates of substitution that are useful for evaluating generic and specific level relationships in plants (Baldwin *et al.*, 1995, see review). There has been an increasing recent interest in reconstructing the phylogeny of Zingiberaceae, mostly by using the ITS region. Searle and Hedderson (2000) reported for the first time the phylogeny within the tribe Hedychieae based on ITS sequences. Their study concentrated on the genera of the '*Kaempferia* group', namely *Kaempferia*, *Boesenbergia*, *Haplochorema*, *Distichochlamys* and *Scaphochlamys*. Another study by Rangsiruji (2000b), investigated the phylogeny of *Alpinia*, the largest genus (about 227 species) in the family, using the ITS region and *trn*L-F spacer of chloroplast DNA. Two other detailed phylogenetic studies of specific relationships in *Roscoea* (Ngamriabsakul *et al.*, 2000) and *Hedychium* (Wood *et al.*, 2000) have been published. These studies have shown the suitability of ITS for the phylogenetic study of ginger plants. In addition to the use of ITS sequences, a region of *trn*L (UAA) 5′ exon to *trn*F (GAA) in the chloroplast genome is also used (Taberlet *et al.*, 1991). The *trn*L-F region can be divided into two subregions, namely *trn*L intron and *trn*L-F spacer. The cpDNA *trn*L-F region has been used widely as a source of phylogenetic markers (Gielly *et al.*, 1996; Sang *et al.*, 1997; Kajita *et al.*, 1998; Bakker *et al.*, 1999). It proves to be useful in reconstructing phylogeny at specific and generic level in these studies. Nonetheless, it was reported that the *trn*L-F spacer in *Alpinia* species was about four times less variable than that of the ITS region (Rangsiruji *et al.*, 2000a), and thus yielded less resolved phylogenetic trees. It is then expected that the region may be more suitable at higher level, i.e. generic level and above for reconstructing phylogeny in Zingiberaceae which is the aim of this study for the Hedychieae.

By ascertaining the phylogeny of the Hedychieae, endless exciting new interpretations of the morphological evolution of the group become possible. For instance, among the twenty-one genera of the tribe, there is a group of five genera, i.e. *Camptandra*, *Cautleya*, *Curcuma*, *Paracautleya* and *Roscoea*, that possesses a versatile anther, an unusual character in the family. The result of this study could

suggest whether this character was derived only once or several times during the evolutionary history of the plants. In addition, the findings of this study may also help in understanding morphological changes in the family as a whole. Although recent morphological studies have noted that the current subdivision of the family is inadequate (Smith, 1980; Larsen and Mood, 1998; Sakai and Nagamasu, 2000), the present study is focused on the tribe Hedychieae as presently circumscribed (Smith, 1981, see Table 1.5). I intend to make a more thorough and balanced sampling of the members of the Hedychieae for this study than the previous studies reported.

2.3 MATERIALS AND METHODS

2.3.1 PLANT MATERIAL

Many of the plant samples were taken from the research glass house of the Royal Botanic Garden Edinburgh (RBGE). Another important source of plant samples was my fieldtrip in Thailand during the months of July and August 1999 (C.N. and M.F.N.). Zingiberaceae researchers also helped me to obtain some samples (see Table 2.1 for the plants sequenced in this study).

The number of species of each genus was sampled to account for the variation within the genus, both in terms of the total number of species described to date and its distribution range. At least 10 per cent of the species in each genus were included. Following are the genus name and, within the parentheses, the number of species described to date and the number included in this study: 1. *Boesenbergia* (50/5), 2. *Camptandra* (4/2), 3. *Caulokaempferia* (10/1), 4. *Cautleya* (2/1), 5. *Cornukaempferia* (2/1), 6. *Curcuma* (50/6), 7. *Distichochlamys* (1/1), 8. *Hedychium* (50/5), 9. *Kaempferia* (40/4), 10. *Paracautleya* (1/1), 11. *Pyrgophyllum* (1/1), 12. *Roscoea* (19/2), 13. *Scaphochlamys* (20/2), 14. *Smithatris* (1/1), 15. *Stahlianthus* (6/1). A species of tribe Zingibereae or *Zingiber* (the only genus in the tribe) (50/1) was also included to test the findings of Searle and Hedderson (2000), Wood *et al.* (2000) and Kress (pers. comm.) that *Zingiber* is found among the genera of

Hedychieae, particularly forming a clade with *Cornukaempferia*.

2.3.2 OUTGROUP TAXA

The phylogenetic relationships (Soltis *et al.*, 2000; Wilf *et al.*, 2000; Wood *et al.*, 2000) within the family unambiguously show that Alpinieae is the basal branch in the family. It is then followed by Globbeae, Hedychieae and Zingibereae. *Zingiber* is also found nesting among the genera of the Hedychieae. I chose three species in Alpinieae: *Alpinia galanga*, *Renealmia battenbergiana* and *Pleuranthodium schlechteri*, as the outgroup, because these plants were available living at RBGE and were used in a previous study (Rangsiruji *et al.*, 2000b).

Table 2.1 Taxa sequenced in this study with source and accession number, and voucher.

Taxon	Source, Accession Number and Voucher
Outgroup *Alpinia galanga* (L.) Willd.	RBGE, 19771077; A. Rangsiruji 3 (E)
Pleuranthodium schlechteri (K.Schum.) R. M. Sm.	WAI, 75p168; C. Cory 5 (E)
Renealmia battenbergiana Cummins ex Baker	RBGE, 19740104; A. Rangsiruji 27 (E), C8482 (E)
Ingroup 1. *Boesenbergia aurantiaca* R. M. Smith	RBGE, 19850843; C. Ngamriabsakul 29 (E)
B. basispicata K. Larsen ex Sirirugsa	RBGE, 19851662; C. Ngamriabsakul 26 (E)
B. gelatinosa K. Larsen	Thailand, the fieldtrip; M.F. Newman 905 (BKF, E)
B. longiflora (Wall.) Kuntze	Thailand, the fieldtrip; M.F. Newman 904 (BKF, E)
B. aff. longiflora	Thailand, the fieldtrip; M.F. Newman 934 (BKF, E)
2. *Camptandra parvula* (King ex Bak.) Ridl.	Malaysia, Prof. Halijah Ibrahim; -
3. *Caulokaempferia thailandica* K. Larsen	Thailand, the fieldtrip; C. Ngamriabsakul 61 (BKF, E)
4. *Cautleya spicata* (Sm.) Baker	RBGE, 19590760; C. Ngamriabsakul 30 (E)
5. *Cornukaempferia longipetiolata* J. Mood & K. Larsen	RBGE, 19991165 (Thailand, the fieldtrip); C. Ngamriabsakul 32 (E)
6. *Curcuma alismatifolia* Gagnep.	Thailand, the fieldtrip; M.F. Newman 944 (BKF, E)
C. amada Roxb.	RBGE, 19810001; M. Ardiyani 27 (E)
C. ecomata Craib	Thailand, the fieldtrip; C. Ngamriabsakul 38 (BKF, E)
C. harmandii Gagnep.	Thailand, the fieldtrip; C. Ngamriabsakul 48 (BKF, E)

C. parviflora Wall.	Thailand, the fieldtrip; C. Ngamriabsakul 32 (BKF, E)
C. rubescens Roxb.	Thailand, Prof. Puangpen Sirirugsa; -
7. *Distichochlamys citrea* M. F. Newman	RBGE, 19901463; C. Ngamriabsakul 24 (E)
8. *Hedychium coccineum* Sm.	RBGE, 19751806; Voucher n.
H. gardnerianum Roscoe	RBGE, 19910120; C. Ngamriabsakul 27 (E)
H. x raffillii	RBGE, 19662631; Voucher n.
H. villosum Wall.	RBGE, 19901454; Voucher n.
H. sp.	Thailand, the fieldtrip; M.F. Newman 916 (BKF)
9. *Kaempferia angustifolia* Roscoe	RBGE, 19621457; Voucher n.
K. elegans Wall.	Thailand, the fieldtrip; M.F. Newman 879 (BKF, E)
K. marginata Carey	RBGE, 19860057; Voucher n.
K. rotunda L.	RBGE, 19590678; C. Ngamriabsakul 28 (E)
10. *Paracautleya bhatii* R. M. Smith	India, Dr K.G. Bhat; K.G.B. 11349 (E)
11. *Pyrgophyllum yunnanensis* (Gagnep.) T. L. Wu & Z. Y. Chen	RBGE, 19901313; C. Ngamriabsakul 33 (E)
12. *Roscoea bhutanica* Ngamriab.	RBGE, 19841747; C. Ngamriabsakul 23 (E)
R. humeana Balf. f. & W. W. Sm.	RBGE, 19871610; C. Ngamriabsakul 8 (E)
13. *Scaphochlamys kunstleri* (Bak.) Holtt.	RBGE, 19643232; C. Ngamriabsakul 25 (E)
S. lanceolata (Ridl.) Holtt.	RBGE, 19782413; Voucher n. (G53)
14. *Smithatris supraneanae* W. J. Kress and K. Larsen	Thailand, Ass. Prof. Yingyong Paisooksantivatana; Y. Paisooksantivatana 00081101 (BK)
15. *Stahlianthus involucratus* (King ex Baker) R. M. Sm.	RBGE, 19981701; C. Ngamriabsakul 34 (E)
16. *Zingiber junceum* Gagnep.	RBGE, 19991169 (Thailand, the fieldtrip); M.F. Newman 954 (BKF, E)

2.3.3 INGROUP TAXA

Thirty-three species of fifteen genera in Hedychieae and *Zingiber junceum* were sequenced (see Table 2.1). At least ten per cent of the species in each genus were sampled to avoid any excess heterogeneity of rates of molecular evolution that may be found. The species were also sampled in such a way as to represent most of the major distribution of each genus. Previous molecular analyses (Wood *et al.*, 2000; Kress, pers. comm.) suggest that *Siliquamomum* and *Siphonochilus* are not members of Hedychieae, instead they are placed within Alpinieae. In addition, *Siphonochilus* appears to be a sister taxon to all the members of the family (Wilf *et al.*, 2000). This is supported by anatomical work by Olatunji (1970). The presence of internal stegmata in the sheath and lamina in *Siphonochilus* and some *Globba* species is similar to all the members of Alpinieae. They are absent from any Hedychieae observed. Thus, I make no further effort to relate this study to these two genera. The ITS sequences of missing Hedychieae genera were also obtained from GenBank (Wood *et al.*, 2000) to include in this present study. These are *Pommereschea lackneri* (2, the number of species in the genus) (GenBank number AF202405), *Rhynchanthus beesianus* (6) (AF202415), *Haniffia cyanescens* (2) (AF202407) and *Hitchenia glauca* (3) (AF202413). The ITS sequences of *Boesenbergia cordata* (AJ388277), a Bornean species and *Camptandra ovata* (AJ388302) were also taken from the GenBank (Searle and Hedderson, 2000). *Curcuma ecomata*'s ITS sequences were kindly provided by Marlina Ardiyani.

Two genera of Hedychieae that are not present in this study are *Haplochorema* (3-4) and *Parakaempferia* (monotypic). *Haplochorema* is morphologically close to *Boesenbergia*. It differs from *Boesenbergia* in that it has a unilocular ovary (instead of mostly trilocular in *Boesenbergia*) and its flower is held flat as opposed to the saccate form in *Boesenbergia*. Nonetheless, it is sometimes found to have a trilocular ovary and is endemic to Borneo, the centre of diversity of *Boesenbergia* (Smith, 1987a). Searle and Hedderson (2000) reported an attempt to amplify the ITS sequences of *Haplochorema* from herbarium sheets, but to no avail. *Parakaempferia* is known only from the type locality in Assam (Rao and Verma,

1969). It resembles *Haniffia* in that it has a rather well-developed pseudostem (up to 60 cm) and the inflorescence arises mainly radically (Larsen and Mood, 2000). Although *Stadiochilus* (monotypic) is of uncertain tribal position (Larsen *et al.*, 1998), it is similar to *Rhynchanthus* and *Hedychium* (Smith, 1980). It is only found in Burma, the centre of diversity of *Pommereschea* and *Rhynchanthus*. Attempts were made to amplify its genomic DNA from herbarium specimens, but these were unsuccessful. While Larsen *et al.* (1998) have placed *Nanochilus* (monotypic) under Alpinieae, the genus besides having the lateral staminodes, shows much resemblance with *Stadiochilus* and *Rhynchanthus* (Smith, 1980). These genera remain the missing pieces in the phylogenetic jigsaws of the Zingiberaceae.

2.3.4 TOTAL GENOMIC DNA EXTRACTION

The CTAB method (Doyle and Doyle, 1987, 1990) was used to obtain the total DNA of the plant cells. Fresh leaf samples were taken and kept in dry silica gel before the DNA extraction. The modified protocol of DNA extraction followed my previous study (Ngamriabsakul *et al.*, 2000). The QIAgen Dneasy kit (QIAGEN, 1997) with liquid nitrogen was also used to give high quality total DNA with a few modifications. Times of incubation were increased to thirty and ten minutes, instead of ten and five minutes in steps three and four of the protocol, respectively.

2.3.5 PCR AMPLIFICATION AND DNA SEQUENCING

Each PCR reaction was 50 µl in volume. The PCR reaction mix was prepared before aliquoting to each tube and adding template DNA as the last component. The components and the conditions of the PCR followed Ngamriabsakul *et al.* (2000), but with a decrease of primers down to 2 µl, instead of 5 µl. No significant reduction in products was detected. The ITS1, 5.8S and ITS2 complete region was amplified by using primers '5P' and '8P' (Möller and Cronk, 1997a). ITS1 and ITS2 had to be amplified separately for some species. Primer '5P' and Primer '2K' (Rangsiruji, 1999) were then used to amplify ITS1, while primer '3P' and primer '8P' were used for ITS2.

PCR amplification of *trn*L-F with primers 'c' and 'f' (Taberlet *et al.*, 1991) was noted to contain more than one distinct band in some species, i.e. two when using the conditions as described for the ITS (Ngamriabsakul *et al.*, 2000). Different conditions of the PCR reaction were then tried. It was found that using primers 'c' and 'f' to amplify some ginger plants DNA, a stricter condition was needed than those of ITS, including a well calibrated thermocycler. In cases that the second band could still be observed after amplifications, the *trn*L-F region was thus amplified by using two sets of primers. Primers 'c' and 'd', along with 'e' and 'f' were used for amplifications of *trn*L intron and *trn*L-F spacer separately, respectively. All the products of primers, 'c' and 'f' (a complete region of *trn*L intron and *trn*L-F spacer), 'c' and 'd' (*trn*L intron), 'e' and 'f' (*trn*L-F spacer) were successfully obtained each as a single band. PCR products were purified before automated cycle sequencing by using a QIAquick™ PCR purification kit. Forward and reverse sequencings, using the same primers as in PCR reactions, were performed for sequence confirmation as described in Ngamriabsakul *et al.* (2000). The primer sequences used in this study are (5′ to 3′), 5P = GGA AGG AGA AGT CGT AAC AAG G, 8P = CAC GCT TCT CCA GAC TAC A, 2K = GGC ACA ACT TGC GTT CAA AG, 3P = GCA TCG ATG AAG AAC GTA GC, c = CGA AAT CGG TAG ACG CTA CG, d = GGG GAT AGA GGG ACT TGA AC, e = GGT TCA AGT CCC TCT ATC CC, f = ATT TGA ACT GGT GAC ACG AG.

2.3.6 SEQUENCE ANALYSIS

All sequences were verified by comparison of their forward and reverse sequences in Autoassembler™ (Applied Biosystems Division) which was used to form the single nucleotide strands of each sequence. Most base-calling disagreement could be resolved unambiguously by eye. When this was not possible, IUPAC codes were used for ambiguous nucleotides. Sequence boundaries of the range of ITS1, 5.8S and ITS2 of all taxa were determined by comparison with published sequence data of *Roscoea* species (Ngamriabsakul *et al.*, 2000) and *Alpinia* species (Rangsiruji *et al.*, 2000a). Sequences of the *trn*L-F region started from nucleotide position 41,

from the last nucleotide of primer 'c' and continued until nucleotide position 3, before the site of primer 'f'. This region was chosen because most of the species have a complete sequence. All sequences will be submitted to GenBank. A transition/transversion ratio was determined by MacClade version 3.07 (Maddison and Maddison, 1992) using one of the most parsimonious trees from the unweighted initial analysis. The G + C content and sequence divergence among taxa were calculated using Base Frequencies and Show Pairwise Distance options in PAUP* Version 4.0b4 (Swofford, 1998).

The sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997; Hickson *et al.*, 2000) with default values (e.g. gap opening cost = 15) and manual adjustment in only the first alignment. Because of the high similarity in length and nucleotides of the *trn*L-F sequences (see Table 2.2), sensitivity test of alignment was performed only for the ITS data set by varying the gap opening cost to 5, 10, 20 and 25 to yield four other different alignments (Jeanmougin *et al.*, 1998). The alignments were directly submitted to parsimony analysis. This test was to find the effect of alignment and gaps of the ITS data set to phylogenetic estimates resulting from the use of the alignment. Character congruence is advocated as both an internal criterion (Bogler and Simpson, 1996) and an external criterion (Giribet and Wheeler, 1999) for choosing the best alignment based on parsimony criterion. Thus, I chose rescaled consistency (RC) index of each analysis (Bogler and Simpson, 1996) and *P*-values of the homogeneity test of each of the differently aligned ITS data sets and the *trn*L-F data set as indicators of the optimal alignment.

2.3.7 PHYLOGENETIC ANALYSIS

Phylogenetic trees were generated using PAUP* Version 4.0b4 (Swofford, 1998), run on a Power Mac G4 with character states unordered and initially equally weighted. The heuristic search was set to 1000 replicates with random addition sequence and TBR (Tree Bisection-Reconnection) branch swapping. Polymorphic characters were treated as uncertain. Gaps were treated as missing values. MULPARS, COLLAPSE and STEEPEST DESCENT were the options selected.

ACCTRAN (accelerated transformation) was chosen for character optimisation.

A partition homogeneity test (Farris *et al.*, 1994), also known as the Incongruence Length Difference, ILD, test was performed, in PAUP* with the heuristic search set to 1000 replicates, 10 replicates of random addition sequence, TBR and MULPARS, to test the hypothesis that the two data sets, ITS and *trn*L-F, contain the same phylogenetic information. The ITS data set was reduced to 26 taxa to match with the 26 taxa *trn*L-F data set for the test. This test is a bootstrap approach which randomly partitions characters and tests the null hypothesis that a given partition of a data set (for example, ITS and *trn*L-F) represents an arbitrary subdivision of one large data set. If two data sets are highly incongruent, then the sum of their minimal trees should be significantly shorter than that of the sum of treelengths from random partitions of the combined data set and the null hypothesis will be rejected. The result of the test suggests that both data sets are congruent (*P*-value > 0.05) and can be combined. A combined analysis of both data sets was performed utilising the same phylogenetic methods and parameters as above.

Successive weighting searches were performed, using Rescaled Consistency index (RC, mean value) (Swofford, 1993) until the resulting tree length remained unchanged in two consecutive rounds. Due to the high value of transitions found in the ITS data matrix (65%), the transition/transversion ratio (ts/tv = 1/2) was applied to a parsimony analysis of the data set to weight transversion over transition.

Descriptive statistics reflecting the fitness of the data sets to the shortest trees were given by the consistency index (CI) (Kluge and Farris, 1969), retention index (RI) (Farris, 1989) and branch length. Support for individual clades was given by two statistics, bootstrap value (Felsenstein, 1985) and decay index (Bremer, 1988; Donoghue *et al.*, 1992). Bootstrap analysis was performed using PAUP*, set to heuristic search with 1000 replicates, TBR and ten random addition sequence replicates per heuristic search. In the results and discussion presented here, clades with bootstrap values of 50-74% represent weak support, 75-84% moderate support and 85-100% strong support (Richardson *et al.*, 2000). The decay index was

calculated using Autodecay version 4.0 (Eriksson, 1998) with ten random addition sequence replicates per heuristic search.

Maximum Likelihood analysis was performed for the ITS data set in PAUP* by applying a model, TrN+G (Tamura and Nei, 1993, G = Gamma distribution). The model was determined to be the best fit model to the data set by the likelihood ratio test (Huelsenbeck and Rannala, 1997) using MODELTEST version 3.0 (Posada and Crandall, 1998). The substitution model used allows unequal base frequencies, unequal transition and transversion rates and among-site rate heterogeneity.

2.4 RESULTS

The ITS sequences of thirty-six species were obtained in this study, leaving out one taxon, *K. marginata* whose the sequences were unreadable. The ITS sequences of other six ingroup taxa were taken from GenBank. In total, there are forty-two taxa in the ITS data matrix and twenty-six taxa in the *trn*L-F data matrix. The reasons why the *trn*L-F data matrix is smaller than the ITS data matrix are: first, the taxa that have ITS sequences in GenBank have not been sequenced for *trn*L-F or the sequences are not yet available; second, my own DNA samples of some species proved to be difficult for the amplification and sequencing of the *trn*L-F region.

2.4.1 SEQUENCE COMPARISON WITH PREVIOUS STUDY

ITS1 and ITS2 sequences of *Renealmia battenbergiana* and *Pleuranthodium schlechteri* in this study were identical to the sequences of the same taxa obtained by Rangsiruji *et al.* (2000a). Only one nucleotide of ITS2 is observed to be different for *Alpinia galanga*. For the *trn*L-F spacer, the sequences of *Alpinia galanga* and *Pleuranthodium schlechteri* were identical to the sequences of Rangsiruji *et al.* (2000a). However, the first thirty-seven nucleotides of the spacer of *Renealmia battenbergiana* of Rangsiruji *et al.* (2000a) were different from this study. Nine

unmatched nucleotides and one gap of eight nucleotides were observed in this region. Apart from this, the sequences differed by only two nucleotides. Each sequence obtained in this study was a complete region of *trn*L intron and *trn*L-F spacer by the sequencing of all four primers products (c, d, e and f). The sequence difference of *Renealmia battenbergiana* observed in Rangsiruji *et al.* (2000a) and this study may be the result of multiple copies of the region in the genome. Different PCR conditions prefer different sites of the region. In addition, the problematic site is near the beginning of the primer which makes it more difficult to obtain the correct sequence by only one primer sequencing.

2.4.2 THE BEST ALIGNMENT OF THE ITS DATA SET

The alignment of the ITS data set with default values (i.e. gap opening cost = 15) in Clustal X gave the highest Rescaled Consistency (RC) value when the data set was analysed to find the most parsimonious trees. Four other values of gap opening in Clustal X, i.e. 5, 10, 20 and 25 gave different alignments from the default value. RC values of these different alignments by parsimony analysis, were lower than that of the first alignment without manual adjustment (data not shown). The default value alignment that gave the highest value of RC was further improved by manual adjustment and when analysed the resulting RC was a bit higher than the alignment without manual adjustment (data not shown).

The *P*-value of the initial homogeneity test of both data sets suggests that the phylogenetic signals contained in the data sets are homogeneous and can be combined (*P*-value > 0.05). It is assumed that the alignment of the ITS data set that yields the highest *P*-value when used in the homogeneity test represents the best alignment of the ITS data set. The assumptions are that the data sets are parts of the one big data set of the taxa and however partitions should lead to the same phylogenetic estimates. The *P*-value of the homogeneity test of *trn*L-F data set and the first alignment of ITS data set with default value and manual adjustment, was the highest value compared to that of other different alignments of the ITS data set (data not shown). Thus, the best alignment of ITS data set that is chosen in this study is

722 bp in length and its characteristics are presented in Table 2.2.

2.4.3 SEQUENCE ANALYSIS OF THE ITS REGION

Alignment of ITS sequences of the 42 taxa analysed resulted in a 722-bp long data matrix. 60 bp were excluded because of alignment ambiguities, so a data matrix 662-bp long was subject to analyses. Its characteristics are given in Table 2.2. Two sequences, *Boesenbergia cordata* and *Camptandra ovata* taken from GenBank were missing the first 23 and 25bp of ITS1, respectively. *Scaphochlamys kunstleri* and *S. lanceolata* lacked most of their 5.8S sequences.

The lengths of the complete ITS sequences were on average 591.24bp. The lengths of aligned ITS1, 5.8S and ITS2 were 265, 158 and 299bp respectively. Of these aligned sites, 320 (48.34%) were constant, 213 (32.17%) had at least two nucleotide states in two or more sequences and were potentially informative phylogenetically, and 129 (19.49%) were autapomorphies (Table 2.2).

The sequence divergence of ITS1, 5.8S and ITS2 among ingroup species ranged from 0.0-23.9% whereas sequence divergence between the ingroup and the outgroup ranged from 10.0-23.8%. The maximum sequence variation among ingroup species was 23.9% between *Kaempferia angustifolia* and *Scaphochlamys lanceolata*. The maximum sequence variation between the ingroup and the outgroup was 23.8% *Alpinia galanga* and *Scaphochlamys lanceolata*. Apart from the identical ITS sequences of *Hedychium coccineum*, *Hedychium gardnerianum* and *Hedychium x raffillii*, the least sequence variation among ingroup species was 0.09% between *Curcuma alismatifolia* and *Curcuma parviflora*.

The sequence of *K. elegans* is the longest found in this study (672 bp) and the highest variation of ITS within a genus belongs to *Kaempferia* (17.93%, between *K. angustifolia* and *K. elegans*). The maximum level of variation of ITS within other genera are 15.37% (*Scaphochlamys kunstleri* and *S. lanceolata*), 11.83% (*Boesenbergia cordata* and *B. gelatinosa*), 7.06% (*Camptandra ovata* and *C.*

parvula), 6.96% (*Curcuma alismatifolia* and *C. ecomata*), 2.75% (*Roscoea bhutanica* and *R. humeana*), 1.88% (*Hedychium coccineum* and *H.* sp.).

2.4.4 SEQUENCE ANALYSIS OF THE *trn*L-F REGION

Alignment of *trn*L-F sequences of the 26 taxa analysed resulted in a data matrix 1008-bp long. Its characteristics are given in Table 2.2. Ranges of the sequence at the primers sites (d and e) in three taxa, *Cornukaempferia longipetiolata*, *Hedychium* sp. and *Kaempferia rotunda* were missing, 25, 66 and 32bp, respectively. The sequence of *Distichochlamys citrea* lacked the last 126bp.

The lengths of the complete *trn*L-F were on average 913.04bp. The lengths of aligned *trn*L intron and *trn*L-F spacer were 595 and 413bp respectively. Of these aligned sites, 885 (87.80%) were constant, 38 (3.77%) had at least two nucleotide states in two or more sequences and were potentially informative phylogenetically, and 85 (8.43%) were autapomorphies (Table 2.2).

The sequence divergence of *trn*L-F intron and *trn*L-F spacer among ingroup species ranged from 0.1-2.5% whereas sequences divergence between the ingroup and the outgroup ranged from 1.8-3.9%. The maximum sequence variation among ingroup species was 2.5% between *Kaempferia angustifolia* and *Pyrgophyllum yunnanensis*. The maximum sequence variation between the ingroup and the outgroup was 3.9% *Renealmia battenbergiana* and *Curcuma alismatifolia*. The least sequence variation among ingroup species was 0.1% between *Boesenbergia aurantiaca* and *Caulokaempferia thailandica*. However, there are two indels present when comparing the sequences of these two taxa, 1 and 7bp in size.

Table 2.2 Sequence characteristics of nuclear ribosomal DNA (ITS1, 5.8S, ITS2) and chloroplast DNA (*trn*L-F). * 662 bp is the length of ITS data set for analyses.

Parameter	ITS1, 5.8S, ITS2	*trn*L-F
Length range (total) (bp)	573-672	894-960
Length mean (total) (bp)	591.24	913.04
Length range (ingroup) (bp)	577-672	894-960
Length mean (ingroup) (bp)	592.00	913.52
Length range (outgroup) (bp)	573-591	906-914
Length mean (outgroup) (bp)	582.00	909.33
Aligned length (bp)	722 (662)*	1008
G + C content range (%)	52.30-59.82	31.35-33.41
G + C content mean (%)	55.71	32.78
Sequence divergence (ingroup) (%)	0.00-23.89	0.11-2.50
Sequence divergence (in/outgroup) (%)	9.98-23.75	1.79-3.88
Number of variable sites (%)	342 (51.66)*	123 (12.20)
Number of constant sites (%)	320 (48.34)*	885 (87.80)
Number of informative site (%)	213 (32.17)*	38 (3.77)
Number of autapomorphic sites (%)	129 (19.49)*	85 (8.43)
Transitions (unambiguous)	483	30
Transversions (unambiguous)	258	38
Transitions/transversions (ts/tv)	1.87	0.79
Average number of steps per character	1.414	0.149

2.4.5 PHYLOGENETIC ANALYSIS OF THE ITS REGION

Twenty-three most parsimonious trees from two islands, size 2 and 21, were obtained with the parsimony analysis of the 42 taxa ITS1, 5.8S and ITS2 data set, with a length of 936, CI = 0.5417, RI = 0.6374 and RC = 0.3452. The strict consensus tree of the twenty-three most parsimonious trees is given in Figure 2.1 with bootstrap values and decay indices. The average number of nucleotide substitutions per character was high, with 1.414 compared to 0.149 for the *trn*L-F data set.

The strict consensus tree strongly supports the hypothesis that *Zingiber* is a member of Hedychieae (BS = 95, DI = 6). There are four major clades recognised in the tree, namely the *Pyrgophyllum* clade, the *Curcuma* clade, the *Camptandra* clade and the '*Hedychium* clade'. Apart from the *Pyrgophyllum* clade as the sister clade to all the rests, their relationships are unresolved. Only two major clades are strongly supported, i.e. the *Curcuma* clade (BS = 100, DI = 9) and the *Camptandra* clade (BS = 99, DI = 10). The resolutions within the *Curcuma* clade are rather high and show that *Curcuma* is paraphyletic. The *Curcuma* clade comprises, besides *Curcuma*, four other morphologically very similar genera, namely *Hitchenia*, *Paracautleya*, *Smithatris* and *Stahlianthus*. *Curcuma ecomata* and *Smithatris supraneanae* form a subclade separated from the rest of the clade but with weak support (BS = 52, DI = 1). *Stahlianthus* is found as the sister group of *Curcuma* subgenus *Hitcheniopsis* (BS = 100, DI = 13). The clade of *Hitchenia/Paracautleya* is the sister clade to *Curcuma* subgenus *Curcuma* (BS = 84, DI = 3).

Although the relationships within the '*Hedychium* clade' are not resolved, there are some well-supported clades. The strict consensus tree shows that *Caulokaempferia* forms a clade with *Boesenbergia aurantiaca* and *B. cordata* (BS = 99 and DI = 8). However this clade is not grouped with the other four *Boesenbergia* taxa with any support. The other four taxa of *Boesenbergia*: *B. basispicata*, *B. gelatinosa*, *B. longiflora* and *B. af. longiflora*, are weakly supported as a clade (BS =

70, DI = 1). *Cautleya spicata* is found to be the sister group to *Roscoea* (BS = 98, DI = 6). *Pommereschea* and *Rhynchanthus* form a weakly supported clade (BS = 73, DI = 3). *Hedychium* species are grouped as a clade with strong support (BS = 100, DI = 14). *Kaempferia* species are grouped as a clade with weak support (BS = 57, DI = 2) while *Scaphochlamys* species are grouped as a clade with strong support (BS = 94, DI = 6).

Successive weighting analyses produced a single most parsimonious tree (Figure 2.3). However this tree is not one of the twenty-three shortest trees resulting from an unweighted analysis (Figure 2.2 shows one of the twenty-three most parsimonious trees). Besides the clear patterns of relationships in the successive weighting tree, the positions of *Pyrgophyllum* and *Camptandra*, in the successive weighting tree and the strict consensus tree of an unweighted analysis, when compared are the most significant differences.

The weighting of transversion over transition by an observed ratio (2/1) of the data set produced fourteen most parsimonious trees (CI = 0.5620, RI = 0.6342, RC = 0.3564). The strict consensus tree of these fourteen trees is nearly identical to the strict consensus tree of an unweighted analysis, but with higher resolutions, particularly within the '*Hedychieae* clade' (Figure 2.4).

The maximum likelihood analysis recovered two optimal trees (ln-likelihood = 5551.712). The strict consensus tree of the two optimal trees is presented in Figure 2.5. Two main subclades, as found in the strict consensus tree of transition/transversion ratio applied search, can be recognised, namely the '*Hedychium* clade' and the '*Curcuma* clade'. Within the '*Curcuma* clade', *Pyrgophyllum* is identified as the sister group to *Camptandra* and thus the clade *Pyrgophyllum/Camptandra* is the sister group to the *Curcuma* complex. Topologies of the complex are identical to those found in the strict consensus tree of the ts/tv-applied search. Within the '*Hedychium* clade', the clade of *Cautleya/Roscoea/Pommereschea/Rhynchanthus* is found to be the sister clade of *Hedychium* species. In turn, this *Cautleya/Roscoea/Pommereschea/Rhynchanthus/*

Hedychium clade is the sister clade to the *Boesenbergia* group. *Haniffia* is suggested to be the sister group to the remaining taxa. One difference of the topologies found here in maximum likelihood tree and the ts/tv-applied tree is the swapping of the clade of *Distichochlamys/Scaphochlamys* and the *Kaempferia* clade while the clade of *Boesenbergia* in the two trees, which also has *Caulokaempferia* nested, is the last branch and identical.

Figure 2.1. The strict consensus tree of the twenty-three most parsimonious trees resulting from the analysis of 42 taxa ITS data set. Upper numbers are bootstrap values of 1000 replicates. Lower numbers are decay indices (CI = 0.542; RI = 0.637; RC = 0.345).

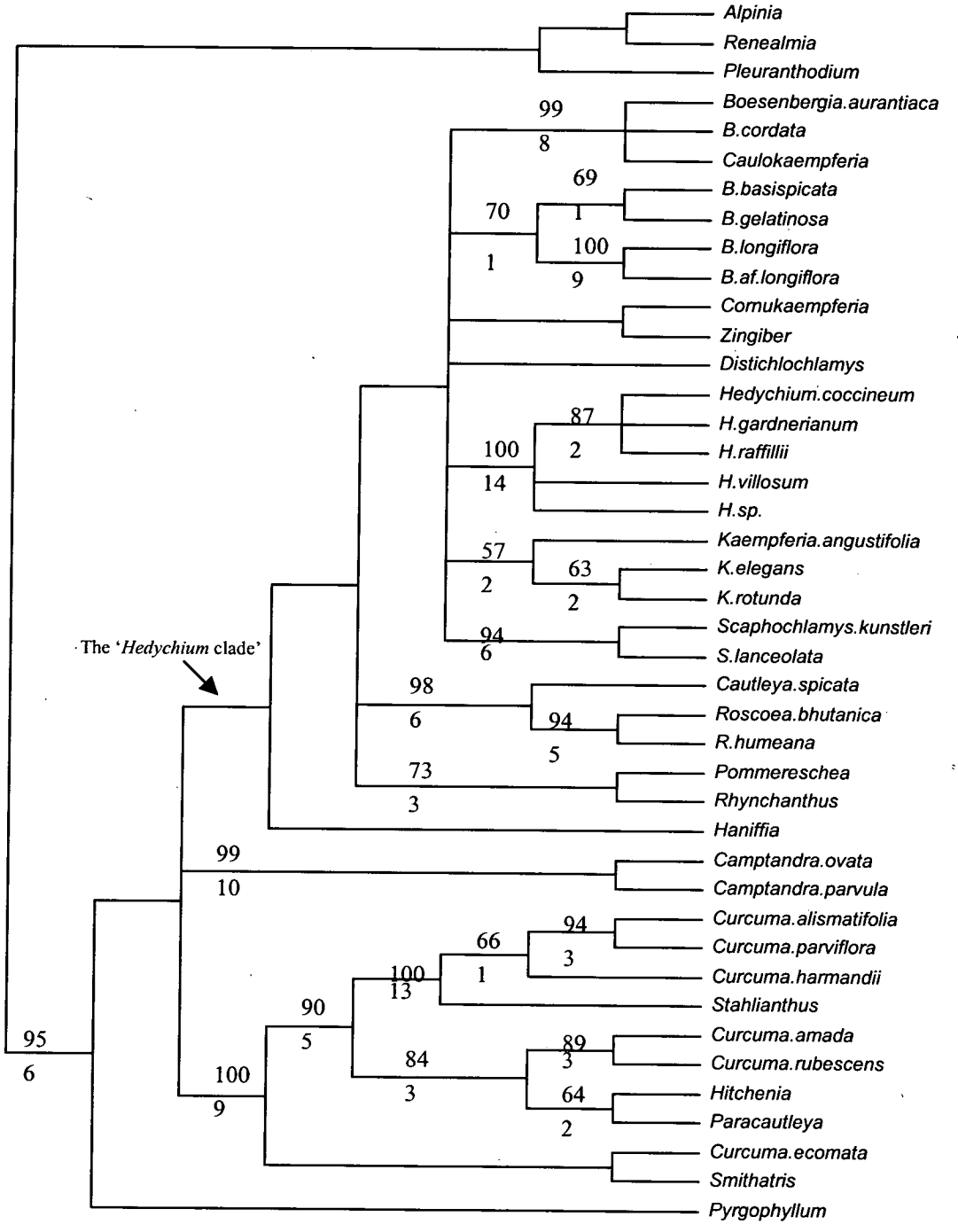


Figure 2.2. One of the twenty-three most parsimonious trees resulting from the unweighted analysis of 42 taxa ITS data set.

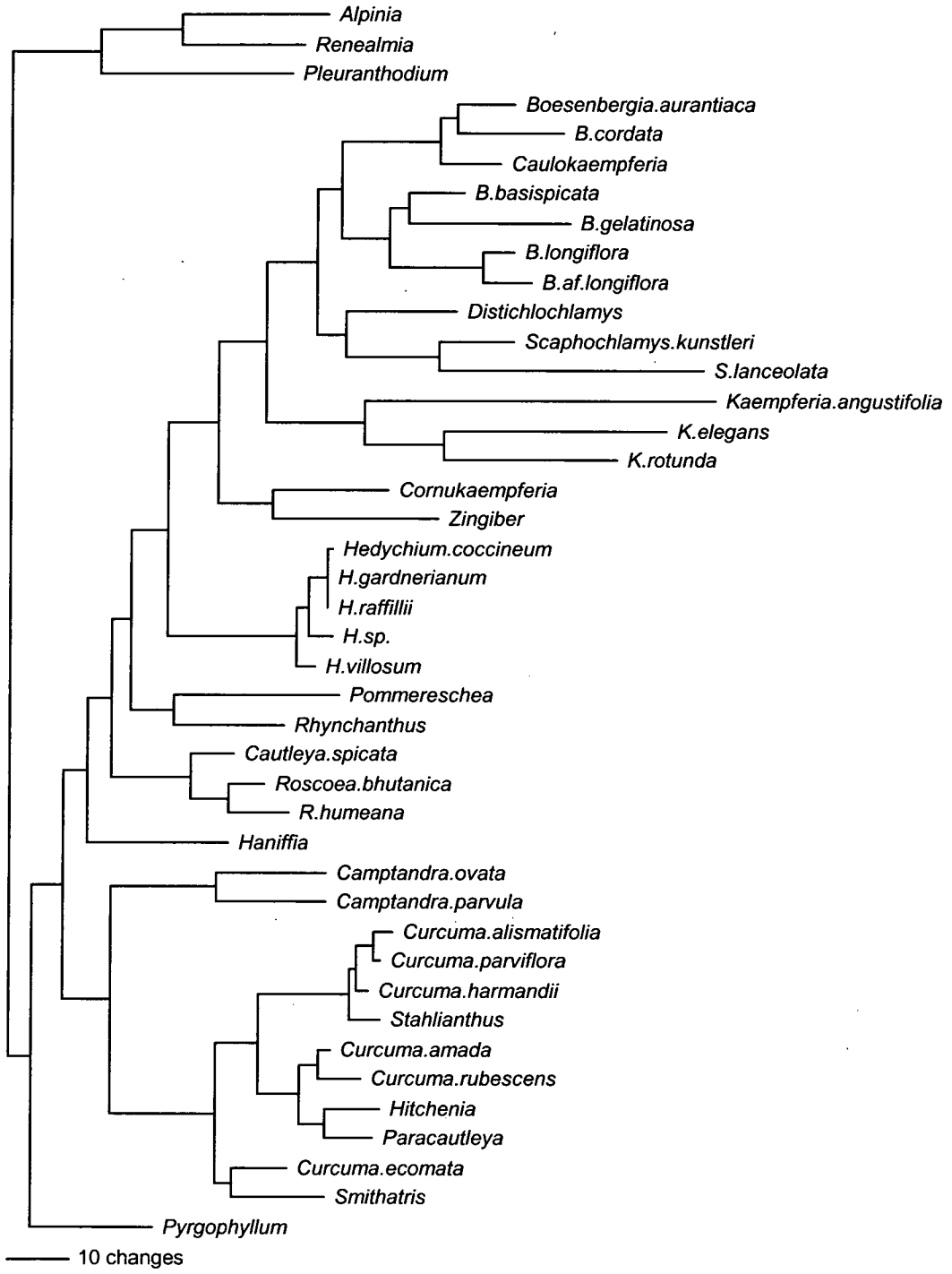


Figure 2.3. The single most parsimonious tree resulting from the successive weighting searches of 42 taxa ITS data set using RC. Note that the tree is not one of the twenty-three most parsimonious trees from an unweighted search.

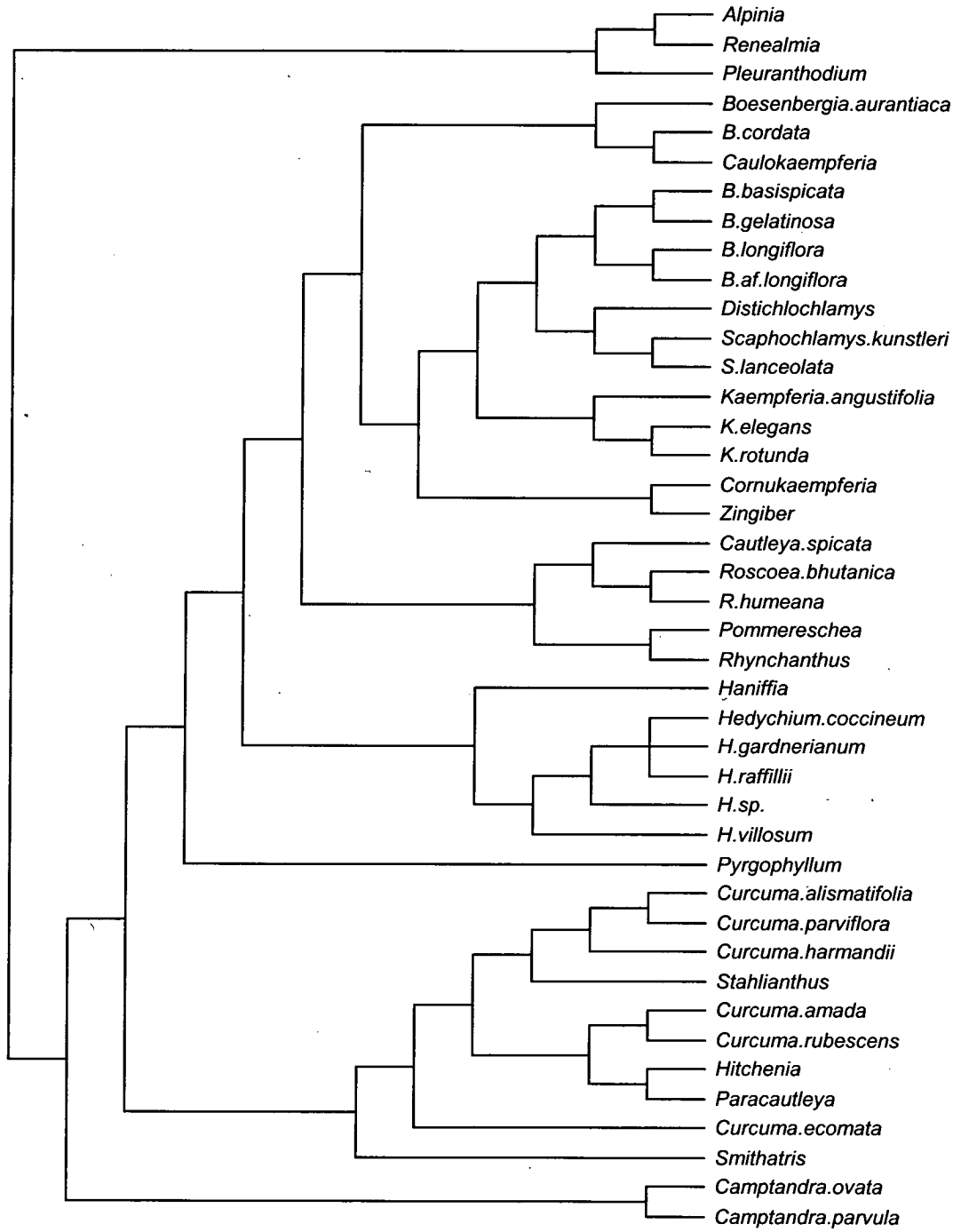


Figure 2.4. The strict consensus tree of the fourteen most parsimonious trees resulting from the transition/transversion ratio applied analysis of 42 taxa ITS data set. The basic chromosome numbers shown are representative, i.e. not all the species in this tree are known.

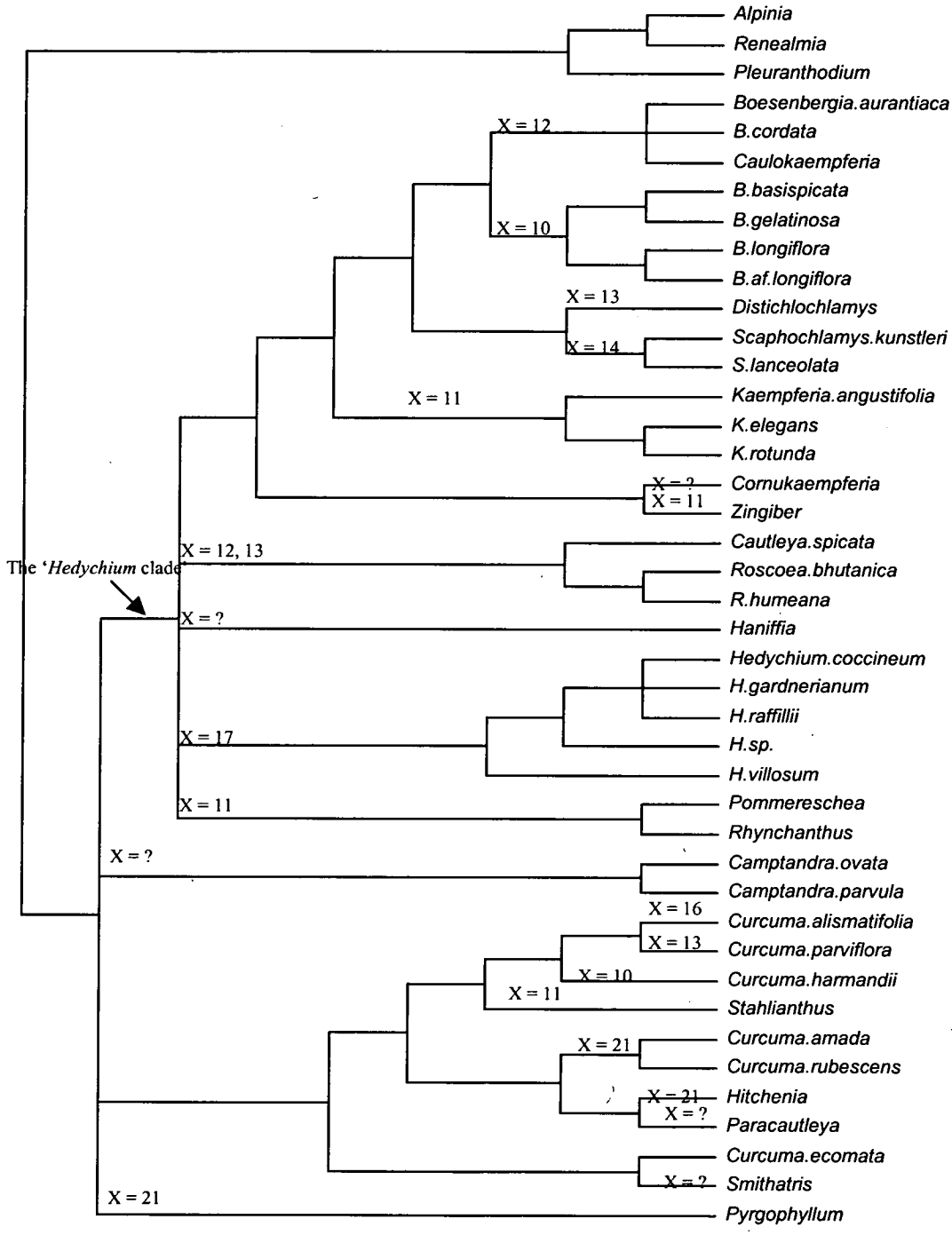
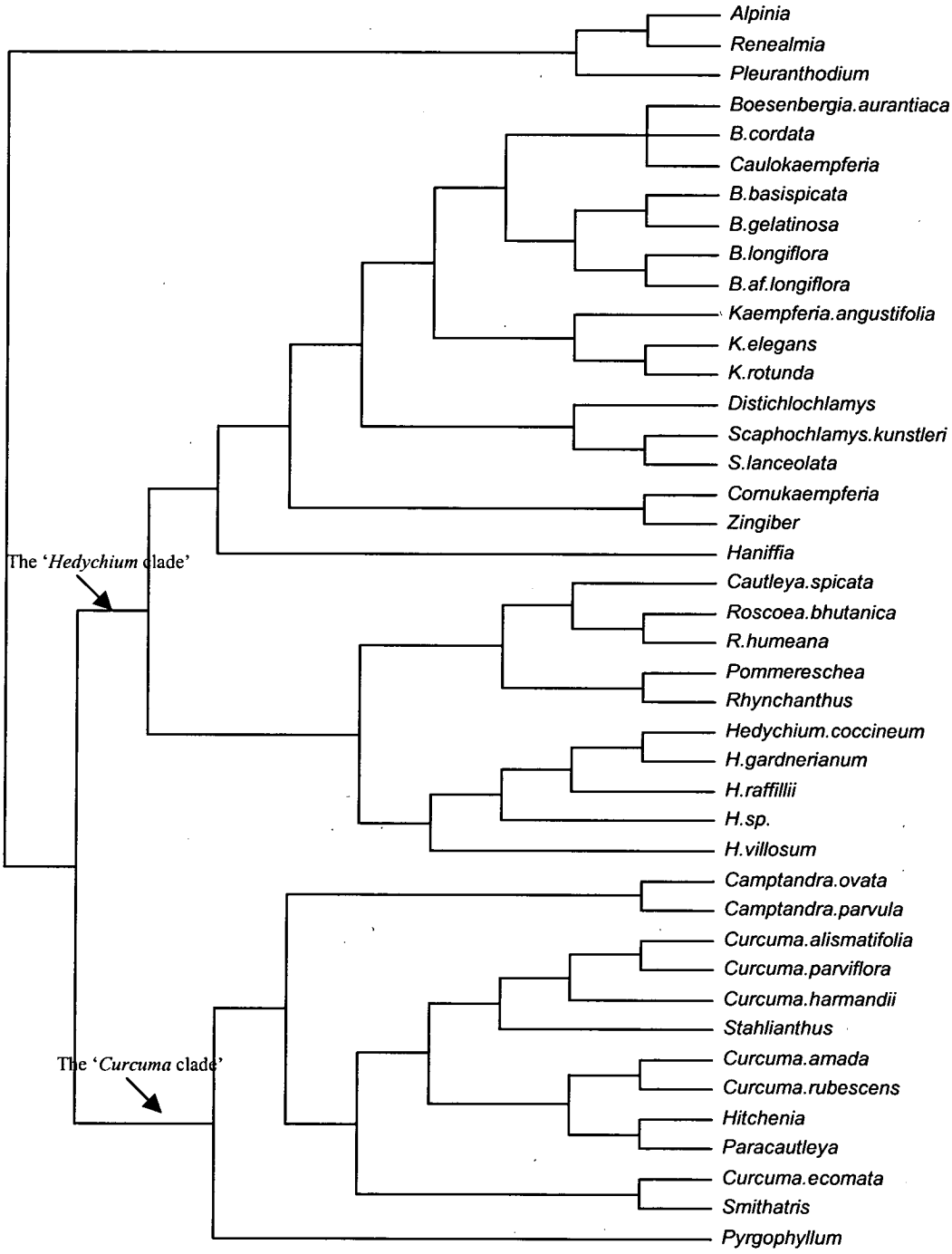


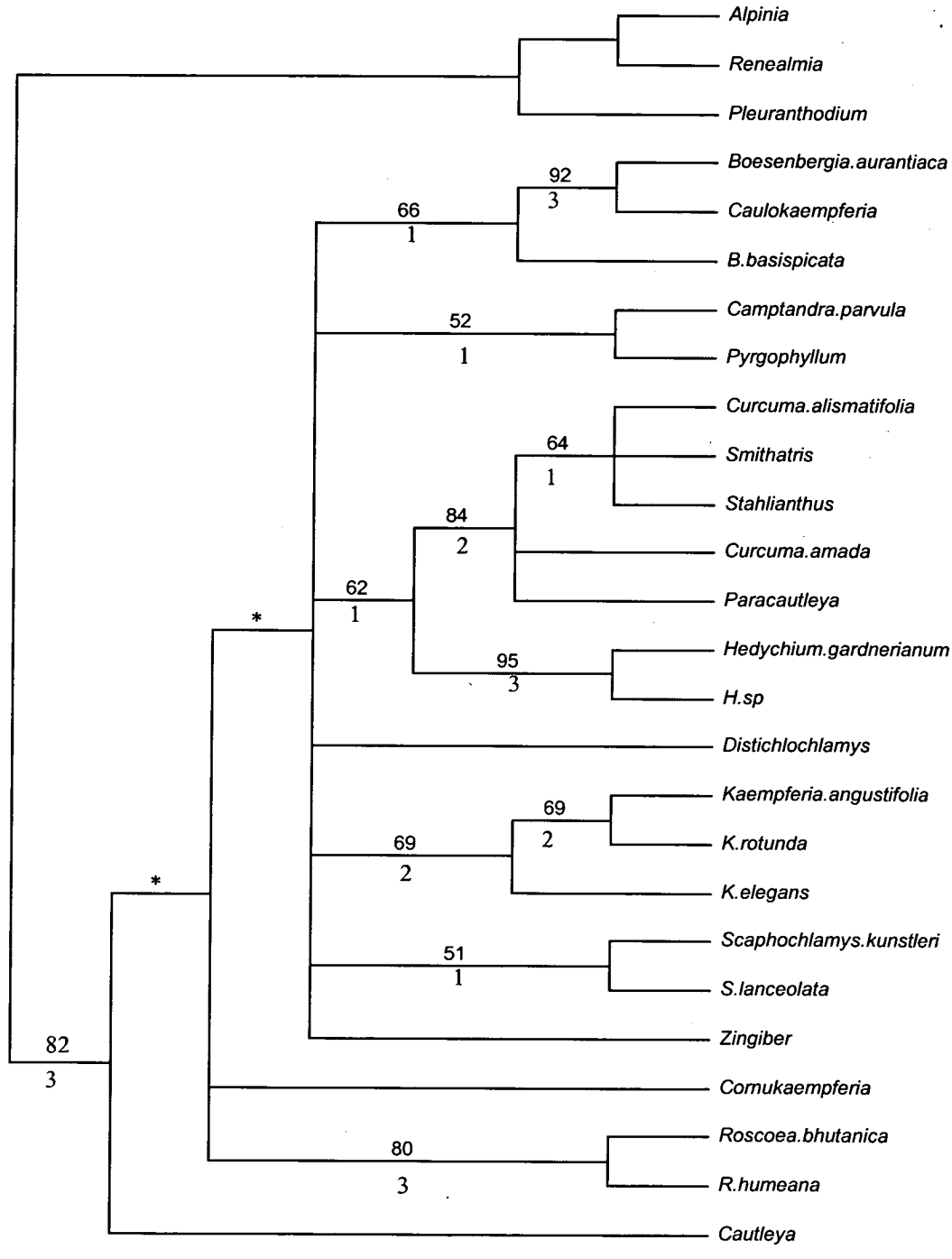
Figure 2.5. The strict consensus tree of two equally optimal trees resulting from the maximum likelihood analysis of 42 taxa ITS data set (ln-likelihood = 5551.712).



2.4.6 PHYLOGENETIC ANALYSIS OF THE *trn*L-F REGION

Five most parsimonious trees of an island were obtained with the parsimony analysis of the 26 taxa *trn*L-F complete region data set, with a length of 150, CI = 0.9067, RI = 0.7879 and RC = 0.7143. Successive weighting analyses produced the same set of trees as found in the unweighted analysis. The majority consensus tree of the five most parsimonious trees was given in Figure 2.6 with bootstrap values and decay indices. Although there is less resolution in the consensus tree compared to that of the ITS data set, the tree of *trn*L-F data set gives some phylogenetic information. It moderately supports that *Zingiber* is a member of Hedychieae (BS = 82, DI = 3). It also confirms that *Caulokaempferia* is derived within *Boesenbergia* (BS = 66, DI = 1). An obscure relationship, not found in the strict consensus tree of ITS, was also revealed when *Camptandra parvula* and *Pyrgophyllum yunnanensis* were grouped together, though with weak support (BS = 52, DI = 1). *Curcuma* complex genera, as found in the strict consensus tree of ITS, were again retrieved by the *trn*L-F data set (*Curcuma*, *Paracautleya*, *Smithatris* and *Stahlianthus*) with moderate support (BS = 84, DI = 2). *Curcuma* subgenus *Hitcheniopsis*, *Smithatris* and *Stahlianthus* were further supported, though weakly as a clade (BS = 64, DI = 1). *Hedychium* was suggested as the sister group to the *Curcuma* complex genera by the *trn*L-F data set, yet with weak support (BS = 62, DI = 1). The members of each of the genera, *Kaempferia*, *Roscoea* and *Scaphochlamys* were grouped together, though with weak to moderate support, i.e. BS = 69, DI = 2 in *Kaempferia*, BS = 80, DI = 3 in *Roscoea* and BS = 51, DI = 1 in *Scaphochlamys*.

Figure 2.6. The majority consensus tree of the five most parsimonious trees resulting from the analysis of 26 taxa *trn*L-F data set. Upper numbers are bootstrap values of 1000 replicates. Lower numbers are decay indices (CI = 0.907; RI = 0.788; RC = 0.714). * denotes collapse branch in the strict consensus tree.



2.4.7 PHYLOGENETIC ANALYSIS OF THE COMBINED DATA SETS

The *P*-value, 0.734, resulting from the partition homogeneity test of both data sets indicates that there is considerable congruence in the phylogenetic information contained within the ITS and *trn*L-F data sets. Thus the data sets were combined for a simultaneous parsimony analysis. Two most parsimonious trees from an island were obtained, with a length of 882, CI = 0.6406, RI = 0.5681 and RC = 0.3639. The strict consensus tree is shown in Figure 2.7. The tree recognised the monophyly of Hedychieae including *Zingiber* with strong support (BS = 99, DI = 11). Three major clades were identified in the Hedychieae, the clade of *Cautleya/Roscoea* (BS = 99, DI = 7), the *Curcuma* complex (BS = 100, DI = 11) and the '*Hedychium* clade' (BS = 63, DI = 3). However, there is no strong support to the relationships of these clades. *Cautleya* is identified as the sister group to *Roscoea* (BS = 99, DI = 7). The clade of *Camptandra/Pyrgophyllum* is suggested as the sister group of the *Curcuma* complex, though the bootstrap value is less than 50 per cent. Within the *Curcuma* complex, *Smithatris* is moderately supported as the sister group to the rest of the complex (BS = 88, DI = 5). *Stahlianthus* is grouped with *Curcuma* subgenus *Hitcheniopsis* (BS = 100, DI = 10) while *Paracautleya* is grouped with *Curcuma* subgenus *Curcuma* (BS = 75, DI = 2).

The '*Hedychium* clade' is weakly supported (BS = 63, DI = 3) and has *Hedychium* as the sister genus to the rest of the clade. The monophyly of the genus *Hedychium* is strongly supported (BS = 100, DI = 20) as so the genus *Kaempferia* (BS = 91, DI = 7) and the genus *Scaphochlamys* (BS = 98, DI = 9). Here is also found the clade of *Boesenbergia aurantiaca* and *Caulokaempferia thailandica* with strong support (BS = 100, DI = 11). Nevertheless, the relationships among these genera are not resolved with any real support in this combined analysis.

The successive weighting searches of the combined data set by using Rescaled Consistency (RC) index produced a single most parsimonious tree (Figure 2.8). Two major clades can be recognised, namely the '*Curcuma* clade' and the

'*Hedychium* clade'. In the '*Curcuma* clade', *Camptandra* and *Pyrgophyllum* are found as the sister clade to a set of four morphologically very similar genera, *Curcuma*, *Paracautleya*, *Smithatris* and *Stahlianthus*. *Smithatris* is found to be the sister group to the clade of *Paracautleya/Curcuma* subgenus *Curcuma* and *Stahlianthus/Curcuma* subgenus *Hitcheniopsis*. Within the '*Hedychium* clade', the clade of *Cautleya/Roscoea* is the sister group to all the rest of the clade. *Hedychium* is next separated as the sister group of the genera of '*Boesenbergia* group': *Boesenbergia*, *Caulokaempferia*, *Cornukaempferia*, *Distichochlamys*, *Kaempferia*, *Scaphochlamys* and *Zingiber*. *Boesenbergia* is found to be paraphyletic. *Caulokaempferia* forms a clade with *Boesenbergia aurantiaca*. *Distichochlamys* is the sister group to *Scaphochlamys*. *Cornukaempferia* and *Zingiber* are sister group to each other.

2.5 DISCUSSION

2.5.1 THE EVOLUTION OF ITS AND *trn*L-F

The rate of mutation in ITS of the Hedychieae is about nine times faster than that of the *trn*L-F region. As a result, the phylogenetic relationships among Hedychieae revealed by ITS are observed to be more fully resolved than those revealed by *trn*L-F region. This was also recorded in *Gentiana*, a perennial herb genus of dicotyledon whose ITS sequences gave a distinctively higher resolution in the parsimony analysis than the *trn*L-F region (Gielly *et al.*, 1996).

The ITS1, 5.8S and ITS2 sequences in *Hedychium* are found to be markedly less variable than those of other genera in the Hedychieae. Their usefulness as phylogenetic markers in the genus is thus minimal as also observed in Wood *et al.* (2000). There are two possible explanations. Firstly it may be attributed to the exceptionally low mutation rate of the sequences in the genus compared to other genera in the family. The other explanation is that the diversity of morphology found in the genus is large and outstrips the mutation rate of the ITS genes (rapid

radiation). The latter is thought to be more likely to occur in the genus. The phenomenon is explained in that morphology is normally held in equilibrium, by stabilising selection for much of evolutionary time, but with punctuation by relatively rapid speciation events (Bateman, 1999). This phenomenon may also happen in *Curcuma* subgenus *Hitcheniopsis* where ITS sequence variation is low, but the morphology of the species cannot be mistaken. Another example can be found in *Aframomum* of the Alpinieae where ITS variation within this medium sized genus (50 species) is exceptionally low, 0-2.74% (Harris *et al.*, 2000). The mechanism is further explained by the species ecological factors. Most *Aframomum* species are found on the edges of forests and savannas and the ecological constraints of these habitats are normally large and have much effect on the morphology of the species. The different edges have rather specific conditions and these differences could be a driving force for speciation. It may also be assumed that the distribution of an ancestor species had been restricted, thus giving rise to a few species, peripheral isolation or fragmentation.

On the contrary, the sequence in *Kaempferia* is very variable. *Kaempferia* has the highest mutation rate of the genera in this study. Its fast evolving ITS regions cannot be ascribed solely to its perennial habit, as all other genera of the Hedychieae in this study are also perennial in habit and shed leaves during the dry season. However, it is noticeable that the ITS sequences of *Kaempferia* are polymorphic implying that there may be more than one copy of the ribosomal gene or low molecular drive to homogenise the gene. This would allow the presence of different copies of the gene and relaxation of the homogenisation process, giving rise to the very variable ITS sequences found among *Kaempferia* species. The big deviation of the ITS mutation rate in *Kaempferia* and *Scaphochlamys* from the mean rate in other genera of the Hedychieae poses a potential problem of long branch attraction when analysed under a parsimony criterion (Felsenstein, 1978). Nonetheless, no implausible groupings in the trees are observed based on morphological grounds. This may be due to the fact that the sampling in this study is quite representative.

Figure 2.7. The strict consensus tree of the two most parsimonious trees resulting from the analysis of the combined data set, ITS and *trn*L-F, of 26 taxa. Upper numbers are bootstrap values of 1000 replicates. Lower numbers are decay indices (CI = 0.641; RI = 0.568; RC = 0.364).

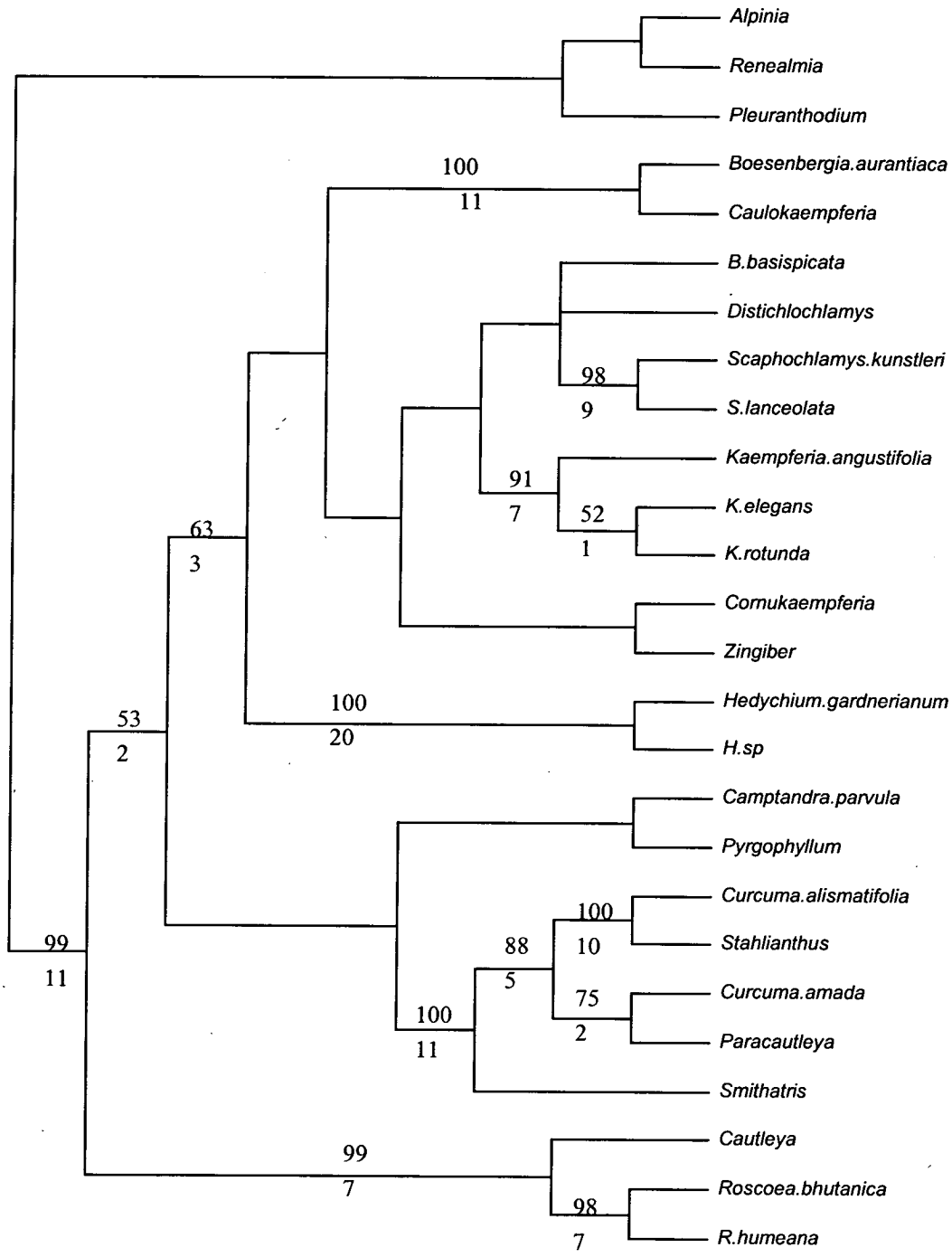
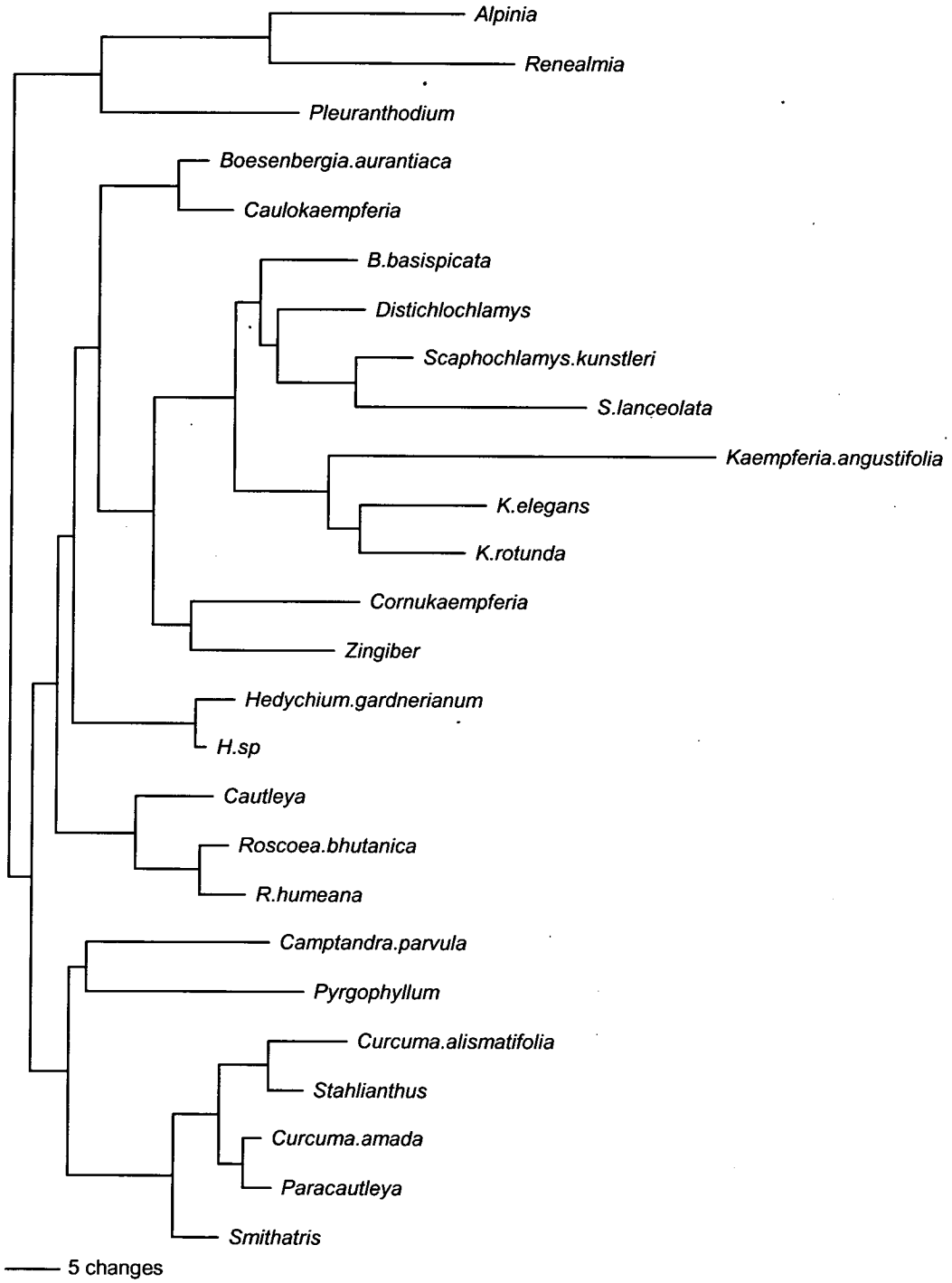


Figure 2.8. The single most parsimonious tree resulting from the successive weighting searches of 26 taxa combined data set, ITS and *trn*L-F, using Rescaled Consistency index.



I believe that ITS analyses give closer trees to the real tree than *trn*L-F analyses because there are more taxa and more informative sites in the ITS data matrix than the *trn*L-F data matrix. In addition, there is no strongly contradictory clade revealed by the analyses of the two genomes. Thus, the following discussion is based mainly on the trees resulting from the ITS while the results of the *trn*L-F analyses are used as supporting evidence.

2.5.2 THE TRIBE ZINGIBEREAE

Molecular analyses of the two data sets, ITS (nrDNA) and *trn*L-F (cpDNA), in this study strongly show that the tribe Zingibereae or *Zingiber* is derived within Hedychieae. It is also suggested that *Zingiber* is a member in the '*Hedychium* clade', particularly in the *Boesenbergia* group. Although the relationships among the *Boesenbergia* group are still not certain according to the present data, *Cornukaempferia* is shown to be the sister group to *Zingiber*. The synapomorphy of these two genera is the large, narrow and curved anther crest that encloses the style and the undivided labellum. Other morphological characters that are distinctive of *Zingiber*, are the fusion of the lateral staminodes with the labellum, forming a 3-lobed structure and the well-developed pseudostem. These characters are also shared with other members of Hedychieae, particularly in the '*Hedychium* clade'. *Boesenbergia longiflora* is an example of the fusion of lateral staminodes with the labellum (Larsen, 1997). The well-developed pseudostem can be found in *Hedychium*. To date, *Zingiber* can be regarded as a derived genus within the Hedychieae and seemingly no tribal rank is needed for the genus. Moreover, cytological evidence does not support the treatment of *Zingiber* as a separate tribe (Beltran and Kam, 1984). Nonetheless, two autapomorphic characters that can be found in *Zingiber* are a pulvinus-like petiole and vascular bundle with collenchymatous sheath. In other Hedychieae observed by Burtt and Olatunji (1972), the vascular bundle has a sclerenchymatous sheath.

2.5.3 *POMMERESCHEA/RHYNCHANTHUS* AND THE TRIBAL POSITIONS

Pommereschea/Rhynchanthus is the sister clade of *Cautleya/Roscoea* in the Hedychieae based on the ITS analyses (Figures 2.3 and 2.5). For years, they have been placed within the Alpinieae based primarily on their lack of lateral staminodes (Smith, 1981; Larsen *et al.*, 1998). Only recently, cladistic analyses of molecular characters have suggested that they belong to the tribe Hedychieae, forming a clade together (Wood *et al.*, 2000). The present analysis, with emphasis on the tribe Hedychieae confirms this hypothesis.

In addition, the chromosome numbers of these two genera do not support a relationship with other members of Alpinieae. All Asian Alpinieae have 2n = 48, or the basic chromosome number is x = 12 (Chen and Huang, 1996, see review). In Alpinieae only *Renealmia*, of Africa and South America has 2n = 22, 44 or the basic chromosome number is x = 11. *Pommereschea lackneri* has 2n = 22, while *Rhynchanthus beesianus* has 2n = 44, so the basic chromosome number of these two genera can be deduced as x = 11. Chen and Huang (1996) proposed to transfer the two genera to Hedychieae based on their chromosome numbers and parallel plane of distichy of leaves to the rhizome. Another evidence of the parallel plane of the leaves to the rhizome is found in *Rhynchanthus longiflorus* (Tripathi and Prakash, 1998). As far as our knowledge of the family goes, all members of Hedychieae possess the parallel plane of the distichy of leaves to the rhizome that is usually associated with the occurrence of petaloid staminodes (Smith, 1980; Larsen *et al.*, 1998; Sakai and Nagamasu, 2000). A study of seed coat in Zingiberaceae (Liao and Wu, 2000) shows that in Alpinieae the endotesta type is sclerenchymatous while that of Globbeae, Zingibereae and Hedychieae (including *Pommereschea*) is parenchymatous. These lines of evidence are in accordance with the molecular analyses and suggest that the proper placement of the two genera be within the tribe Hedychieae. In fact, Smith (1980) already gave a convincing statement of the petaloid staminodes and the classification of Zingiberaceae that the staminodes alone do not justify the placement of the genus, especially whether it be Hedychieae or Alpinieae. She went on to

hypothesise that *Pommereschea*, *Stadiochilus*, *Rhynchanthus* and *Nanochilus*, with the *Hedychium* resemblance and the lack of a close affinity in the Alpinieae, the correct tribe of these genera is Hedychieae.

The lack of petaloid staminodes in *Pommereschea* and *Rhynchanthus* can be seen as a derived character loss. This may be the case in the monotypic Burmese genus, *Stadiochilus* which also lacks petaloid staminodes. *Stadiochilus* resembles *Rhynchanthus* and *Hedychium* in many morphological characters (Smith, 1980). Probably *Stadiochilus* and *Nanochilus* are members of the Hedychieae and close to *Pommereschea* and *Rhynchanthus* or *Hedychium*. The lateral staminodes are found to be postero-lateral members of the outer whorl of the androecium whereas the anterior member of this whorl is always suppressed and absent (Rao *et al.*, 1954; Kirchoff, 1997; 1998). The producing of the lateral staminodes may be controlled by a gene or a set of genes that only a shift of gene control or expression can result in the presence or absence of the staminodes.

On the contrary, interpretation based on recent molecular cladistic analyses (Searle and Hedderson, 2000; Wood *et al.*, 2000; Kress, pers. comm.) points to another fact that non-member of Hedychieae in the family can sometimes have petaloid staminodes. This is shown in *Siphonochilus* and *Siliquamomum*. The two genera both with petaloid staminodes are found to be allies with Alpinieae. In addition, *Siphonochilus* appears to be the sister clade to all the rest of the family (Wilf *et al.*, 2000). Although *Siliquamomum* has petaloid staminodes, its narrow elongated capsule (at least 10x1 cm) is not shared with any other member of the Hedychieae (Smith, 1981). Rather, the elongated capsule is found also in other two genera of Alpinieae: *Burbidgea* and *Siamanthus* (Larsen and Mood, 1998). Its chromosome number, 2n = 48, is an additional evidence suggesting a close relationship to Alpinieae (Wu and Larsen, 2000).

In the case of *Siphonochilus*, its conventional placement in Hedychieae also means that the genus is the only member of Hedychieae found outside Asia, i.e. Africa and Madagascar. *Siphonochilus*'s position as the basal clade on the family

phylogenetic tree (Wilf *et al.*, 2000) appears to be more closely related to the Alpinieae clade than to the Hedychieae clade. A recent discovery of a new genus, *Tamijia*, in Borneo has given additional evidence on morphological evolution of the family (Sakai and Nagamasu, 2000). Although *Tamijia* is placed in Alpinieae based on the transverse plane of the distichy of leaves to the rhizome and other floral characters shared with *Elettaria* and *Elettariopsis*, the genus has distinctively petaloid staminodes as in *Siphonochilus*. Interestingly, *Siphonochilus* and *Tamijia* share also other morphological characters, i.e. stigma not ciliate along the rim, broad and petaloid anther crest and short filament (Sakai and Nagamasu, 2000). However, the phylogenetic relationships of these two genera are not yet known.

The only morphological character of the traditional classification of the family into tribes left intact, or in other words, not showing to date any homoplasy, is the plane of the distichy of the leaves to the rhizome (Smith, 1981). It is transverse in Alpinieae whereas it is parallel in Globbeae and Hedychieae. However, it is not always possible to observe the character as often encountered in very short rhizome species, for instance in, *Kaempferia* and *Siphonochilus*. Finding a tribe for a species in Zingiberaceae, however, is not as hopeless as it may seem. Many workers already use a combination of characters for the critical species, for example: *Siamanthus*, (Larsen and Mood, 1998) and *Tamijia* (Sakai and Nagamasu, 2000).

2.5.4 *CAUTLEYA* AND *ROSCOEA*

The predominantly circum-Himalaya genera *Cautleya* and *Roscoea* are found to form a clade in this study and others (Searle and Hedderson, 2000; Wood *et al.*, 2000). The most detailed study of the phylogeny of *Roscoea* and its relationship to *Cautleya* is that of Ngamriabsakul *et al.* (2000). It confirms the monophyly of *Roscoea* and that *Cautleya* is the sister group to the genus. The synapomorphies of the two genera include the closed leaf-sheath (Spearing, 1977), the versatile anthers and the absence of bracteoles. The closed leaf-sheath and small tuber roots that grow deep in the soil in the two genera can be seen as adaptations to the extreme climate (Chen, 1989).

2.5.5 THE *BOESENBERGIA* GROUP

Boesenbergia, *Caulokaempferia*, *Cornukaempferia*, *Distichochlamys*, *Kaempferia*, *Scaphochlamys* and *Zingiber* are found as a polytomy clade in the ITS strict consensus tree (Figure 2.1), though also with the clade of *Hedychium* species. The morphological similarity of the group is, indeed obvious. Most of them are small plants in habit, the pseudostem being poorly developed, except in *Caulokaempferia*, *Zingiber* and some species of *Boesenbergia*, for example *B. pulcherrima* and *B. acuminata* (Sirirugsa, 1992a). Some species of *Boesenbergia*, *Kaempferia* and *Scaphochlamys* also have unilocular ovary in contrast to the uniform occurrence of trilocular ovary in all other Hedychieae. Nonetheless, floral development study in *Scaphochlamys kunstleri* indicates that the unilocular ovary observed is strictly derived from a trilocular ovary (Kirchoff, 1998).

The maximum likelihood tree suggests that *Haniffia* is the sister group to the clade of '*Boesenbergia* group' (Figure 2.5) while the relationships in ts/tv strict consensus tree are unresolved (Figure 2.4). Then the clade of *Cornukaempferia/Zingiber* is the sister clade to the clade of remaining genera in both trees. *Distichochlamys* is found to be the sister group of *Scaphochlamys*. Whereas *Kaempferia* is the sister clade of *Boesenbergia* in the maximum likelihood tree (Figure 2.5) the clade of *Distichochlamys/Scaphochlamys* is suggested as the sister group to *Boesenbergia* in ts/tv tree (Figure 2.4). *Caulokaempferia* is derived within *Boesenbergia* in both trees.

Distichochlamys is morphologically close to *Scaphochlamys* (Newman, 1995). In each bract of these two genera, there is a cincinnus that has up to 3 flowers (only one flower in *Scaphochlamys biloba*). The character is a synapomorphy of the two genera compared to others in the *Boesenbergia* group: *Boesenbergia*, *Caulokaempferia*, *Kaempferia*, *Cornukaempferia* and *Zingiber*. The bracts in the latter genera only bear a single flower (except in *Caulokaempferia*, *Zingiber clarkei*). *Distichochlamys* is identified as the sister group of *Scaphochlamys* in this study. The same relationship is also found in Searle and Hedderson (2000) where they included

five species of *Scaphochlamys*. Two floral characters and a character of ovary separate the two genera. Morphological differences in the two genera include the arrangement of the bracts and the form of the bracteoles. The bracts in *Distichochlamys* are arranged distichously while the bracts of *Scaphochlamys* appear spiral. The first bracteole in *Distichochlamys* is tubular whereas it is open to the base and often keeled in *Scaphochlamys*. In fact, the form of all bracteoles is as the first one in both genera. Chromosome number as a character in a parsimony analysis prefers the topology of the *Boesenbergia* group in the ts/tv tree (5 steps) over the maximum likelihood tree (6 steps) (see Figure 2.4). The close relationship between *Kaempferia* and *Zingiber* is supported, besides the same basic chromosome number x = 11, by the similar size of the chromosomes (2.4-5.8 μm in *Kaempferia*, the biggest in Tribe Hedychieae; 2.1-4 μm in *Zingiber*) (Beltran and Kam, 1984).

Unlike the RC-weighted tree of ITS data set, all *Boesenbergia* species are found to form a clade in the maximum likelihood tree and the transition/transversion ratio applied tree. The clade is further subdivided into two subclades. These two subclades seem to correspond well with the origin of the species and the chromosome numbers. The subclade of *Boesenbergia aurantiaca* and *B. cordata* has the origin in Borneo and the basic chromosome number is x = 12. In contrast, the subclade of *B. basispicata, B. gelatinosa, B. longiflora* (formerly known as *Curcumorpha*) and *B. aff. longiflora* has the origin on the continental Southeast Asia and the basic chromosome number is x = 10. Note also that in Searle and Hedderson (2000), while the Bornean *Boesenbergia* species were strongly supported as a clade, *B. plicata*, a continent species was actually left out to be the sister taxon to the clade.

Although *B. longiflora* seems to have spirally arranged bracts, a few bracts of the inflorescence appear two-ranked or distichous (Larsen, 1997). Larsen (1997) stated that the flowering pattern (basipetal floral development as opposed to acropetal floral development) of the genus is the most reliable character in *Boesenbergia*. This study confirms that *B. longiflora*, or formerly *Curcumorpha*, is actually a taxon in *Boesenbergia*.

2.5.6 *BOESENBERGIA* AND *CAULOKAEMPFERIA*

Boesenbergia is morphologically a well-defined genus by its distichously arranged bracts and the basipetal (meaning towards base) flowering pattern (Smith, 1987a; Larsen, 1997). In this analysis, *Caulokaempferia thailandica*, from North Thailand is grouped with two *Boesenbergia* species from Borneo. The general synapomorphies of the two genera are distichous bracts, basipetal flowering pattern, undivided labellum and bracteole open to the base. The two genera, however, are stated to have some morphological differences (Larsen and Smith, 1972). The entire labellum of *Caulokaempferia* is never saccate in shape characteristic of *Boesenbergia*. Many *Boesenbergia* are found to have a short tube resulting from the base of labellum combining with the filament while there is no evidence of this in *Caulokaempferia*. There is also a prominent anther crest in *Caulokaempferia* that is rarely found or minute in *Boesenbergia*. The bracts in *Boesenbergia* subtend a single flower, but up to a few flowers are found in *Caulokaempferia*. Nonetheless, these morphological characters may prove to be very variable in both genera when more studies are conducted. More sampling of *Caulokaempferia* species and studies on other lines of evidence are needed before suggesting that *Caulokaempferia* better be treated as a subgroup within *Boesenbergia*, possibly a subgenus.

It is more difficult to ascertain the relationships of the two genera based on the chromosome numbers. Chromosome numbers show that the continental Southeast Asian *Boesenbergia* species in this study (*B. basispicata* and *B. longiflora*) have 2n = 20 while that of *Boesenbergia aurantiaca*, a Bornean species, is 2n = 24, the number also found in *Caulokaempferia alba* and *C. coenobialis* (see Table 2.3). It may suggest that *Caulokaempferia* has a common ancestor with *Boesenbergia* species that have x = 12. However, *Caulokaempferia saxicola* has 2n = 20. Geographically, they are far apart in present distribution ranges. The lowest recorded latitude in distribution range of *Caulokaempferia* species is that of *C. saksuwaniae* in South Thailand (~ 08° 27′ N, Phangnga Province) (Larsen, 1973) and most of the species occur in tropical Himalaya, Southwest China and North Thailand. The Sunda Shelf which refers to continental Southeast Asia and the Malesian archipelago was a

continuous landmass for a long period of time (50 MBP), until about 5 MBP, when the gulf of Thailand was created (Hall, 1998). This would cause the separation of the distribution ranges of many plant species, possibly including the ancestor of *Boesenbergia* and *Caulokaempferia*.

Table 2.3. Recorded chromosome numbers of *Boesenbergia* and *Caulokaempferia* species.

Origin/Species (distribution range)	n	2n	Sources
Continental Southeast Asia *Boesenbergia basispicata* K. Larsen ex Sirirugsa (Peninsular Thailand)	-	20	(Newman, 1988)
B. curtisii (Hook. f.) Schltr. (Malay Peninsular, Java, India)	-	24	(Eksomtramage *et al.*, 1996)
B. fallax Loes. (Yunnan)	-	36	(Chen *et al.*, 1988)
B. longiflora (Wall.) Kuntze (India, Burma, Thailand)	-	20	(Eksomtramage *et al.*, 1996)
B. longipes (Ridl.) Schltr. (Malay Peninsular)	-	20	(Newman, 1988)
B. plicata (Ridl.) Holtt. (Malay Peninsular, India)	10	20	(Beltran and Kam, 1984; Newman, 1988; Eksomtramage *et al.*, 1996)
B. prainiana (Baker) Schltr. (Malay Peninsular)	10	20	(Beltran and Kam, 1984; Eksomtramage *et al.*, 1996)
B. rotunda (L.) Mansf. (cultivated)	-	36	(Chen *et al.*, 1988; Sirirugsa, 1992b)
Borneo *B. aurantiaca* R. M. Smith	-	24	(Newman, 1988)
B. belalongensis A. D. Poulsen	-	24	(Poulsen, 1993)
B. burttiana R. M. Smith	-	24	(Poulsen, 1993)
B. orbiculata R. M. Smith	-	36	(Poulsen, 1993)
B. pulchella (Ridl.) Merr.	-	20	(Newman, 1988)
Continental Southeast Asia *Caulokaempferia alba* K. Larsen & R. M. Smith (N Thailand)	-	24	(Larsen and Smith, 1972)
C. coenobialis (Hance) K. Larsen (China)	12	-	(Chen *et al.*, 1988)
C. saxicola K. Larsen (C Thailand)	-	20	(Larsen, 1964)

2.5.7 THE VERSATILE ANTHER GROUP

Five genera in the Hedychieae possess versatile anthers, namely *Camptandra*, *Cautleya*, *Curcuma*, *Paracautleya* and *Roscoea* (Smith, 1981). It is shown in this study that the character in these genera has probably convergent origins. Versatile anther has lost many times in the '*Curcuma* clade', i.e. that is found in, *Hitchenia*, *Pyrgophyllum* and *Stahlianthus*. On the contrary, the versatile anther in *Cautleya/Roscoea* has arisen independently.

However, there is another genus in the family, *Nanochilus* that possesses versatile anther (Smith, 1980, figure 2). While Larsen *et al.* (1998) have placed the monotypic genus of Sumatra under Alpinieae, *Nanochilus*, besides having the lateral staminodes, shows much resemblance with *Stadiochilus* and *Rhynchanthus* (Smith, 1980). As Smith's hypothesis is supported by the molecular analyses, that *Pommereschea* and *Rhynchanthus* are actually members of Hedychieae, the tribal position of *Nanochilus* may well also be Hedychieae. It is interesting to test the position of *Nanochilus* in the family phylogenetic tree based on Smith's morphological observation. Whether it is within the clade of *Pommereschea/Rhynchanthus/Roscoea/Cautleya* awaits future study.

2.5.8 THE POUCH BEARING GROUP: THE '*CURCUMA* CLADE'

Although, in some species of *Boesenbergia*, *Scaphochlamys* and *Zingiber*, similar water-holding pouches can be formed by the leaf bases or the bracts (Larsen *et al.*, 1998), the bracts are normally free from the axis of the inflorescence and do not fuse in members of the '*Hedychium* clade'. By contrast, the bracts of the majority number of species of the '*Curcuma* clade' are adnate to each other and form pouches. In this clade, it is also noted that the basic chromosome number of the majority of the members is x = 21. While the basic chromosome number is x = 21 or 2n = 42, 63 in most of the *Curcuma* subgenus *Curcuma* species (Chen *et al.*, 1984; Joseph *et al.*, 1999, for example), the numbers in *Curcuma* subgenus *Hitcheniopsis* are variable, e.g. 2n = 20 in *C. harmandii* (Eksomtramage *et al.*, 1996), 2n = 26 in *C. parviflora*

and 2n = 32 in *C. alismatifolia* (Saensouk *et al.*, 1998). *Stahlianthus involucratus* which may be the sister group of *Curcuma* subgenus *Hitcheniopsis* has 2n = 22 (Bisson *et al.*, 1968) and 2n = 33 (Sirirugsa, 1992b). *Hitchenia* (Ramachandran, 1969) and *Pyrgophyllum* (Chen *et al.*, 1988) have the same basic chromosome number of x = 21.

2.5.8.1 MAINLY ONE SINGLE POUCH: *PYRGOPHYLLUM* AND *CAMPTANDRA*

Pyrgophyllum yunnanensis was originally described in *Kaempferia* subgenus *Pyrgophyllum* by Gagnepain (1901). It was then transferred to *Camptandra* subgenus *Pyrgophyllum* following Ridley's establishment of the genus (Gagnepain, 1902). Schumann (1904) subdivided *Camptandra* into two sections: *Eucamptandra* and *Pyrgophyllum*. *P. yunnanensis* was later transferred to *Caulokaempferia* (Larsen and Smith, 1972). The taxon was finally separated out from *Caulokaempferia* to be recognised as a distinct genus *Pyrgophyllum* based on morphological, anatomical and cytological grounds (Wu and Chen, 1989). The molecular findings in this study support the recognition of the genus. *Camptandra* and *Pyrgophyllum* share two main characters of the inflorescence. Firstly a single large concave bract whose base is adnate to the inflorescence axis, is usually present in the inflorescence (or up to 2-3 bracts in succession in both genera). Secondly the main axis of the inflorescence extends beyond the insertion of the uppermost bract into a short slender sterile tip (Larsen and Smith, 1972). In each bract in both genera, there is a cincinnus of flowers. These characters are unique to the two genera. In addition, the labellum of the two genera is divided, in contrast to the entire labellum of *Caulokaempferia*. The leaves of *Camptandra* and *Pyrgophyllum* are also noticeable of differing degrees of unequal division. Interestingly, the high elevation of their habitats, above 1000 metres (except, *Camptandra parvula*) is in common. The morphological differences between the two genera are that there are a lamina-like extension of the bract and a well-developed anther crest in *Pyrgophyllum* whereas there is no epigynous gland in *Camptandra*.

2.5.8.2 MULTIPLE BRACTS OR POUCHES: THE *CURCUMA* COMPLEX

Most inflorescences appear to comprise only a large single bract in *Pyrgophyllum* and *Camptandra*. On the other hand, genera in the *Curcuma* complex, *Curcuma*, *Hitchenia*, *Paracautleya*, *Smithatris* and *Stahlianthus*, bear an inflorescence composed of free or connate bracts. The single involucral bract of *Stahlianthus* can be regarded as two bracts joining together (Wood *et al.*, 2000; Searle and Hedderson, 2000). In addition, *Stahlianthus*, instead of having anther appendages at the base of the thecae rendering the anthers versatile, has a large and entire anther crest resembling that of *Kaempferia* species. Generally, the whole group has bracts forming the inflorescence. In the *Curcuma* complex, either the bracts fuse to one another (*Curcuma* and *Stahlianthus*) or stay separately on the axis of the inflorescence (*Hitchenia*, *Paracautleya* and *Smithatris*).

Hitchenia and *Paracautleya* share a character of free bracts on the inflorescence. The differences between *Hitchenia* and *Paracautleya* are the protruding nature of the corolla tube of *Hitchenia*, tubular bracteole and the non-versatile anther. *Paracautleya* has no bracteole and its anther is versatile. However, the separation of *Hitchenia* from *Curcuma* has never seemed adequate on the grounds of exserted flowers and non-versatile anthers (Wood *et al.*, 2000). Olatunji (1970) came to the conclusion that *Curcuma* and *Hitchenia* are very similar in anatomical characters. The genus *Curcuma* itself, though long thought to comprise two subgroups, namely subgenus *Curcuma* and subgenus *Hitcheniopsis* based largely on the spurs of the anthers, needs to be thoroughly examined to prove that it is not just a degree of extension of the spurs on the anthers for dividing the genus.

All the presently recognised genera in the *Curcuma* complex: *Curcuma*, *Hitchenia*, *Paracautleya*, *Smithatris* and *Stahlianthus*, may be regarded as a single genus, *Curcuma*, though there are some morphological characters supporting the distinction of each taxon. These morphological characters, however, are autapomorphic as suggested by the present data. The acceptance of *Hitchenia*,

Paracautleya, *Smithatris* and *Stahlianthus* each as a distinct genus renders the genus *Curcuma* paraphyletic within which the relationships are more complicated. *Smithatris* may be regarded as a distinct genus, yet more sampling of *Curcuma* species may prove otherwise. If classification should reflect the phylogeny of the members, *Curcuma* is best to be the only recommended generic name for all the genera mentioned above with an adjusted circumscription to cover all various subgroups.

Position of the inflorescence in *Curcuma* subgenus *Curcuma* may be a good taxonomic character. To date, only members of *Curcuma* subgenus *Curcuma*, in the whole '*Curcuma* clade', are found to have a radical inflorescence, i.e. on a leafless side-shoot from the rhizome, with sometime a later terminal one. It is not known yet whether other members of the '*Curcuma* clade' may have a radical inflorescence. The character, however, is found to have only relative value throughout the family, i.e. a species or genus can have both types of the inflorescences. In '*Hedychium* clade', *Haniffia* and *Zingiber* are examples. *Haniffia* produces mainly radical inflorescence, but terminal one is sometimes found (Larsen and Mood, 2000). While most species of *Zingiber* bear radical inflorescences, some species possess both types of the inflorescence e.g. *Z. junceum* and *Z. gramineum* (Theilade, 1999). *B. longiflora*, *B. basispicata* and *B. prainiana* are examples of deviation from the norm of the genus. They have radical inflorescences versus the terminal one of all the rest in the genus (Sirirugsa, 1992a). It has also been observed in some species of terminal inflorescence genera, *Renealmia* and *Alpinia* in Alpinieae (Sakai and Nagamasu, 2000). In Globbeae, *Mantisia*, once thought bearing only radical inflorescence genus, is found to possess also a terminal inflorescence (Burtt and Smith, 1968; Newman and Jong, 1986).

CHAPTER THREE: MOPHOLOGICAL STUDY OF THE VERSATILE ANTHER GROUP IN THE HEDYCHIEAE

3.1 ABSTRACT

Scanning electron micrographs of anther development in *Cautleya spicata* show that the appendages develop from the joint connective tissue where at the one end the anther develops first, well before the other end much later turns into the appendages. The anther with appendages is thus basifixed in mature plant in *Cautleya spicata* while observation of *Curcuma* species reveals that the anther is dorsifixed, and the appendages are derived from the thecae of the anther. Mapping this character of the anther in the five genera that possess versatile anther in Zingiberaceae, namely *Camptandra*, *Cautleya*, *Curcuma*, *Paracaulteya* and *Roscoea*, onto the ITS based phylogeny of the tribe suggests that the dorsifixed versatile anther of the *Curcuma* complex has been lost independently in *Hitchenia* and *Stahlianthus*, while the basifixed versatile anther has arisen independently in *Camptandra* and *Cautleya/Roscoea*.

3.2 INTRODUCTION

The inflorescence of Zingiberaceae plants is usually a thyrse, sometimes with large coloured bracts (Endress, 1996; Larsen *et al.*, 1998). A thyrse is a densely branched inflorescence with the main branch racemose, but the lateral branches cymose (Harris and Harris, 1994). The bracts of the inflorescence subtend a short cincinnus of flowers (Smith, 1981). In some taxa, the cincinni are reduced to a single flower, thus resulting in a raceme or spike (Larsen *et al.*, 1998). The flowers of Zingiberaceae are zygomorphic or monosymmetric and most last only for a day. They are tubular and contain nectar. The most outstanding parts of the flower are petaloid staminodes. Only one stamen is fertile while the remaining five stamens are transformed or absent. The lip of the flower is composed of the two staminodes of

the inner whorl whereas, if present, the two lateral petaloid staminodes are those of the outer whorl. The anterior member of the outer whorl is always suppressed and absent (Kirchoff, 1997, 1998). The two-locular anther is attached to the filament mostly basally and along its whole length. The connective is sometimes produced apically into a structure called the anther crest that may be large and petaloid, as in *Kaempferia*. The connective near the joint of the filament and anther is sometimes also structured into a special base that is termed anther appendages or spurs. These anther appendages give the anther versatility. They can be found in *Cautleya*, *Roscoea* and *Camptandra*. In other cases, the anther is dorsifixed, as found in *Curcuma* and *Paracautleya*, and thus also giving rise to the versatile anthers. Unlike the anther appendages in *Cautleya*, *Roscoea* and *Camptandra*, *Curcuma* and *Paracautleya* have anther appendages that are formed from the bases of the thecae of the anther, not distinctly so from the connective as in the former group. In all, the versatile anther is observed in five genera of the Hedychieae namely: *Cautleya*, *Camptandra*, *Curcuma*, *Paracautleya* and *Roscoea*.

However, note that there is another genus, *Nanochilus*, that possesses pronounced anther appendages (Smith, 1980). The relative position of the anther appendages in *Nanochilus*, however, is in line with the anther and it seems that no such mechanism for pollination is present as is found in the five genera mentioned above (Smith, 1980). The versatile anther in these genera of Zingiberaceae resembles that of the dicotyledon genus *Salvia* (Labiatae). The anther appendages act as a lever when the pollinator enters the floral tube foraging for nectar, its head will pull the anther down bringing it into contact with the pollinator's back. Nonetheless, the success of pollination depends on the pollinator visiting another flower and transfering pollen from its back to the stigma of the second flower. It seems likely that versatile anthers in Zingiberaceae are a mechanism acquired through coevolution of the plants and the pollinators.

Pollination syndrome or pollination system is a term for the descriptive interrelation between flower and pollinator. Five aspects of a flower may be considered for any given type of syndrome. They are floral colour, scent, time of

flowering, structure and rewards. On the pollinator side, three main aspects are involved, namely sensory capacity, behaviour and diet. These factors are interrelated in the success of any pollination. Rewards for pollinators from flowers are mainly of two kinds, nectar or pollen. It is thought that nectar is the main reward in Zingiberaceae. The nectar of Zingiberaceae usually contains a high concentration of sugar, 6-32 % (Kato, 1996), 15.5-35.5 % (Sakai *et al.*, 1999).

Reports of pollinators in the family are scant. Endress (1996) compiled a list of pollinators. These are hawkmoths and butterflies which visited *Hedychium coronarium* and *H. coccineum*, respectively. Large bees (*Euglossine*, *Centris*, and *Bombus*) are found to be the pollinators of the flower of *Alpinia zerumbet* and *Xylocopa* species are the pollinators of *A. malaccensis* and *A. hookeriana*. *Etlingera elatior* is found to be visited by a bird of Nectariniidae and butterflies. *Renealmia* species are pollinated by hummingbirds (Maas, 1977). *Etlingera brevilabris* and *Hornstedtia tomentosa* have *Arachnothera* species (spiderhunters; Nectariniidae) as the pollinator (Kato, 1996). The latter species of Zingiberaceae both have red flowers and basal inflorescences. Small traplining bees (*Nomia* and *Trinchostoma* of Halictidae) are also observed on *Amomum polycarpum* and three species of *Boesenbergia*. Medium sized traplining bees (*Amegilla*) are the pollinators of *Amomum gyrolophos*, *Plagiostachys crocydocalyx* and *Globba brachyanthera* (Kato, 1996). A recent study by Sakai *et al.* (1999) identified three pollination groups, namely spiderhunters (two species), *Amegilla* bees (two species) and halictid bees (four species) as the pollinators of 29 species of Zingiberaceae in Borneo. Eight plant species (all of Alpinieae) were pollinated by spiderhunters, eleven species (two *Costus* species, *Globba brachyanthera*, *Zingiber longipedunculatum* and the rest of Alpinieae) by medium-sized *Amegilla* bees and ten species (three *Boesenbergia* species and the rest of Alpinieae) by small halictid bees. They also found that there were significant correlations between floral morphology and pollination guilds.

Here I attempt a preliminary study of the ontogeny of the two types of anther appendages using Scanning Electron Microscopy and direct observation. The result will be discussed with the phylogenetic findings of the plants. As shown in Chapter

Two, the versatile anther genera in Hedychieae involve three distinct lineages namely the clade of *Cautleya/Roscoea*, the *Curcuma* complex clade and the separate clade of *Camptandra*. *Cautleya spicata* representing the clade of *Cautleya/Roscoea*, was studied for the growth and development of the anther appendages by SEM. *Roscoea* species and *Curcuma* species were observed from fresh material, spirit collection and drawings. *Camptandra* and *Paracautleya* are not in cultivation at Royal Botanic Garden Edinburgh, only spirit material and drawings were available for observation.

3.3 MATERIALS AND METHODS

A plant sample of *Cautleya spicata* was obtained from the cultivated stocks of the Royal Botanic Garden Edinburgh for scanning electron microscope study. The accession number and the voucher specimen number are RBGE 19590760 and C.Ngamriabsakul 30. Living plant observation was also made on *Roscoea* species, *Curcuma* species in the garden in addition to the spirit collection and available drawings. *Camptandra* and *Paracautleya* were studied from spirit material and drawings.

The material of *Cautleya spicata* was fixed in FAA (9 parts 70% ethanol: 0.5 parts glacial acetic acid: 0.5 parts formaldyhyde) overnight. Then the material was passed through a series of an increased concentration ethanol to absolute ethanol and finally acetone to dehydrate it (70% ethanol for 15 minutes, 95% ethanol for 10 minutes, 100% ethanol for 5 minutes and 100% acetone for 5 minutes twice). The material was next dried in an Emitech K850 critical point dryer. Dried parts were mounted with carbon discs on 1.25-cm Agar Scientific aluminium stubs, and further dissected. The stubs were sputter coated with gold-palladium using an Emscope sc500. Specimens were viewed using a Zeiss DSM962 SEM at a working distance of 8-13 mm, and operating at 5 kV. Digital photographs were taken. Phylogenetic findings in the previous chapter were also used as an additional basis for the evolutionary interpretation.

It was intended that a sample taxon of *Roscoea* would be present for the study. Unfortunately, at the time I started to collect the material, it was found that *Roscoea* had already developed inflorescences and flowers. Although no leaf or a lack of the elongation of pseudostem were observed in *Roscoea*, the inflorescences and the flowers were already well advanced in development. The rates of inflorescence and floral development in *Roscoea* species are generally faster than those of *Cautleya* species. Vegetative and reproductive growth seem to be concomitant in *Roscoea* whereas *Cautleya spicata* seems to develop quite a few leaves and a long stem before the maturation of its inflorescence and flowers. Thus only *Cautleya spicata*, whose stages of inflorescence and floral development were available, was suitable for this development study.

3.4 RESULTS

Although it has been observed that five genera in Hedychieae possess versatile anthers (Smith, 1981), the nature of the versatile anthers has not been given much attention or has not been mentioned at all. It is rather interesting why this character of the anther has managed to escape attention as it can be observed by the naked eye or with a hand lens. Light microscopy could be used to confirm the character. In this present study, visual inspection was confirmed by SEM that the type of the connection of the filament and the anther in *Cautleya* is basifixed. Observation in *Camptandra*, *Curcuma*, *Paracautleya* and *Roscoea* revealed that the versatile anther of *Camptandra* and *Roscoea* is basifixed whereas it is dorsifixed in *Curcuma* and *Paracautleya*. Figure 1.3 in Chapter One and Figure 5.4 in Chapter Five can be consulted.

The development of the versatile anthers in *Cautleya spicata* suggests that the appendages were developed at the base of the connection of the anther and the filament. The appendages were observed much later in comparison to the growth of the thecae (Figure 3.1-3.9). The thecae were already big and developed when the

appendages were initiated. Then later, the connection at the thecae side extended pushing the thecae further away from the appendages (Figure 3.10, 3.11). The dried plant material gave also a clear distinction between the thecae and the appendages (Figure 3.7, 3.8 and 3.12). There appeared a groove in the middle on along the appendages whereas the thecae were slightly changed in form.

In *Curcuma* species, the appendages are produced from the base of the thecae compared to the appendages from the connection tissue of *Cautleya* and *Roscoea*. Despite the lack of the appendages in some *Curcuma* species e.g. *C. alismatifolia*, *C. harmandii* and *C. parviflora*, the anthers in these species are still versatile because of the dorsifixed attachment of the anthers to the filament. It should be noted here also that the thecae of some *Curcuma* species are fertile only in part while the thecae in *Cautleya* and *Roscoea* are fertile throughout.

Legend for Figures 3.1-3.12. St denotes stigma while Sty = style, Ant = anther, App = appendages, EpiG = epigynous gland, Stm = staminodes, Ova = ovary.

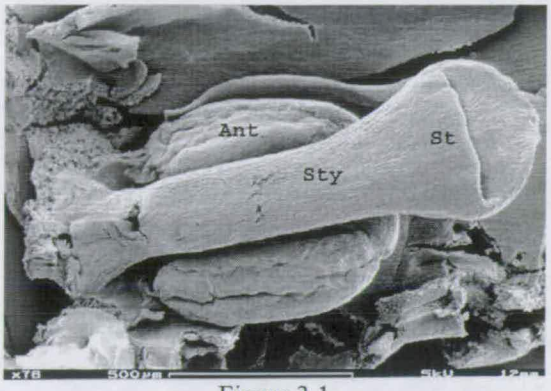


Figure 3.1

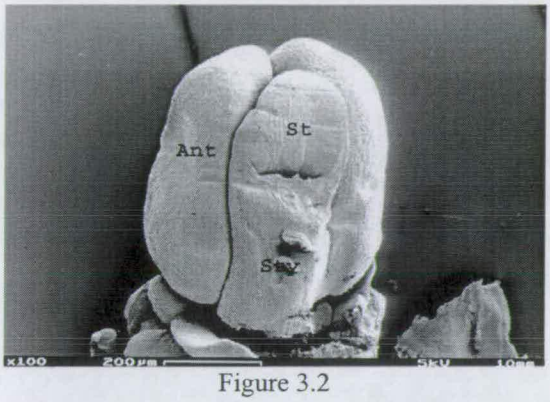


Figure 3.2

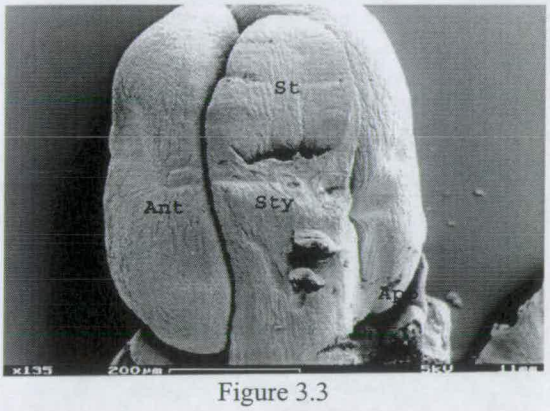


Figure 3.3

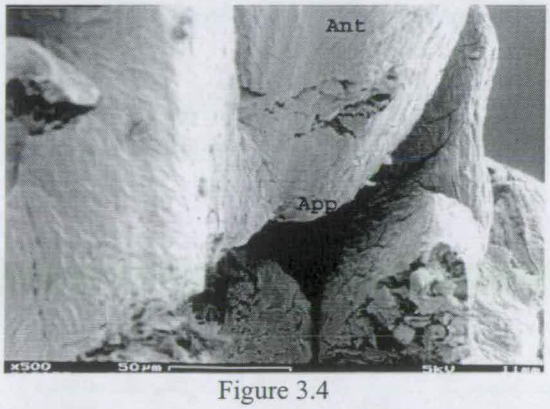


Figure 3.4

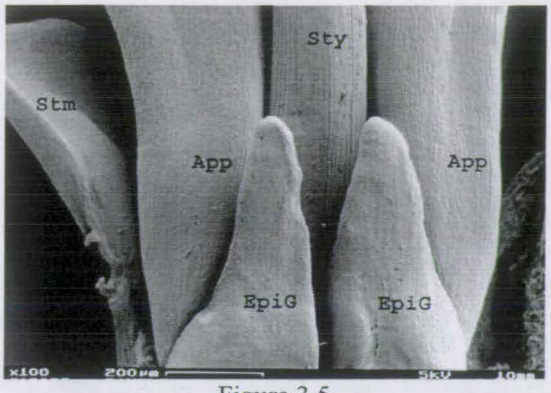


Figure 3.5

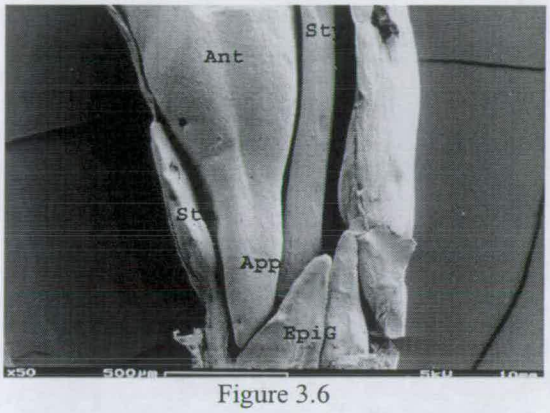


Figure 3.6

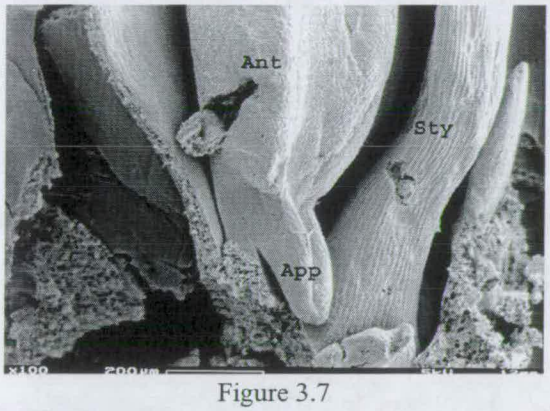


Figure 3.7

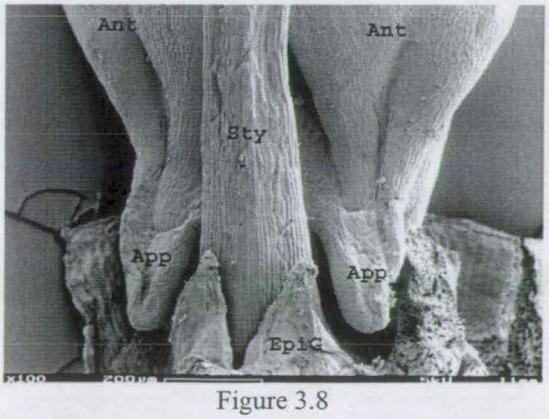


Figure 3.8

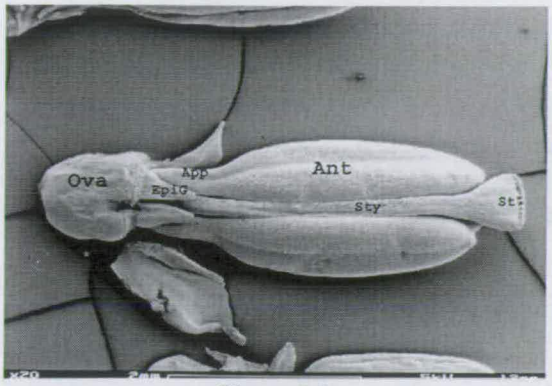


Figure 3.9

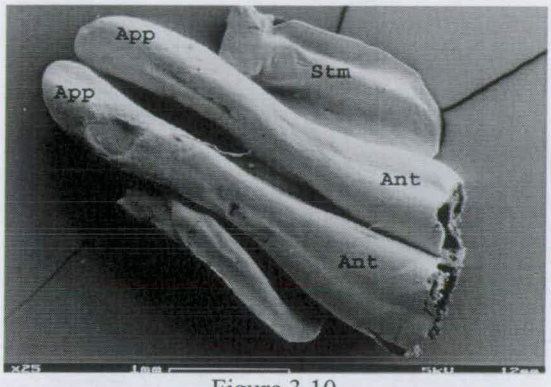


Figure 3.10

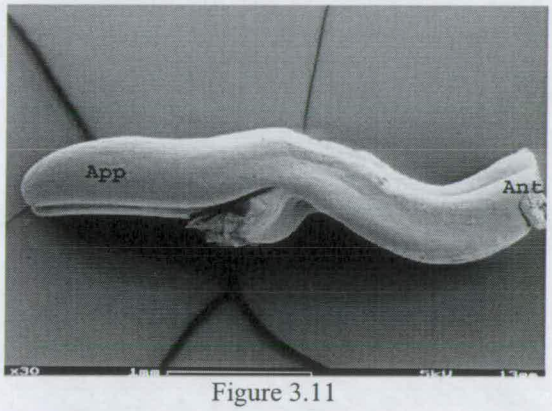


Figure 3.11

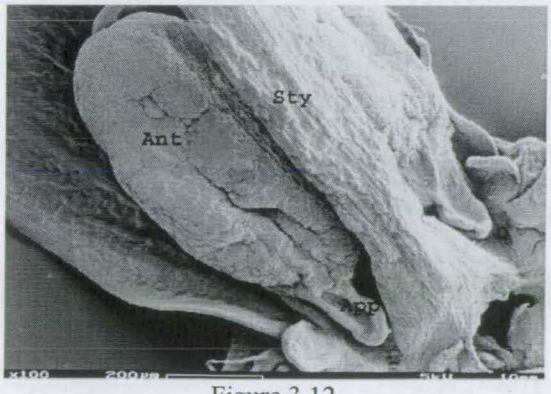


Figure 3.12

3.5 DISCUSSION

Developmental studies of the inflorescence and flower of Zingiberaceae, especially members of Hedychieae, have been carried out by Kirchoff (1997, 1998). The results of these studies reveal that, even in a very short period of time, differences in morphological changes through time (heterochrony) are observed in two closely related species *Hedychium coronarium* and *H. gardnerianum* (Wood *et al.*, 2000). The study of ontogeny, or the series of developmental processes through time, is of pivotal value to the study of phylogeny and systematics. It may demonstrate that slight differences in development can lead to dramatic differences in mature organ structures (divergence). On the other hand, different pathways can also lead to invariant mature floral morphology (convergence).

The phylogenetic findings based on ITS and *trn*L-F sequences presented in the previous chapter, suggested that the basifixed versatile anther in the clade of *Cautleya/Roscoea* and *Camptandra* had been derived independently. The convergence of basifixed versatile anther in the two distinct lineages in Hedychieae may have resulted from adaptation to similar pollination syndromes in different habitats. Floral structure, including the anther and the appendages indicate that the pollinators of *Cautleya*, *Roscoea* and *Camptandra* are bee species which forage for the nectar of the flower. However, there is no report of pollination studies in these genera. The pendulous lip of the flower is thought to be a platform for the pollinator to enter and in so doing the appendages will be pushed and bringing down the anther into contact with the back of the pollinator. Fruits of *Roscoea* are often observed in the Royal Botanic Garden Edinburgh where there is probably no true pollinator of *Roscoea* as in its wild habitat. Garden bees may be pollinating the flowers, leading to the formation of fruits. Because *Roscoea* grows as a clump of individuals, possibly other insects or wind may also play a part in the pollination.

The appendages can grow into varying shapes and sizes in *Curcuma* species (Mangaly and Sabu, 1993; Sirirugsa, 1996) and *Roscoea* species (Cowley, 1982;

Ngamriabsakul *et al.*, 2000). Not only are they useful taxonomically, but also may be a clue suggesting the pollinators of the species.

The molecular phylogenetic findings also suggested that the dorsifixed versatile anther of the *Curcuma* complex has been lost independently in *Hitchenia* and *Stahlianthus*. These may have further obscured the patterns of morphological changes in Hedychieae which otherwise would be more revealing for the students of Zingiberaceae. Holttum (1950) who studied the Zingiberaceae of Malay Peninsula, however, with meticulous conduct, came to notice the differences of the anther appendages in *Camptandra* (and *Roscoea*) and *Curcuma* as well as suggesting the implication of their function as quoted below.

"In *Camptandra* (and apparently also in *Roscoea*) the pollen-sacs are much produced basally into the sterile appendages which are inclined forwards away from the filament, thus giving a versatile character to the anther. In *Curcuma* also the anther is versatile, being attached usually about the middle of the pollen-sacs, and at the same time there is usually a sterile outgrowth from the back of the base of each pollen-sacs. These outgrowths are usually called spurs, and they function in the same way as the basal appendages in *Camptandra* as a mechanism for cross-pollination. A visiting insect pushes against the spurs on entering the flower, and in so doing brings the pollen-sacs into contact with its back." (Holttum, 1950, p. 46-47)

CHAPTER FOUR: PHYLOGENY AND DISJUNCTION IN *ROSCOEA* (ZINGIBERACEAE)

(Materials in this chapter have been published in 'Ngamriabsakul, C., Newman, M.F. and Cronk, Q.C.B. (2000) Phylogeny and disjunction in *Roscoea* (Zingiberaceae). *Edinburgh Journal of Botany*, 57, 39-61.')

4.1 ABSTRACT

A phylogenetic study of *Roscoea* (Zingiberaceae) – a subtropical, high altitude genus of an otherwise tropical, lowland plant family- was undertaken using sequence data from the internal transcribed spacers (ITS) of the nuclear ribosomal DNA (nrDNA). Two species of *Cautleya* and two species of *Curcuma* were used as outgroups. This resulted in an aligned matrix of 436 bp (ITS1, 203 bp; ITS2, 233 bp). Sequence divergence of ITS1 and ITS2 within the ingroup ranged from 0-13.9% and 0-7.6% respectively.

The results suggest that *Roscoea* is monophyletic (BS = 99%; DI = >3) with the genus *Cautleya* as sister group. *Roscoea* itself is divided into two sister clades which correlate with geography: a 'Chinese' clade (BS = 67%; DI = +2) and a 'Himalayan' clade (BS = 59%; DI = +1). These two groups are disjunct across the 'Brahmaputra gap', a region in which no *Roscoea* spp. have been recorded. The only species which occurs on both sides of the Brahmaputra gap is *Roscoea tibetica*. However, the western populations of *Roscoea tibetica* (from Bhutan) show numerous morphological differences. It is therefore possible that Bhutanese *R. tibetica* represents a distinct taxon, possibly more closely allied to Himalayan species.

Seventeen morphological characters of *Roscoea* were analysed cladistically to explore the usefulness of the characters. Morphology was found to contain too much homoplasy to be usefully analysed on its own. The strict consensus tree of a

hundred and sixty-six equally most parsimonious trees of the morphological data analysis of seventeen species was compared with the strict consensus tree of four equally most parsimonious trees of the ITS analysis of the same set of taxa. A combined analysis of the ITS and morphological data of seventeen species gave twenty-six most parsimonious trees. The most parsimonious tree, resulting from rounds of weighting searches of ITS data using mean rescaled consistency index as a weight, was used as a backbone constraint to a later search of morphological data. The evolution of morphological traits in *Roscoea* were then studied on the strict consensus tree of three equally most parsimonious trees, resulting from the backbone constraint search of all *Roscoea* species morphological data.

4.2 INTRODUCTION

Roscoea is one of a group of five genera in *Hedychieae* (Zingiberaceae) which possess versatile anthers. The members of the group are *Camptandra, Cautleya, Curcuma, Paracautleya,* and *Roscoea.* They all occur in tropical regions or low altitude sites, except the truly alpine genus, *Roscoea. Cautleya* and *Roscoea* have sometimes been confused by inexperienced observers. Indeed, these two genera occur in similar habitats, and have a similar habit with orchid-like flowers. Nevertheless, there are many characters separating these genera as pointed out by Cowley (1982), e.g. lateral petals are free from the claw of labellum in *Roscoea* while they are joined to the labellum for about half their length in *Cautleya. Roscoea,* the high altitude genus of Zingiberaceae, comprises 18 species (Cowley, 1982; Cowley and Baker, 1996). It occurs along the Himalaya from the west (Pakistan), to the east (Southwest China), between 1200 and 4880 metres (Cowley, 1982). *Roscoea* grows in drier and cooler environments than other Zingiberaceae, in places that are more exposed to extremes of climate. Unlike some other members of Zingiberaceae, *Roscoea* has closed leaf-sheaths (Spearing, 1977).

The internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA are well established as being useful in systematics (Baldwin, 1992). ITS regions have

rates of substitution that are useful for evaluating generic and species level relationships in plants (Baldwin *et al.*, 1995). Many investigations have been carried out using these regions, for instance in Asteraceae (Baldwin, 1992), Apiaceae (Downie and KatzDownie, 1996), Gesneriaceae (Möller and Cronk, 1997a; Möller and Cronk, 1997b), and Araliaceae (Wen *et al.*, 1998). In Zingiberaceae, there are currently phylogenetic studies going on at Royal Botanic Garden Edinburgh (RBGE) using ITS regions as a source of phylogenetic information. These regions have proved to be useful for studying the evolutionary relationships of the family at the species level (e.g. *Curcuma* spp., Ardiyani, 1997; *Alpinia* spp., Rangsiruji, 1999).

This molecular part of the study aimed to confirm the monophyly of *Roscoea*, and the relationship between *Roscoea* and *Cautleya*, using living collections in the Royal Botanic Gardens (Edinburgh and Kew). It was hoped that, by combining data from ITS regions with distribution records and information on geological history, the study would give insights into the evolution of *Roscoea* and its sister genera: how a tropical plant family has colonized temperate regions.

There is no report of morphological cladistic analysis in Zingiberaceae. Here I explore the usefulness of the morphological data of *Roscoea* in cladistic analysis. The most important step in the cladistic analysis of morphology is the delimitation of characters or character coding. Unlike qualitative or discrete morphological data which is readily accepted and used in the cladistic study, it has been a topic of debate whether quantitative or continuous characters should be used and, if so, how they should be delimited into character states. Almeida and Bisby (1984) presented a simple method for defining multistate characters from measurement data by using bar diagrams. There are various other methods for coding variable morphological features (Archie, 1985; Chappill, 1989; Thiele, 1993) which advocate using continuously varying characters in phylogenetic analysis. This practice seems logically sound, or otherwise illogical to discard characters *a priori*, to employ as much information as possible of the taxa being studied for inferring their phylogeny. However, it is likely that these continuously varying characters are often totally abandoned because there is no really objective means of delimiting states within

them (Pimentel and Riggins, 1987; Stevens, 1991). Besides, it has been shown that differing presentations of these soft or continuous characters have played a major role in individual's perception of the characters, hence one's own delimitation of the character states (Gift and Stevens, 1997) which confound the cladistic study using these characters. Many so-called qualitative or discrete characters are also in fact based on a quantitative phenomenological base and thus face the same problem as quantitative characters (Stevens, 1991).

It is normally assumed that the morphological terminology used in the study of cladistics is uniform among various authors, either in a group of plants or within a particular journal. I have never found any disclosing statement in publications regarding the source of the terminology used which at least to me is a helpful source of reference. I here follow the guidelines from Cowley's works with two additional other published references: Systematics Association Committee for descriptive biological terminology (1962) and Harris and Harris (1994).

4.3 MATERIALS AND METHODS

4.3.1 INGROUP TAXA

Eight species of *Roscoea* cultivated in the RBGE were verified by using the identification key and species descriptions of (Cowley, 1982). Fresh leaf material of one plant representing each accession was taken for a total DNA extraction. Multiple samples of some species were also used. Voucher specimens were prepared, flowers were also preserved in Kew cocktail (water 5.5 units; methanol 3.5 units; glycerol 0.5 units) and both were deposited at the Royal Botanic Garden Edinburgh herbarium (E). DNA extracts of another eight species were taken from living plants at the Royal Botanic Gardens Kew (RBGK), and DNA aliquots were kindly provided by Dr Mark Chase (Table 4.1). These species represent all major areas of distribution of the genus (Figure 4.1). The remaining species that are no longer in cultivation are *Roscoea nepalensis*, *Roscoea forrestii* and *Roscoea debilis*. Attempts were made

several times to acquire DNA of *Roscoea nepalensis* and *Roscoea forrestii* from dried herbarium specimens but, unfortunately, these failed. *Roscoea nepalensis* is an endemic species of central Nepal, near Jumla. It is thought that this species might be allied to others from central Nepal, such as *Roscoea capitata* and *Roscoea ganeshensis*. On the other hand, *Roscoea forrestii* is one of the species that only occurs in southcentral China. Although the DNA sample of *Roscoea debilis* from RBGK was thought to be genuine, it turned out to be a variant of *Roscoea tibetica* after closer examination of the plant. These 15 species comprise most (83%) of the genus (total 18 species).

Table 4.1. Accessions of *Curcuma*, *Cautleya* and *Roscoea* examined for ITS1 and ITS2 sequence variation. [a] Number as shown in Figure 4.1 the distribution map of *Roscoea*. [b] RBGE is Royal Botanic Garden Edinburgh; RBGK is Royal Botanic Gardens Kew. The distribution is given first and the locality of the plant sampled in this study is then given in brackets.

(number)[a] Taxon	Distribution (locality sampled)	Royal Botanic Garden[b] accession number	Genbank accession number	
			ITS1	ITS2
(1) *Curcuma amada* Roxb.	SE. Asia, India (Kerala)	RBGE 1981 0001	AF192218	AF192219
(2) *Curcuma parviflora* Wall.	Burma, Thailand (Sukhothai)	RBGE 1985 1661	AF192220	AF192221
(3) *Cautleya gracilis* (Sm.) Dandy	India, Nepal, Bhutan, China, Burma, Thailand (not known)	RBGE 1982 0532	AF192222	AF192223
(4) *Cautleya spicata* (Sm.) Baker	India, Nepal, Bhutan, China, Burma (Nepal)	E00061739 (RBGE herbarium specimen)	AF192224	AF192225
(5) *Roscoea alpina* Royle	India, Nepal, Tibet, Bhutan (Himachal Pradesh)	RBGE 1986 1108	AF192226	AF192227
(6) *Roscoea auriculata* K. Schum.	India, Nepal, Tibet (not known)	RBGE 1969 9652	AF192228	AF192229
(7) *Roscoea australis* Cowley	Burma (Mount Victoria)	RBGE 1983 0913	AF192230	AF192231
(8) *Roscoea aff. brandisii* (Baker) K. Schum.	India: Meghalaya (not known)	RBGK 1997 5649	AF192232	AF192233
(9) *Roscoea capitata* Sm.	Nepal (Ganesh Himal)	RBGK 1992 2299	AF192234	AF192235
(10) *Roscoea cautleoides* Gagnep.	China (Yunnan)	RBGE 1991 0649	AF192236	AF192237

(11) *Roscoea ganeshensis* Cowley & W. J. Baker	Nepal (Ganesh Himal)	RBGK 1992 2303	AF192238	AF192239
(12) *Roscoea humeana* Balf. f. & W. W. Sm.	China (Yunnan)	RBGE 1985 1160	AF192240	AF192241
(13) *Roscoea praecox* K. Schum.	China (Yunnan)	RBGK 1994 3511	AF192242	AF192243
(14) *Roscoea purpurea* Sm.	India, Nepal, Bhutan (Ganesh Himal)	RBGK 1992 2310	AF192244	AF192245
(15) *Roscoea schneideriana* (Loes.) Cowley	China (Yunnan)	RBGK 1990 3345	AF192246	AF192247
(16) *Roscoea scillifolia* (Gagnep.) Cowley	China (not known)	RBGE 1979 4045	AF192248	AF192249
(17) *Roscoea tibetica* Batalin	Tibet, Bhutan, China (Yunnan)	RBGE 1985 1159	AF192250	AF192251
(18) *Roscoea tumjensis* Cowley	Nepal (Ganesh Himal)	RBGK 1992 2301	AF192252	AF192253
(19) *Rosocea wardii* Cowley	India, Tibet, Burma (Yunnan)	RBGE 1987 1608	AF192254	AF192255

4.3.2 OUTGROUP TAXA

There are currently 5 accepted names in *Cautleya*, though there may be fewer than five species (Kumar, 1994; Larsen *et al.*, 1998). *C. carthcartii* is probably just a robust form of *C. gracilis* while *C. robusta* may be synonymous with *C. spicata* (Smith, 1994). The number of *Curcuma* spp. is less certain, partly because many species of *Curcuma* have long been widely cultivated, causing doubts on the justification of these species. Nonetheless, it is estimated at 50 species worldwide (Larsen *et al.*, 1998). Two species of *Cautleya* (*C. gracilis* and *C. spicata*) and two species of *Curcuma* (*C. amada* and *C. parviflora*) were chosen as the outgroup because living collections of these plants are available at RBGE. As mentioned in the introduction, *Cautleya* is morphologically very similar to *Roscoea*. Its strong affinity with *Roscoea* necessitates further outgroup species which are distantly enough related to allow unequivocal rooting of the phylogenetic tree. *Curcuma* spp. were then chosen on the grounds that they possess versatile anthers, a shared distinct character of five genera in *Hedychieae*, including *Roscoea* and *Cautleya*, but are clearly different from *Roscoea* and *Cautleya* in other characters. An attempt was made to obtain *Paracautleya*'s DNA, a monotypic genus from South India, from a dried herbarium specimen. Unfortunately, this was not successful. Fresh leaf material

of *Camptandra* (4 species, Larsen *et al.*, 1998) from Malaysia (Ibrahim, pers. comm.) was not available, so it was not included in this study.

4.3.3 DNA EXTRACTION

Fresh leaf materials were kept in silica gel-filled plastic bags and stored at 0 °C overnight in a refrigerator before extraction, to destarch the leaf tissue. Starch may interfere with subsequent operations performed using the DNA. Total DNA extraction was carried out using the modified CTAB procedure of Doyle & Doyle (1987) sometimes with further purification using a QIAquick™ PCR purification kit (Qiagen Ltd, Dorking, Surrey, UK). All the samples of the study were obtained from fresh leaves, except *Cautleya spicata* which was taken from a dried herbarium specimen.

Small scale total genomic DNA extraction using CTAB (Doyle and Doyle, 1987) as a detergent gives lower levels of enzyme inhibition than other methods (Scott and Bendich, 1994). The modified protocol is as follows:

A portion of leaf about 1 cm^2 was cut into many small pieces, and put into a 1.5-ml microcentrifuge tube and about 50 mg of purified sand and 200 µl of 2x CTAB extraction buffer were added. The leaf tissue was ground with a plastic pestle until a homogeneous slurry was formed. A further 800 µl 2x CTAB was then added. The contents were mixed gently, and the tube was incubated at 65 °C for 30 minutes. The tube was allowed to cool to ambient temperature before adding 200 µl of wet chloroform (chloroform 24 units; octan-1-ol 1 unit).

The solution was mixed gently 4 or 5 times and centrifuged for 2 minutes at 13000 rpm. The aqueous (upper) phase was removed to a clean tube and re-extracted with 200 µl wet chloroform. Again this was mixed gently to obtain a momentary single phase and centrifuged for 2 minutes at 13000 rpm. In another clean tube with the aqueous phase, 600 µl cold (-20 °C) propan-2-ol was added and the contents were mixed gently to precipitate the nucleic acids. After 10-15 minutes at room

temperature, the pellet of nucleic acids was precipitated by centrifuging for 2 minutes at 13000 rpm. The supernatant was removed and 1 ml of wash buffer (76% ethanol, 10 mM ammonium acetate) was added. The tube was left for at least 30 minutes to remove the 2x CTAB from the pellet. The supernatant was then aspirated as much as possible after the tube was centrifuged for 2 minutes at 13000 rpm. Next, the pellet was dried completely by using an incubator drying oven for 10 minutes at 50 °C. Lastly the pellet was dissolved in 30-50 µl of sterile distilled water to obtain a DNA concentration of 10-30 ng/µl and stored at –20 °C until required.

4.3.4 PCR AMPLIFICATION AND SEQUENCING STRATEGY

Double-stranded DNAs of the complete ITS regions in each genomic DNA were amplified by the polymerase chain reaction method (PCR) using 2 primers, ITS 5P and ITS 8P (Möller and Cronk, 1997a). The primer sequences were (5′ to 3′), ITS 5P = GGA AGG AGA AGT CGT AAC AAG G and ITS 8P = CAC GCT TCT CCA GAC TAC A. The reaction (total volume = 50 µl) contained (in order of addition) 32.5 µl of sterile distilled water, 5.0 µl of 10x Dynazyme™ reaction buffer (1X: 10 mM Tris-HCl, pH 8.8 at 25 °C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100; Finnzymes Oy, Espoo, Finland), 1.0 µl of a mix of each dNTP at 10mM (final concentration 200 µM) (Sigma Chemicals, Poole, Dorset, UK), 5.0 µl of each primer at 10µM (final concentration 1µM) (Oswel DNA Service, Southampton, UK), a 1.0 µl aliquot of unquantified total genomic (template) DNA and 0.5 µl (1U) of Dynazyme™ II thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland). PCR amplification of the ITS region was carried out in 0.2-ml microcentrifuge tubes in a Perkin Elmer thermal cycler. Each PCR reaction cycle proceeded as follows: (1) 1 minute at 94 °C to denature the double-stranded template DNA; (2) 2 minutes at 55 °C to anneal primers to single-stranded template DNA; and (3) 1 minute at 72 °C to extend primers. The first cycle was preceded by an initial denaturation step of 3 minutes at 94 °C. Each set of reactions was monitored by the inclusion of a negative (no template DNA) control. Five microlitres of each double-stranded DNA PCR product were resolved by electrophoresis in 1.5% agarose gel using 1x TBE as the

gel buffer. Successful PCR resulted in a single band of ethidium bromide corporated-DNA viewed under ultraviolet (UV) light corresponding to approximately 700 base pairs. The PCR product was then purified using the QIAquick™ PCR purification kit.

Purified PCR products were sequenced using a dye terminator cycle-sequencing ready-reaction kit (Perkin Elmer, Applied Biosystems Division, Warrington, UK), with AmpliTaq® DNA polymerase, FS, according to the manufacturer's recommendation. Sequencing products were analyzed on an ABI 377 Prism Automatic DNA Sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA), according to the manual supplied. Each reaction was 20 µl in volume and contained (in order of addition) 6 µL of sterile distilled water, 8 µl of Reaction Mix, 1 µl of primer at 3.2 ρM and 5 µl of purified PCR product. For each taxon forward and reverse sequencing reactions were performed for sequence confirmation. Sequencing primers were ITS 5P, ITS 8P and in addition ITS 3P (Möller and Cronk, 1997a) and a modification suitable for Zingiberaceae, ITS 2K (Rangsiruji, 1999) were also used. All primers were synthesized by and purchased from Oswel DNA Service, Southampton, UK. The primer sequences were, ITS 3P = GCA TCG ATG AAG AAC GTA GC and ITS 2K = GGC ACA ACT TGC GTT CAA AG.

4.3.5 SEQUENCE ANALYSIS

All sequences were verified by comparison of their forward and reverse sequences. Sequence boundaries of both internal transcribed spacers of all taxa were determined by comparison with published rDNA sequence data for *Daucus carota*, *Vicia faba* (Yokota *et al.*, 1989) and *Alpinia* spp. (Rangsiruji, 1999). ITS1 and ITS2 of each species are deposited in GenBank (accession numbers AF186195-AF186213, see Table 4.1). Both ITS regions were aligned using the CLUSTAL option in the multiple alignment program Sequence Navigator™ Version 1.0.1 (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA), with minor manual adjustments. The G + C content and transition/transversion ratio were determined by

inspection, using MacClade Version 3.0.1 (Maddison and Maddison, 1992). Sequence divergences among taxa were calculated using the DISTANCE MATRIX option in PAUP Version 3.1.1 (Swofford, 1993).

4.3.6 PHYLOGENETIC ANALYSIS

Phylogenetic trees were generated using PAUP Version 3.1.1 (Swofford, 1993), run on a Power Macintosh 6400/200 computer with character states unordered. The branch-and-bound search option, which guarantees to find the shortest tree or trees, was selected with MULPARS and furthest addition sequence options.

Bootstrap analyses (Felsenstein, 1985) were performed using PAUP, set to branch-and-bound search option and 1000 replicates. Decay indices (DI) (Bremer, 1988; Donoghue *et al.*, 1992) for individual clades were obtained by comparing the strict consensus of all equal-length trees up to four steps longer than the shortest tree, using the branch-and-bound search option. Descriptive statistics reflecting the amount of phylogenetic signal in the parsimony analyses were given by the consistency index (CI) (Kluge and Farris, 1969), retention index (RI) (Farris, 1989), and the resulting rescaled consistency index (RC) (Swofford, 1993). Additionally, the g_1 statistics (Hillis and Huelsenbeck, 1992) were obtained by calculating the tree-length distribution of 10000 random trees using RANDOM TREES under PAUP to assess the amount of phylogenetic signal in the data set, in comparison to random noise.

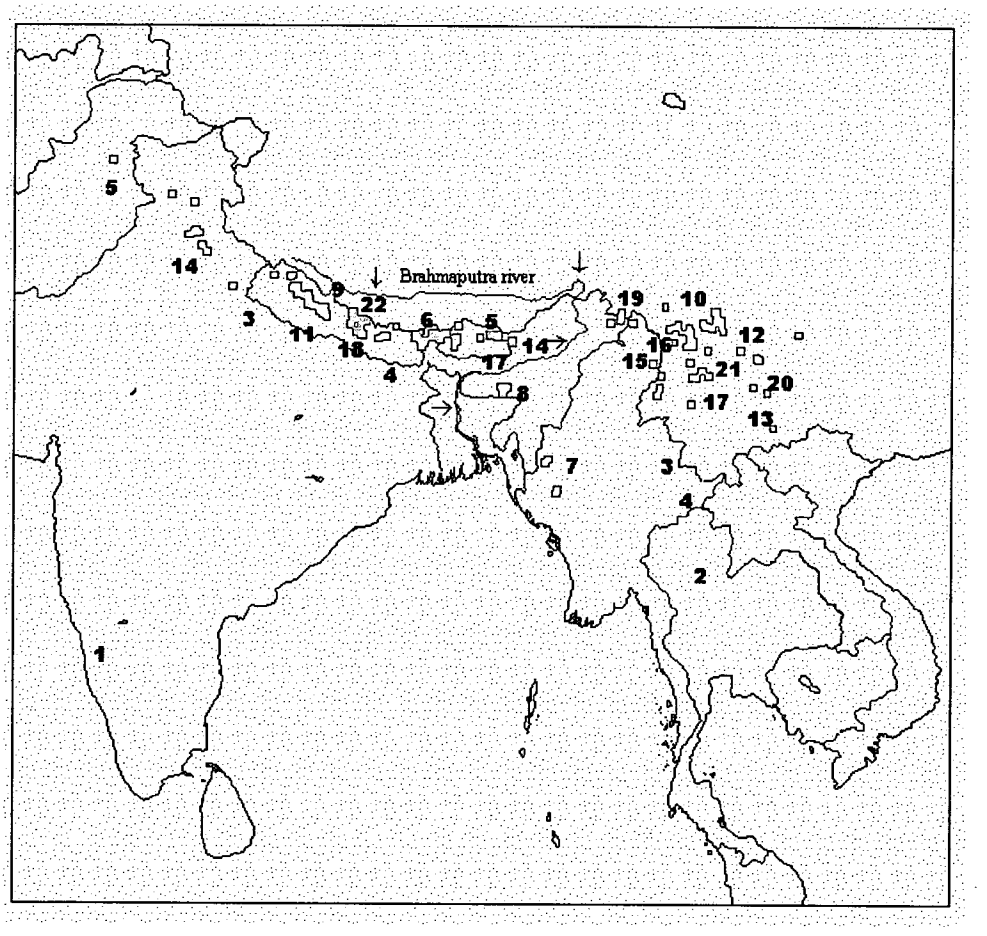
For all analyses of sequence data, gaps (indels) were treated as missing data (Soltis and Kuzoff, 1995; Susanna *et al.*, 1995; Downie and KatzDownie, 1996). Indels were scored as a separate presence/absence character and added to the sequence data matrix (Wojciechowski *et al.*, 1993; Oxelman and Liden, 1995). To investigate the effect of these additional data, a separate analysis without indels scored as characters was undertaken. Character state changes were weighted equally, except for one analysis in which character-state weighted parsimony was

implemented: transversions were weighted over transitions by a factor of 1.7, corresponding to an average of the transition/transversion ratio of ITS1 and ITS2.

4.3.7 MAPPING THE DISTRIBUTION AREA OF *ROSCOEA*

Three hundred and eleven records of locations of *Roscoea* spp. were taken from all the herbarium sheets at E, including extra locations taken from a revision of *Roscoea* (Cowley, 1982). These data were entered into PANDORA (a taxonomic database system by Richard Pankhurst and Martin Pullan, RBGE: www.rbge.org.uk/research/pandora.home) at RBGE (see Appendix Four for all the records). The latitude-longitude format data in PANDORA were then exported and modified for use with MapPad (a freeware program by John Keltner at National Oceanic and Atmospheric Administration (NOAA), Paleoclimatology Program: www.ngdc.noaa.gov/ paleo/softlib.html). The simplified distribution map of *Roscoea* is shown in Figure 4.1 (including the outgroups).

Figure 4.1. Simplified geographical distribution of *Roscoea* species described to date (number 5 to 19 referring to the species listed in Table 4.1, number 20 = *Roscoea debilis*, number 21= *Roscoea forrestii*, number 22 = *Roscoea nepalensis*). The position of the number is an indication of the species. Note: *Roscoea alpina* and *Roscoea purpurea* are widespread along the Himalaya. *Cautleya gracilis* (number 3) and *Cautleya spicata* (number 4) occur both in the Himalaya and China. *Curcuma* species numbers (1 and 2) only indicate the origin of samples. Arrows show the course of the Brahmaputra river.



4.3.8 MORPHOLOGICAL METHODS

I scored seventeen morphological characters for all nineteen species of *Roscoea* (Table 6.1) and one outgroup species, *Cautleya gracilis*. All the characters investigated should cover the full range of variation within species. This was done by checking the literature of the species and genus (Cowley, 1982, 1994, 1997a, 1997b, 1998; Cowley and Baker, 1994, 1996; Cowley and Wilford, 1998, 2000), and by personal observation of herbarium specimens and living collection.

Cautleya gracilis was chosen as the outgroup based on its close affinity with *Roscoea*, as suggested by molecular analyses in the previous chapters and morphology.

4.3.8.1 *ROSCOEA* CHARACTER CODING

The criteria for selecting the following putative synapomorphies are, firstly that they have uniform and constant occurrence or absence among the terminal taxa, implying that they are not likely to be environmentally plastic and that differences among populations are fixed. Therefore they are thought to be intrinsic attributes of the taxa. Secondly, continuously varying or overlapping quantitative characters are here mostly omitted because it is difficult to put them into discrete states. Although I have found colour of flower useful for species identification, it is rather difficult to group the species meaningfully according to all the variation of each species. Thus this character was not used in the analysis. The data matrix is presented in Table 6.1.

1. Bladeless sheathing leaf number

(0) 0-2 (1) 3-5

This number sometimes overlaps among species. However the modal number separates them and is thus used here as the representative number for species. *R. alpina* and *R. australis* have 2-3 sheathing leaves, so are given 01.

2. Leaf number

(0) up to 4 (1) usually 5 or more

3. Leaf form

(0) linear to elliptic (1) elliptic-lanceolate to oblong-ovate (2) ovate to ovate-lanceolate.

4. Leaves forming a tuft or rosette

(0) absent (1) present

5. Leaf bases

(0) shortly petiolate (1) decurrent (2) slightly auriculate or first leaf auriculate (3) all bases auriculate

6. Inflorescence

(0) peduncle showing (1) peduncle not showing

7. Flowering precociously (leaves hardly developed at anthesis)

(0) no (1) yes

R. cautleoides, *R. tumjensis* and *R. alpina* show considerable variation. I scored their character state as 01.

8. Number of flowers open at a time

(0) a few flowers to many (1) usually only one

9. Bract length compared with calyx length

(0) shorter to equal (1) equal to longer

Although the character states seem continuous, the proportional length of the two parts of the flower (bract and calyx) in all species is consistent as observed in two states as above.

10. Bract tip

(0) acuminate (1) acute (2) obtuse-truncate

11. Lowest bract tubular

(0) absent (1) present

12. Dorsal petal form

(0) elliptic (1) broadly elliptic to obovate or obcordate (2) circular

13. Labellum

(0) deflexed (1) not deflexed

14. Labellum claw

(0) present (1) absent

15. Staminode form

(0) obliquely spathulate (1) circular (2) asymmetrically obovate to elliptic

16. Angle of appendages to thecae

(0) 90 (1) 135 (2) 180

17. Appendages tip

(0) pointed (1) obtuse (2) ball

R. schneideriana has an unusual form of appendage tip, globular or ball-shaped. This is clearly an autapomorphy.

Table 4.2. Morphological character coding in *Roscoea*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Cautleya gracilis	01	01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R. alpina	01	0	0	1	2	1	01	1	0	2	0	2	1	1	1	2	0
R. auriculata	0	1	0	0	3	1	0	1	0	1	0	1	0	0	2	0	0
R. australis	01	0	1	1	2	1	0	1	0	2	0	1	0	0	2	1	01
R. bhutanica	1	0	1	1	2	1	0	1	0	1	0	1	0	0	0	0	0
R. brandisii	0	1	0	0	2	1	0	1	1	1	0	0	1	1	2	0	0
R. capitata	0	1	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0
R. cautleoides	1	0	0	0	1	0	01	0	0	1	1	1	0	0	2	1	1
R. debilis	1	0	1	0	0	1	0	0	0	12	1	0	0	0	2	1	01
R. forrestii	1	0	1	1	1	1	0	0	0	2	0	0	0	0	2	1	1
R. ganeshensis	0	1	2	0	2	1	0	1	1	1	1	0	1	0	0	1	0
R. humeana	1	0	2	1	1	1	1	0	0	2	0	1	0	0	2	1	1
R. nepalensis	0	0	2	1	0	1	0	1	0	1	0	0	1	1	1	2	0
R. praecox	1	0	0	1	1	1	1	0	0	1	1	0	0	0	2	01	1
R. purpurea	0	1	0	0	2	1	0	1	1	1	0	0	01	0	0	0	0
R. schneideriana	1	1	0	1	1	0	0	1	1	1	1	0	0	0	2	0	2
R. scillifolia	0	0	0	0	1	0	0	1	1	12	1	0	1	1	2	01	1
R. tibetica	1	0	1	1	2	1	1	1	0	1	0	0	01	0	2	1	1
R. tumjensis	1	0	2	0	3	1	01	1	0	1	0	1	1	0	1	0	0
R. wardii	1	0	0	0	01	0	0	1	0	2	1	1	0	0	2	01	1

4.3.8.2 MORPHOLOGICAL ANALYSIS

To allow comparison and combination of the morphological and molecular characters, the same phylogenetic methods and parameters were used for analyses of both data sets. Phylogenetic trees were generated using PAUP* Version 4.0b4 (Swofford, 1998), run on a Power Mac G4 with character states unordered and initially equally weighted. Polymorphic characters were treated as uncertain. The branch-and-bound search option was selected. Then successive weighting searches were applied, using Rescaled Consistency index (RC, mean value) (Swofford, 1993) until the length of the resulting tree remained unchanged.

Bootstrap analyses (Felsenstein, 1985) were performed using PAUP*, set to branch-and-bound search option and 1000 replicates, or heuristic search with 1000 replicates, tree bisection-reconnection (TBR) branch swapping and random taxon addition sequence. Descriptive statistics reflecting the amount of phylogenetic signal in the parsimony analyses were given by the consistency index (CI) (Kluge and Farris, 1969), retention index (RI) (Farris, 1989), and branch length.

Because there are only 16 *Roscoea* species in ITS data matrix, morphological characters are scored for the same species as in the ITS data matrix. The data matrices are coded **ITS17** and **Mor.17**, respectively. The morphological data matrix of all nineteen species of *Roscoea* is called **Mor.20**. The ITS data matrix of *Roscoea*, *Cautleya* and *Curcuma* species presented in Table 4.1 is here re-analysed with the addition of *R. bhutanica*'s sequences. Thus, there are now 20 species in this new ITS data matrix or **ITS20**. The **ITS17** and **Mor.17** data matrices are combined, **Com.17**, for a simultanious analysis. A constraint analysis, **Con.20**, of **Mor.20** on the most parsimonious tree of Rescaled Consistency Index weighted **ITS20** analysis is also performed. This should also suggest the positions of ITS missing species, i.e. *R. debilis*, *R. forrestii* and *R. nepalensis* in the evolutionary history of the genus. The evolution of characters in *Roscoea* were then studied by using MacClade (Maddison and Maddison, 1992) with ACCTRAN (accelerated transformation) option, presenting the morphological changes on the branching trees.

4.4 RESULTS

4.4.1 SEQUENCE ANALYSIS

Alignment of internal transcribed spacer sequences of the 19 taxa analyzed resulted in a 436-bp long data matrix (Figure 4.2); its characteristics (including G + C content) are given in Table 4.3. The number of unresolved bases ranged from 0 to 5 bp per sequence.

The lengths of ITS1 and ITS2 were, on average, 189.7 and 225.1 bp. Alignment of all taxa required the insertion of 14 gaps of 1 to 5 bp length, 8 in ITS1 and 6 in ITS2 of which 5 and 2, respectively, were potentially informative. The lengths of aligned ITS1 and ITS2 regions were 203 and 233 bp respectively. Of these aligned sites, 296 (67.90%) were constant, 70 (16.05%) had at least two nucleotide states in two or more sequences and were potentially informative phylogenetically, and 70 (16.05%) were autapomorphies (Table 4.3).

Sequence divergence of ITS1 and ITS2 between ingroups ranged from 0-13.9% and from 0-7.6% respectively. Sequence divergence between ingroups and outgroups showed that ITS2 was marginally more variable at 4.5-21.0% than ITS1 at 3.2-19.2%. Pairwise comparison of individual taxa across both spacer regions revealed 0-9.7% sequence divergence within the ingroup and 4.6-18.4% divergence between ingroup and outgroup taxa analyzed (Table 4.3). The maximum sequence variation between *Roscoea* accessions was 9.7% (40 character changes) between *R. praecox* and *R. ganeshensis*. Sequences of *R. cautleoides*, *R. wardii* and *R. humeana* were identical.

Figure 4.2. Sequence data matrix of aligned ITS1 and ITS2 regions of nuclear ribosomal DNA of 19 taxa of Zingiberaceae. Nucleotide sequences are displayed from 5′ to 3′. ITS1 ranges from site 1 to 203 and ITS2 ranges from site 204 to 436. Uncertain nucleotide states are coded according to PAUP conventions (Swofford, 1993): n = A/C/T/G, k = G/T, r = A/G, s = C/G, w = A/T, y = C/T, m = A/C; hypens denote alignment gaps; numbers in italic print above the nucleotide matrix, ranging from 1 to 14, indicate the number and position of alignment gaps; numbers in square brackets at the end of sequences indicate the actual spacer length of the combined region of ITS1 plus ITS2.

```
                          10        20        30        40        50        60        70        80        90
alignment      ITS1       .         .         .         .         .         .         .         .         .
gaps                      .    1 2  .         .         .         .         .    3    4         .         .

Curcuma amada             TTGTTGAGAGAGCATAGCATGGAATGAGGGAGGATTGGGAATGTGTGAACGTGACCCTTTCSTYKKCCCATCCCATGTTGGTGGGCGATT   [90]
Curcuma parviflora        TTGTTGAGAGAGCATA---TAGAATGACGGATGAATGTGAATGTGTGAACGTGACCCTTTCTTT-----AGCCCATGTTGGTGGGCGATT   [82]
Cautleya gracilis         TTGTTGAGAGAGCT-----TAGAATGATGGATGGTTGTGAATGTGTAAATGTGCCCCTTTCCTT-----TCCCCATGTTGGTGGGCGATT   [80]
Cautleya spicata          TTGTTGAGAGAGCA-----TAGAATGATGGATGGTTGTGAATGTGTAAATGTGCCCCTTTCCTT-----TCCCCATGTTGGTGGGCGATT   [80]
R.cautleoides             TTGTTGAGAGAGCA-----CAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.wardii                  TTGTTGAGAGAGCA-----CAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.humeana                 TTGTTGAGAGAGCA-----CAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.praecox                 TTGTTGAGAGAGCA---'---CAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCTTT------CCCCATATAGGTGGGGGAGA   [79]
R.australis               TTGTTGAGAGAGCA-----CAGAATGACGGATGGTTGTGAATGTGTGAATGTGTCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.scillifolia             TTGTTGAGAGAGCA-----TAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.schneideriana           TTGTTGAGAGAGCA-----TAGAATGATGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATATCGGTGGGCGATT   [79]
R.tibetica                TTGTTGAGAGAGCA-----TAGAATGACGGATGGTTGTGTATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.capitata                TTGTTGAGAGAGCA-----TAGAATGATGGATGGTTGTGAATGTGTGAAKGTGCCCSTTTCCTT------CCCCATCCTGGTGGGTGAKT   [79]
R.tumjensis               TTGTTGAGAGAGCA-----AAGAATGATGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTTGGTGGGGGATT   [79]
R.ganeshensis             TTGTTGAGAGAGCA-----TAGAATGATGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTTGGTGGGCGATT   [79]
R.purpurea                TTGTTGAGAGAGCA-----TAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.brandisii               TTGTTGAGAGAGCA-----TAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.auriculata              TTGTTGAGAGAGCA-----TAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
R.alpina                  TTGTTGAGAGAGCA-----TAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTT------CCCCATCTCGGTGGGCGATT   [79]
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                          100       110       120       130       140       150       160       170       180
alignment                 .         .         .         .         .         .         .         .         .
gaps                      .         .         .         .         .       5.          .    67   .    8    .

Curcuma amada             GACCGTAGCTCGGTGCGATCGGCAMTAAGGAACAACGAAATTGGAAGCAGAGGACCCCCTTAGCGTGAGCGGGG--AGCCCAAT-GCGTC  [177]
Curcuma parviflora        GACCGTAGCTCGGTGCGATCGGCACTAAGGAACAATGAACTTGAAAGCAGAGGGCCCC-TTGGCGTGAGCGGGG--AGCACAAT-GCGTC  [168]
Cautleya gracilis         GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTGGCGTGCGCGGGG-GAGCCCAAT-GCGTC  [167]
Cautleya spicata          GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTGGCGTGCGCGGGG-GAGCCCAAT-GCGTC  [167]
R.cautleoides             GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TCGGCGTGCGCGGGGGGAGCCCGAT-GCGTC  [167]
R.wardii                  GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TCGGCGTGCGCGGGGGGAGCCCGAT-GCGTC  [167]
R.humeana                 GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TCGGCGTGCGCGGGGGGAGCCCGAT-GCGTC  [167]
R.praecox                 GACTCTAGCTCAGAGTGGTTGGCATTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TCGGCGTGCGCGGGGGGAGCCCGAT-GCGTT  [167]
R.australis               GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TCGGCGTGCGCGGGGGGAGCCCAAT-GCGTC  [167]
R.scillifolia             GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTGGCGTGCGCTGGG--AGCCCAAT-GCGTC  [165]
R.schneideriana           GACCGTAGCTCAGTGCGATCGGCATTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTCGCGTGCGCGGGGGGAGCCCAAT-GCGTA  [167]
R.tibetica                GACCGTATCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTGCCGTGCGCGGGG--AGCCCGAT-GCGTC  [165]
R.capitata                GACCGTAGGTCAGTGCGATCGGTACTAAGGMACAATGAAMTCAGAAGCAGAGGGCCCC-TTGGTGTKCCCGGGG--AGCCCAAT-GAGTT  [165]
R.tumjensis               GACCGTAGSTCAGTGCGATCAGCASTAAGGAACAATGAACTCAGAAGCAGATGGCCCC-TTGGCGTKCCCGGGG--AGCCTAAT-GAGTT  [165]
R.ganeshensis             GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCAGAAGCAGAGGGCCCC-TTGGCATTCCCGRGA--AGCCCAATTGAGTY  [166]
R.purpurea                GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTTGCGTTCCCGACG--AGCCCAATTGAGTT  [166]
R.brandisii               GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTGGCGTGCCCGGGG--AGCCCAAT-GCGTC  [165]
R.auriculata              GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCC-TTGGCGTGCCCGGGG--AGCCCAAT-GCGTC  [165]
R.alpina                  GACCGTAGCTCAGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCGGAGGGCCCC-TCGGCGTGCCCGGGG--AGCCCAAT-GCGTC  [165]
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alignment                 .         .    ITS2 .         .         .         .         .         .         .
gaps                      .         .         .         .         .         .         .         .         .

Curcuma amada             GGAGATTCTTCGGAATCAAATGAATCGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCGCGGAAGTTGGCCCCGTGTGCCCTCGGGCA [267]
Curcuma parviflora        GAAGATTCTTCGGAATCAAATGAATTGTCGCTTATGCTTCATGCTTTGTTGGCATTGAGTGCGGAAATTGGCCCCGTGTGCCCTCGGGCA [258]
Cautleya gracilis         GGAGATTTTTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTATTGGCATCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [257]
Cautleya spicata          GGAGATTTTTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTATTGGCATCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [257]
R.cautleoides             GGAGATATCTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [257]
R.wardii                  GGAGATATCTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [257]
R.humeana                 GGAGATATCTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [257]
R.praecox                 GGAGATATCTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [257]
R.australis               GGAGATATCTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [257]
R.scillifolia             GGAGATTTCTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [255]
R.schneideriana           AGAGATTTCTCGAAATCAAATGAATCGTCGCTTTAGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [257]
R.tibetica                GGAGATATCTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [255]
R.capitata                GGAGATTTGTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGCCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGACA [255]
R.tumjensis               GGAGATTTCTCAAAATCAAATGAATCGTCGCTTTCGCTCCATGCGTTGCTGGTGTCGAGCGCGAAAATTGGCCTCGTGTGTCCTCGGGCA [255]
R.ganeshensis             GGAGATTTCTCGAAATCAGATGAATCGTCACTTTTGCTCCATGCGTTGCTGGAGTCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [256]
R.purpurea                GGAGATTTGTCGAAATGAGATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [256]
R.brandisii               GGAGATTTCTCGAAATCAAATGAATCGTCACTTTAGCTCCATGCGTTGCTGGTGTCCAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [255]
R.auriculata              GGAGATTTCTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCATTGCTGGTGTCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [255]
R.alpina                  GGAGATTTCTCGAAATCAAATGAATCGTCGCTTTCGCTCCATGCATTGCTGGTGTCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCA [255]
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                          280       290       300       310       320       330       340       350       360
alignment                 .         .         .         .         .         .         .         .         .
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Curcuma amada             CAGTCGGTCGAAGAGTGGGTAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT   [357]
Curcuma parviflora        TAGTCGGTCGAAGAGTGGGTACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT   [346]
Cautleya gracilis         CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [345]
Cautleya spicata          CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [345]
R.cautleoides             CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGCCGGGCACGACGGGTGTTGGTCGCCTTGAGCGAGAACAGAACGTCGT--CCCCGTCGC   [345]
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R.humeana                 CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGCCGGGCACGACGGGTGTTGGTCGCCTTGAGCGAGAACAGAACGTCGT--CCCCGTCGC   [345]
R.praecox                 CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGCCGGGCACGACGGGTGTTGGTCGCCTTGAGCGAGAACAGAACGTCGT--CCCCGTCGC   [345]
R.australis               CAGTCGGTTGAAGAGCGGGTAGTCCGCAGTCGCCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGC   [345]
R.scillifolia             CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGCCGGGCACGACGGGTGTTGGTCGCCTTGAGCGAGAACAGAACGTCGT--CCCCGTCGC   [343]
R.schneideriana           CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGC   [345]
R.tibetica                CAGTCGGTTGAAGAGTGGGCAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [343]
R.capitata                CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [343]
R.tumjensis               CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [343]
R.ganeshensis             CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAACACAGAACGTCGT--CCCCGTCGT   [344]
R.purpurea                CAGTCGGTTGAAGAGTGGGTAGTCCGAAATCGTCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAAAACAGAACGTCGT--CCCCGTCGT   [344]
R.brandisii               CAGTCGGTTGAAGAGTGGGTAGTCCGAACTCGTCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [343]
R.auriculata              CAGTCGGTTGAAGAGTGGGTAGTCCGAAGTCGTCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [343]
R.alpina                  CAGTCGGTTGAAGAGTGGGTAGTCCGAAGTCATCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAAAACGTCGT--CCCCGTCGT   [343]
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gaps                      01         .          .   2      .          .          .          3 4

Curcuma amada             TTTGGGATGAGTCCTCCAGAGACCCTGTGTGATGATTGCGGAGTCGCGTGAAAGCGCCGCGTCAATCA---TTTGC    [430]
Curcuma parviflora        TTTGGGATGAGTCCTCAAGAGACCCTATGTGAT---TGCAGAGTCGGACGAAAGCGATGTGTCAATCATCATTTGC    [419]
Cautleya gracilis         TTTGGGAAT-GTCCTCAAGAGACCCTGTGTGAT---TGTGATGTCGTGTGAAAGTGCCGTGTCCATCA--AATTGT    [415]
Cautleya spicata          TTTGGGAAT-GTCCTCAAGAGACCCTGTGTGAT---TGTGATGTCGTGTGAAAGTGCCGTGTCCATCA--AATTGT    [415]
R.cautleoides             TTTAGGATT-GTCCTCAAGAGACCCCGTGTGAT---TGTGACGTCGTGCGAAAGTGCCGTGTCCATCA--AATTGT    [415]
R.wardii                  TTTAGGATT-GTCCTCAAGAGACCCCGTGTGAT---TGTGACGTCGTGCGAAAGTGCCGTGTCCATCA--AATTGT    [415]
R.humeana                 TTTAGGATT-GTCCTCAAGAGACCCCGTGTGAT---TGTGACGTCGTGCGAAAGTGCCGTGTCCATCA--AATTGT    [415]
R.praecox                 TTTAGGATT-GTCCTCAAGAGACCCCGTGTGAT---TGTGACGTCGTGCGAAAGTGCCGTGTCCATCA--AATTGT    [415]
R.australis               TTTAGGATT-GTCCTCAAGAGACCCCGTGTGAT---CGTGACGTCGTGCGAAAGTGCCGCGTCCATCA--AATTGT    [415]
R.scillifolia             TTTAGGATT-GTCCTCAAGAGACCCCGTGTGAT---TGCGACGTCGTGCGAAAGTGCCGCGTCCATCA--AATTGT    [413]
R.schneideriana           TTTAGGATT-GTCCTCAAGAGACCCCGTGTGAT---TGTGATGTCGTGCGAAAGTGCCGTGTCCATCA--AATTGT    [415]
R.tibetica                TTTAGGATT-GTCCTCAAGAGACCCCGTGTGAT---CGTGATGTGGTGCGAAAGTGCCGTGTCCATCA--ATTTGT    [413]
R.capitata                TTTAGGATT--TCCTCAAGAGACCCTGTGTGAT---TGTGATATCGTGCGAAAGTGCCGTGTCCATCA--AATTGT    [412]
R.tumjensis               TTTAGGATT--TCCTCAAGAGACCCCGTGTGAT---TGTGATATCGTGTGAAAGTGCCGTGTCCATCA--AATTGT    [412]
R.ganeshensis             ATTACGATT--TCCTCAAGAGACCCTGTGTGAT---TGTGATATCGTGTGAAAGTGCCGTGTCCATCA--AATTGT    [413]
R.purpurea                TTTACGATT--TCCTCAAGAGACCCCGTGTGAT---TGTGATGTGGTGTGAAAGTGCCGTGTCCATCA--AATTGT    [413]
R.brandisii               TTTACGATT--TCCTCAAGAGACCCCGTGTGAT---TGTGATGTCGTGTGAAAGTGCCGTGTCCATCA--AATTGT    [412]
R.auriculata              TTTAGGATT--TCCTCAAGAGACCCCGTGTGAT---TGTGATGCGGTGTGAAAGCCCCGTGTCCATCA--AATTGT    [412]
R.alpina                  TTTAGGATT--TCCTCAAGAGACCCCGTGTGAT---TGTGATGTCGTGCGAAAGTGCCGTGTCCATCA--AATTGT    [412]
```

Table 4.3. Sequence characteristics of ITS1 and ITS2 regions of 19 taxa of Zingiberaceae.

Parameter	ITS1	ITS2	ITS1 and ITS2
Length range (total) (bp)	188-200	224-230	412-430
Length mean (total) (bp)	189.74	225.05	414.79
Length range (ingroup) (bp)	188-190	224-225	412-415
Length mean (ingroup) (bp)	188.93	224.53	413.47
Length range (outgroup) (bp)	190-200	225-230	415-430
Length mean (outgroup) (bp)	192.75	227	419.75
Aligned length (bp)	203	233	436
G + C content range (%)	47.34-55.79	53.07-59.56	51.55-57.35
G + C content mean (%)	52.43	56.64	54.73
Sequence divergence (ingroup) (%)	0-13.86	0-7.58	0-9.75
Sequence divergence (in/outgroup) (%)	3.21-19.22	4.46-21	4.58-18.47
Number of indels (ingroup)	3	1	4
Number of indels (total)	8	6	14
Size of indel (ingroup)	1-2	1	1-2
Size of indel (total)	1-5	1-3	1-5
Number of variable sites (%)	67(33)	73(31.33)	140(32.10)
Number of constant sites (%)	136(67)	160(68.67)	296(67.90)
Number of informative site (%)	27(13.30)	43(18.45)	70(16.05)
Number of autapomorphic sites (%)	40(19.70)	30(12.88)	70(16.05)
Transitions (minimum)	50	71	121
Tranversions (minimum)	40	32	72
Transitions/tranversions	1.25	2.21	1.68
Skewness of tree-length distribution (g_1 value for 10000 random trees)	-1.022	-1.663	-1.509
Average number of steps per character	0.448	0.446	0.447

4.4.2 PHYLOGENETIC ANALYSIS

Parsimony analysis of aligned ITS sequences using equally weighted character states yielded five most parsimonious trees when coded indels were added to the data matrix. The strict consensus tree was computed (Figure 4.3), with 213 steps when all uninformative characters were included, 136 steps with autapomorphies excluded, with CIs of 0.812 and 0.706, respectively. These were higher than the expected empirical values of 0.559 calculated from 60 phylogenetic studies for 19 taxa (Sanderson and Donoghue, 1989). The RI was 0.793, and thus the RC was 0.644 with, and 0.560 without, uninformative characters.

The average number of nucleotide substitutions per character was low, with 0.447 indicating a low saturation of base substitution. The homoplasy index (HI) of the present data matrix was low (HI = 0.188).

Thirty three character changes separated the *Cautleya/Roscoea* clade from *Curcuma* spp. (BS = 99, DI = >3). The ingroup *Roscoea* spp. was separated from *Cautleya* spp. by nine character changes (one indel) (BS = 99, DI = >3). *Roscoea* spp. formed 2 distinct groups, a Chinese clade comprising eight species from China and Burma, separated by two character changes (BS = 67, DI = 2), and a Himalayan clade with seven species from the Himalaya, separated by four character changes (one indel) (BS = 59, DI = 1) (Figure 4.3). In the Chinese clade, the relationship of species was fairly well resolved, with bootstrap values ranging from 59 to 75% and decay index values of 1 to 2. However, the relationship of a terminal branch in this Chinese group was unresolved due to a lack of sequence variation, forming a four species polytomy (*R. cautleoides*, *R. wardii*, *R. humeana*, *R. praecox*) separated from *R. australis* by two character changes (BS = 75, DI = 1). The Himalayan clade contained two subclades, (1) *R. capitata*, *R. tumjensis* and *R. ganeshensis* with seven character changes (BS = 70, DI = 1), (2) *R. auriculata* and *R. alpina* by one character change (BS = 53, DI = 1) with *R. purpurea* and *R. brandisii* unresolved. Of the seven potentially informative indels, four

were congruent with the tree topology of the strict consensus tree.

Exclusion of the coded indels from the combined ITS1 and ITS2 data matrix resulted in eight most parsimonious trees of 195 steps (125 steps excluding uninformative characters; CI = 0.815; RI = 0.787; RC = 0.642). The strict consensus tree differed from the strict consensus tree obtained with the addition of coded indels only in the collapse of the Himalayan clade grouping all seven species from the Himalaya while the two subclades within remained.

The transition/transversion ratio was 1.25 for ITS1 and 2.21 for ITS2, and 1.68 for the combined data matrix. Altering the character weights to 1.7:1 to accommodate this ratio and reanalyzing the data (coded indels excluded) in a parsimony analysis gave a single most parsimonious tree (Figure 4.4).

Figure 4.3. Strict consensus tree based on five most parsimonious trees for 15 *Roscoea*, two *Cautleya* and two *Curcuma* taxa of 213 steps length based on parsimony analysis of the combined ITS1 and ITS2 sequence data plus the coded indels. Upper numbers are bootstrap values of 1000 replicates. Lower (boldface) numbers are decay indices (CI=0.812; RI=0.793; RC=0.644).

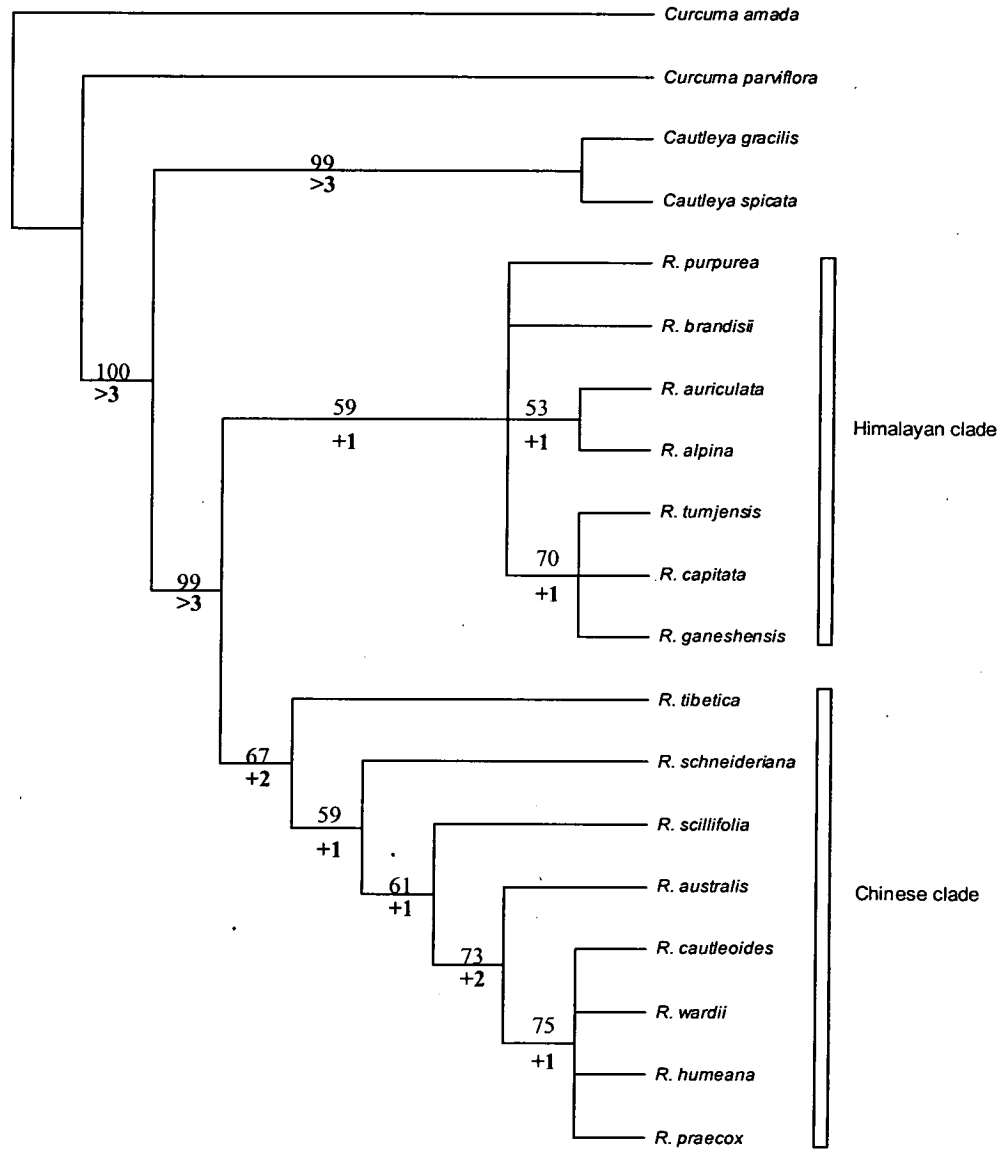
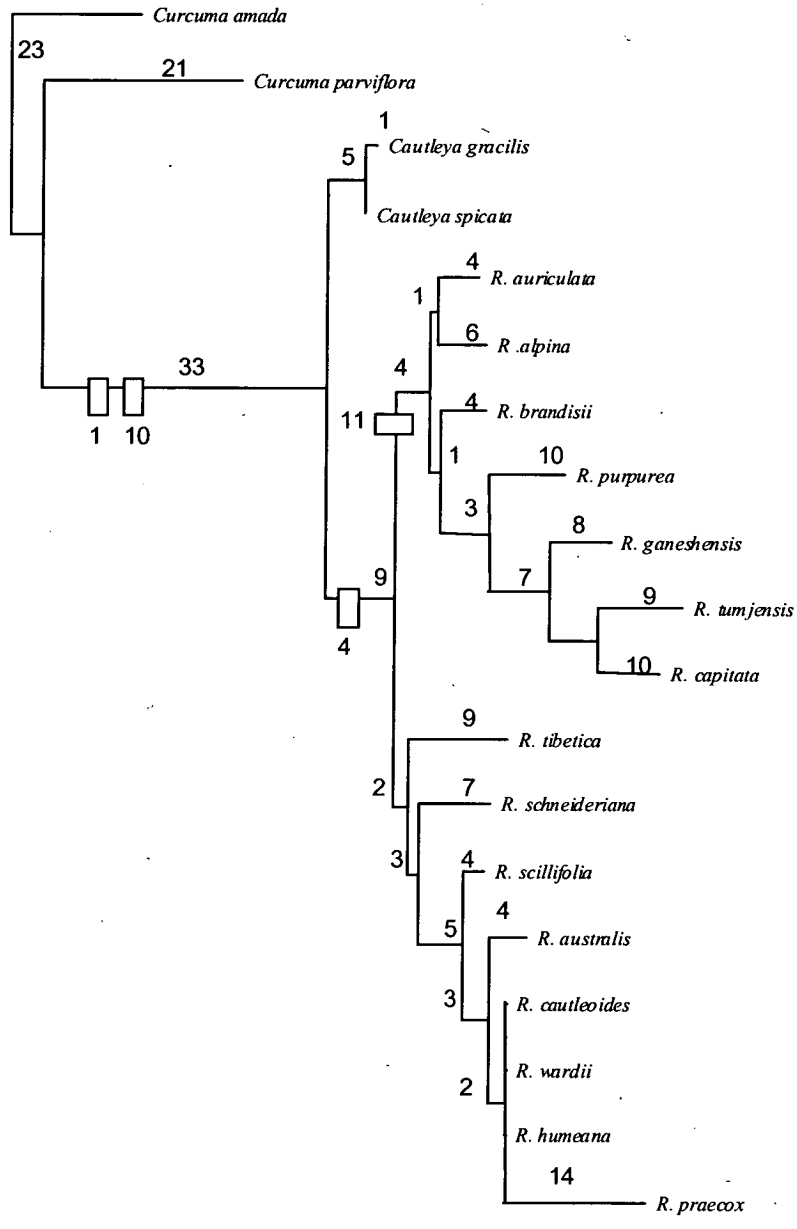


Figure 4.4. The single most parsimonious tree obtained from the weighting of transitions and tranversions (one of the five trees found in the unweighted search). Numbers above branches indicate number of character changes shared amongst taxa (branch length, from unweighted analysis), including autapomorphic changes. Bars and numbers associated indicate the indels and their positions in the sequences (see Figure 4.2).



4.4.3 MORPHOLOGICAL RESULTS

The results of parsimony analyses of all different sets of species and types of data are summarised in Table 4.4. Cladistic analysis of seventeen morphological characters (all potential synapomorphies) of sixteen *Roscoea* species and *Cautleya gracilis* as the outgroup (**Mor.17**) generated 166 equally most parsimonious trees (length = 56; consistency index (CI) = 0.446; retention index (RI) = 0.586). The strict consensus tree (Figure 4.5) preserved five clades, but with no support (all clades had bootstrap values less than 50%). The analysis of these seventeen morphological characters in all *Roscoea* species (nineteen species) and the outgroup (**Mor.20**) yielded six equally most parsimonious trees (length = 62; CI = 0.403; RI = 0.606). The strict consensus tree (Figure 4.6) had somewhat better resolved clades than the strict consensus tree of **Mor.17**, though only one branch had bootstrap value higher than 50% (the clade of *R. alpina* and *R. nepalensis*, with 75% bootstrap value).

The analysis of ITS sequences matrix of the same set of species as in morphology (**ITS17**) gave four equally most parsimonious trees (length = 138) with significantly higher values of CI = 0.753 and RI = 0.725 than in the morphological analysis. The strict consensus tree was presented in Figure 4.7. The re-analysis of the nineteen species ITS data matrix in as presented in Figure 4.3-4.4 with a newly acquired ITS sequences of *R. bhutanica* (**ITS20**) produced fourteen equally most parsimonious trees (length = 217, CI = 0.801, RI = 0.785). The strict consensus tree (Figure 4.8) was very similar to the strict consensus tree of the ITS analysis of nineteen species data matrix (Figure 4.3), only one exception in the Himalayan clade. The subclade of *R. alpina* and *R. auriculata* collapsed while the subclade of *R. tumjensis*, *R. capitata* and *R. ganeshensis* was retained. The support of bootstrap values in this strict consensus tree was also in the same range (57-100%) with the tree in the previous analysis.

Table 4.4 shows the results of cladistic analyses in morphological part of this study from *Roscoea* data matrices.

	Mor.17	**Mor.20**	**ITS17**	**ITS20**	**Com.17**	**Con.20**
Number of steps	56	62	138	217	208	77
Number of trees	166	6	4	14	26	3
CI (Consistency Index)	0.446	0.403	0.753	0.801	0.620	0.324
HI (Homoplasy Index)	0.553	0.596	0.246	0.198	0.379	0.675
CI (excluding uninformative sites)	-	-	0.580	0.686	0.476	-
HI (excluding uninformative sites)	-	-	0.419	0.313	0.523	-
RI (Retention Index)	0.586	0.606	0.725	0.785	0.605	0.446
RC (Rescaled Consistency index)	0.261	0.244	0.547	0.629	0.375	0.145
Number of informative sites	17	17	39	79	56	17

The simultaneous analysis of both morphological and ITS data sets of seventeen species (**Com.17**) yielded twenty-six equally most parsimonious trees (length = 208; CI = 0.620; RI = 0.605). The strict consensus tree (Figure 4.10) differed from the strict consensus tree of the **ITS20** analysis in that the only subclades recognised within *Roscoea* were the clade of *R. capitata*, R. *ganeshensis* and *R. tumjensis* and the clade of five terminal species of the Chinese clade (*R. australis*, *R. cautleoides*, *R. humeana*, *R. praecox* and *R. wardii*). These two clades had bootstrap values of 50 and 63, respectively. In addition, the clade containing all members of the Chinese clade was retrieved with the bootstrap value at 59, though it collapsed in the strict consensus tree.

When Rescaled Consistency index (RC, mean value) was applied to rounds of successive weighting searches of the **ITS20** matrix, a single most parsimonious tree was retrieved. The tree is shown in Figure 4.9 (CI = 0.947; RI = 0.937; RC = 0.888) and it was used as a constraint tree to a later morphological analysis. The constraint analysis of morphological data of all *Roscoea* species (**Mor.20**) by the single most parsimonious tree of the RC weighted tree as a backbone constraint generated three equally most parsimonious trees with the length of 77 steps (Figure 4.11). The descriptive statistics of the phylogenetic signals of the tree were low, i.e. CI = 0.324, RI = 0.446 and RC = 0.145.

Figure 4.5. The strict consensus of 166 trees from a morphological analysis of sixteen *Roscoea* species and the outgroup (**Mor.17**). All clades have less than 50 per cent bootstrap value and Decay Index value is one. Branch length is given under the clades.

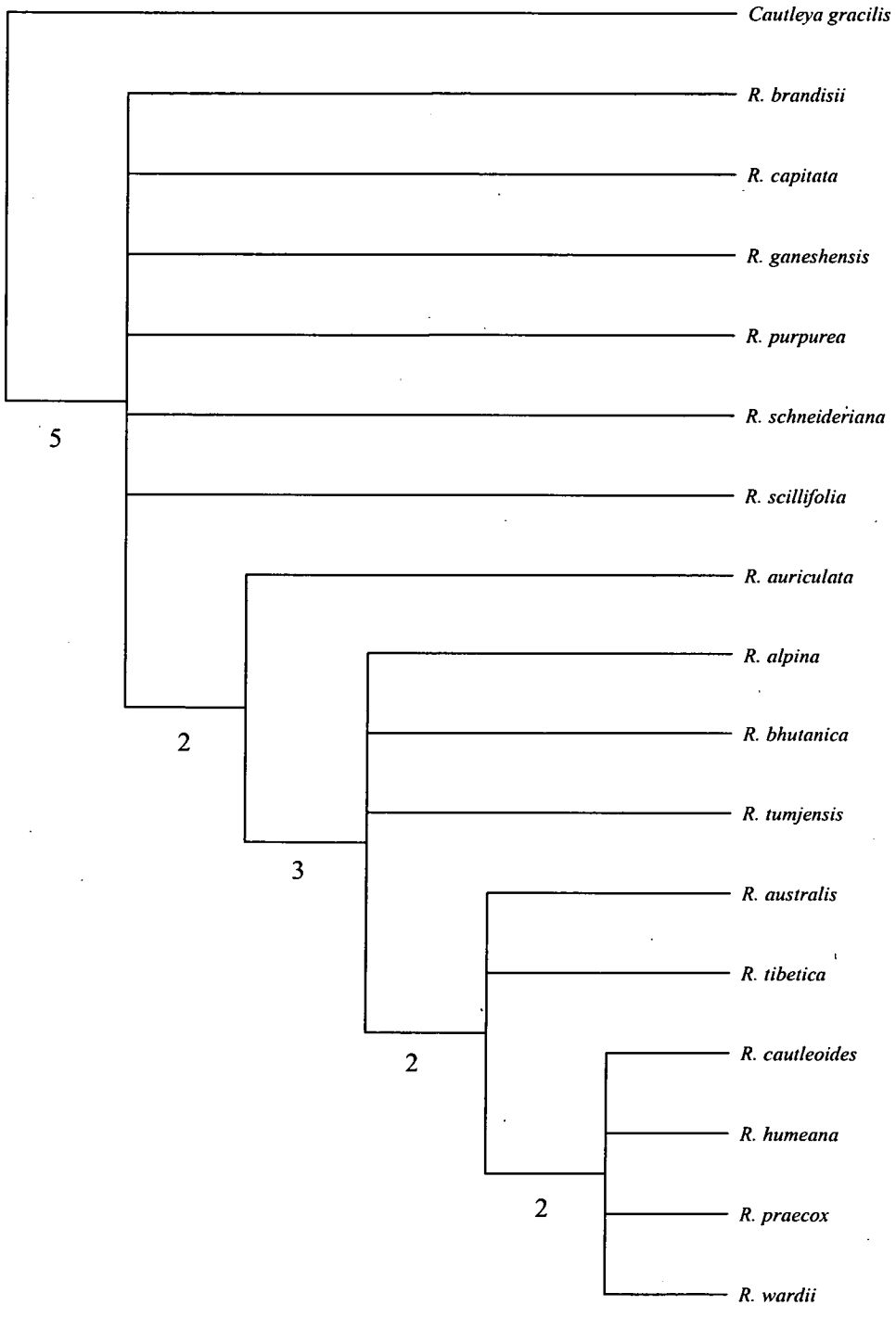


Figure 4.6. The strict consensus tree of 6 trees from a morphological analysis of nineteen *Roscoea* species and the outgroup (**Mor.20**). Only the clade of *R. alpina* and *R. nepalensis* has bootstrap value higher than 50 per cent. All clades have Decay Index value one. Branch length is given under the clades.

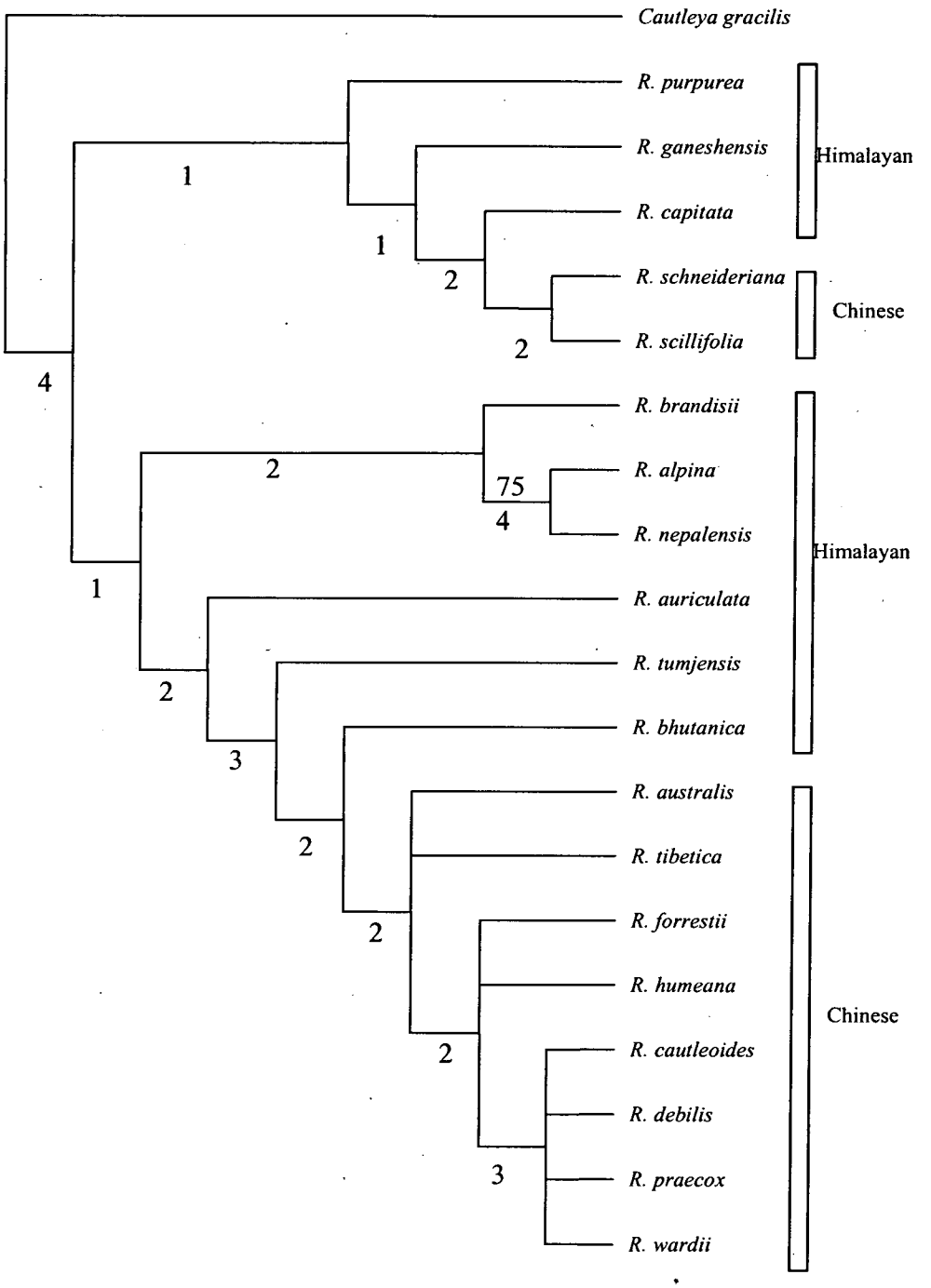


Figure 4.7 The strict consensus tree of 4 trees from an ITS analysis of sixteen *Roscoea* species and the outgroup (**ITS17**). Bootstrap value higher than 50 per cent are given above the branches. Branch length is given under the clades.

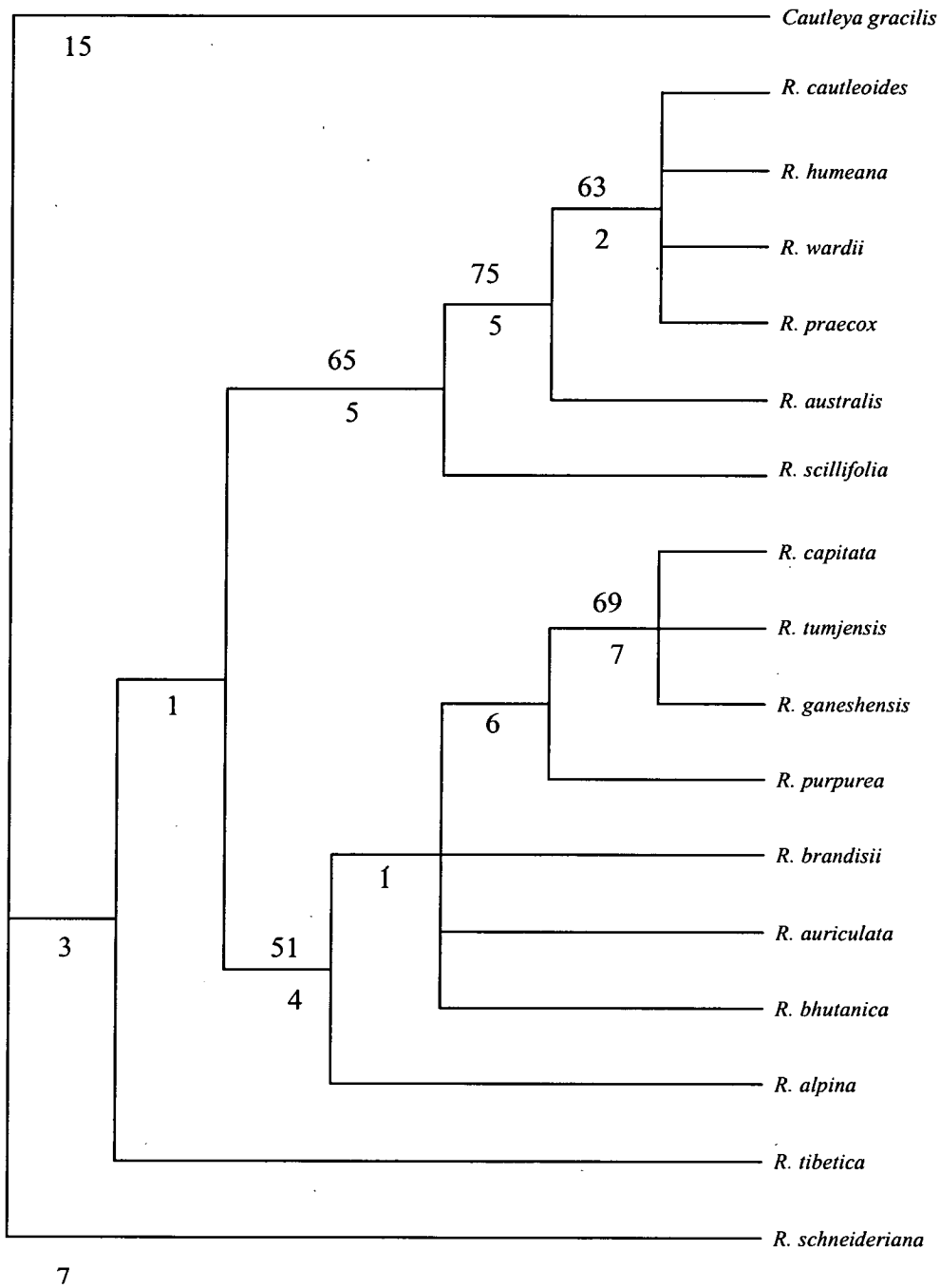


Figure 4.8. The strict consensus tree of 14 trees from an ITS analysis of sixteen *Roscoea* species and four outgroup species (**ITS20**). Bootstrap value higher than 50 per cent is given above the branches. Branch length is given under the clades.

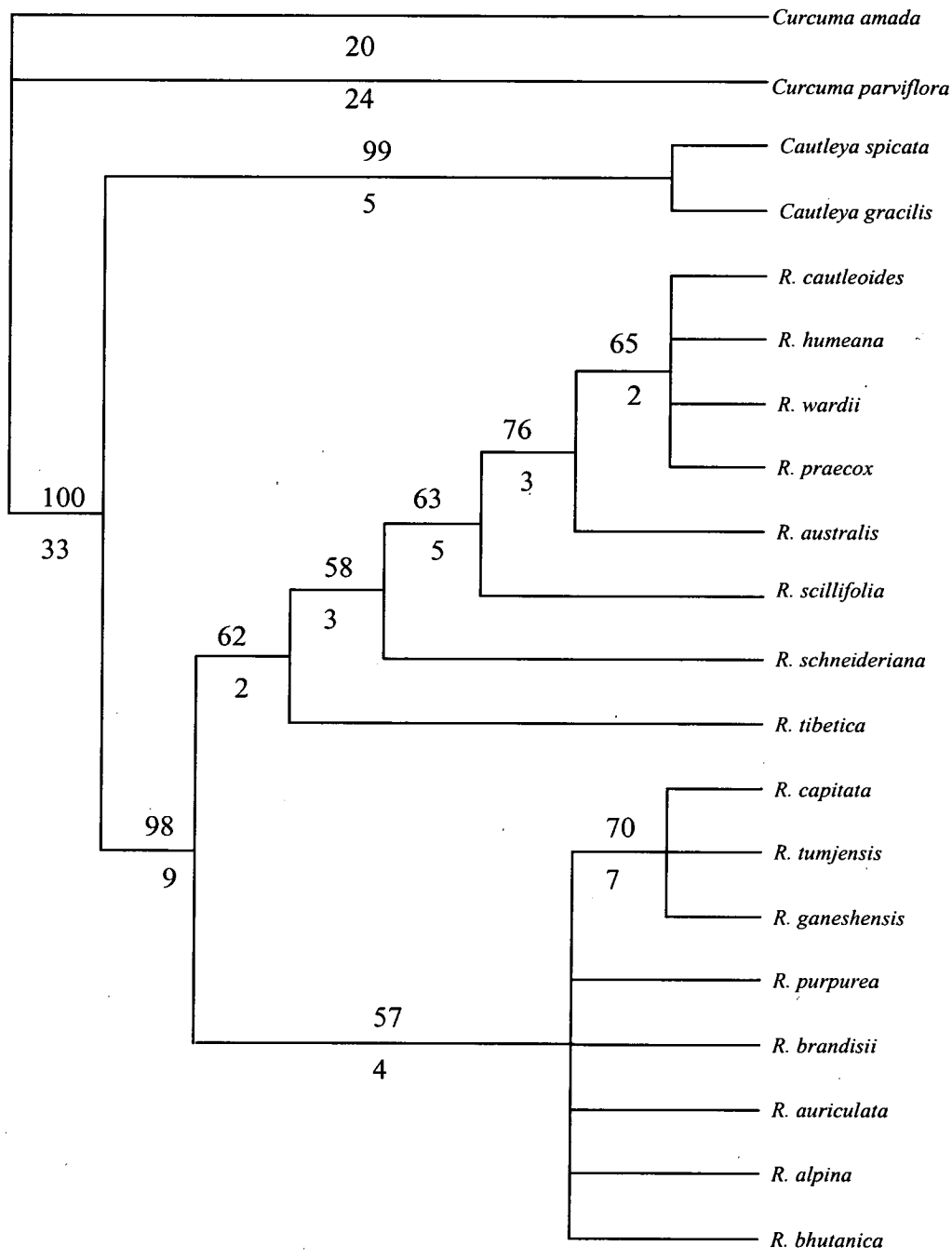


Figure 4.9. The single most parsimonious tree, resulting from an ITS analysis of sixteen *Roscoea* species and four outgroup species **(ITS20)** with Rescaled Consistency index applied. This tree was used as a backbone constraint in a later morphological analysis of all nineteen *Roscoea* species.

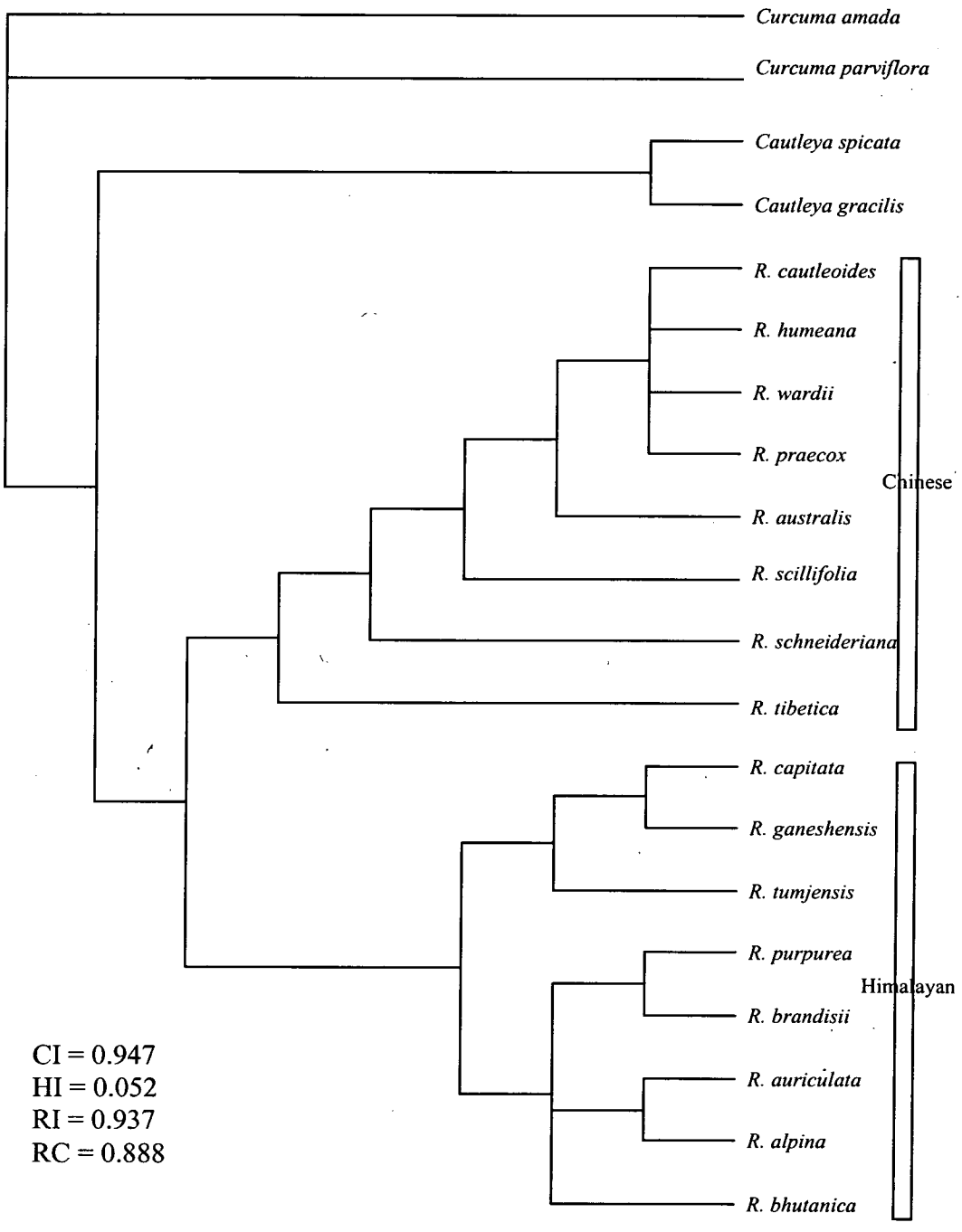


Figure 4.10. The tree shows all clades that have bootstrap value higher than 50 per cent in the analysis of **Com.17**. All clades have Decay Index value one. Branch length is given under the clades. * Note, only the clade of all Chinese species collapses in the strict consensus tree.

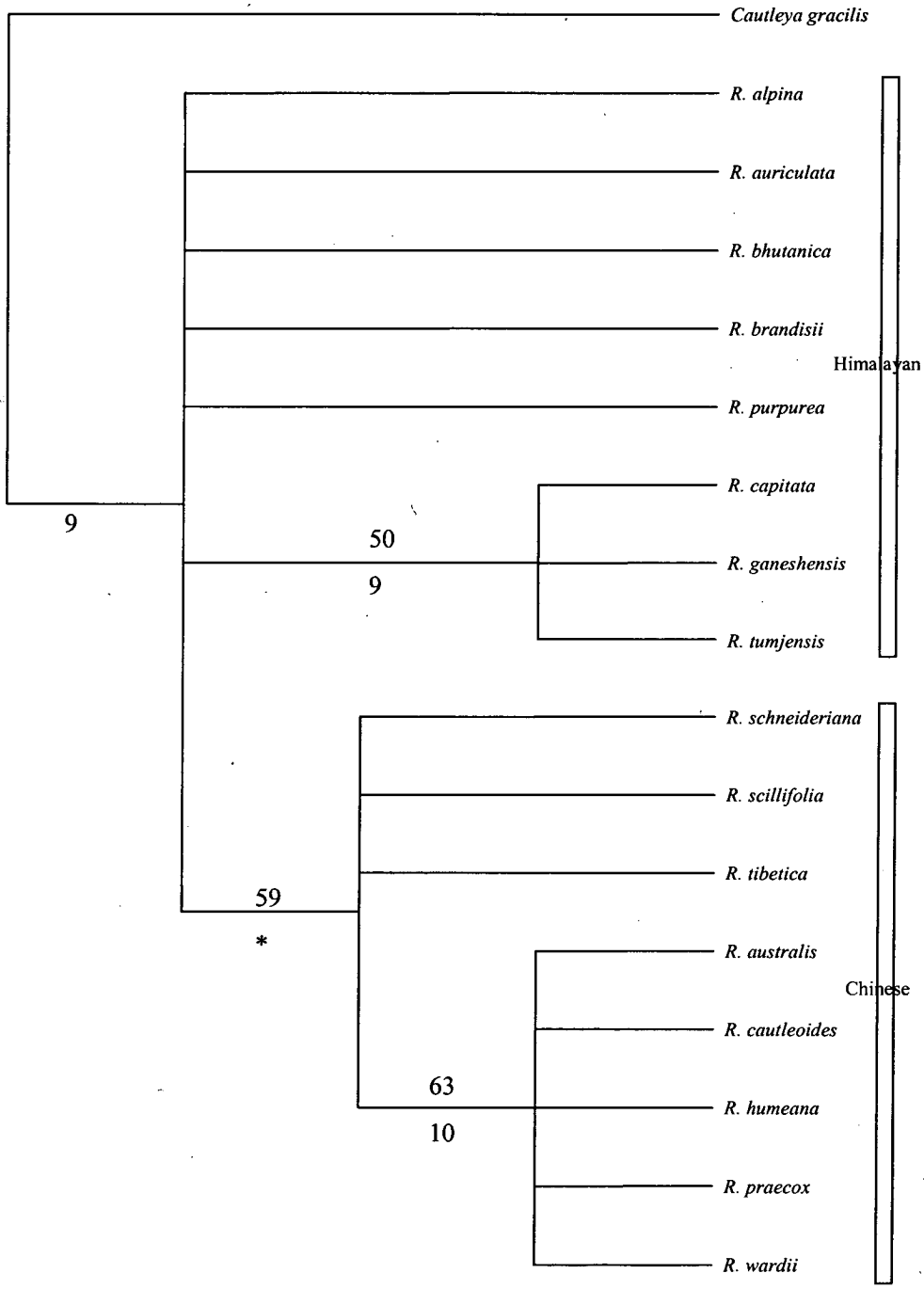
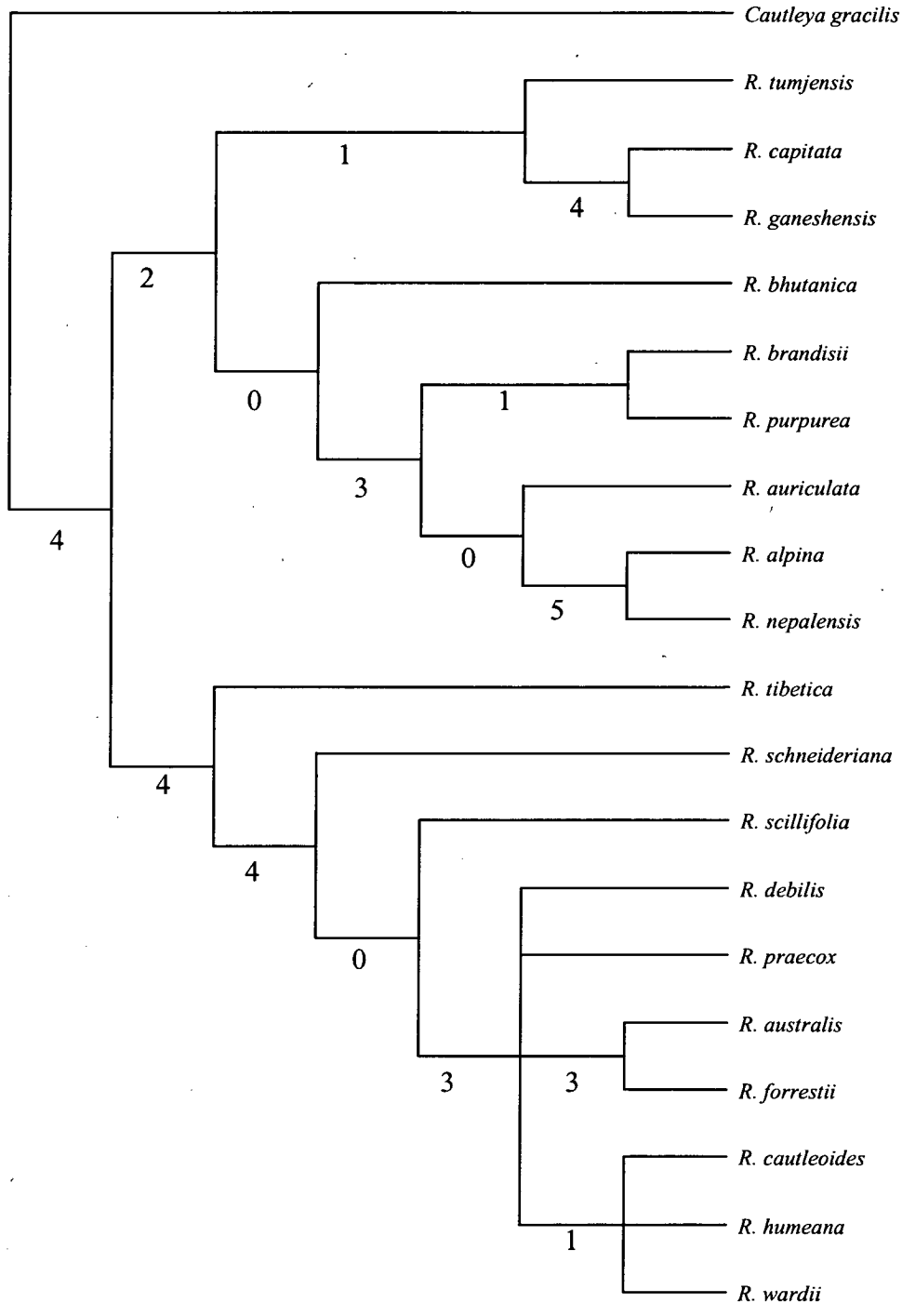


Figure 4.11. The strict consensus tree of 3 most parsimonious trees of a morphological analysis of **Mor.20** with the backbone constraint tree (Figure 4.9) of the **ITS20** analysis applied. Numbers below the clade are branch lengths.



4.5 DISCUSSION

4.5.1 MOLECULAR EVOLUTION OF ITS IN *ROSCOEA*

The internal transcribed spacers of ribosomal DNA of the *Roscoea* spp. investigated have evolved mainly by base substitution. Only four indels occurred in the DNA studied, of 1-2 bases in length. The levels of sequence variation among the *Roscoea* spp. are similar to those infrageneric levels found in other angiosperms. Sequence divergence within the *Roscoea* spp. ranged from 0 to 13.8% for ITS1 and 0 to 7.5% for ITS2. In species of *Saintpaulia* (Gesneriaceae) for example, the range of sequence divergence was from 0 to 17.6% for ITS1 and 0 to 13.9% for ITS2 (Möller and Cronk, 1997b) and in species of *Alpinia* (Zingiberaceae), sequence variation ranged from 0 to 20.9% for ITS1 and 0 to 19.7% for ITS2 (Rangsiruji, 1999). However, a group of four species remained unresolved because the level of sequence divergence was too low for unequivocal phylogenetic resolution. Indeed, three of these species had identical sequences. Other similar studies have such unresolved groups (Kim *et al.*, 1996; Möller and Cronk, 1997b) and this is generally attributed to rapid radiation, especially on islands or in newly created ecological niches. The Chinese and Himalayan mountains apparently represent a recent range extension for the predominantly tropical family Zingiberaceae. This may have induced processes of adaptive radiation similar to those found on islands. These regions have been affected by the continuous uplift of the Himalaya since the collision of the Indian and Asian plates ca. 52 to 45.8 Ma B.P. (Rowley, 1998).

Although the spacers show considerable variation at higher levels of the taxonomic hierarchy, they are thought to be important in post-transcriptional processing, and are thus conserved to some extent (Liu and Schardl, 1994; Van Nues *et al.*, 1994). It is interesting to find that speciation, as in the *Saintpaulia ionantha* complex (Möller and Cronk, 1997b) and in the *Roscoea cautleoides* complex, has been able to outstrip variation in the comparatively fast-evolving ITS region. Möller & Cronk (Möller and

Cronk, 1997b) have suggested that, where divergence times are short compared to rDNA homogenization rates, ITS variation will appear highly conservative; on the other hand, where divergence times are long compared to rDNA homogenization rates, ITS variation will appear disproportionately variable.

4.5.2 *ROSCOEA* AND *CAUTLEYA*

The phylogenetic trees resulting from this study show that *Roscoea* is monophyletic and *Cautleya* is its sister group. This is supported by a preliminary phylogenetic study of *Hedychieae* which included *R. cautleoides*, *R. purpurea*, *C. gracilis* and twenty six species from another ten genera of Hedychieae (Searle and Hedderson, 2000). The relatively low sequence divergence of species of *Cautleya* from those of *Roscoea* suggests a close relationship between the two genera. This is supported by their similar morphology and overlapping distribution area. *Roscoea* spp. can be found between 1200 to 4880 metres and *Cautleya* spp. between 900 to 3100 metres above sea level (Kumar, 1994; Wu and Larsen, 2000). However, Cowley (1982) pointed out that some clear distinguishing features exist. *Roscoea* has no true petiole, its lateral petals are free from the claw of the labellum and it has an elongate capsule, while *Cautleya* has a true petiole, the lateral petals are joined to the claw of the labellum and it has a round capsule. In addition, *Roscoea* has small (ca. 3 x 1 cm), fusiform, fascicled tuber roots, whereas in *Cautleya*, the tuber roots are cylindrical (see Table 4.5, a comparison of the two genera). The closed leaf sheath (Spearing, 1977) of all *Roscoea* spp. and *Cautleya gracilis* also suggests a relationship.

It is also interesting to consider which of the Chinese and Himalayan groups of *Roscoea* is more closely related to *Cautleya*. Morphologically, *Cautleya* spp. are superficially similar to *Roscoea cautleoides*. However, the phylogenetic analyses presented here suggest that neither the Chinese nor the Himalayan clade can be considered as more closely related to *Cautleya*, as they are sister groups.

Table 4.5. A morphological comparison of *Cautleya* and *Roscoea*

*Cautleya** (Royle ex Bentham) Hook. f.	*Roscoea* Smith
Ligule conspicuous	Ligule inconspicuous
Pseudostem packed, small with red dots	Loose without red dots
All 2nd veins merge into midrib of petiole	No real petiole
Leaves elliptic-lanceolate-oblong with apiculate-aristate tip	Leaves vary, acute or acuminate
Rachis elongates, flowers well separated	A head of flowers
Lateral petals joined up to half its length	Lateral petals free
Flower always yellow	Flower mainly purple, few species yellow or white
Stigma under a small anther crest	No anther crest
Roots fusiform	Roots cylindrical
Seeds.sharply angular, adhering in a mass; aril fleshy, lacerate	Seeds not sharply angular, not adhering; aril inconspicuous

* Five valid names of *Cautleya* appear in the literature. Only two species are believed to be really distinct. 1. *Cautleya gracilis* (Smith) Dandy (*Cautleya cathcartii* Baker differs from *C. gracilis* in that it has more flowers on the inflorescence), 2. *Cautleya spicata* (Smith) Baker (*Cautleya robusta* Baker was described from inadequate, fruiting material; *Cautleya petiolata* Baker has fewer flowers on the inflorescence and its bracts are shorter than *C. spicata*) (Kumar, 1994; Smith, 1994; Larsen *et al.*, 1998).

4.5.3 TWO GROUPS IN *ROSCOEA*

The strict consensus tree of five most parsimonious trees resulting from the combined ITS sequences and coded indels clearly shows not only that *Roscoea* is monophyletic, but also that it is divided into two distinct groups. Seven species from China and one species from Burma form the first group (Chinese clade) (Figure 4.3),

while the rest form the second group (Himalayan clade). These two groups are supported by morphological characters as shown in Table 4.6. In order to explain this divergence, we need to examine the distribution of *Cautleya*, the sister group of *Roscoea*. *Cautleya* is not only found with *Roscoea* at lower levels of the Himalaya and in southcentral China, but is also recorded from high altitude sites on nearby tropical mountains, in Burma and in the north of Thailand (Larsen, 1980). However, the geographical centre of the present distribution of *Roscoea* and *Cautleya* is Assam, as shown in Figure 4.1. Assam is also the centre of diversity of the related genus *Hedychium*. There are 39 species of *Hedychium* in India, of which 35 occur in Assam (Jain and Prakash, 1995). It is possible that *Roscoea* originated in Assam, and spread east and west along the nearest mountain ranges, thus accounting for the separate Chinese and Himalayan groups. This is supported by a single maximally likely tree showing that a clade of *Roscoea/Cautleya* shares an ancestor with *Hedychium* species clade (Searle and Hedderson, 2000). Smitinand *et al.* (Smitinand *et al.*, 1970) reported that *Anaphalis margaritacea* (L.) Benth. & Hook. f. ssp. *margaritacea* is mainly distributed in the cool temperate zone of eastern Asia (including the Himalaya), and in North America. On finding the species in northern Thailand, he suggested that the species may have spread southwards along the high mountains of the Indo-China Peninsula to Thailand and Vietnam. Similar migration along mountain dispersal routes may have occured in *Roscoea* and *Cautleya*.

All of the species in the Chinese clade (except *R. australis*, Burma) are found in Yunnan province (mostly in Lijing and Dali) and some extend to parts of Sichuan. The data suggest that this is an area of rapid evolution of a complex of *Roscoea* spp. On the other hand, the area of greatest diversity of the Himalayan clade is in central Nepal. One particular area is Ganesh Himal (Cowley and Baker, 1996) which accounts for up to five species among eight species in the entire Himalayan region. These data give an indication of the priority of land protection and preservation for the authorities concerned.

Table 4.6*. The distinguishing characters of the two groups of *Roscoea* spp.

Chinese group	Himalayan group
1. Sheathing leaf number ca. 3-5, except *R. scillifolia*	Sheathing leaf number ca. 0-2, except *R. bhutanica*, *R. tumjensis*
2. Leaf number 0-4, except *R. schneideriana*	Leaf number > 4*
3. Leaf base not auriculate, except *R. australis*, *R. tibetica*	Leaf base auriculate, except *R. capitata*, *R. nepalensis*
4. Leaves almost forming rosette, except *R. cautleoides*, *R. scillifolia*	Leaves usually not forming rosette, except *R. alpina*, *R. bhutanica*, *R. nepalensis*
5. Corolla tube length < 6 cm , except *R. humeana*, *R. australis*	Corolla tube length > 6 cm
6. Appendage tip obtuse (*R. australis*, *R. scillifolia*: obtuse-pointed, *R. schneideriana*: ball)	Appendage tip pointed
7. Epigynous gland length < 5 mm	Epigynous gland length > 5 mm, except *R. auriculata*, *R. capitata*
8. Seed aril deeply lacerate, except *R. scillifolia*, *R. australis*, *R. wardii*	Seed aril shallowly lacerate
9. Ratio labellum length/dorsal petal length < 1, except *R. schneideriana*, *R. wardii*, *R. praecox*, *R. cautleoides*	Ratio labellum length/dorsal petal length > 1

* See Table 4.2 and Figure 4.13 for exceptions.

4.5.4 THE BRAHMAPUTRA GAP

The distribution of *Roscoea* (Figure 4.1) is strikingly discontinuous. There are no records from that part of Assam where the Brahmaputra river flows south around the eastern end of the Himalayan chain. Interestingly, this gap in the distribution coincides with the boundary between the Chinese and Himalayan clades. Although it is possible that the Brahmaputra gap is an artefact of undercollection, it is also possible that it represents a genuine phytogeographical boundary.

The region of the Brahmaputra gap is known to be undercollected, as the area has been historically inaccessible. Rao (1994) suggests that 30% of northeastern India (not including Arunachal Pradesh) has been only casually surveyed. More collecting in this region is therefore badly needed.

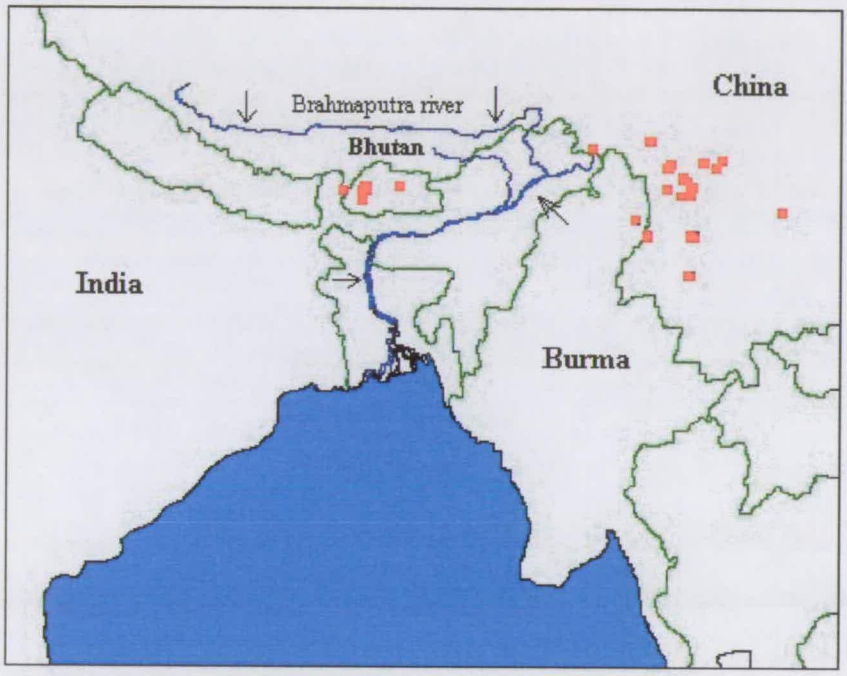
It is also possible that this area really has no *Roscoea* spp.. Although the Himalayan mountains form a continuous, geologically connected chain, here the eastern Himalaya rise rather abruptly from the plain without a distinct sub-Himalayan zone (Rao, 1994). This abrupt rise of the mountain range and its horseshoe shape may serve as a barrier between the two sides of the area. Thus the disjunct distribution of *Roscoea*, between two sides of northeastern India, may be genuine along with other examples of Indian disjunctions (Rao, 1994), e.g. *Nymphaea pygmaea* Ait. (with Siberia, N China), *Illicium cambodiana* Hance (with Southern Indo-China), *Mitrastemon yamamotoi* Makino (with Japan, Sumatra) and *Dendrobium bensoniae* Reichb. (with Burma, Thailand).

4.5.5 *ROSCOEA TIBETICA* AS A TRANSGRESSOR SPECIES

As mentioned above, the species of *Roscoea* fall into two groups, with either an eastern or a western distribution. The only exception to this is *Roscoea tibetica* which

occurs in both the eastern (China and Southeast Tibet) and the western area (Bhutan and in nearby Tibet) (Figure 4.12). There are two possible explanations for this: (1) that *R. tibetica* is a genuine transgressor which crosses the phytogeographical boundary, or (2) that the Bhutanese populations of *R. tibetica* represent a separate species, possibly more closely allied to Himalayan ones.

Figure 4.12. Distribution map of *Roscoea tibetica* showing the discontinuity between Chinese and Bhutanese populations. Arrows show the course of the Brahmaputra river.



The accession used in this study comes from China and groups with the Chinese clade. It would be very interesting to obtain material from Bhutan. There is some evidence of morphological difference between the Bhutanese and Chinese specimens (Table 4.7). Further studies on Bhutanese *Roscoea tibetica* remain a priority.

4.5.6 *ROSCOEA BRANDISII*: UNCERTAIN IDENTITY

Jill Cowley (RBGKew) has recently informed me that *R. brandisii* at Kew used in this study is unlikely to come from Meghalaya, the only recorded distribution area of the taxon (see also Figure 4.1). The plant was donated by a Dutch businessman who acquired it through local plant hunters. Its origin is unknown. The molecular findings in the present study suggest that the plant belongs to the Himalayan clade, though Meghalaya is southwestern to the Brahmaputra river. Geographically speaking, this species of western side of the Brahmaputra river is predicted to form a clade with other Chinese and Burmese species. The molecular phylogeny of *Roscoea* suggests that this plant may have its origin on the eastern side of the Brahmaputra river, not from the western side or the type locality. I sent these findings back to Jill Cowley and she decided to make another close observation of the plant. She found that the plant is probably not *R. brandisii*, by various morphological differences. However, the true identity of the plant has yet to be further confirmed.

Table 4.7. The distinguishing characters of the two geographically distinct populations of *Roscoea tibetica.*

Chinese populations	Bhutanese populations
1. Leaf number ≈ 2	Leaf number ≥ 3
2. Calyx longer than bract	Bract and calyx equal
3. Corolla tube long, exserted from calyx	Corolla tube short, within calyx
4. Labellum shorter than lateral petals	Labellum longer than lateral petals
5. Labellum usually divided more than half	Labellum divided less than half
6. Labellum drying dark purple or pink (in herbarium specimens)	Labellum drying purple (in herbarium specimens)
7. Labellum throat with white lines	Labellum throat without white lines
8. Lateral petal tip acute	Lateral petal tip obtuse
9. Appendage tip obtuse	Appendage tip pointed
10. Stigma with long hairs at tip	Stigma with short hairs at tip

4.5.7 MORPHOLOGICAL DISCUSSION

4.5.7.1 MORPHOLOGY AS A SOURCE OF PHYLOGENETIC INFORMATION

The morphological characters treated here were mostly hard characters or qualitative ones. It has been suggested that only such characters should be used to discern the phylogeny of taxa (Bateman, 1999). In addition, some other characters that were useful in the identification of the species were included in the study, such as the relative length of the bract and the calyx.

Although the morphological analyses of *Roscoea* species performed rather well in terms of CI, RI and RC values (CI = 0.446, RI = 0.586, RC = 0.261 in **Mor.17** and CI = 0.403, RI = 0.596, RC = 0.244 in **Mor.20**), the molecular analyses of the same taxa were better fitted with their resulting trees as indicated by the same descriptive statistics with higher values (CI = 0.753, RI = 0.725, RC = 0.547 in **ITS17** and CI = 0.801, RI = 0.785, RC = 0.629 in **ITS20**). The resolution of the strict consensus tree of the morphological analysis was also less than that of the molecular analysis. It can be seen that there are more homoplasies in morphological data than in molecular data, resulting in more discrepancy in each morphological trait in the best trees. This might be explained by environmental factors which play a crucial role in plants. Plant morphological plasticity is well known and thought to have occurred within a species. Adding more morphological characters, particularly continuously varying ones may increase the resolution of the phylogenetic tree, yet it may also reduce the CI, RI and RC values of the tree because of the added homoplasies.

The character coding of morphological data in a cladistic study is also problematic. There is no real objective means for delimitation of morphological data. For qualitative and non-overlapping characters, it is rather straightforward to put them into discrete character states, yet the polarity of the character states may need more explanation. Clear biological explanation of morphological states can help investigators

to study the evolution of characters. It becomes more complicated when quantitative or continuously varying characters are employed in cladistic analysis, including some so-called qualitative characters such as leaf form and dorsal petal form. These so-called characters are based on a quantitative phenomenological base filtered through the reified semantic discontinuities of botanical terminology (Stevens, 1991). Thus, they face the same problem as continuously varying characters. There are several methods for delimiting these continuously varying characters into states, but all are subject to criticism, based on the methods and statistics used, and the presentation of these characters to investigators (Gift and Stevens, 1997). Nonetheless, the situation is not totally hopeless in using morphology in cladistic analysis. Gift and Stevens (1997) suggested that all measurements and the variation of each character should be presented as well as a character coding table. This will help justification for the use and the delimitation of the characters. It should also demonstrate whether the study suffers from sampling error or not.

4.5.7.2 SIMULTANEOUS ANALYSIS

The consensus trees of both the morphological and molecular analyses (**Mor.17** and **ITS17**) showed no real discrepancy. The morphology just could not identify the Himalayan clade as in the molecular data. In both strict consensus trees, two of the members of the Chinese clade identified by the analysis of **ITS20** were left out. This indicated less informative sites in the data matrices. The strict consensus tree of **Mor.17** also had less credence in terms of bootstrap values, in comparison to the tree of **ITS17.** However, they both grouped the five terminal species of the Chinese clade.

The combined analysis of both morphology and ITS of the data sets gave twenty six equally most parsimonious trees. The consensus tree (Figure 4.10) was less resolved than that of the consensus tree of **ITS20** analysis. It did not recognise the two subclades, i.e. the Himalayan and the Chinese clades in *Roscoea*, though the 59 bootstrap value of the Chinese clade was retrieved. This suggested that among the twenty-six equally most

parsimonious trees, the topologies within were highly discordant. The CI, RI and RC of the combined analysis were less than those of the molecular analysis alone (**ITS17**) and more trees were generated. The results showed that adding the morphological characters into the molecular data increased homoplasies in the most parsimonious trees, and reduced resolution of the phylogeny.

4.5.7.3 MORPHOLOGICAL EVOLUTION AS SEEN BY MOLECULAR TREE

The morphological data set was analysed according to the RC weighted **ITS20** tree by backbone constraint option in PAUP (**Con.20**). The three equally most parsimonious trees were seventy-seven steps which were fifteen steps longer than the resulting trees of the morphological analysis alone. It implied that molecular data detected more changes in the morphological evolutionary history of the genus. A summary of descriptive statistics of morphological characters is given in Table 4.8. Figure 4.13 (character one to seventeen) shows morphological changes in the phylogeny of *Roscoea*. Based on the core topology of *Roscoea*'s phylogenetic relationships, the following morphological discussion was focused at the division of the Chinese and the Himalayan clades.

From Figure 4.13, Character one, it was observed that there was a trend in reducing of the number of bladeless sheathing leaf in *Roscoea*. Terminal taxa in the Himalayan clade had only a few sheathing leaves (0-2), with exception in *R. tumjensis* and *R. bhutanica*. Only *R. scillifolia* in the Chinese clade had a few sheathing leaves. Character five, leaf bases, is one of the most distinguishable characters among species in *Roscoea*. Nearly all species in the Himalayan clade had either first leaf base auriculate or all leaves base auriculate, with the exception of leaves base shortly petiolate in *R. nepalensis* and decurrent in *R. capitata*. In the Chinese clade, the majority of species had decurrent leaf bases while *R. tibetica* and *R. australis* showed slightly auriculate leaf bases and *R. debilis* had slightly petiolate leaf bases. Character six, only *R. capitata* had showy pedunculate inflorescence in the Himalayan clade whereas three species in the

Chinese clade possessed the character (*R. schneideriana*, *R. scillifolia* and *R. cautleoides*). Character eight, the flowers of the members of the Himalayan clade all seem to open one after another in succession whereas in the Chinese clade, five species at the terminal taxa showed a simultaneous flowering pattern of a few flowers to many flowers. Character fifteen, all the species in the Chinese clade had asymmetrically obovate or elliptic staminode. This character in the Himalayan clade was variable among species with no discernible trend. Character sixteen, most of the species in the Himalayan clade had anther appendages at right angle to the thecae. Two species, *R. alpina* and *R. nepalensis*, however, had very small appendages or unnoticeable and they were in line with the thecae (180°). In the Chinese species, they were mostly placed more or less at the obtuse angle to the thecae. Character seventeen, the tip of the appendages was pointed or tapering toward the end in the Himalayan species whereas it was rather obtuse in the Chinese species, but with one extra form *in R. schneideriana* whose appendages tip was globular.

For other morphological characters that were not included in the analyses, there were some useful characters for the identification of the species. Colour of flowers as discussed earlier, was difficult to put them all meaningfully in discrete character states, though colour yellow was only found at three terminal species in the Chinese clade (*R. cautleoides*, *R. forrestii* and *R. humeana*). The majority of colour was purple with different shade variation while white and pink were less found forms. Hairiness of any parts of the plant was found not a consistent character in general, so it was not possible to assign a character state to all species. It was, however, useful to confirm some species identification, such as *R. capitata*, *R. ganeshensis* and *R. tumjensis*. Despite the measurement of the plant was a wealthy source of information and was used indispensably in plant systematics, it was not readily useable and subject to justification in the cladistic analysis of morphology.

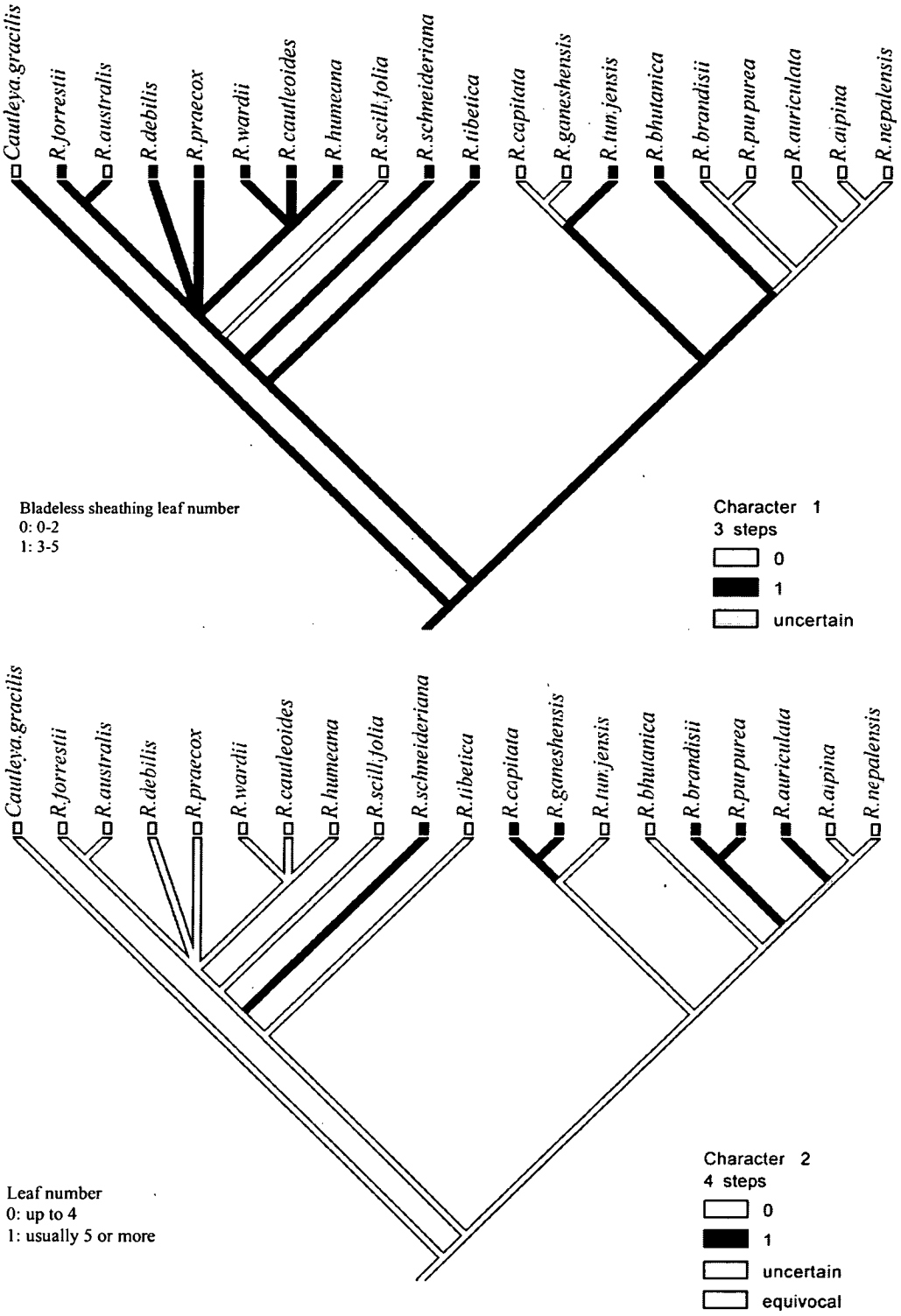
A more detailed study of the morphology of *Roscoea* may yield many more characters that are suitable to use in the cladistic analysis. In addition, continuously

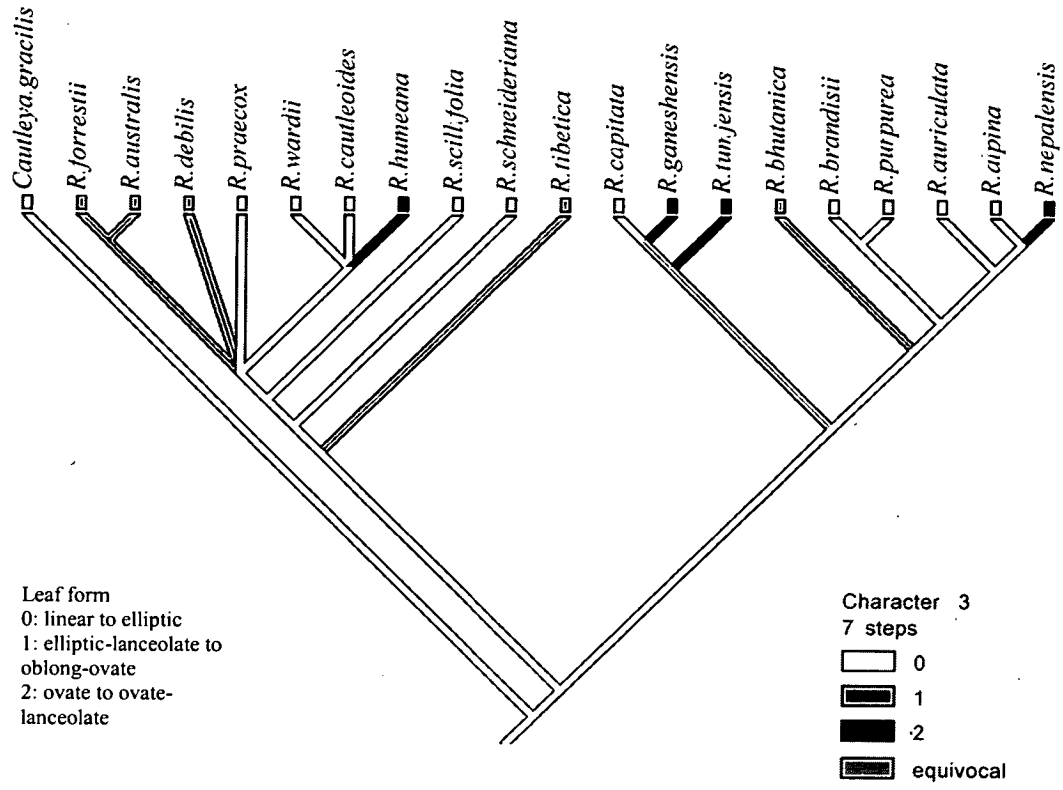
varying characters may be included in the study. This will give a comparison opportunity of how each data, i.e. molecular data, qualitative morphological characters and all morphological characters, perform. The more the well-studied morphological characters are used in the analysis, the better may be the resolution of the phylogenetic tree of the genus. The delimitation of the morphological characters may also be improved.

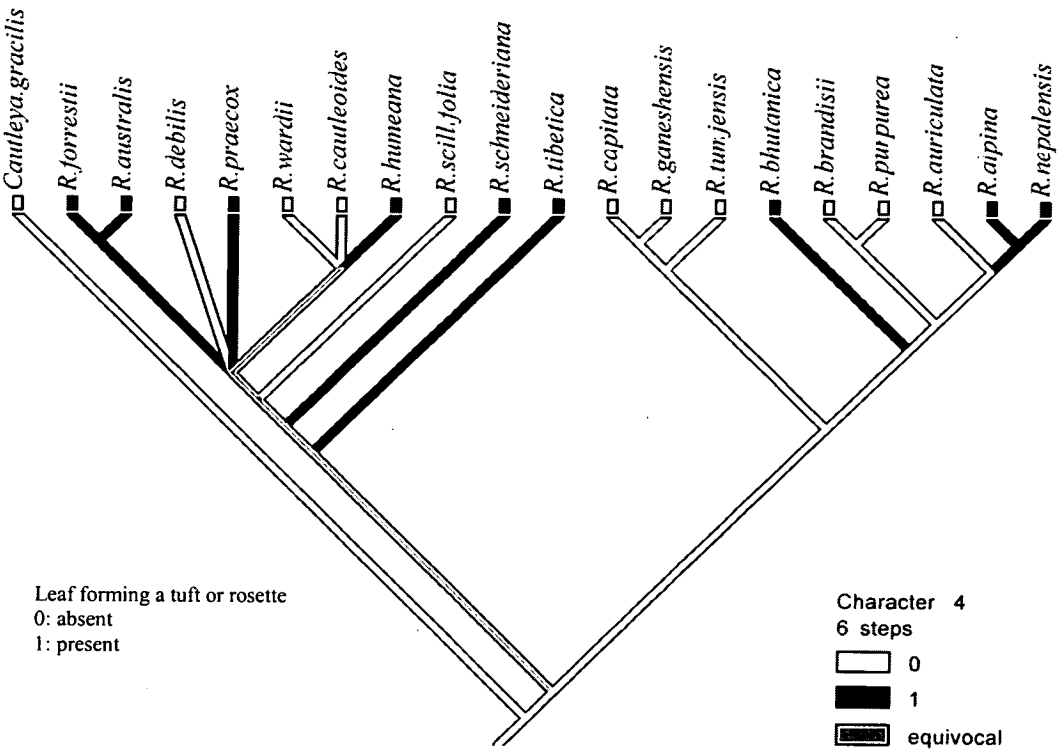
Table 4.8 shows the statistics of morphological characters on the molecular tree (Figure 4.13).

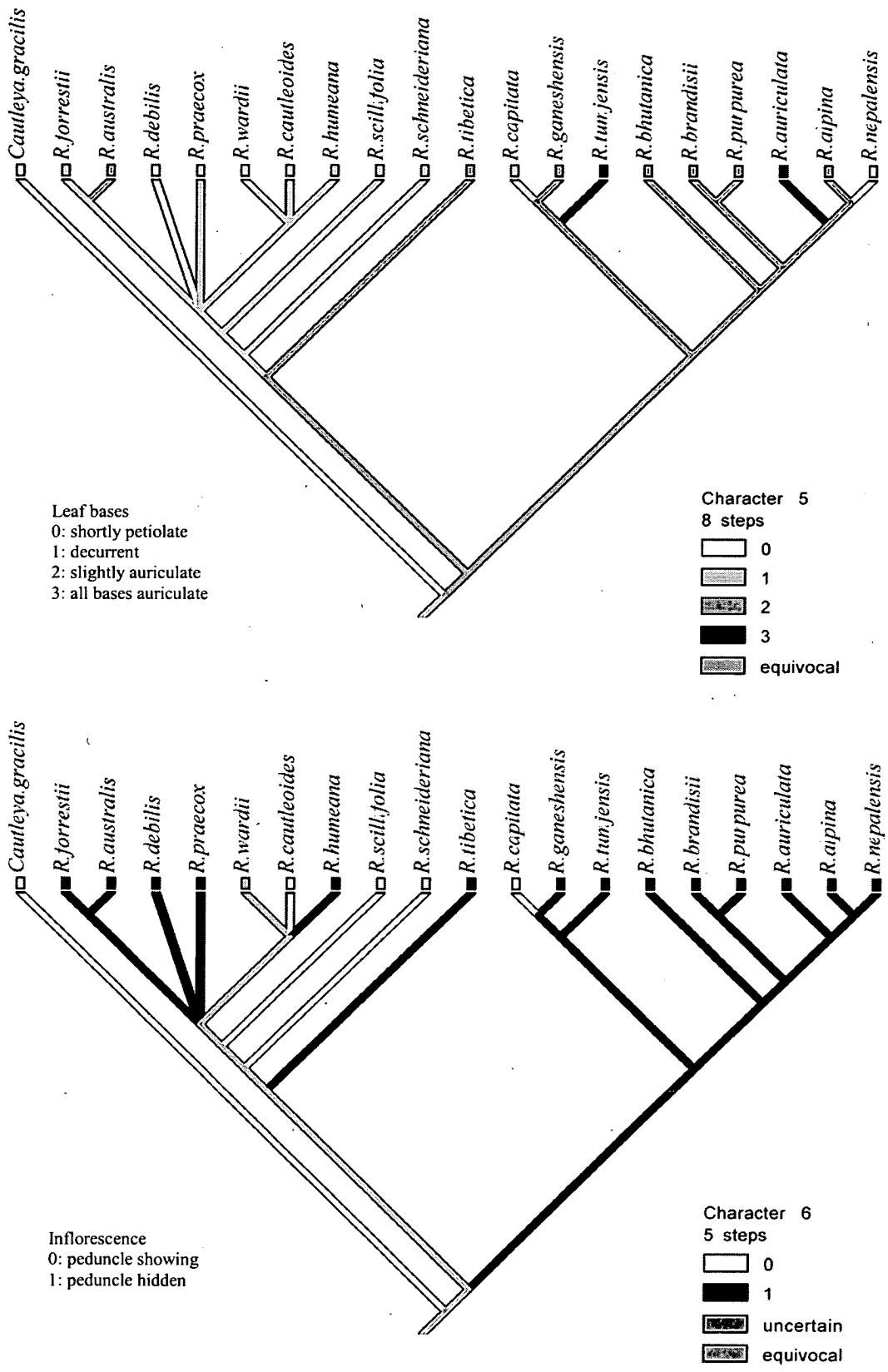
Character	Consistency Index (CI)	Retention Index (RI)	Rescaled Consistency Index (RC)	Homoplasy Index (HI)
1. Sheathing leaf number	0.33	0.66	0.22	0.66
2. Leaf number	0.25	0.40	0.10	0.75
3. Leaf form	0.25	0.14	0.03	0.75
4. Leaves forming a tuft or rosette	0.16	0.37	0.06	0.83
5. Leaves base	0.37	0.44	0.16	0.62
6. Peduncle of Inflorescence	0.20	0.00	0.00	0.80
7. Flowering precociously	0.50	0.00	0.00	0.50
8. Number of flowers	0.25	0.40	0.10	0.75
9. Bract length cf. calyx length	0.25	0.40	0.10	0.75
10. Bract tip	0.50	0.50	0.25	0.50
11. Lowest bract tubular	0.25	0.57	0.14	0.75
12. Dorsal petal form	0.33	0.33	0.11	0.66
13. Labellum deflex	0.20	0.20	0.04	0.80
14. Labellum claw	0.33	0.33	0.11	0.66
15. Staminode form	0.40	0.50	0.20	0.60
16. Appendages angle	0.50	0.71	0.35	0.50
17. Appendages tip	1.00	1.00	1.00	0.00

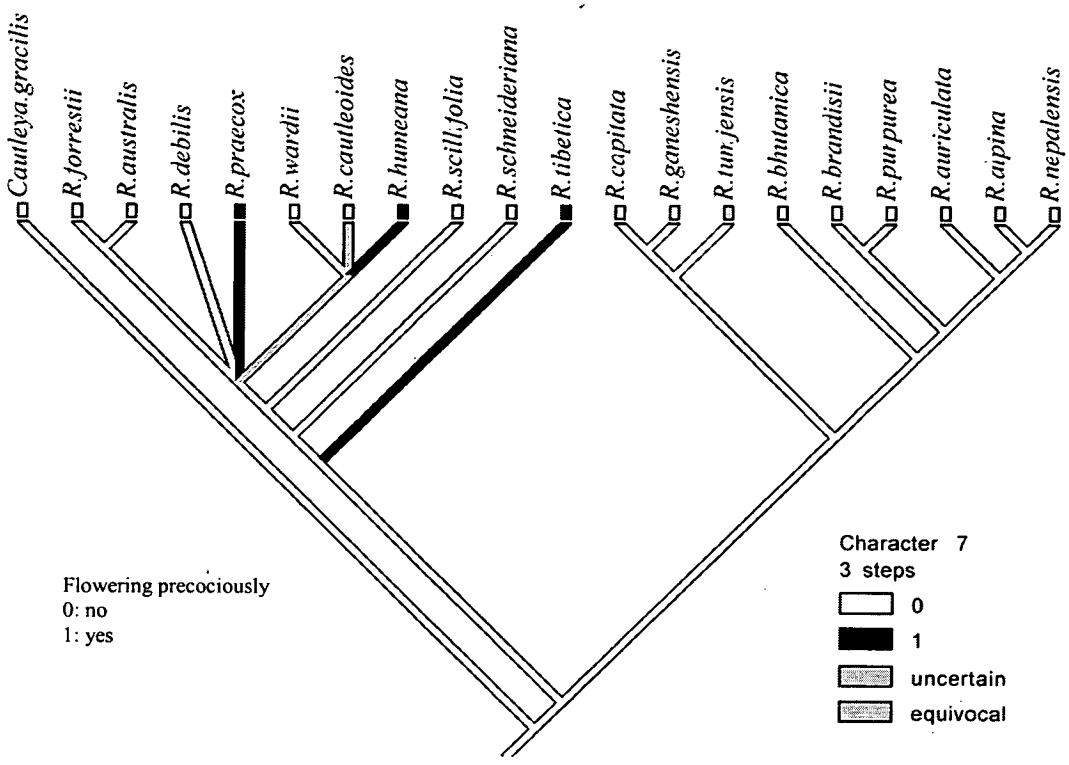
Figure 4.13 shows morphological changes in *Roscoea*. See '*Roscoea* character coding' section in the text for character description and states, and Table 4.8 for a summary of the statistics.




Caulleya.gracilis
R.forrestii
R.australis
R.debilis
R.praecox
R.wardii
R.caulleoides
R.humeana
R.scill.folia
R.schmeideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Leaf form
0: linear to elliptic
1: elliptic-lanceolate to oblong-ovate
2: ovate to ovate-lanceolate
Character 3
7 steps
0
1
2
equivocal


Caulleya.gracilis
R.forrestii
R.australis
R.debilis
R.praecox
R.wardii
R.caulleoides
R.humeana
R.scill.folia
R.schmeideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Leaf forming a tuft or rosette
0: absent
1: present
Character 4
6 steps
0
1
equivocal


Cautleya.gracilis
R.forrestii
R.australis
R.debilis
R.praecox
R.wardii
R.caulleoides
R.humeana
R.scill.folia
R.schneideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Leaf bases
0: shortly petiolate
1: decurrent
2: slightly auriculate
3: all bases auriculate
Character 5
8 steps
0
1
2
3
equivocal
Inflorescence
0: peduncle showing
1: peduncle hidden
Character 6
5 steps
0
1
uncertain
equivocal


Caulleya.gracilis
R.forrestii
R.australis
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R.praecox
R.wardii
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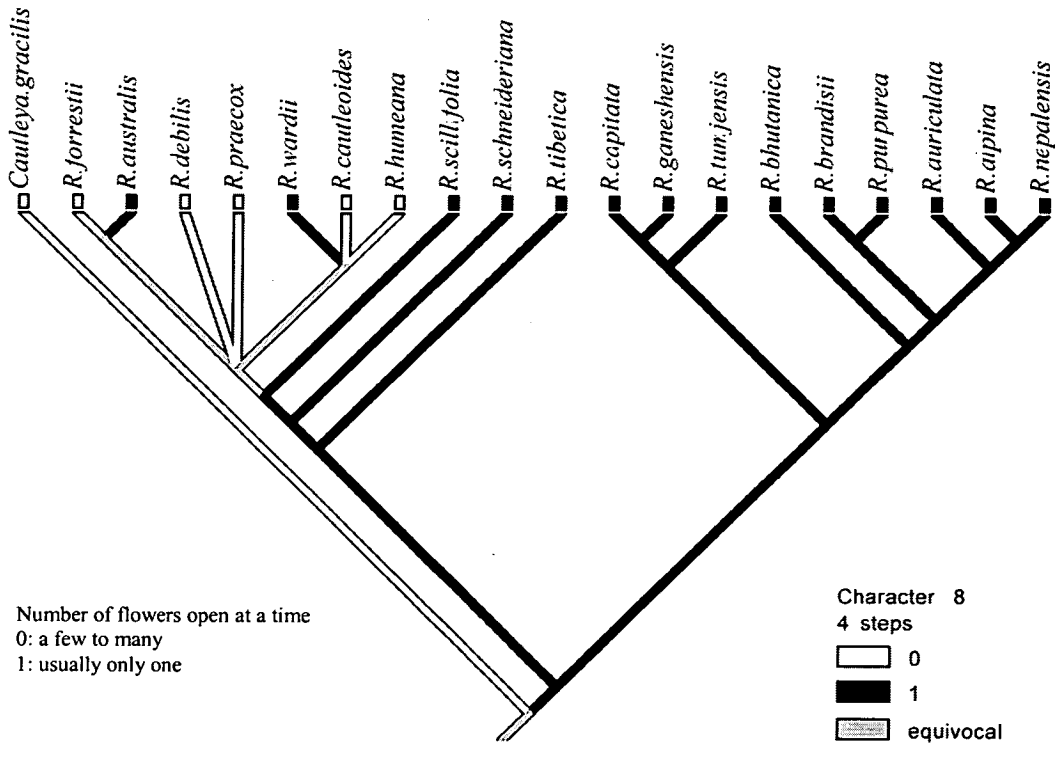
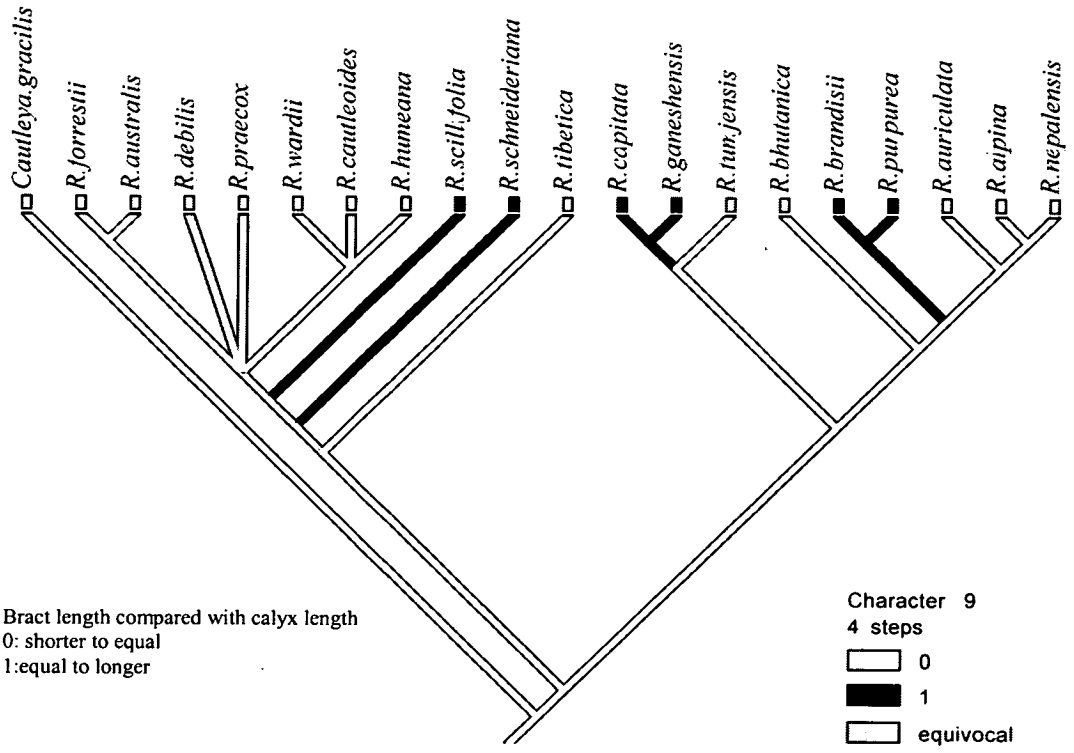
R.scill.folia
R.schneideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Flowering precociously
0: no
1: yes
Character 7
3 steps
0
1
uncertain
equivocal


Caulleya.gracilis
R.forrestii
R.australis
R.debilis
R.praecox
R.wardii
R.caulleoides
R.humeana
R.scill.folia
R.schneideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Number of flowers open at a time
0: a few to many
1: usually only one
Character 8
4 steps
0
1
equivocal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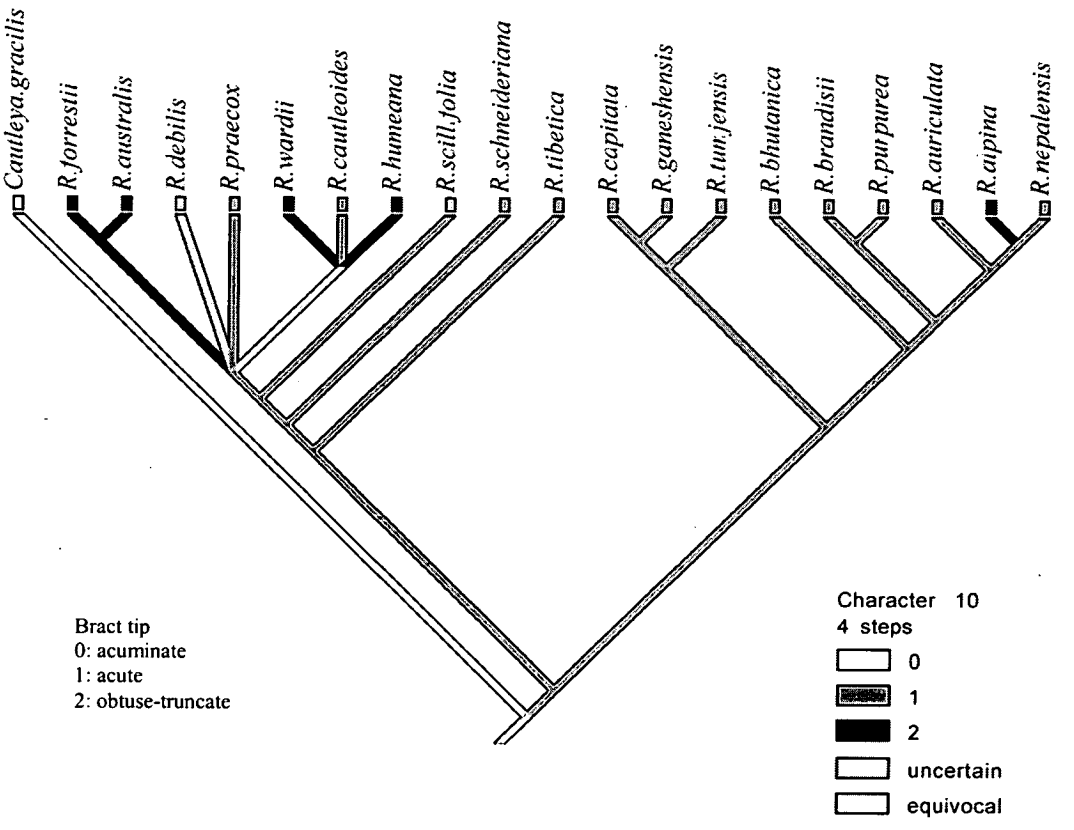
R.debilis
R.praecox
R.wardii
R.cauleoides
R.humeana
R.scill.folia
R.schneideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Bract length compared with calyx length
0: shorter to equal
1:equal to longer
Character 9
4 steps
0
1
equivocal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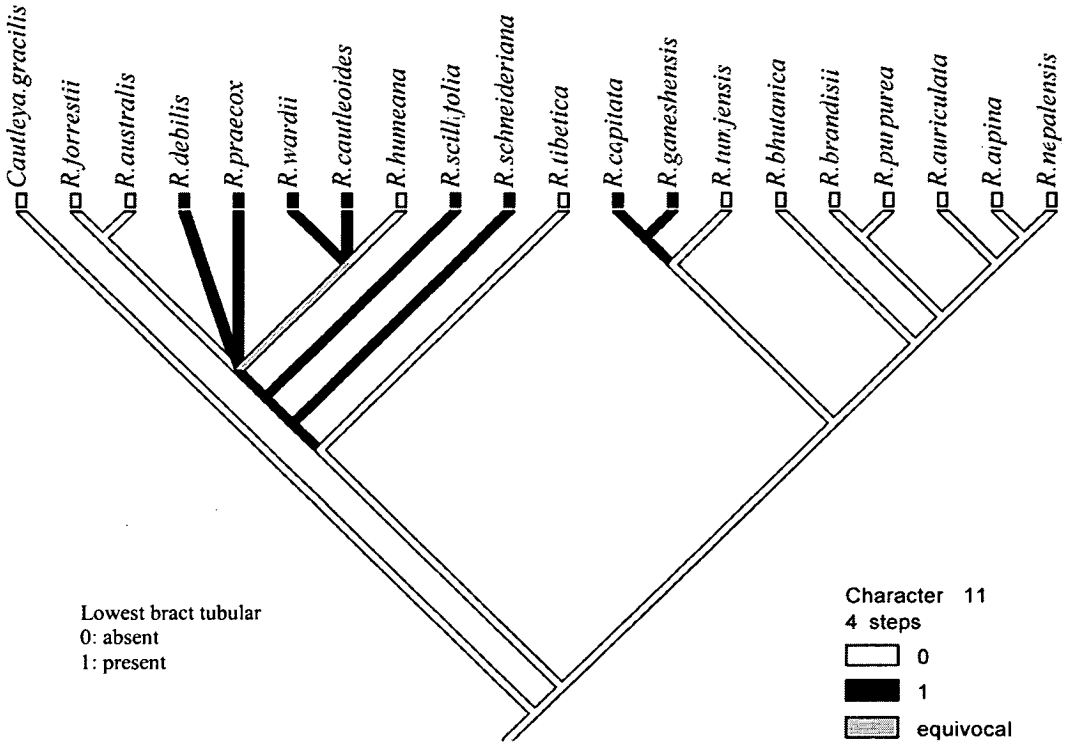
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Bract tip
0: acuminate
1: acute
2: obtuse-truncate
Character 10
4 steps
0
1
2
uncertain
equivocal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R.scill.folia
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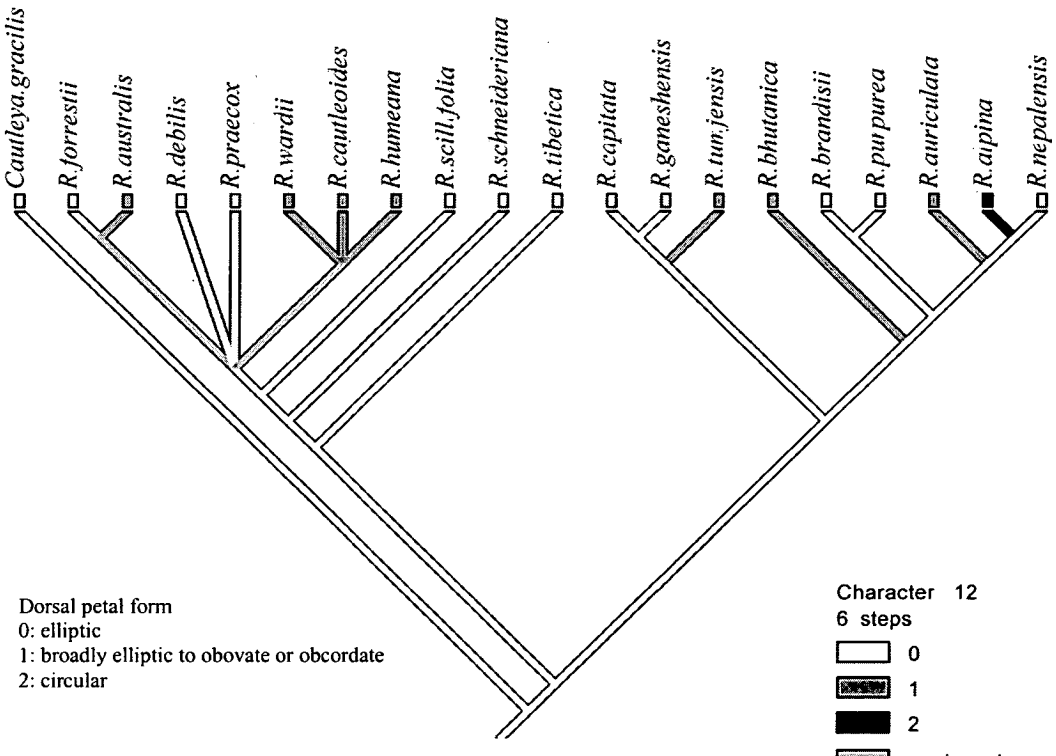
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.pur purea
R.auriculata
R.aipina
R.nepalensis
Lowest bract tubular
0: absent
1: present
Character 11
4 steps
0
1
equivocal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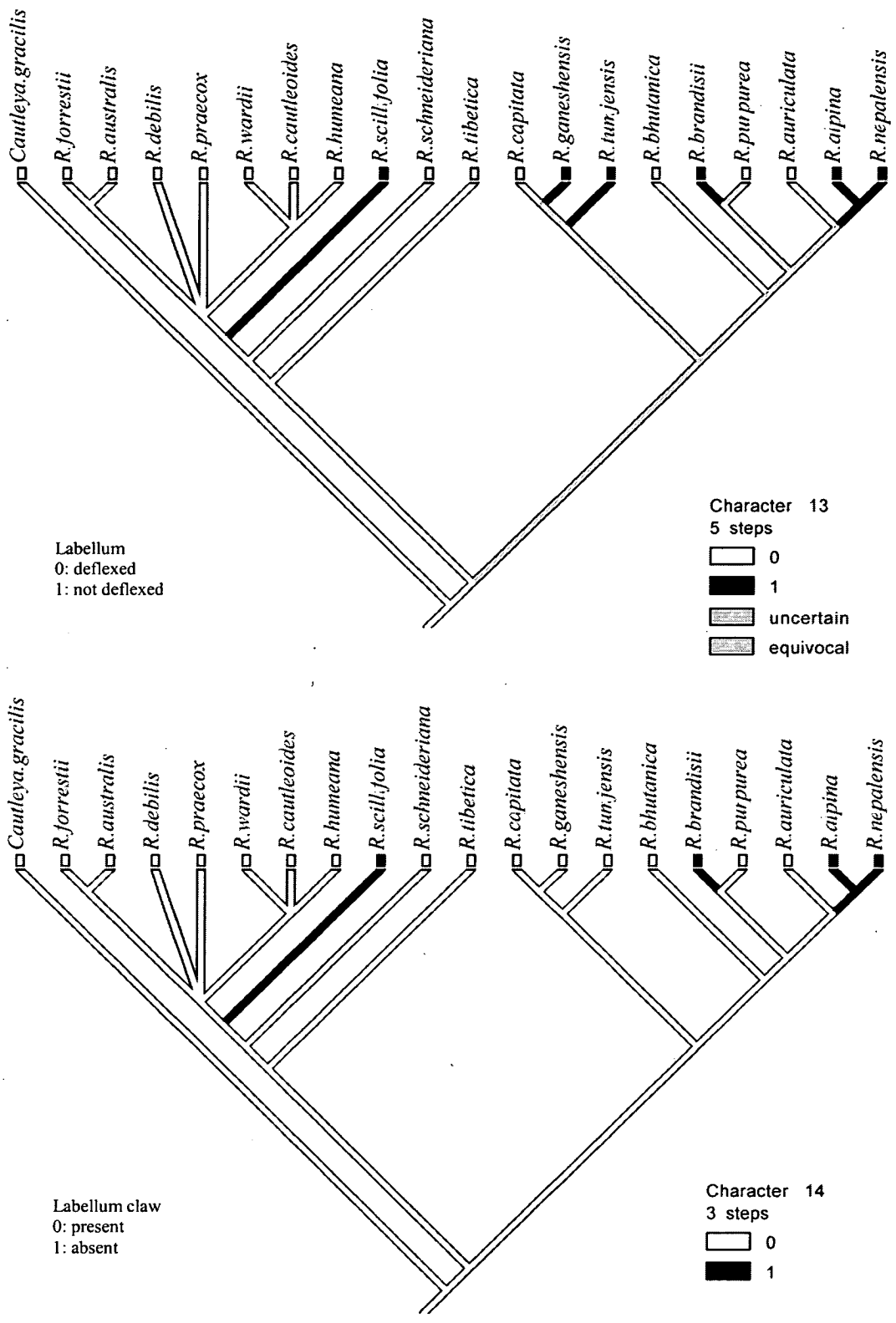
R.schneideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.pur purea
R.auriculata
R.aipina
R.nepalensis
Dorsal petal form
0: elliptic
1: broadly elliptic to obovate or obcordate
2: circular
Character 12
6 steps
0
1
2
equivocal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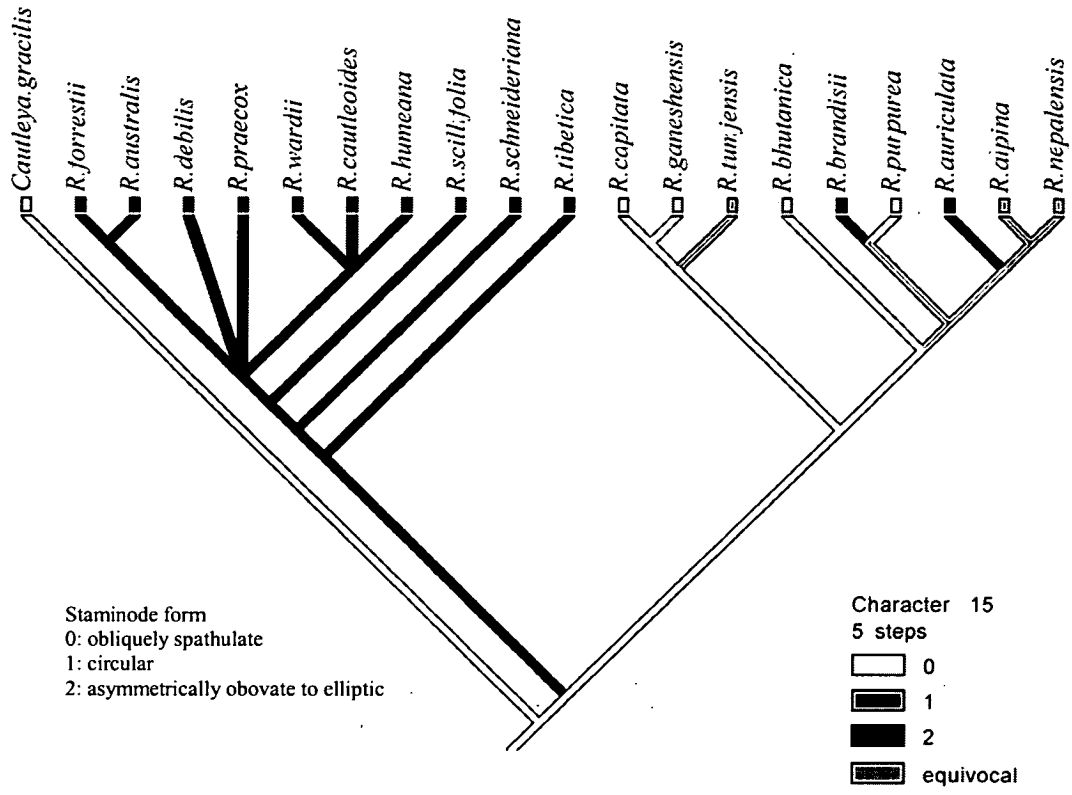
R.scill.folia
R.schmeideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.pur purea
R.auriculata
R.aipina
R.nepalensis
Labellum
0: deflexed
1: not deflexed
Character 13
5 steps
0
1
uncertain
equivocal
Labellum claw
0: present
1: absent
Character 14
3 steps
0
1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R.praecox
R.wardii
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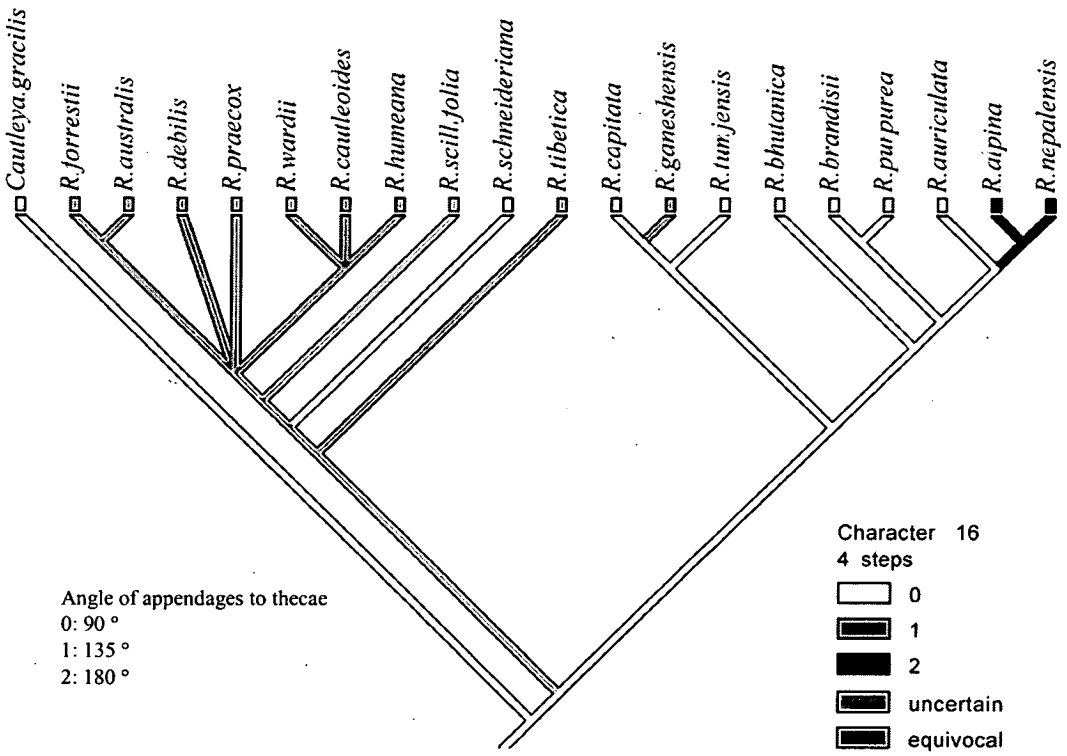
R.scill.folia
R.schmeideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Staminode form
0: obliquely spathulate
1: circular
2: asymmetrically obovate to elliptic
Character 15
5 steps
0
1
2
equivocal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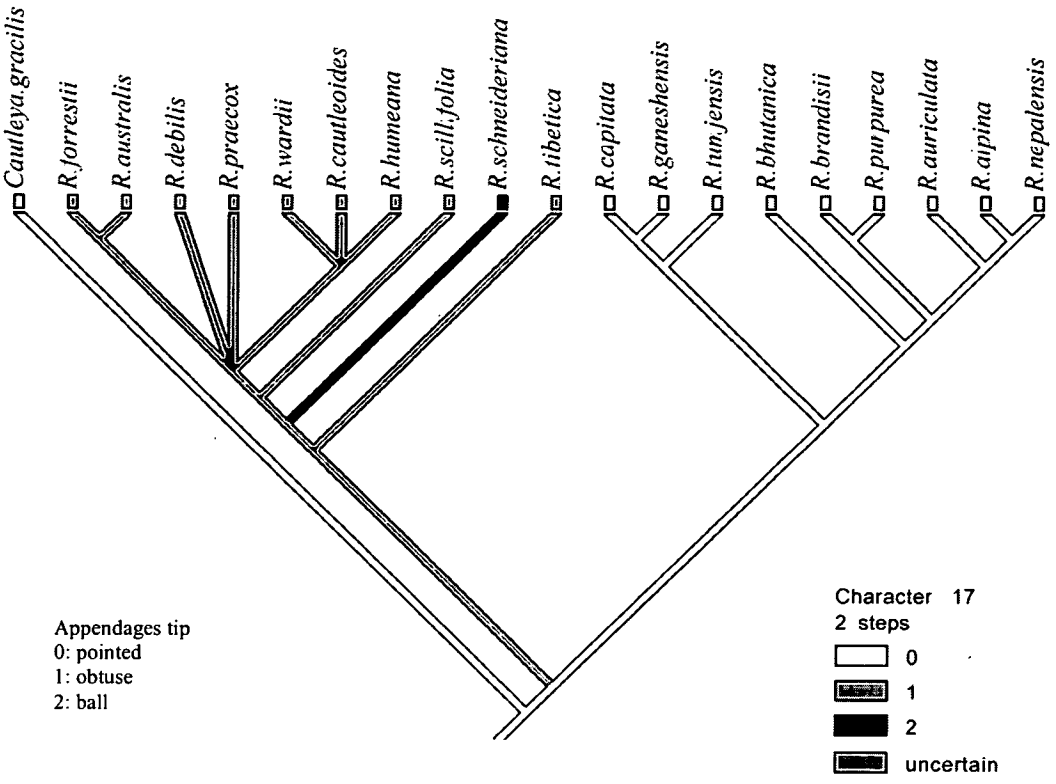
R.caulleoides
R.humeana
R.scill.folia
R.schmeideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Angle of appendages to thecae
0: 90 °
1: 135 °
2: 180 °
Character 16
4 steps
0
1
2
uncertain
equivocal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R.australis
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R.cautleoides
R.humeana
R.scill.folia
R.schneideriana
R.tibetica
R.capitata
R.ganeshensis
R.tun.jensis
R.bhutanica
R.brandisii
R.purpurea
R.auriculata
R.aipina
R.nepalensis
Appendages tip
0: pointed
1: obtuse
2: ball
Character 17
2 steps
0
1
2
uncertain

CHAPTER FIVE: TAXONOMIC STUDY OF *ROSCOEA*

(Materials in this chapter have been published in 'Ngamriabsakul, C. and Newman, M.F. (2000) A new species of *Roscoea* Smith (Zingiberaceae) from Bhutan and Southern Tibet. *Edinburgh Journal of Botany*, 57, 271-278.')

5.1 ABSTRACT

A new species of *Roscoea* from Bhutan and South Tibet, *R. bhutanica* Ngamriab., formerly included in *R. tibetica* Batalin, is described and a new key to all species of *Roscoea* is provided. While studying the phylogeny of *Roscoea*, I discovered that there is a correspondence between phylogeny and biogeography (Ngamriabsakul *et al.*, 2000). There are two distinct areas of distribution in *Roscoea*, namely the Himalaya and China. Only *R. tibetica* has been recorded in both areas. Cowley (1982) indicated that this species was very variable and suggested that it might be divided. I now propose to name a new species, *R. bhutanica*, based on observation of living and herbarium material at the Royal Botanic Garden Edinburgh (RBGE) and a molecular systematic study. A morphological table comparing *R. tibetica* with *R. bhutanica* is given, along with the ITS sequences of *R. tibetica*, *R. bhutanica* and *R. auriculata*. The identification key to *Roscoea* species largely follows our phylogenetic tree (Ngamriabsakul *et al.*, 2000).

5.2 INTRODUCTION

The name of the genus *Roscoea* first appeared in taxonomic literature in 1804. J.E.Smith (Smith, 1804) described *Roscoea purpurea* (Figure 5.1), a new species of a new genus, commemorating William Roscoe (1753-1831), one of the founders of Liverpool Botanic Garden who had a keen interest in the Zingiberales and was known to have had several collections in cultivation. William Roscoe's authoritative interest in the Zingiberales can be seen in his publication 'Monandrian Plants' which contains 112 coloured illustrations of Zingiberaceae, Cannaceae and

Marantaceae (Cullen, 1973). Later on, there were many additional collections and new names published in the genus expanding through the region (see Cowley, 1982), but it was not until 1904 that K. Schumann thoroughly revised *Roscoea*, as a part of his monumental monograph of the whole family. Schumann's account written for Das Pflanzenreich and based mainly on herbarium investigation (Burtt, 1972, p. 155; Cowley, 1997b, p. 3) recognised 13 species of *Roscoea* (Schumann, 1904). The difficulties encountered when trying to identify dried specimens of the Zingiberaceae are well known. J.M. Cowan (Cowan, 1938) mentioned these difficulties in *Roscoea*, "the species are difficult to delineate and the criteria used to distinguish them are quite unreliable".

Therefore, in the most important recent work on *Roscoea*, Cowley (1982) tried to employ as much living material as possible. Cowley (1982), however, underlined that it is still desirable that a more detailed study of this genus including fieldwork be carried out. Cowley (1982) recognised 17 species and 2 varieties. The number of species has been added to 19 species in two later publications: *R. ganeshensis* (Cowley and Baker, 1996), *R. bhutanica* (Ngamriabsakul and Newman, 2000) (Table 5.1).

A new species from Kunming (*Roscoea kunmingensis*) (Tong, 1992) which is closely related to *R. praecox*, has been described in a Chinese publication. This species is smaller in size than *R. praecox* and has smaller bracts and labellum.

Figure 5.1. The first *Roscoea* to be given a name, *R. purpurea*.

Table 5.1 shows altitude and flowering time of *Roscoea* spp. (Cowley, 1982; Cowley and Baker, 1996; Ngamriabsakul and Newman, 2000).

Species	Altitude (metres above sea level)	Flowering Time
1. *R. alpina* Royle	2130-4270	May-August
2. *R. auriculata* K.Schum.	2130-4880	May-September
3. *R. australis* Cowley	2130-2820	May-July
4. *R. bhutanica* Ngamriab.	2130-3510	May-August
5. *R. brandisii* (Baker) K.Schum.	1520-3050	July-August
6. *R. capitata* Smith	1200-2600	June-September
7. *R. cautleoides* Gagnep.	2130-3350	May-August
8. *R. debilis* Gagnep.	1670-2440	June-August
9. *R. forrestii* Cowley	2000-3350	May-July
10. *R. ganeshensis* Cowley & W.J.Baker	1900	August
11. *R. humeana* Balf.f. & W.W.Sm.	2900-3800	May-July
12. *R. nepalensis* Cowley	2240-3050	June-July
13. *R. praecox* K.Schum.	1520-2300	April-June
14. *R. purpurea* Smith	1520-3100	June-September
15. *R. schneideriana* (Loes.) Cowley	2600-3350	July-August
16. *R. scillifolia* (Gagnep.) Cowley	2740-3350	June-August
17. *R. tibetica* Batalin	2130-4270	May-August
18. *R. tumjensis* Cowley	2740-3050	May-July
19. *R. wardii* Cowley	2240-3960	June-August

"*Roscoea kunmingensis* S.Q. Tong, sp. nov.

Species *R. praecox* K. Schum. affinis, sed flore minutiore, labello 1.6-2.1 cm longo, 1-1.5 cm lato, profundo-bilobo, bracteis brevioribus, 5-7 mm longis, staminodiis lateralibus anguste obovato-cuneatis differt"

Because of the wide range of variation in a species, Cowley (1996) stated that one has to be somewhat cautious when studying this genus and judgement on the validity of new species has to be reserved until good specimens can be studied. There is a report, 'Notes on the Zingiberaceae for the Flora of China' (Wu, 1997), confirming the high variation in a species from China. Wu (1997) sank two new species of *Roscoea*, namely *Roscoea pubescens* Z.Y. Zhu under *Roscoea cautleoides var. pubescens* and *Roscoea sichuanensis* Miau under *Roscoea humeana* on the grounds that one only has slightly different morphological characters (pubescent sheaths, abaxial leaf surfaces and slightly longer fruit) and the other one is indistinguishable. Nonetheless, in Flora of China, it seems that *Roscoea kunmingensis* is now accepted as a distinct species (Wu and Larsen, 2000). In this study, because of lack of materials, it is not possible to include it.

5.3 A NEW SPECIES OF *ROSCOEA* FROM BHUTAN AND SOUTH TIBET

5.3.1 VARIATION IN MATERIAL PREVIOUSLY IDENTIFIED AS *ROSCOEA TIBETICA*: EVIDENCE FOR SEPARATION OF EASTERN AND WESTERN POPULATIONS

It is evident that *R. tibetica* is very variable. Cowley (1982) wrote, "there is also a very wide range of variation within this species which needs further study and may reveal the need to divide the taxon into subspecies". A later study of *Roscoea* (Ngamriabsakul *et al.*, 2000) noted the significant disjunct distribution of materials identified as *R. tibetica* across the 'Brahmaputra gap' (Figure 5.2), and the morphological differences between eastern and western populations. There is one living population from Bhutan identified as *R. tibetica* in the Royal Botanic Garden

Edinburgh. This Bhutanese plant was grown from seed collected by Ian Sinclair and David Long on their expedition to Bhutan in 1984 (accession number RBGE 19841747). Molecular phylogenetic analysis of ITS sequences of *Roscoea* (Ngamriabsakul *et al.*, 2000), revealed two clades, the Himalayan clade and the Chinese clade. *R. tibetica* from China was placed within the Chinese clade, but *R. tibetica* from Bhutan was not sequenced. The Bhutanese material has now been sequenced and when this is added to the previous phylogenetic analysis, it is found to be nested in the Himalayan clade. All this evidence taken together (ITS sequence, distribution range and morphology) persuades me that this plant from Bhutan is a new taxon. The ITS sequence of the Bhutanese material, now called *R. bhutanica*, which is more similar to sequences from species in the Himalayan clade than to those from species in the Chinese clade, is shown in Figure 5.3 along with *R. tibetica* (Chinese clade) and *R. auriculata* (Himalayan clade) for comparison.

5.3.2 NEW SPECIES

Roscoea bhutanica Ngamriab., **sp. nov. Figure 5.4.**

R. tibeticae Batalini affinis sed floribus generaliter majoribus, tubo corollae vix exserto, staminodiis spatulatis et appendicibus acutis ab thecis antherarum angulo recto divergentibus.

Type: Bhutan: Bumthang Dist., Bumtang Chu, Byakar, wooded valley above Lami Gompa, 27° 33′ N, 90° 42′ E, alt. 3050 m, 12 vi 1979, *Grierson & Long* 1826 (holo. E)

Plants 8–14cm tall. Roots tuberous, oblong-fusiform. *Sheathing leaves* 2–4, apex obtuse. *Leaf blades* usually 2–4 (–6) at flowering time, lanceolate-ovate to oblong, slightly auriculate, 4–21 × 1–4.5cm, glabrous, crowded together at the base. *Inflorescence* enclosed in leaf sheaths. *Flowers* opening just above leaves tuft, purple, one open at a time. *Bracts* 4.5–8 × 1–1.6cm, oblong to spathulate, acute. *Calyx* 5–6.5cm, apex more or less equal to bract, bidentate, teeth 1–3 (–9)mm long, split by 1–1.5cm. *Corolla tube* 5–6.5cm long, usually longer than calyx by up to 1cm, rarely equal to or shorter than it. *Dorsal petal* narrowly oblanceolate, 2.3–2.6 ×

1.1–1.3cm, apiculate. *Lateral petals* linear-oblong, 2.4–2.8 × 0.4–0.6cm, obtuse. *Labellum* slightly deflexed, 2.5–3.2 × 1.6–2cm, obovate, lobed less than ½ its length, without white lines at claw. *Lateral staminodes* obliquely spathulate, 1.6–1.9 × 0.5–0.6cm. *Anther* white, thecae 6–7mm long, at right angles to connective elongation and pointed appendages. *Ovary* 1–1.7 × 0.3cm. *Epigynous glands* 4–5mm. *Style* pinkish-white. *Stigma* white. *Seed aril* shallowly lacerate.

OTHER SPECIMENS SEEN

Bhutan, cultivated material: RBGE accession number 19841747, originating from Bhutan, Thimphu Dist., Dechhenphu, N of Thimphu. 27° 32′ N, 89° 38′ E. In cleared *Pinus wallichiana* forest amongst *Artemisia*, alt. 2480m, 5 ix 1984, *Sinclair & Long* 4829.

Bhutan, herbarium specimens: Ha Dist.: Damthang, Ha Valley, alt. c. 3050m, 2 vi 1933, *Ludlow, Sherriff* 50 (BM). Thimphu Dist.: 6km N of Thimphu Dzong, alt. 2450m, 9 vii 1975, *Grierson & Long* 116 (E); Dotena Chu, alt. c. 3050m, 27 v 1949, *Ludlow, Sherriff & Hicks* 16377 (E, BM); Pumo La, alt. c. 3350m, 8 vii 1938, *Gould* 925 (K); Tsalimaphe, alt. c. 2440m, 8 vii 1938, *Gould* 912 (K); Tsalimaphe, alt. c. 2440m, 28 v 1938, *Gould* 251 (K); Phajudin, alt. c. 2740m, 13 viii 1914, *Cooper* 2526 (E, BM); Zado La, alt. c. 2740m, 29 vii 1914, *Cooper* 3252 (E, BM); Tashichu, alt. c. 2380m, 12 vii 1914, *Cooper* 1512 (E); Chapcha, alt. c. 2130m, 6 vii 1914, *Cooper* 1300 (E, BM). Punakha Dist.: Kotaka, Wangdi Phodrang, alt. c. 2590m, 24 v 1966, Bowes–Lyon 3244 (BM); Mara Chu Valley, alt. c. 2440-3050m, 28 v 1937, *Ludlow, Sherriff* 3123 (BM). Tongsa Dist.: Chendebi, alt. c. 2290m, 2 vi 1938, *Gould* 356 (K); Bumthang Dist.: Takhung, Bumthang Tang, alt. c. 3050m, 20 v 1949, *Ludlow, Sherriff & Hicks* 18911 (BM).

S. Tibet, herbarium specimens: Kyimpu (Chayul to Charwe), alt. c. 3510m, 3 vii 1936, *Ludlow, Sherriff* 2275 (BM); Chumbi, Ta-ssi-cheu-doow, 16 vi 1884, *King's collector* 454 (K); Chumbi, 26 vi 1878, *Dungboo* 56 (K); Chumbi, 21 vii 1877, *Dungboo* 4244 (K).

Figure 5.2. Distribution map of *R. bhutanica* and *R. tibetica* showing the separation in ranges of these two species over the 'Brahmaputra gap'.

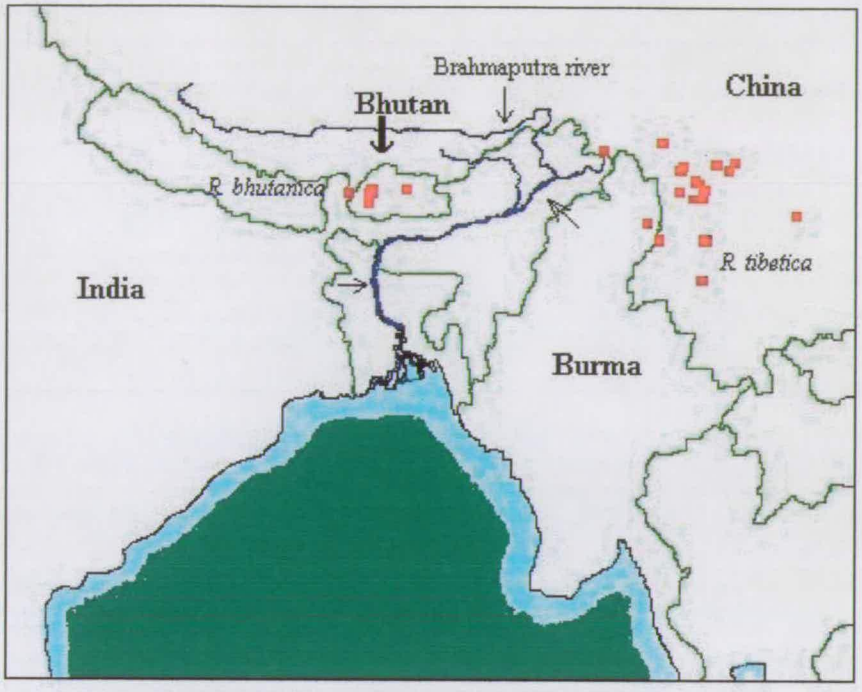

Figure 5.3. ITS sequences of *R. bhutanica* compared with *R. tibetica* (Chinese Clade) and *R. auriculata* (Himalayan clade). Asterisks mark variable bases. The similarity between *R. bhutanica* and *R. auriculata* can be seen.

```
                        10        20        30        40        50        60        70        80        90
alignment       ITS1    .         .         .         .         .         .         .         .         .

R.tibetica      TTGTTGAGAGAGCATAGAATGACGGATGGTTGTGTATGTGTGAATGTGCCCCTTTCCTTCCCCATCTCGGTGGGCGATTGACCGTATCTC   [90]
R.auriculata    TTGTTGAGAGAGCATAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTTCCCCATCTCGGTGGGCGATTGACCGTAGCTC   [90]
R.bhutanica     TTGTTGAGAGAGCATAGAATGACGGATGGTTGTGAATGTGTGAATGTGCCCCTTTCCTTCCCCATCTCGGTGGGCGATTGACCGTAGCTC   [90]
                                                  *                                                   *

                         100       110       120       130       140       150       160       170       180
                         .         .         .         .         .         .         .         .         .

R.tibetica      AGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCCTTGCCGTGCGCGGGGAGCCCGATGCGTCGGAGATATCTCGAAA   [180]
R.auriculata    AGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCCTTGGCGTGCCCGGGGAGCCCAATGCGTCGGAGATTTCTCGAAA   [180]
R.bhutanica     AGTGCGATCGGCACTAAGGAACAATGAACTCGGAAGCAGAGGGCCCCTTGGCGTGCCCGGGGAGCCCAATGCGTCGGAGATTTCTCGAAA   [180]
                                                        *     *         *         *

                         190       200       210       220       230       240       250       260       270
                        ITS2       .         .         .         .         .         .         .         .

R.tibetica      TCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCAAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCACAGTCGGTTGAAGAG   [270]
R.auriculata    TCAAATGAATCGTCGCTTTTGCTCCATGCATTGCTGGTGTCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCACAGTCGGTTGAAGAG   [270]
R.bhutanica     TCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGTCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGGGCACAGTCGGTTGAAGAG   [270]
                                          *         *
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                    280       290       300       310       320       330       340       350       360
                    .         .         .         .         .         .         .         .         .

R.tibetica          TGGGCAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGTCCCCGTCGTTTTAGGATTGTCCTCAA   [360]
R.auriculata        TGGGTAGTCCGAAGTCGTCGGGCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGTCCCCGTCGTTTTAGGATT-TCCTCAA   [359]
R.bhutanica         CGGGTAGTCCGAAGTCGTCGGCCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGTCCCCGTCGTTTTAGGATT-TCCTCAA   [359]
                    *   *       *         *     *                                                       *

                              370       380       390       400       410
                              .         .         .         .         .

R.tibetica          GAGACCCCGTGTGATCGTGATGTGGTGCGAAAGTGCCGTGTCCATCAATTTGT   [413]
R.auriculata        GAGACCCCGTGTGATTGTGATGCGGTGTGAAAGCCCCGTGTCCATCAAATTGT   [412]
R.bhutanica         GAGACCCCGTGTGATTGTGATGTGGTGTGAAAGTGCCGTGTCCATCAAATTGT   [412]
                              *       *     *     **            *
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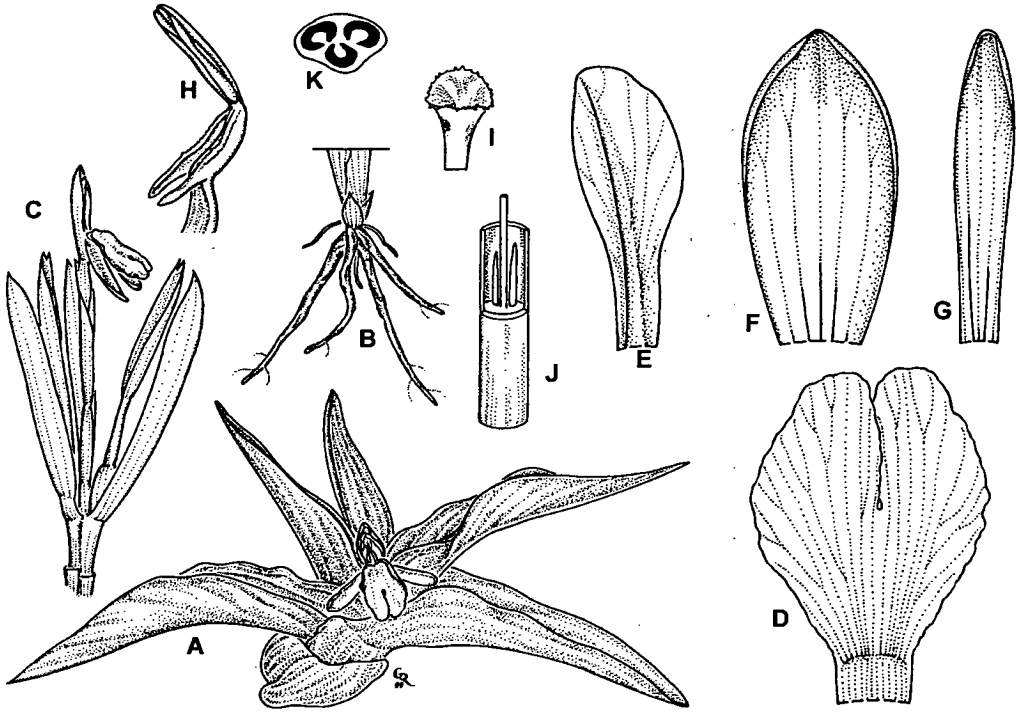
Figure 5.4. *Roscoea bhutanica* Ngamriab. A, habit (×1/3); B, roots (×1/3); C, inflorescence (×2/3); D, labellum (×2); E, staminode (×2); F, dorsal petal (×2); G, lateral petal (×2); H, stamen (×3); I, stigma (×10); J, ovary and base of style with epigynous glands (×3); K, ovary, transverse section (×6); drawn from plant in cult. RBGE 19841747 by Glenn Rodrigues.



This new species resembles both *R. purpurea* and *R. auriculata* (Himalayan clade) in floral characters. *R. purpurea* and *R. auriculata* are bigger plants with a well-developed stem, usually more than 25 cm in length, thus the leaves are not crowded together. *R. bhutanica*'s staminodes are intermediate in colour and shape between those of *R. auriculata*, which are white and rather asymmetrically obovate, and those of *R. purpurea*, which are purple and spathulate. They are purple with a long claw, thus the proportion of staminode length to width is greater, closer to that of *R. purpurea* than to that of *R. auriculata* which has a short claw. *R. bhutanica* generally has smaller flowers than *R. purpurea* or *R. auriculata*. The confusion with *R. tibetica* (Chinese clade) in the past resulted from their superficial similarities; they are both small plants with crowded leaves at the base. In most of the herbarium specimens, *R. tibetica* shows only one or two small leaves (some with no leaf at all) while *R. bhutanica* usually shows two or three leaves at flowering time and can have up to 6 leaves. Young plants of both species with very few leaves are not easily distinguished, especially when they are pressed on herbarium sheets. Nevertheless, at a later stage of growth *R. bhutanica* clearly shows a distichous leaf arrangement whereas *R. tibetica* remains a rosette. From observations in herbaria and of living plants at RBGE, it seems that *R. tibetica* flowers slightly earlier and usually precociously while *R. bhutanica* generally starts to flower after producing several leaves. In addition, *R. bhutanica* can be distinguished by its bracts being equal to or longer than the calyx, shortly exserted corolla tube, narrowly elliptic dorsal petal, the labellum being large compared to the rest of the flower, usually divided for less than half its length and lacking white lines at the throat, the pointed appendages, and anther thecae at right angles to the connective elongation and appendages. Table 5.2 shows the morphological comparisons between *Roscoea tibetica* and *Roscoea bhutanica*.

Table 5.2. The distinguishing characters of *Roscoea tibetica* and *Roscoea bhutanica*

Roscoea tibetica	*Roscoea bhutanica*
1. Calyx longer than bract	Calyx equal to or shorter than bract
2. Corolla tube long, exserted from calyx	Corolla tube short, usually within calyx
3. Labellum shorter than lateral petals	Labellum longer than lateral petals
4. Lateral petal tip acute	Lateral petal tip obtuse
5. Appendage tip obtuse	Appendage tip pointed

5.4 AN IDENTIFICATION KEY TO *ROSCOEA* SPECIES

1a. Labellum longer than dorsal petal; anther appendages pointed or tapering toward tips; staminodes obliquely spathulate or circular to elliptic; thecae at right angles or in line with appendages; flowers purple, red, white never yellow; the Himalaya 2

2a. Leaves usually 2–3 (–6) at flowering time, forming a tuft; plant usually less than 20cm high 3

3a. Staminodes circular to elliptic 4

4a. Leaves linear, first leaf slightly auriculate; bracts obtuse *R. alpina*

4b. Leaves obovate, all leaves slightly petiolate; bracts acute *R. nepalensis*

3b. Staminodes obliquely spathulate *R. bhutanica*

2b. Leaves usually more than 3 at flowering time, well spread; plant usually more than 20cm high 5

5a. Leaves auriculate throughout; bracts equal to or shorter than calyx 6

6a. Bracts exserted, equal to or slightly shorter than calyx; staminodes white *R. auriculata*

6b. Bracts hidden, much shorter than calyx; staminodes purple *R. tumjensis*

5b. Leaves generally not auriculate, rarely lower leaves auriculate; bracts equal to or longer than calyx 7

7a. First bract tubular, soon splitting or not, bracts ciliate; calyces ciliate 8

8a. Inflorescence on exserted peduncle, capitulate; thecae at right angles to appendages; lateral petal linear to oblong *R. capitata*

8b. Inflorescence hidden; thecae ± in line with appendages; lateral petal elliptic *R. ganeshensis*

7b. First bract not tubular, bracts glabrous; calyces glabrous 9

9a. Leaves lanceolate to oblong-ovate; dorsal petal narrowly elliptic, length > 3cm *R. purpurea*

9b. Leaves linear to narrowly lanceolate; dorsal petal elliptic to broadly elliptic, length < 3cm *R. brandisii*

1b. Labellum mostly shorter than dorsal petal; anther appendages obtuse or globular, never really pointed; staminodes asymmetrically obovate, rhombic or elliptic; thecae at obtuse angles with appendages; flowers purple, yellow or white; southcentral China or Burma 10

10a. Leaves bases petiolate or slightly auriculate 11

11a. Leaves petiolate; bracts equalling calyces *R. debilis*

11b. Leaves auriculate; bracts shorter than calyces 12

12a. Bracts acute; dorsal petal elliptic; lowest bract not tubular *R. tibetica*

12b. Bracts obtuse; dorsal petal obovate; lowest bract tubular *R. australis*

10b. Leaves bases decurrent 13

13a. Bracts longer than calyces 14

14a. Leaves crowded together in a fan shape; inflorescence not capitulate, peduncle hidden in leaf sheaths *R. schneideriana*

14b. Leaves rather evenly spaced up the stem; inflorescence capitulate, peduncle visible *R. scillifolia*

13b. Bracts shorter than or equal to calyces 15

15a. Leaf blade abaxially glaucous; flowers deep purple *R. wardii*

15b. Leaf not as above; flowers purple, yellow or white 16

16a. Bracts obtuse; lowest bract not tubular 17

17a. Dorsal petal obovate to obcordate; bracts much shorter than calyces *R. humeana*

17b. Dorsal petal broadly elliptic; bracts shorter than or equal to calyces *R. forrestii*

16b. Bracts acute; lowest bract tubular 18

18a. Peduncle hidden; dorsal petal elliptic to narrowly elliptic

R. praecox

18b. Peduncle visible; dorsal petal obovate to obcordate

R. cautleoides

NB. Lead number 1, 8, 9 and 18 of this key reflect the phylogenetic findings.

China possesses 13 species of *Roscoea*, the largest number of any one country. The main distribution is in Yunnan, along with neighbouring areas including Southeast Tibet. I include also two other identification keys of *Roscoea* for the purpose of comparative study as appendices. The first one is taken from Flora of China (Wu and Larsen, 2000). The second one is a translation from the Chinese version (Tong, 1992).

CHAPTER SIX: CYTOLOGICAL STUDY IN *ROSCOEA* AND *CAUTLEYA*

6.1 ABSTRACT

Chromosome counts of *Roscoea alpina*, *R. auriculata*, *R. purpurea* and *Cautleya spicata* are presented. My counts of two species: *R. auriculata*, *R. purpurea* confirm the widely reported number of 2n = 24. However, I found that both *R. alpina* and *C. spicata* have a chromosome number of 2n = 26. The chromosome number, 2n = 24, of *R. auriculata* is reported for the first time. Chromosome structures of the species studied are metacentric.

6.2 INTRODUCTION

Roscoea is a small genus, distributed mainly in temperate regions, with nineteen species in a tropical plant family, Zingiberaceae (Cowley, 1982; Cowley and Baker, 1996; Ngamriabsakul and Newman, 2000). It is found along the Himalaya, from Pakistan in the west to Southwest China in the east. Molecular phylogenetic studies of the tribe Hedychieae (Searle and Hedderson, 2000; Chapter Two in this thesis) and the genus *Roscoea* (Ngamriabsakul *et al.*, 2000) find that *Cautleya* is the sister group to *Roscoea* and *Roscoea* is monophyletic. In addition, *Roscoea* is further divided into two subclades, namely the Himalayan clade and the Chinese clade (Ngamriabsakul *et al.*, 2000).

The chromosomes of *Roscoea* have been relatively well studied by comparison with those of other genera in the family Zingiberaceae. The first recorded chromosome count is of *R. alpina* (Sharma and Bhattacharyya, 1959), a widespread species along the Himalaya (Ngamriabsakul *et al.*, 2000). The diploid number of the species is reported to be twenty-four. All the counts, up to the present are summarised in Table 6.1. In all, eight taxa (42%), eighteen lineages are reported.

While most of the counts report 2n = 24, *R. purpurea* (five lineages) from the Himalaya being the only species is found to have 2n = 26, besides one lineage of 2n = 24 (Table 6.1). An incidence of polyploidy, 2n = 48, was also observed in an unidentified *Roscoea* species (Mahanty, 1970).

A recent study by West & Cowley (1993) reports that all four Chinese *Roscoea* species investigated: *R. cautleoides*, *R. debilis*, *R. schneideriana* and *R. tibetica* (seven lineages) have uniform chromosome morphology and number, i.e. metacentric and 2n = 24. The sizes of the chromosomes are in the range of 1-2 μm. Moreover, they mentioned that the 2n = 26 number of *R. purpurea* (Mahanty, 1970) may actually be a result of false impression of two of the chromosomes separated into chromatids at late metaphase. For unknown reason, Mahanty (1970) did not mention *R. purpurea* and the number in his discussion, though it was written 2n = 26 in the legend of *R. purpurea*'s photograph. It is interesting to know whether other Himalayan species also have 2n = 26 populations as found in most of *R. purpurea* populations reported. To find out, the chromosome counts of three Himalayan *Roscoea* species: *R. alpina*, *R. auriculata* and *R. purpurea* are carried out. The count of *R. auriculata* is reported for the first time. The present study of Himalayan species may confirm the aberrant chromosome number and give new evidence to the systematic study of *Roscoea*.

6.3 MATERIALS AND METHODS

6.3.1 COLLECTION AND STORAGE OF ROOT TIPS

Root tips of three species of *Roscoea* from the Himalaya, namely *R. alpina*, *R. auriculata* and *R. purpurea* and *Cautleya spicata* were taken from Royal Botanic Garden Edinburgh around midday (11.30-12.30 p.m.) (see Table 6.3 for plants in this study). This time has been found to give high numbers of cells at metaphase in Zingiberaceae (Lim, 1972; Newman, 1990). The root tips were then washed with tap water a few times and once with distilled water.

Table 6.1 A summary of reported chromosome counts in *Roscoea*.

Species	Place of origin	Number of chromosomes		Author(s)
1. *Roscoea cautleoides* Gagnep.	China	2n = 24	-	(Mahanty, 1970; Chen *et al.*, 1987; West and Cowley, 1993)
2. *Roscoea debilis* Gagnep.	China	2n = 24	-	(West and Cowley, 1993)
3. *Roscoea humeana* Balf. f. & W. W. Sm.	China	2n = 24	-	(Mahanty, 1970; Chen *et al.*, 1986)
4. *Roscoea schneideriana* (Loes.) Cowley	China	2n = 24	-	(West and Cowley, 1993)
5. *Roscoea tibetica* Batalin	China	2n = 24	-	(Chen *et al.*, 1988; West and Cowley, 1993)
6. *Roscoea alpina* Royle	India, Nepal, Bhutan and China (Xizang)	2n = 24	n = 12	(Sharma and Bhattacharyya, 1959; Malik, 1961; Mahanty, 1970)
7. *Roscoea purpurea* Sm. (syn. *Roscoea procera* Wall.)	India, Nepal and Bhutan	2n = 24	-	(Bisson *et al.*, 1968)
Roscoea purpurea Sm. (syn. *Roscoea procera* Wall.)	India, Nepal and Bhutan	2n = 26	n = 13	(Bhattacharyya, 1968; Mahanty, 1970; Mehra and Sachdeva, 1971, 1976, 1979)
8. *Roscoea* species	-	2n = 48	-	(Mahanty, 1970)

Table 6.2 A summary of reported chromosome counts in *Cautleya*.

Species	Place of origin	Number of chromosomes	Author(s)
Cautleya gracilis (Sm.) Dandy (syn. *Cautleya lutea* Royle)	China (Sichuan, Yunnan, Xizang), India and Nepal	n = 12	(Mehra and Sachdeva, 1979)
Cautleya gracilis (Sm.) Dandy (syn. *Cautleya lutea* Royle)	China (Sichuan, Yunnan, Xizang), India and Nepal	n = 13	(Mehra and Sachdeva, 1979)
Cautleya spicata (Sm.) Baker	China (Guizhou, Sichuan, Yunnan, Xizang), India, Nepal and Myanmar	n = 13	(Mehra and Sachdeva, 1971, 1976, 1979)

6.3.2 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). The root tips were treated in either 1-bromonaphthalene (MBN saturated aqueous solution, at 4°C, for 24 hours) or 8-hydroxyquinolene (OQ aqueous solution, 0.002-0.02M, at 13 °C, for 5-7 hours). Pre-treatment is very important since the success rate of staining depends directly on the number of good metaphases rather than on the dyes used in the staining stage (Newman, 1988). Fixation is necessary to kill the material rapidly in such a way that the internal structures are preserved in a life-like form. In this study, the root tips were treated in Farmer's fluid for 24 hours. Dyer (1979) suggests a fixation period of 5 minutes to 24 hours. Freshly prepared Farmer's fluid (Schiff's reagent) contains 3 parts absolute ethanol and 1 part glacial acetic acid (Jong, 1997).

6.3.3 HYDROLYSIS AND STAINING

The cell wall is softened using an acid to make the cells easier to squash. The acid used in this study is 5N HCl and the hydrolysis is for 30 minutes. Additional softening with enzymes can be employed in a later step depending on the schedule used (Jong, 1997). In this study, 4% cellulase and pectinase is used after the staining stage at 60 °C for 30 minutes. In this study, Feulgen which is a dye made mainly from pararosaniline, is used to stain the chromosomes. Of all staining methods employed for the study of chromosomes, the Feulgen reaction is considered to be the most effective (Sharma and Sharma, 1999). Two commonly used methods to prepare the reagent are given in Jong (1997). The dye is light sensitive and thus the staining is carried out in a dark room, for 3 hours. DNA is stained a deep magenta colour while the other cell components remain unstained.

6.3.4 SLIDE PREPARATION, SQUASH AND OBSERVATION

The root tip was cut off into a small piece and placed on a clean slide. It was macerated with 2% acetic-orcein or 2% aceto-carmine using a brass tapping rod. A number 1 coverslip was placed on the material and the material was warmed over a flame. Squashing was done by pressing the slide firmly and suddenly between sheets of blotting paper or filter paper. The edges of the coverslip were sealed immediately with rubber solution. The material may be heated again over a lamp to increase the intensity of the stain. Slides were observed under a light microscope. Slides were made permanent by a quick freeze method using liquid nitrogen (Conger and Fairchild, 1953; Jong, 1997). A block of aluminium was immersed in liquid nitrogen for equilibrating the temperature of the aluminium to that of the liquid nitrogen. The aluminium was then placed in a block of polystyrene and the slide to be frozen was stood on the cold aluminium block for two minutes. Next the coverslip was flicked off and the slide and the coverslip were both dehydrated in 95% ethanol for two minutes and 100% ethanol for two minutes. One drop of Euparal, a permanent mountant, was allowed near but not on top of the material. Slides were left to dry on a slide warming plate for a few days.

Chromosome information generally can be divided into three groups, namely chromosome number, chromosome structure and chromosome behaviour (Stace, 1989). Mitotic studies of *Roscoea* and *Cautleya* in the present investigation, only chromosome number and chromosome structure can be obtained. Whenever possible, chromosome counts are based on as many cells as can be found in the slides. However, only one population and a few individuals of each species were sampled in this study. Some workers suggest numbers of cells and individual root tips to base the counts. These include Chen (1992) who suggests counting at least 30 cells of at least 5 individuals and the number finalised should represent in more than 85% of the cells counted.

The most commonly utilised aspect of chromosome structure is the position of centromeres, i.e. the arm-length ratio of each chromosome in the genome. A

system of chromosome classification of Levan *et al.* (1964) based on the ratio of the lengths between the long arm and the short arm of the chromosome has been widely followed. The system recognises 5 forms of the chromosomes. They are metacentric (the ratio = 1.0-1.7), submetacentric (1.7-3.0), subacrocentric (3.0-7.0), acrocentric (more than 7.0) and telocentric (where centromere is at the terminal).

6.4 RESULTS

Chromosome numbers of all four species were determined. The results are 2n = 24 in *Roscoea purpurea* and *R. auriculata*, 2n = 26 in *R. alpina* and *Cautleya spicata*. The chromosomes are mainly metacentric, with occasional submetacentrics (Figures 6.1-6.14). The size of the chromosomes ranges between 1-2 μm. The pre-treatment of the root tips by 1-bromonaphthalene (MBN) gives a slightly higher percentage of metaphase cells in the plants studied than 8-hydroxyquinolene (OQ). Feulgen stain gives well-stained chromosomes in this study.

Figure 6.1 *Roscoea purpurea*

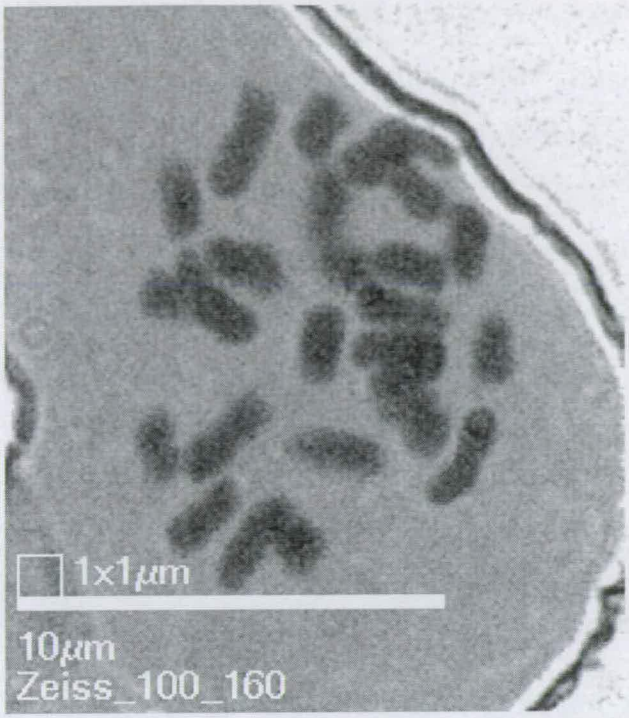


Figure 6.2 *R. purpurea*

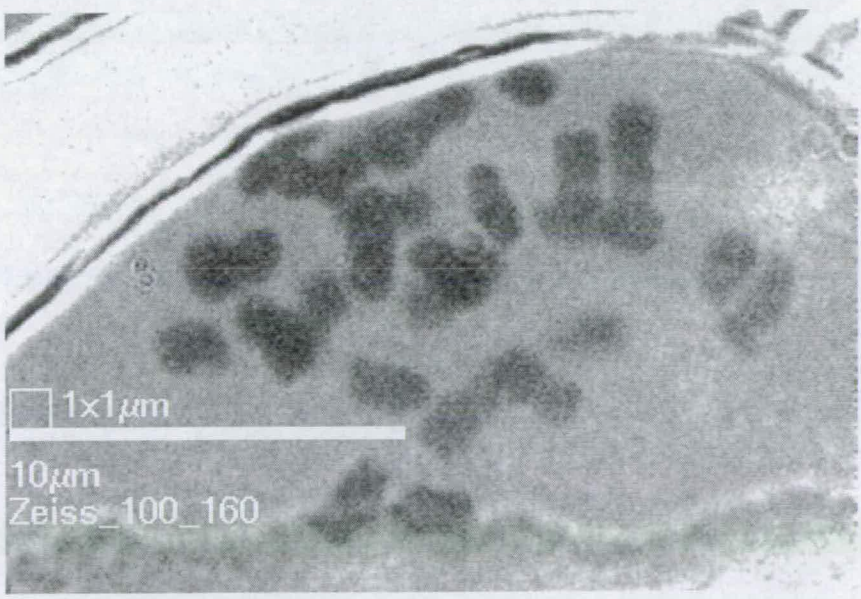


Figure 6.3 *R. purpurea*

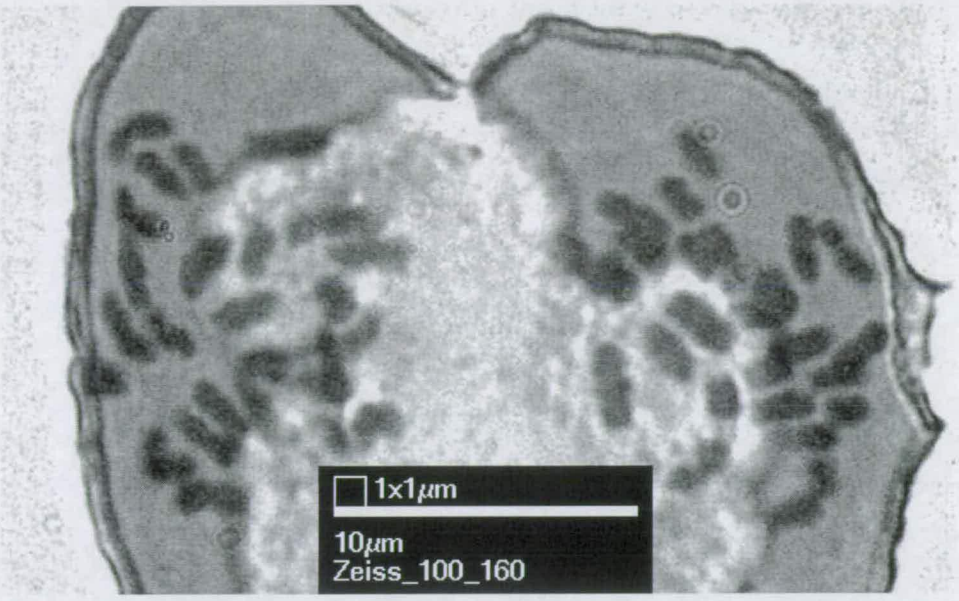


Figure 6.4 *R. purpurea*

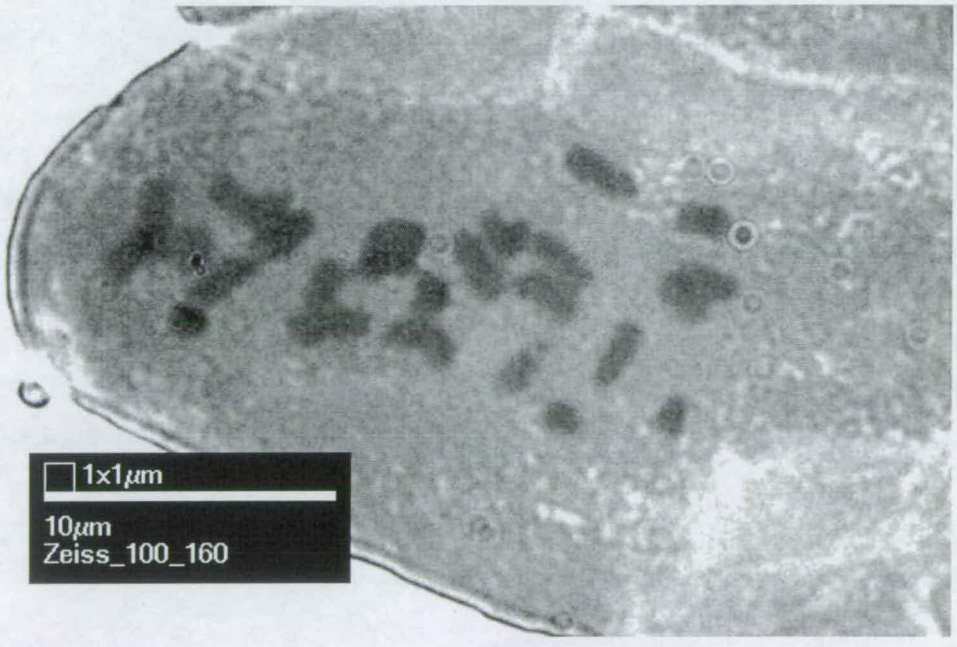


Figure 6.5 *Roscoea alpina*

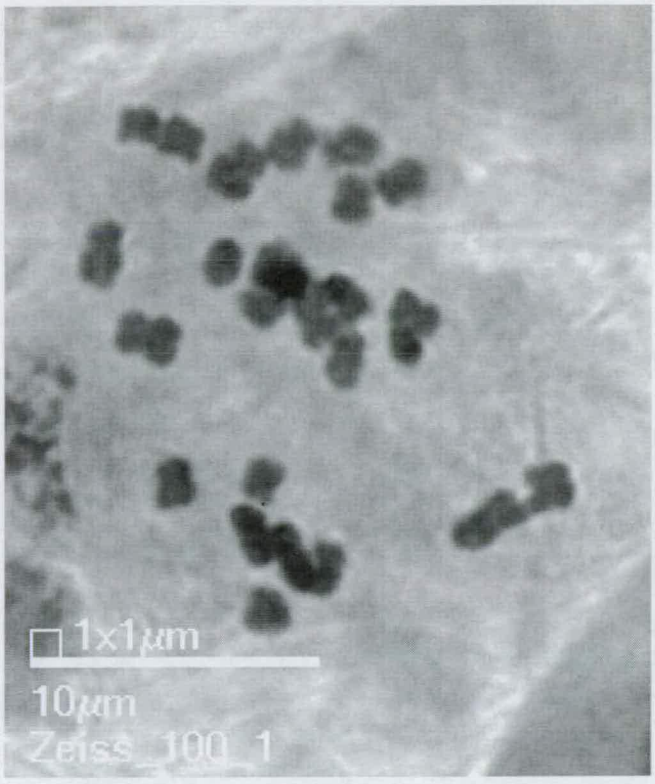


Figure 6.6 *R. alpina*

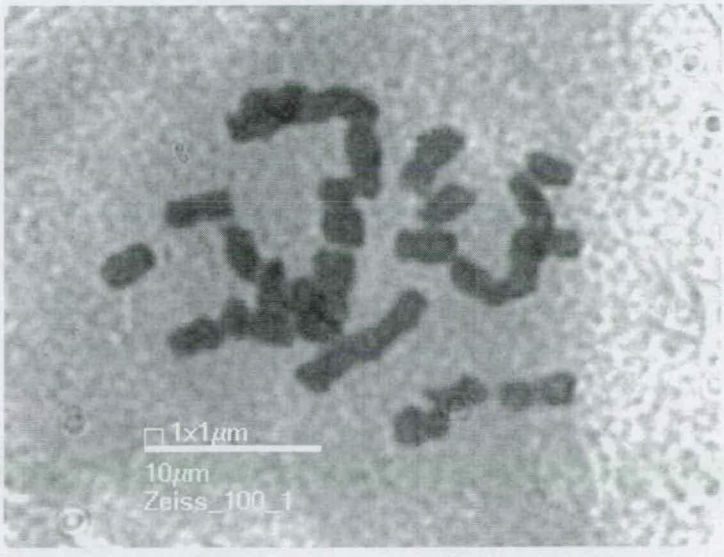


Figure 6.7 *R. alpina*

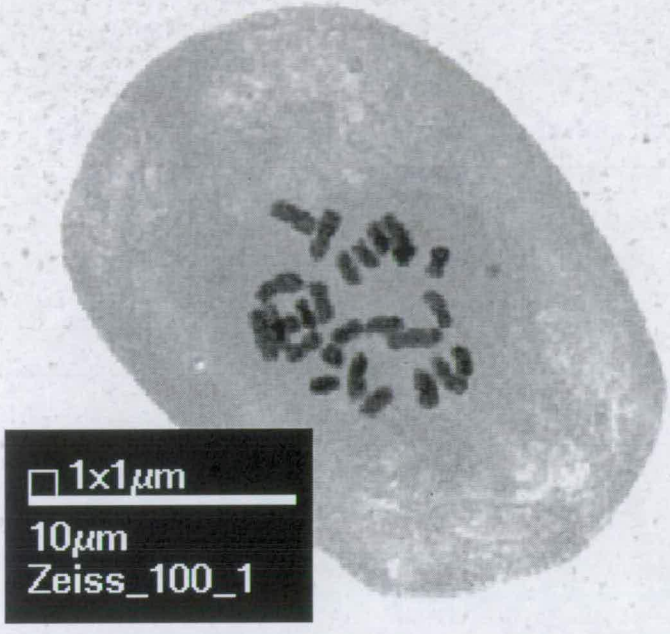


Figure 6.8 *R. alpina*

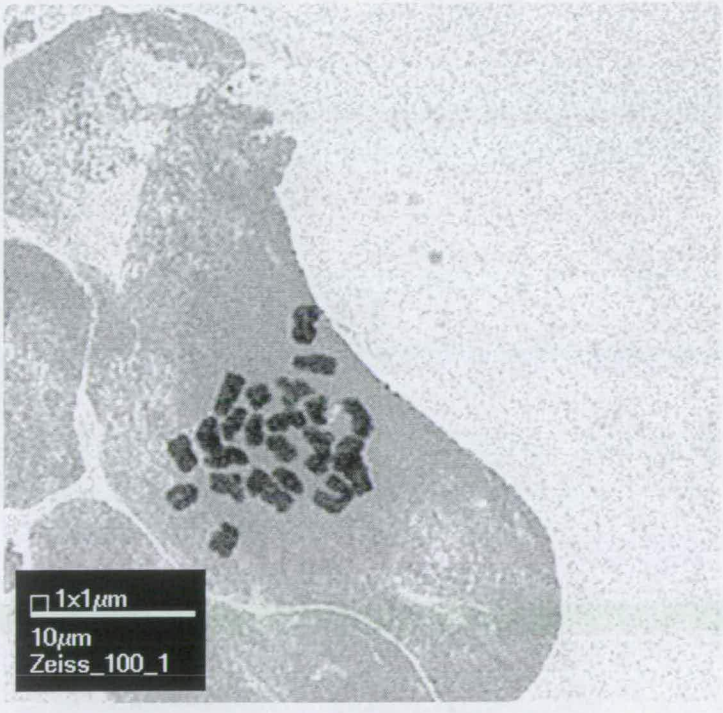


Figure 6.9 *R. auriculata*

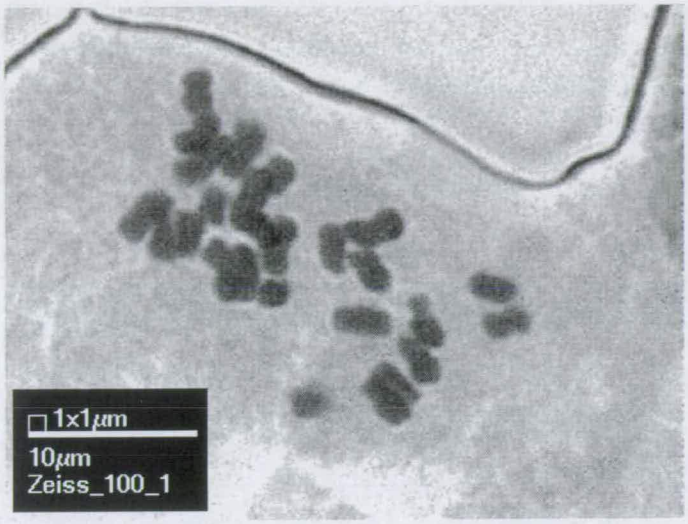


Figure 6.10 *R. auriculata*

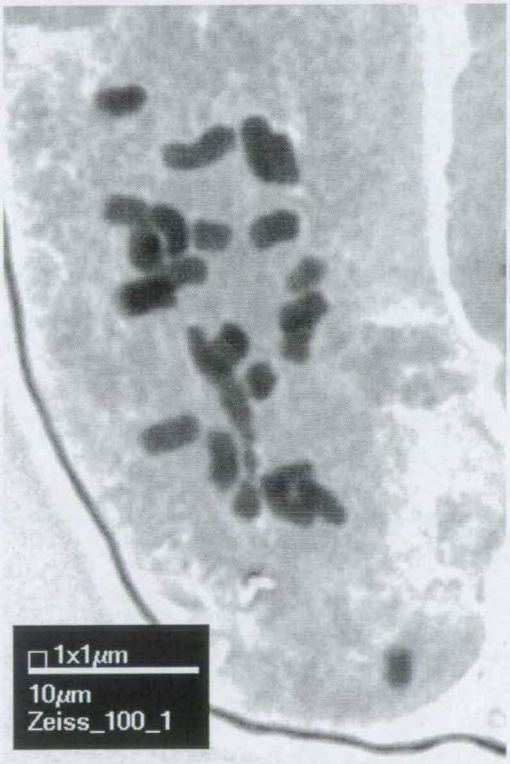


Figure 6.11 *R. auriculata*

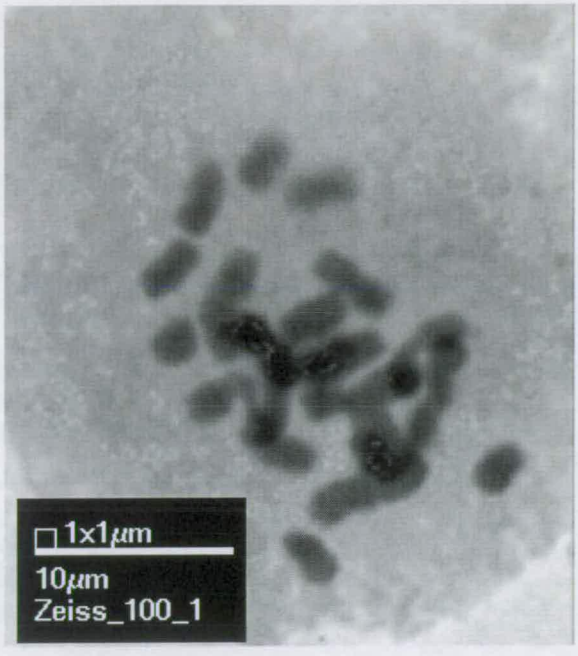


Figure 6.12 *R. auriculata*

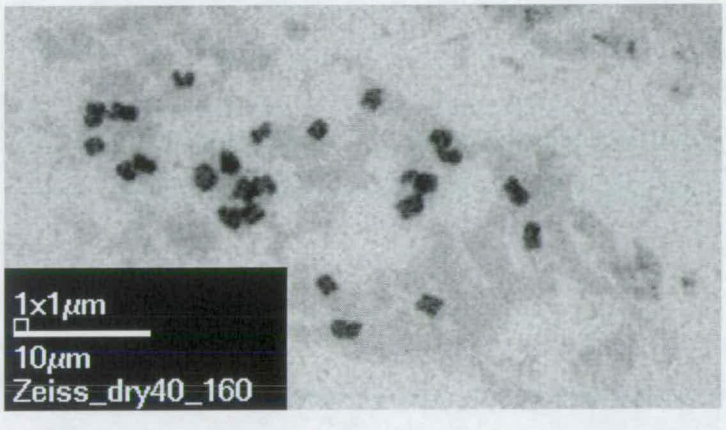


Figure 6.13 *Cautleya spicata*

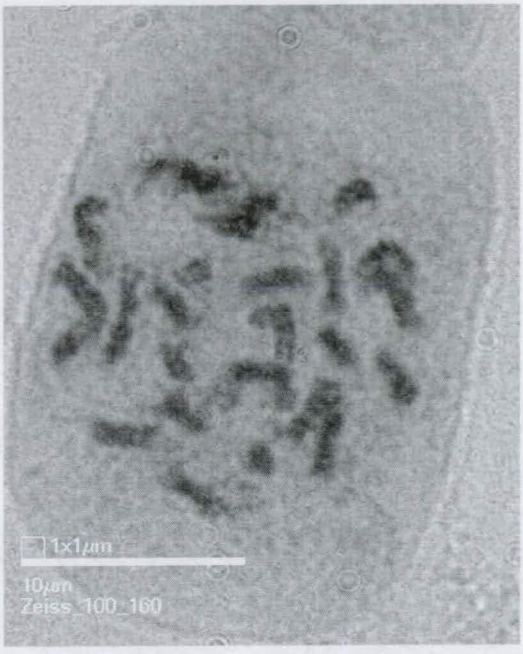
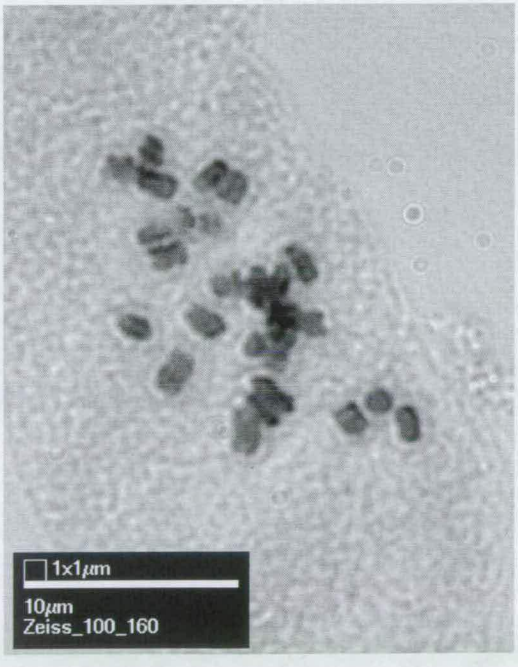


Figure 6.14 *C. spicata*



6.5 DISCUSSION

6.5.1 TIMING OF ROOT TIP COLLECTION

There are two times of day at which it is best to collect root tips. West and Cowley (1993) collected the root tips of four Chinese *Roscoea* species between 9 and 10 am whereas, in this study, the root tips were taken between 11.30 and 12.30 am. Both periods were found to give adequate numbers of metaphases. However, the midday period is widely followed in the field of cytology, both for Zingiberaceae (Lim, 1972; Newman, 1990) and other families (Jong, 1997). Midday is known to be at the peak of cell division in many plants and thus will yield the highest numbers of metaphases when fixed for cytological observation. The time recommended proves to be generally satisfactory in all plant families (Jong, 1997). Nonetheless, the midmorning period, 9-10 am, is preferred by Chen (1992). Lim (1972) collected root tips at midday for mitotic studies, but at 9-11 a.m. for meiotic studies of flower buds. No systematic study of the relationship of the two periods and the metaphases of root tips of *Roscoea* species is conducted in this study.

6.5.2 PRE-TREATMENT AND STAINING

Literature review shows that workers in cytotaxonomic studies of Zingiberaceae have used various pre-treatment and staining chemicals. Chen (1992) stated that 1-bromonapthalene (MBN) and paradichlorobenzene (PDB) are better at treating the material of Zingiberaceae plants than other chemicals. West and Cowley (1993) used MBN and obtained plenty of metaphases. The pre-treatment of the root tips by 1-bromonaphthalene (MBN) gives a slightly higher percentage of metaphase cells than 8-hydroxyquinolene (OQ) in this study. However, 8-hydroxyquinolene (OQ) is preferred in the cytological lab of RBGE by two other workers on *Curcuma* species (Ardiyani, pers. comm.; Nasir, pers. comm.). Recent papers on cytological studies of *Zingiber officinale* (Rai *et al.*, 1997; Das *et al.*, 1998) and *Curcuma* species (Joseph *et al.*, 1999), show that OQ and PDB are preferred for the pre-treatment. In addition, there is one report of using colchicine as the pre-treatment

chemical in the study of *Zingiber officinale* (Dhamayanthi, 1998).

Feulgen has proved so far to be effective in staining the chromosomes of Zingiberaceae. Examples are Lim (1972), Newman (1990), West and Cowley (1993). Feulgen gives also well-stained chromosomes in this study. Chen (1992) used and recommended a derivative of basic fuchsin, carbolo fuchsin for its convenience and reliability. In other plant families, Jong (1993) for example, successfully used Feulgen to stain the chromosomes of tribe Manuleae, Scrophulariaceae. Feulgen reagent is known as the most useful stain, but perhaps also one that causes the most disappointment (Jong, 1997). However, the state of the root tips is observed to be far more important than the stain (Newman, 1988). The healthy state of root tips collected for the study is the main reason to the well-stained chromosomes observed (Newman, 1988). Other dye, such as Haematoxyline is found to stain components of the cell as well as the chromosomes in *Curcuma* species, thus failing to yield well distinct-coloured chromosomes from the background (Ardiyani, pers. comm.).

6.5.3 THE CHROMOSOME NUMBER

The chromosome number of individuals is sometimes found to be different to the number of the species because of factors, such as chromosome fission and misdivision of the paired chromosomes at meiosis. An example is *Crepis tectorum* (2n = 8) where in 4000 plants, 10 plants, 4 plants and 4 plants have 2n = 9, 10 and 11, respectively (Navashin, 1926 as cited in Briggs and Walters, 1997). The chromosome numbers 2n = 26 of *Roscoea alpina* in this study and *R. purpurea* in other studies may be attributed to **centric fission** of one of a pair of the chromosomes. The event is thought to derive from centromere breakage without reunion giving rise to two telocentrics or iso-chromosomes. However, meiotic studies of the species are needed before any such statement can be confirmed. The pairing of the homologous chromosomes during meiosis will be the first evidence for any conclusion. It may be noted here that n = 12 and n = 13 populations of *Cautleya gracilis* show correlations with some vegetative and floral characters (Mehra and Sachdeva, 1979). Plants with n = 13 are shorter, possess smaller leaves and bracts

and have fewer flowers per spike, in comparison to plants with n = 12. However the size of the flower is almost the same in both groups. In addition, plants with n = 13 are always found at higher altitudes, 2250-2500 m, in comparison to those with n = 12 that occur between 2000-2200 m. Flower colour in *Roscoea* populations has not been found to correlate with the chromosome information (West and Cowley, 1993).

The sister clade of *Roscoea/Cautleya* is a clade of *Pommereschea* and *Rhynchanthus* (Wood *et al.*, 2000; Kress, pers. comm.; Chapter Two in this thesis). The basic chromosome number of *Pommereschea* is x = 11 or 2n = 22 (Larsen, 1973b), while that of *Rhynchanthus* is x = 22, 2n = 44 (Chen *et al.*, 1987). This suggests that the basic chromosome number of the clade of *Pommereschea/Rhynchanthus* is x = 11. In the *Roscoea/Cautleya* clade, the basic chromosome number is x = 12 and 13 (see Tables 6.1-6.3). Within the context of Hedychieae evolution and its chromosomal changes, these numbers imply that the ancestor of *Roscoea/Cautleya* and *Pommereschea/Rhynchanthus* had a basic chromosome number of x = 11, with later the addition of chromosomes in the clade of *Roscoea/Cautleya*. A pollen character that seems to support the relationships among the genera is the type of spine on the surface of the pollen. Pollen grains in *Pommereschea* and *Rhynchanthus* are spineless whereas pollens of *Roscoea* and *Cautleya* are long-spined (Chen, 1989).

6.5.4 THE CHROMOSOME SIZE

The chromosomes of Zingiberaceae are of small to medium size, 0.24-5.8 μm, compared with those of other angiosperms where small is ≤ 2 μm and large is ≥ 10 μm (Stace, 2000). Most are metacentric in shape with submetacentrics and occasional subacrocentrics (Newman, 1988). West and Cowley (1993) found that chromosomes in *Roscoea* are uniform by metacentric and the total length range of the chromosomes is 1-2 μm. Chromosome sizes of *Roscoea* species in this study,1-2 μm, conform to those found by West and Cowley (1993). The size of the chromosomes of *Roscoea* is rather small compared to those found in *Kaempferia*, 2.4-5.8 μm, the biggest chromosomes in Hedychieae (Beltran and Kam, 1984). The

smallest chromosomes found in Hedychieae are those of *Curcuma*, a genus with the highest basic chromosome number, x = 21, in Zingiberaceae. Chromosome sizes in *Curcuma* range between 0.24-0.99 μm in six species studied by Joseph *et al.* (1999).

6.5.5 ASPECTS FROM LITERATURE REVIEW

An incident of the chromosome number 2n = 34 of *Cautleya spicata* (Sharma and Bhattacharyya, 1959) has had quite an impact on cytotaxonomic interpretation of the family as a whole. Apart from three other reports (Mehra and Sachdeva, 1971, 1976, 1979) that all recorded n = 13 for *Cautleya spicata*, Chen (1989) followed the number of 2n = 34 for *Cautleya spicata* in his review of cytology and pollen structure of Asian Zingiberaceae. Although, Chen and his colleagues had published a series of chromosome counts of Zingiberaceae in six papers (Chen *et al.*, 1982; Chen *et al.*, 1984; Chen *et al.*, 1986; Chen *et al.*, 1988; Chen *et al.*, 1987; Chen *et al.*, 1989), *Cautleya* species is not one of the species counted. Mehra & Sachdeva (1979) pointed out that the *Cautleya spicata* count of Sharma & Bhattacharyya (1959) appeared to be erroneous since they recounted the plant from the same locality of Sharma & Bhattacharyya and found the chromosome number of *Cautleya spicata* to be n = 13. Molecular phylogenetic studies (Searle and Hedderson, 2000; Wood *et al.*, 2000; Chapter Two in this thesis) show that *Cautleya* is the sister group to *Roscoea* and the basic chromosome number of the clade is x = 12 and 13. In light of the molecular phylogenetic findings, it also suggests that the basic chromosome number x = 17 appears only in *Hedychium* (Mukherjee, 1970; Chen *et al.*, 1984) in the family which is confirmed to be a monophyletic group within the tribe (Wood *et al.*, 2000).

There is also another assumption that seems to be incorrect in Chen's evolutionary interpretation paper of the chromosome numbers in Zingiberaceae (Chen, 1989). Two species of *Boesenbergia*, namely *B. fallax* (endemic to Yunnan) and *B. rotunda* (widely cultivated), from the three species that are found in China (with *B. albomaculata*, another endemic to Yunnan, Wu and Larsen, 2000) were counted with 2n = 36 (Chen *et al.*, 1988). These numbers and few others of

Boesenbergia species counts of 2n = 20 available up to the time persuaded Chen to deduce that the basic chromosome number of *Boesenbergia* is x = 9 while x = 10 species are the minority and perhaps derived by aneuploidy of x = 9. New cytological studies of *Boesenbergia*, particularly those of Poulsen (1993) and Eksomtramage *et al.* (1996) with new numbers of 2n = 24 and 36, indicate that the likely basic chromosome number of Chinese *Boesenbergia* is x = 12 and not x = 9. The 2n = 36 *Boesenbergia* species are probably triploids. The basic chromosome numbers of x = 10 and x = 12 are supported in the molecular phylogenetic studies of the Hedychieae in Chapter Two. Two distinct lineages of *Boesenbergia* species are revealed in accordance with the basic chromosome numbers, i.e. x = 10 and x = 12. Only mitotic events were investigated in the two Chinese *Boesenbergia* species (Chen *et al.*, 1988). It is important that the meiotic behaviour study of the two species and other *Boesenbergia* species of 2n = 36 is conducted for further information, particularly for the ploidy level.

Table 6.3 *Roscoea* and *Cautleya* species in this cytotaxonomic study.

Species	Place of origin	RBGE accession number	Number of chromosomes
Roscoea alpina Royle	India (Himachal Pradesh)	19861108	2n = 26
R. auriculata K. Schum.	Not known	19699652	2n = 24
R. purpurea Sm.	Nepal (Sing Gompa)	19962515	2n = 24
Cautleya spicata (Sm.) Baker	Not known	19590760	2n = 26

CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUSIONS

It is always challenging for man to understand the variation of biological diversity. Not only is it for his own curiosity as an entity in the biological world, but also the knowledge of the all living beings is fundamental for his own survival. Advances of the theory and practice in cladistics, molecular techniques and computer technology enable systematists to have much more rigorous and accountable tools for studying biological variation and producing rigorous hypotheses of the relationships of plants. However, differences in the phylogeny or branching patterns uncovered by different sources or methods, remind the reader that we do not know the true evolutionary history of the plants. We were not there to see speciation or extinction. The best we can do is to have the closest tree to the real tree based on available data.

This thesis presents an attempt to understand the evolution of the Zingiberaceae, by using mainly two sources of phylogenetic information, i.e. molecules and morphology. The internal transcribed spacers of nuclear ribosomal DNA (ITS) have proved to be informative and useful in the reconstruction of the phylogeny of the Zingiberaceae plants, both at generic and specific levels as shown in the Hedychieae study (Chapter Two) and the *Roscoea* study (Chapter Four). Although the nucleotide substitution rate in *trn*L-F is low compared to that in ITS sequences, it gives some phylogenetic information for the Hedychieae study confirming parts of the ITS trees.

Two main subclades can be recognised in the tribe Hedychieae, namely the '*Hedychium* clade' and the '*Curcuma* clade'. Two genera are found to be paraphyletic, namely *Boesenbergia* and *Curcuma*. It is also clearly shown that the tribe Zingibereae should be combined with the tribe Hedychieae, reducing the numbers of tribes in the family to three, namely Hedychieae, Globbeae and Alpinieae. Since the type genus of the family, *Zingiber*, is included in what is

currently called tribe Hedychieae, the tribe must be renamed to Zingibereae according to article 19.4 of the International Code of Botanical Nomenclature (Greuter *et al.*, 2000). The proper tribal placement of *Pommereschea* and *Rhynchanthus* should be made clearly in the Hedychieae. While the traditional classification of the family as comprising the three tribes, is likely to be a reflection of the family evolutionary history, an explicit adjusted circumscription of the three tribes should be laid out synthesising from all the available data. A new classification of Zingiberaceae based on this study and others is presented in Table 7.1.

Lateral staminodes, as suggested by the molecular analyses in this study and others (Searle and Hedderson, 2000; Wilf *et al.*, 2000; Wood *et al.*, 2000), appear to be present and fused with the labellum first in *Siphonochilus*, as found also in members of the Costaceae. It is likely that this character, a fusion of lateral staminodes with the labellum is plesiomorphic in the family Zingiberaceae. Nonetheless, the labellum in Costaceae is composed of all five staminodes (two from the inner whorl and all three from the outer whorl) different from that of the Zingiberaceae. Then within the Alpinieae they are wanting or reduced to very small tooth-like appendages (but petaloid and fused with the labellum in *Tamijia*). Note that the homology interpretation of the tooth-like appendages in Alpinieae is not conclusive among the members of the family (Burtt, 1972). In Globbeae, they are again petaloid with a notable position of the staminodes in the lower part of the filament in *Mantisia*. Nearly all the members of Hedychieae possess petaloid and free staminodes, except *Pommereschea*, *Rhynchanthus* and *Stadiochilus* without the staminodes. *Boesenbergia longiflora* which was once held to be a distinct genus, *Curcumorpha*, has again the fused staminodes with the labellum (Larsen, 1997) and so has the so-called tribe Zingibereae or *Zingiber* species.

Although, the ideal morphological synapomorphies of the tribes readily observable in the field are only a few, if any, the correlated characters consideration of a given species seems to be the best possible measurement for the moment. The classification of Zingiberaceae seems never to be adequate basing on a few morphological characters. Besides, as more and more molecular data have become

available for the phylogenetic investigation, the result is making it clear that the convergence and reversal evolution of many morphological characters in the family is more likely to occur than previously thought.

The relationships among *Boesenbergia* species may be better resolved with additional molecular characters and/or more samples of the species. Ideally, the species of *Caulokaempferia* are also highly in need for the investigation. But what is clearly shown here is that *Boesenbergia* is paraphyletic in respect to *Caulokaempferia*. The phylogenetic relationships revealed here and the distribution ranges of *Pyrgophyllum* and *Camptandra*, *Boesenbergia* and *Caulokaempferia* suggest that the progenitor of these genera may have the ancient, wider and connected distribution range. But it was later restricted to certain areas, i.e. mountain ranges that act as a reservoir for the species.

Smithatris may be the true sister group of the *Curcuma* complex that manifests the paraphyly of *Curcuma*. A detailed molecular phylogenetic study of the members in the complex is badly needed in order to discern and confirm the relationships found in this thesis. All genera in the complex, namely *Curcuma*, *Hitchenia*, *Paracautleya*, *Smithatris* and *Stahlianthus* may be recognised under a single genus *Curcuma*. This proposal could be supported by more studies on both molecules and morphology.

There are two types of anther appendages in the Hedychieae. One is derived from the joint of the anther and the filament, as found in basifixed versatile anther of *Cautleya/Roscoea* and *Camptandra*. The other is derived from the base of the thecae of the anther, as found in dorsifixed versatile anther of *Curcuma* and *Paracautleya*.

Wood (1991, as cited in Wood *et al.*, 2000) hypothesised that the origin of the family Zingiberaceae may have been in West Gondwanaland before the effective separation of South America and Africa. The progenitor of the plants was then rafted on the Indian subcontinent to Asia. The hypothesis seems to fit well with all molecular results available to date, i.e. with the basal placement of *Siphonochilus* in

the phylogenetic tree of the family and Alpinieae as the only tribe whose members are pantropical. On the other hand, Globbeae and Hedychieae are confined to Asia. In addition, the preponderance of the members of Costaceae, the sister family of Zingiberaceae, in neotropics and Africa supports the hypothesis.

The molecular phylogenetic trees obtained not only suggest the likely pathways of evolution or phylogeny of the plants studied, but also give us a clue about the origin of some of the well known spices in Zingiberaceae such as *Curcuma longa* or turmeric and *Zingiber officinale* or ginger. The branching patterns of the trees in Chapter Two coupled with the distribution ranges of the species and the genus, suggest that the origin place of *Curcuma longa* lies roughly in the Indian subcontinent while the place of origin of *Zingiber officinale* is confined within Indochina, highly possibly in Thailand. The clue for the origin of the true ginger comes from the fact that *Cornukaempferia*, a newly found genus (Mood and Larsen, 1997, 1999), is the sister group of *Zingiber*. The distribution ranges of the two species of *Cornukaempferia* lie within North and Northeast Thailand. Although *Zingiber* has become widespread in tropical Asia, it suggests that *Zingiber* may have originated in the area. As *Curcuma longa* or turmeric is a species placed in *Curcuma* subgenus *Curcuma* that is the sister clade of *Paracautleya/Hitchenia*, it suggests that the clade of *Curcuma* subgenus *Curcuma* may have originated in peninsular India where *Paracautleya* is endemic to the Western Ghats in Kerala and *Hitchenia caulina* is endemic to the next state, Karnataka (Jain and Prakash, 1995). Two other species of *Hitchenia*, namely *H. careyana* and *H. glauca* have wider distribution ranges that are as far as Himalayan India and Burma (Kress, 2000). On the other hand, species in the clade of *Stahlianthus/Curcuma* subgenus *Hitcheniopsis* are well presented in North and Northeast Thailand and adjacent countries. These patterns of relationships and the distribution records suggest that the '*Curcuma* clade' may have originated in continental Thailand and later two distinct lineages appeared. One is the diversification of species in more or less the same area (*Stahlianthus/Curcuma* subgenus *Hitcheniopsis*). The other is well adapted in the Indian subcontinent (*Paracautleya/Hitchenia/Curcuma* subgenus *Curcuma*). The division of these two subclades is coincident with the species distributions of the two subclades of a

monophyletic genus *Roscoea*, namely: the 'Himalayan clade' and the 'Chinese clade' that are separated at Northeast India.

These line of evidence suggest that the likely place of origin of the Hedychieae may have been Northeast India and that the tribe later diversified in both directions, i.e. eastward to Southeast Asia and westward to peninsular India. The hypothesis is also supported by the high endemism and rich species diversity of *Hedychium*, an early-branched genus in one of the two subclades in the tribe phylogeny (Chapter Two), in Northeast India (Jain and Prakash, 1995).

The basic chromosome number x = 11 seems to be the first shared number of tribe Hedychieae. In the '*Curcuma* clade', the number of x = 21 is thought to derive from x = 11 by either 11×2-1 or 11+10. The numbers in the clade of *Stahlianthus /Curcuma* subgenus *Hitcheniopsis* appear to be variable whereas it is rather constant at x = 21 within the clade of *Hitchenia/Paracautleya/Curcuma* subgenus *Curcuma*. In the '*Hedychium* clade', the changes of the basic chromosome number appear to be exclusively by aneuploidy of x = 11 either losing (x = 10) or adding up (x = 12, 13, 14 and 17) the chromosomes. In spite of the numerous chromosome studies in Zingiberaceae, some genera are still not yet counted, for instance, *Camptandra, Cornukaempferia, Paracautleya, Smithatris, Haniffia, Stadiochilus* and *Nanochilus*. The missing numbers of these genera are very important evidence to the study of the evolutionary history of tribe Hedychieae and the family as a whole. In addition, comparative cytological study in *Boesenbergia* and *Caulokaempferia* may shed light on the evolution of the two genera.

Table 7.1. A new classification of Zingiberaceae based on this study and others (Searle and Hedderson, 2000; Wood *et al.*, 2000; Kress, pers. comm.). (see also Table 1.6)

Alpinieae A. Rich. (21 genera, ~788 species)	**Globbeae** Meisn. (4 genera, ~110 species)	**Zingibereae** Meisn. (24 genera, ~395 species)
Aframomum K. Schum. (50)	*Gagnepainia* K. Schum. (3)	*Boesenbergia* Kuntze (60)
Alpinia Roxb. (227)	*Globba* L. (100)	*Camptandra* Ridl. (4)
Amomum Roxb. (150)	*Hemiorchis* Kurz (3)	*Caulokaempferia* K. Larsen (10)
Aulotandra Gagnep. (5)	*Mantisia* Sims (4)	*Cautleya* (Benth.) Hook. f. (2)
Burbidgea Hook.f. (8)		*Cornukaempferia* J. Mood & K. Larsen (2)
Cyphostigma Benth. (1)		*Curcuma* L. (50)
Elettaria Maton (7)		*Distichochlamys* M. F. Newman (1)
Elettariopsis Baker (10)		*Haniffia* Holttum (2)
Etlingera Giseke (70)		*Haplochorema* K. Schum. (3-4)
Geocharis (K. Schum.) Ridl. (7)		*Hedychium* J. König (50)
Geostachys (Baker) Ridl. (18)		*Hitchenia* Wall. (3)
Hornstedtia Retz. (50)		*Kaempferia* L. (40)
Leptosolena C. Presl (1)		*Nanochilus* K. Schum. (1)
Plagiostachys Ridl. (20)		*Paracautleya* R. M. Sm. (1)
Pleuranthodium (K. Schum.) R. M. Sm. (25)		*Parakaempferia* A. S. Rao & D. M. Verma (1)
Renealmia L.f. (75)		*Pommereschea* Wittm. (2)
Riedelia Oliv. (60)		*Pyrgophyllum* (Gagnep.) T.-L. Wu & Z.-Y. Chen (1)
Siamanthus K. Larsen & J. Mood (1)		*Rhynchanthus* Hook. f. (6)
Siliquamomum Baill. (1)		*Roscoea* Sm. (19)
Tamijia S. Sakai & Nagam. (1)		*Scaphochlamys* Baker (30)
Vanoverbergia Merr. (1)		*Smithatris* W. J. Kress & K. Larsen (1)
		Stadiochilus R. M. Sm. (1)
		Stahlianthus Kuntze (6)
		Zingiber Boehm. (100)

A genus of uncertain placement:

Siphonochilus J. M. Wood & Franks (15)

It should be borne in mind that the genes sampled in this study constitute only a minute part of the big pool of molecular information contained in the plant genome. With the completion of the sequencing project in *Arabidopsis thaliana*, we now know that there are 25498 genes in its genome (The Arabidopsis Genome Initiative, 2000). The ideal scenario may be that as many genes as possible be used to infer the phylogeny of the plants, preferably from all the three genomes: nuclear, chloroplast and mitochondrial. Other genes or parts of the genome that may be sampled for phylogenetic information of Zingiberaceae plants include, for example, *mat*K (Hilu and Liang, 1997). Kress (pers. comm.) has sequenced this noncoding region in chloroplast DNA, and found that the region is informative and suitable for the study of phylogeny in Zingiberaceae at generic level. Apart from the two common sources of molecular phylogenetic information, nuclear and chloroplast DNA, to be complete with the molecules of a cell, a region in mitochondria should be included. Because of the very low rate of nucleotide substitution in mitochondrial DNA, this may take sometimes to find out which region is informative enough and suitable for phylogenetic study at specific or generic levels (Palmer, 1992b). The exceptional high rate of nucleotide substitution of *nad*1 b/c in *Pelargonium*, is an example that shows the usefulness of the mitochondria (Bakker *et al.*, 2000). New genes may give more support to those clades in the molecular trees in this thesis that are only weakly supported, i.e. bootstrap value less than 75%.

Morphology, however, should not be ignored. The evidence of morphological variation in plants is always the first that we can see and appreciate. Searching for hard morphological characters as well as documenting all variable characters will be pivotal to the interpretation of the study of morphological evolution, whether the study is based on molecular evidence and/or morphological evidence alone. In addition, the study of the development of morphological traits or ontogeny, will be the basis for homology assessment. However, It is found that the morphological characters of *Roscoea* species contain too much homoplasy to be usefully analysed on their own. Nonetheless, one character, anther appendages tip, is completely congruent with the molecular phylogeny, dividing the genus into Himalayan and Chinese clades.

Distribution ranges of the species have long been used, along with morphology, to define the delimitation of the taxon. The geographical boundaries of the species are now also a crucial part of the study of their evolutionary history. The distribution patterns of the plants provide another clue of how the species are related and derived. There are various ways to incorporate biogeographical data into phylogeny (Conran, 2000). As has been shown in helping to define a new taxon, *Roscoea bhutanica* in Chapter Five, herbarium records are invaluable in this respect. More plant collecting in less explored areas is badly needed to fill in the gaps in the study of the evolutionary history of the group. An exploration in a new or already visited area may discover new taxa and give new evidence to the study of Zingiberaceae phylogeny. Live samples of the taxa not yet sequenced, such as *Stadiochilus* and *Nanochilus* are valuable as well. Complete collections, including leaf samples in silica gel, colour photographs of the flowers, flowers in spirit, dried pressed plants and possibly also living plants of a given taxon in Zingiberaceae is highly recommended for the collectors.

Other traditional lines of evidence, for instance, cytology, anatomy and breeding systems (including pollination biology) may be further pursued for the plants. As all these are different aspects of a single taxon, they will find no greater value than in the context of evolutionary history of the taxon. Searching for the closest phylogenetic tree of the true tree will go on for as long as we are not certain yet with the current ones. In addition, fossil records and ecological information should be sought in order to explain better the branching patterns found.

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APPENDIX ONE: MOLECULAR TECHNIQUES IN ZINGIBERACEAE

A1.1 DNA EXTRACTION

Fresh leaf material was harvested and kept in silica gel-filled plastic bags and stored at 0 °C at least overnight in a refrigerator before extraction for plants in cultivation at RBGE, to destarch the leaf tissue (starch may interfere with subsequent operations performed using the DNA). Field collected samples were also kept in silica gel (Chase and Hills, 1991) and used after returning to the laboratory.

Generally, there are two methods of extracting plant DNA in this study. A modified CTAB procedure of Doyle and Doyle (1987) was followed for most of the plants studied. The QIAGEN DNeasy Plant Mini Kit (QIAGEN, 1997a), with a little modification, was used to obtain purer DNA from herbarium specimens and some species which proved difficult to amplify in later PCR reactions. The former method is sometimes followed by purification using the QIAGEN QIAquick PCR Purification Kit.

Considering the two methods above, it is found that DNA extraction work by the Kit is easier, quicker and at the same time gives relatively higher quality (fewer impurities, such as carbohydrates, protein, and salts) of DNA. However, the modified CTAB procedure works just as well in general for most species and is cheap to use.

CTAB PROCEDURE

1. A portion of leaf c. 1 cm^2 was cut into many small pieces, and put into a 1.5-ml microcentrifuge tube and c. 50 mg of purified sand and 200 µl of 2x CTAB extraction buffer were added. The leaf tissue was ground with a

plastic pestle until a homogeneous slurry was formed.

2. A further 800 µl of 2x CTAB was then added. The contents were mixed gently, and the tube was incubated at 65 °C for 30 to 60 minutes with optional gentle swirling.
3. The tube was allowed to cool to ambient temperature before adding 200 µl of wet chloroform. The solution was mixed gently 4 or 5 times and centrifuged for 2 minutes at 13000 rpm.
4. The aqueous upper phase was removed to a clean tube and re-extracted with 200 µl of wet-chloroform. (Extracting DNA by wet-chloroform can be improved by using a shaking platform for c. 10 minutes each)
5. Again this was mixed gently to obtain a momentary single phase and centrifuged for 2 minutes at 13000 rpm.
6. In another clean tube with the aqueous phase, 600 µl of cold (-20 °C) propan-2-ol was added and the contents were mixed gently to precipitate the nucleic acids. After at least 30 minutes at room temperature, the pellet of nucleic acids was precipitated by centrifuging for 5 minutes at 13000 rpm. (Longer period of precipitation increases the yield, i.e. overnight)
7. The supernatant was removed and 1 ml of wash buffer was added. The tube was left for at least 30 minutes to remove the 2x CTAB from the pellet.
8. The tube was centrifuged for 5 minutes at 13000 rpm and the supernatant was then aspirated as much as possible.
9. Next, the pellet was dried completely by using an incubator drying oven for 10 minutes at 50 °C. Lastly the pellet was dissolved in 30-50 µl of sterile distilled water and stored at –20 °C until required. (DNA concentration is normally between 10-30 ng/µl)

STOCK SOLUTIONS

2x CTAB (500 ml):

10 g CTAB (Hexadecyltrimethylammonium bromide), 140 ml 5M NaCl, 25 ml 2M Tris-HCl (pH 8.0), 20 ml 0.5M EDTA, with optional 1% PVP-40T (5

g) and 0.2% Beta-Mercaptoethanol or DTT (added immediately prior to use, 1/50 dilution)

Adjust to pH 8.0 with either NaOH or HCl and autoclave.

Wet Chloroform:

Chloroform 24 units, Octan-1-ol 1 unit

Wash Buffer:

76% ethanol, 10 mM Ammonium Acetate

CTAB is a cationic detergent that aids in the lysis of cell membranes and will form complexes with nucleic acids. NaCl aids in the formation of nucleic acid-CTAB complexes. EDTA chelates divalent ions, particularly Ca^{2+} and Mg^{2+} and prevents the activity of metal-dependent nucleases. PVP-40T forms complexes with secondary plant products, particularly with polyphenols, tannins and quinones. Beta-mercaptoethanol and DTT are reducing agents that protect DNA against quinones, disulphides, peroxidases and polyphenol oxidases. The chloroform is described as 'wet' because the addition of isoamylalcohol (or octan-1-ol) changes its properties making it slightly more hydrophilic and therefore, capable of precipitating proteins and carbohydrates more effectively. The purpose of the chloroform stage is to remove proteins and carbohydrates. The wash buffer stage reduces the salt concentration in the extraction buffer, therefore the CTAB-nucleic acid complex is precipitated. The effect of the wash buffer is to dissolve the CTAB from the CTAB-nucleic acid complex.

DNEASY KIT PROCEDURE

1. Plant tissue was ground under liquid nitrogen to a fine powder using a plastic pestle in a 2-ml microcentrifuge tube. Without leaving to thaw, the sample was continued immediately with step 2.
2. 400 µl of Buffer AP1 and 4 µl of Rnase A stock solution (100 mg/ml) were added to a maximum of 100 mg of ground plant tissue and mixed by spinning with a vortex shortly and vigorously.
3. The mixture was incubated for at least **30** (instead of 10) minutes at 65 °C

and was mixed 2-3 times during incubation by inverting tube. (Longer period of incubation gives higher yield)

4. 130 µl of Buffer AP2 was added to the lysate and mixed. The mixture was incubated for **10** (instead of 5) minutes. (This step precipitates detergent, proteins and polysaccharides)
5. Next, the lysate was applied to the QIAshredder spin column (lilac) sitting in a 2-ml collection tube. The tube was then centrifuged for 2 minutes at maximum speed (13000 rpm).
6. A flow-through fraction from step 5 was transferred to a new tube without disturbing the cell-debris pellet. (Typically 450 µl of lysate are recovered)
7. 225 µl of Buffer AP3 and 450 µl of ethanol (96-100%) were added to the cleared lysate and mixed by pipetting. (or 0.5 volume of Buffer AP3 and 1 volume of ethanol in corresponding to a flow-through amount)
8. 650 µl of the mixture from step 7 were applied, including any precipitate which may have formed, onto DNeasy mini spin column sitting in a 2-ml collection tube. The tube was then centrifuged for 1 minute at 8000 rpm and a flow-through was discarded.
9. Step 8 was repeated with remaining sample. A flow-through and a collection tube were then disposed.
10. A DNeasy column was placed in a new 2-ml collection tube. 500 µl of Buffer AW were added onto the DNeasy column and the tube was centrifuged for 1 minute at 8000 rpm. A flow-through was discarded but the collection was reused in step 11.
11. 500 µl of Buffer AW were added to DNeasy column and the tube was centrifuged for 2 minutes at 13000 rpm to dry the column membrane.
12. A DNeasy column was then transferred to a new 2-ml microcentrifuge tube. **50** (instead of 100) µl of preheated (65 °C) Buffer AE were added directly onto the DNeasy column membrane and incubated for 5 minutes at room temperature. Next, the tube was centrifuged for 1 minute at 8000 rpm to elute.
13. Step 12 was repeated once as described.

POINTS TO NOTE

1. Do not underestimate the importance of this very first step of working on DNA. High yield and purity of DNA will make things a lot more easier in later uses of this DNA.
2. Using liquid nitrogen in the grinding step is a very effective technique to break down cells of plant tissues. Using sand requires more vigorous force and skill. The first step of grinding of each protocol below can be interchanged.
3. If working on many samples at the same time, an electric drill can be used for the grinding step.
4. It is always better to use fresh material or silica gel-dried sample from fresh material for DNA extraction. If this is not possible, try to use the most recent herbarium sheet with a healthy, green colour.
5. The procedures presented here are a total genomic DNA extraction method yielding both nuclear DNA and chloroplast DNA that are later used successfully in this study.
6. Isopropanol, propan-2-ol and 2-propanol are the different names of one chemical used interchangeably that I came across in a literature of protocols.

A1.2 AGAROSE GEL ELECTROPHORESIS

The DNA obtained in section A should be visualised to check its presence, size and conformation (quality) and relative density (quantity). Agarose gel electrophoresis is used throughout this study, not only for DNA checking in the first step of DNA extraction, but also for a later step of PCR result checking. This method employs the fact that DNA is overall negatively charged by phosphates along its backbone. By applying an electric field to a supporting layer (in this case, agarose gel), different sizes of DNA will travel through this layer at different rates according to their relative sizes. The method is also used for purification of some PCR products

(Gel Extraction) when PCR products contain more than one distinct band on a checking gel.

PROCEDURE

1. An appropriate amount of agarose and a volume of 1× TBE were prepared for an optimal concentration of the gel depending on the size of DNA fragments to be analysed. These were mixed and heated in a microwave oven to make a well melted solution (usually c. 2 minutes).
2. A gel mould was prepared by sealing its open ends with tape and a gel comb was aligned vertically in the mould.
3. The gel solution was allowed to cool for 2-3 minutes, then 1μ of ethidium bromide was added to 50 ml of the gel solution. The mixture was poured onto the gel mould.
4. The gel mould was left to set for c. 20 minutes. This could be accelerated by running tap water onto the outside of the flask holding the hot gel solution prior to step 3.
5. Once the gel had set which could be seen by its opacity, the adhesive tape and gel comb were removed to a special bin designated hazardous. The gel mould then was placed in an electrophoresis tank containing TBE buffer at the same concentration as the gel.
6. Samples were mixed with 1 μl of loading solution and then loaded into the gel plus a DNA marker.
7. Electricity was then applied, usually 80 V for one hour.
8. The DNA fragments in the gel were visualized under UV light and then photographed using a digital camera.

STOCK SOLUTIONS

10× TBE (Tris-Borate-EDTA) Buffer:

108 g Tris base, 55 g boric acid, 9.5 g EDTA, disodium salt, 750 ml dH_2O. Adjust pH to 8.3 with NaOH or HCl. Filter and adjust final volume to 1L.

DNA Markers:

1. Lambda HinDIII (33 ng/μl) (for DNA checking)

   1 part Lambda HinDIII stock (0.33 mg/ml): 3 parts loading solution: 6 parts dH_2O

2. 123 ladder (0.1 μg/μl) (for PCR products checking)

   1 part 123 bp ladder stock (1 μg/μl): 3 parts loading solution: 6 parts ladder buffer

Loading solution (SIGMA):

0.05% bromophenol blue (serves as tracking dye), 40% sucrose (add density and facilitate sample loading), 0.1 M EDTA pH 8.0 (terminate the action of enzymes that require divalent cations), 0.5% sodium lauryl sulphate (SDS) (dissociate DNA-protein complexes)

Ladder buffer:

10 mM Tris-HCl (pH. 7.5), 50 mM NaCl, 0.1 mM EDTA

Ethidium bromide (10 mg/ml)

POINTS TO NOTE

1. In this study, Gel moulds were usually prepared at 1% for DNA checking and 1.5% for PCR products checking (low agarose concentrations are used to separate large DNA fragments, while high agarose concentration allow resolution of small DNA fragments). The products of primers 'ITS 5P' and 'ITS 8P' are approximately 700 bp long, while that of primer 'c' and primer 'f' are approximately 1000 bp long both of which are suitable for analysing in 1.5% gel.
2. If the volume of liquid reduces considerably during heating due to evaporation, make up to the original volume with distilled water.
3. Ethidium bromide is a radioactive agent which is harmful upon contact.
4. Make sure that there are no air bubbles in the gel or trapped between the wells.
5. TBE buffer in a gel tank should slightly (1 mm) cover an agarose gel. Too little buffer will hinder the flow of electricity, whereas the electricity will

pass over an agarose gel, rather than go through the gel, with too much buffer.

6. Low V and longer time, for example 60 V and two hours, can be used as a combination to well separate DNA fragments.

A1.3 POLYMERASE CHAIN REACTION (PCR)

In order to decode or sequence a segment of DNA, current technologies require an adequate amount of the target segment that will be analysed. The polymerase chain reaction (PCR) (Mullis and Faloona, 1987; Mullis, 1990) which has been a driving force behind molecular biology researches since its invention is widely used to amplify *in vitro* a segment of DNA that lies between two regions of known sequence. The principle of this method is straightforward; the DNA segment flanked by two oligonucleotide primers is amplified *in vitro* by repeating a cycle of (1) heat denaturation of the DNA, (2) annealing the primers to their complementary sequences, and (3) extension of the primers with heat stable DNA polymerase. The components of every single PCR reaction that are DNA templates, primers, a DNA polymerase (with buffer to keep it active during the cycles) and free nucleotides each play a great role of whether a PCR reaction will be successful (with plenty of products) or not. Concentrations of each component in the specific volume must be optimal, along with an optimal profile of temperature used, in order to amplify successfully and correctly the target segment of DNA. This optimality is usually a subject of individual species or groups of species.

Despite of a careful handle of the PCR procedure, contamination sometimes arises and is a serious problem. Any amount of foreign DNA present will theoretically be amplified competing with the target DNA. A good laboratory will have a specific area and designated equipments used especially for the PCR. All equipments and chemicals should be autoclaved before use if it is possible. Reagents that are used regularly for PCR should be aliquoted into smaller amount tubes, making it easier to work with and can be easily discarded when contamination has

been detected. Every set of PCR reactions should have a negative control which has all the reagents as in others except a DNA template. A positive control which has a high quality of DNA template (highly amplifiable) should be included in the case of working with difficult species or herbarium specimen-DNA.

In this study, two main regions in the genome were amplified. The internal transcribed spacers (ITS), the 5.8 gene, and flanking regions of the 18S and 26S genes in nuclear DNA, and a segment comprising *trn*L intron and *trn*L-F of chloroplast DNA were amplified from total genomic DNA. Double-stranded DNAs of the complete ITS regions in each genomic DNA were amplified using initially 2 primers, 'ITS 5P' and 'ITS 8P' (Möller and Cronk, 1997). Whereas the region of cpDNA was amplified using 2 primers, 'c' and 'f' (Taberlet *et al.*, 1991). Later reactions and in sequencing step also used other primers i.e. 'ITS 1', 'ITS 3P', 'ITS 4' (Möller and Cronk, 1997); 'ITS 2K' (Rangsiruji, 1999); 'd' and 'e' (Taberlet *et al.*, 1991).

PROCEDURE

The reaction (total volume = 50 µl) contained (in order of addition)

1. 32.5 µl of sterile distilled water
2. 5.0 µl of 10x Dynazyme™ reaction buffer
3. 1.0 µl of a mix of each dNTP at 10mM (final concentration 200 µM) (Sigma Chemicals, Poole, Dorset, UK)
4. 5.0 µl of each primer at 10µM (50 ρmol, final concentration 1µM) (can decrease down to 2 µl without significant decrease of the products)
5. 0.5 µl (1U) of Dynazyme™ II thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland)
6. a 1.0 µl aliquot of unquantified total genomic (template) DNA

The reaction solution can be prepared as a master mix for a set of run and aliquoted into each tube prior to adding the last component, DNA template. PCR amplification was carried out in 0.2-ml microcentrifuge tubes in a thermal cycler.

Each PCR reaction cycle usually proceeded to a 30 cycles of: (1) 1 minute at 94 °C to denature the double-stranded template DNA; (2) 1 minute at 55 °C to anneal primers to single-stranded template DNA; and (3) 1 minute and a half at 72 °C to extend primers. The first cycle was preceded by an initial denaturation step of 3 minutes at 94 °C and the last cycle was followed by a completion of extension at 72 °C for 7 minutes. This temperature profile was normally successfully used with both regions, ITS and *trn*L, *trn*L-F. Each set of reactions was monitored by the inclusion of a negative (no template DNA) control. Three microlitres of each double-stranded DNA PCR product were resolved by electrophoresis. Successful PCR resulted in a single band of ethidium bromide corporated-DNA viewed under ultraviolet (UV) light corresponding to approximately 700 bp in ITS and 1000 bp in *trn*L, *trn*L-F.

STOCK SOLUTIONS

10x Dynazyme™ reaction buffer (Finnzymes Oy, Espoo, Finland):

1X: 10 mM Tris-HCl, pH 8.8 at 25 °C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100

Primers (Oswel DNA Service, Southampton, UK):

The primer sequences are (5′ to 3′)

'ITS 1' = TCC GTA GGT GAA CCT GCG G

'ITS 2K' = GGC ACA ACT TGC GTT CAA AG

'ITS 3P' = GCA TCG ATG AAG AAC GTA GC

'ITS 4' = TCC TCC GCT TAT TGA TAT GC

'ITS 5P' = GGA AGG AGA AGT CGT AAC AAG G

'ITS 8P' = CAC GCT TCT CCA GAC TAC A

'c' = CGA AAT CGG TAG ACG CTA CG

'd' = GGG GAT AGA GGG ACT TGA AC

'e' = GGT TCA AGT CCC TCT ATC CC

'f' = ATT TGA ACT GGT GAC ACG AG

Nature Taq DNA polymerase is isolated from *Thermus aquaticus* (thermophilic bacterium) found growing in hotsprings in Yellowstone National Park

(USA). One unit of DNA polymerase is defined as the amount of the enzyme that will incorporate free nucleotides into the extension of primer at the rate of 10 nmoles in 30 minutes at 74 °C under the stated assay conditions.

POINTS TO NOTE

1. If you are fortunate to have more than one thermal cylcer in your lab, it is wise to stick to only one thermal cycler. Different thermal cyclers have different temperature control qualities. A parameter of thermal cyclers that can be adjusted but often ignored is a ramping time during two different set temperatures, i.e. heating up or cooling down. Besides, the pace of diffusing heat to the reaction tubes is also a factor of its quality. A poorly calibrated thermal cycler can have a dramatic effect on the sensitivity, specificity, and reproducibility of PCR. Some targets require precise cycling condition to prevent formation of secondary amplification products. Secondary products can form from hybridization of primers either to a pseudogene with a sequence that is similar to that of the actual target, or to a non-specific target.
2. A reaction volume can be either 25 µl or 50 µl. This volume of a PCR reaction is not really a factor when working with a good template DNA. However, a reaction volume of 50 µl is more likely to be successful than a reaction of 25 µl for difficult species or herbarium specimen-DNA. This may account to a fact that greater amount of template DNA increases a chance of amplification.
3. Preparing the reaction solution as a master mix and then aliquoting it into each tube is really a better idea. Measuring a very minute amount of PCR's contents can be difficult and doing it all over for each individual tube is relatively time consuming. In addition, any set of reaction tubes can be sure that the contents are all the same (standard control), allowing a comparison of the products of the set.
4. Primers should be heated up prior to use from time to time if they have been kept for an extended period.

5. Please note the number of ITS primers, odd numbers are used for a forward direction and even numbers are used for a reverse direction. (see Figure 1.3 and 1.4 for the approximate position of each primer)
6. In this study, primers 'ITS 5P' and 'ITS 8P' were initially used for amplifying a region of ITS1, 5.8S and ITS2. Later attempts were followed by using a set of primers 'ITS 1' and 'ITS 4' for the whole region which is shorter than that of primers 'ITS 5P' and 'ITS 8P' about a hundred of base pairs. Otherwise, an individual spacer, i.e. ITS1 and ITS2, could be amplified by a set of primers 'ITS 5P' and 'ITS 2K' for ITS1, and 'ITS 3P' and 'ITS 8P' for ITS2. There were again alternatives set of primers, 'ITS 1' and 'ITS 2K' for ITS1, and 'ITS 3P' and 'ITS 4' for ITS2. It was normally successful at the first attempt of amplification when working with a good DNA. However, it was found that some DNA required more than once on amplification.
7. Although there has never been observed in PCR products of ITS having more than one distinct band (presumably one product), using the same amount of primers in *trn*L, *trn*L-F caused some products having more than one clear band. The amount of primers was then reduced and it was observed on later trials that this factor has played a pivotal role in the PCR reaction of Zingiberaceae species (primer purity is also another factor).
8. Temperature profile in *trn*L, *trn*L-F is also an important factor in its amplification. Usually it was adjusted to increase stringency in the PCR reaction, i.e. increasing an annealing temperature to up to 62 °C and reducing the time to 30 second, in later reactions. The whole reaction could also be reduced to 25 cycles, instead of 30.
9. It should be noted that the second band generated by a set of primer 'c' and 'f' in *Alpinia* study (Rangsiruji, 1999) is at the approximate length of 800 bp, apart from the referred one at 1000 bp. Whereas in this study, it is a much shorter one at about 300 bp.
10. The problem of the second band in *trn*L-F may be further tackled by: 1. The PCR techniques 'hot start' or 'step down' could be employed; 2. Gel

purification, select only the right size band i.e. about 1000 bp and discard the second one (in this study, the band is about 300 bp); 3. Primer redesigning for the region in the problematic species of Zingiberaceae e.g. *Hedychium* spp., to increase the stringency of the annealing stage.

A1.4 PURIFICATION OF PCR PRODUCT

The efficiency of the sequencing reaction can be improved by purifying the PCR-generated DNA template prior to sequencing. Contaminants in the templates, such as residual primers and nucleotides reduce the quality of sequence data. In this study, the templates were purified by using a QIAGEN QIAquick PCR Purification Kit. This kit is designed to purify single- or double-stranded PCR products ranging from 100-10000 base pair from primers, nucleotides, polymerases and salts using the QIAquick spin columns in a microcentrifuge (QIAGEN, 1997b).

PROCEDURE

1. 5 volumes of Buffer PB were added and mixed to 1 volume of the PCR reaction.
2. A QIAquick spin column was placed in a provided 2-ml collection tube.
3. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 minute.
4. The flow-through was discarded. The QIAquick column was then put back into the same tube.
5. To wash, 0.75 ml Buffer PE were added to the column and centrifuged for 1 minute.
6. Again, the flow-through was discarded and the QIAquick column was placed back in the same tube. It was centrifuged for an additional 1 minute.
7. The QIAquick column was then placed in a clean 1.5-ml microcentrifuge tube.

8. To elute DNA, 50 µl Buffer EB were added (10 mM Tris-Cl, pH 8.5) or H_2O to the centre of the QIAquick column and the tube was centrifuged for 1 minute. Alternatively, for increased DNA concentration, a reduced Buffer EB or H_2O at 30 µl was added instead to the center of the QIAquick column. Then it was left for 1 minute and centrifuged.

Ethanol (96-100%) was added to Buffer PE before use (see bottle label for volume). All centrifuge steps were at 13000 rpm in a conventional tabletop microcentrifuge. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within the range, and store DNA at –20 °C as DNA may degrade in the absence of a buffering agent.

A1.5 AUTOMATED CYCLE SEQUENCING

Purified PCR products were sequenced using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Kit (Perkin Elmer, Applied Biosystems Division, Warrington, UK), with AmpliTaq® DNA polymerase, FS, according to the manufacturer's recommendations. The chemistry of dye terminator method is that the PCR products that are to be analysed then are fluorescently labeled by incorporating fluorescently labeled dideoxynucleotides. Each different dideoxynucleotides is labeled with a different fluorophore so the sequencing reaction can be performed in a single tube provided with all the components of a normal PCR reaction plus all of the four fluorescently labeled dideoxynucleotides. As a result of incorporating the fluorescently labeled dideoxynucleotides, the extension of the sequencing primer terminates. In general, DNA fragments of the same size generated in the sequencing reaction are labeled at the 3′ end with the same dye. Then they are separated by running through a gel. As the sequencing products pass through the gel at a fixed point, the fluorophore is excited by a laser and the fluorescence emission is produced and measured making up a sequence of DNA. The PCR products were sequenced in both strands to reduce any potential error. Sequencing products were analysed on an

ABI 377 Prism Automatic DNA Sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA), according to the manual supplied (This stage was carried out by Nicola Preston, ICMB's former sequencing staff, and later Dr Michelle Hollingsworth and Alexandrea Ponge at RBGE).

PROCEDURE

Each reaction was 20 µl in volume and contained (in order of addition) 6 µl of sterile distilled water, 8 µl of Reaction Mix, 1 µl of primer at 3.2 µM (3.2 ρmol) and 5 µl of purified PCR product. For each taxon forward and reverse sequencing reactions were performed for sequence confirmation. Sequencing primers were those used in the PCR amplification.

The automated cycle sequencing profile is a cycle of, the denaturation of the PCR products at 96 °C for 10 seconds, the annealing of the sequencing primer at 50 °C for 5 seconds and the extension of the sequencing product at 60 °C for 4 minutes. This cycle was repeated 25 times and followed with keeping the final sequencing products at 4 °C till required.

POINTS TO NOTE

1. Originally all sequencing reactions were carried out at a 20 µl scale. It was later reduced to a 10 µl scale upon finding that the result sequences were as good as at the original scale. However, it was found that any variation occurred in the reaction tube in a 10 µl scale had greater affect to the result sequence than in a 20 µl scale. It is thus best to use a 20 µl scale for a sequencing of long stretch of DNA (in this case, *trn*L which is about 600 bp in Zingiberaceae). Sequencing of ITS1, ITS2 (both about 200 bp) and *trn*L-F (about 300 bp) posed no problem at the 10 µl scale. It is also possible to reduce the reaction mix to 6 µl in a 20 µl scale and use as a standard scale.

2. Try to use internal primers for sequencing reactions. For example, primers 'ITS 2K' and 'ITS 3P' are used in sequencing reactions of the products originally generating from a set of primers 'ITS 5P' and 'ITS 8P'.
3. The amount of purified DNA template in the procedure is roughly calculated, from a clear band in the checking gel, to be optimal for the sequencing reaction. Each purified PCR products has different concentration of DNA template, so it should be individually determined of how much is optimal for the sequencing reaction. This needed amount of DNA template for the sequencing reaction is normally suggested by the sequencing kit's company used (ABI). Normally, 5-10 ng of amplified frangment are required to any 100 bp. sequencing.

A1.6 PRECIPITATION OF CYCLE SEQUENCING PRODUCT

After finishing the thermal cycler run, the next step was the removal of excess dye. Ethanol precipitation aims to discard the excess, unincorporated dye terminators from the extension products.

PROCEDURE 1

1. A 0.75-ml microcentrifuge tube was prepared for each reaction by adding the followings: 2 µl 3M Sodium acetate, pH 4.6 and 50 µl 100% ethanol.
2. The entire 20 µl contents of the tubes from the cycle sequencing reaction were transferred to the ethanol solution and shortly spinned with a vortex.
3. The tube was then placed on ice for 10 minutes.
4. Next, it was centrifuged at 13000 rpm for 30 minutes.
5. The ethanol solution was carefully aspirated with a micropipetter. The solution was removed as completely as possible.
11. The pellet was rinsed by adding 250 µl 70% ethanol, and left for 1 minute.

12. The tube was then centrifuged at 13000 rpm for 2 minutes.
13. Again, the alcohol solution was carefully aspirated. Be careful not to disturb the pellet that may or may not be visible.
14. The pellet was dried in a vacuum centrifuge at medium temperature for 3-5 minutes.
15. The sample was kept at -20 °C before proceeding to an electrophoresis process in a sequencing machine (ABI 377).

PROCEDURE 2

This protocol is intended for use with AmpliTaq® DNA Polymearase, FS (Taq FS) dye terminator chemistry. The ABI PrismTM Dye Terminator Cycle Sequencing Kits with AmpliTaq® DNA Polymearase, FS use much lower amounts of dye terminator than kits with AmpliTaq® DNA Polymearase. As a consequence, a simple ethanol precipitation protocol can now be used for the removal of unincorporated dye terminators from the extension products.

Use of this protocol may leave some residual dye-labeled terminators in the sample, but only small peaks should be observed in the electropherogram and should not affect the base calling above base 40. The use of procedure 2.1 (not pursued in this study) will result in no residual dye-labeled terminators in the sample. 70% ethanol with 0.5 mM $MgCl_2$ is made by mixing 70% ethanol and 0.5 M $MgCl_2$ at 1000:1 volumetric ratio.

1. A 0.75-ml microcentrifuge tube was prepared for each reaction by adding 37 µL 70% ethanol with 0.5 mM $MgCl_2$.
2. The entire 10 µl contents of the sequencing product were transferred to the microcentrifuge tube containing the ethanol solution. The tube was then spinned with a vortex briefly.
3. The solution tube was left at room temperature for 15 minutes to precipitate the extension products. (A precipitation time of less than 5 minutes will result in loss of very short extension products, precipitation

time greater than 24 hours will increase the precipitation of the unincorporated dye terminators)

4. The solution tube was centrifuged at 13000 rpm. for 15 minutes.
5. The supernatant was discarded immediately after step 4. The supernatant must be completely removed (This is because unincorporated dye-labeled terminators are dissolved in the supernatant).
6. The sample tube was visually inspected, if there was any residual supernatant, it was briefly centrifuged (5-10 seconds) and then aspirated.
7. The pellet was dried in a vacuum centrifuge for 1-3 minutes.

Procedure 2 gives a slightly better sequence than the procedure 1 when other control factors are all the same.

PROCEDURE 2.1

(Optional further reduction of unincorporated dye-labeled terminators by shrimp alkaline phosphatase (SAP) digestion)

At the end of dye terminator sequencing reaction add 2 μL of SAP (1 unit/μL) and 18 μL of SAP buffer to the reaction tube. Re-seal the reaction tube and incubate at 37 °C for 30 minutes. The SAP digested reaction is then ethanol precipitated using the above method with the following modifications.

1. After completion of the SAP reaction, add 150 μL of 70% ethanol with 0.5 mM $MgCl_2$ to this tube (or add 40 μL of 2 mM $MgCl_2$ and then 110 μL of 95% ethanol). Cap the tube and spin with a vortex briefly.
2. Go to step 3 in the method above.

POINTS TO NOTE

All the methods presented above can be further consulted at Qiagen website, www.qiagen.com/literature, which iucludes many useful handbooks, application guides and newsletter.

A1.7 SEQUENCE ANALYSIS

All sequences were verified by comparison of their forward and reverse sequences simultaneously using Factura™ version 2 and later Autoassembler™. Sequence boundaries of both internal transcribed spacers of all taxa were determined by comparison with published rDNA sequence data for *Daucus carota*, *Vicia faba* (Yokota *et al.*, 1989) and *Alpinia* spp. (Rangsiruji, 1999). Both ITS regions were aligned using the CLUSTAL option in the multiple alignment program Sequence Navigator™ Version 1.0.1 and CLUSTAL X, with minor manual adjustments. A transition/transversion ratio was determined using MacClade Version 3.0.1 (Maddison and Maddison, 1992). Sequence characteristics, such as sequence divergence, number of constant sites, variable sites and G+C content were calculated in PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b4 (Swofford, 1998).

The sequence boundaries of the *trn*L-F region started at the base number 41 counted from the end of primer 'c' and stopped at the base positioned 16 away from the beginning of primer 'f'. The sequences in this range were, in most of the species, complete and unambiguously alignable. These sequences were also determined with Alpineae species (Rangsiruji, 1999). All computer programs and methods for the sequence analyses followed those of the ITS sequence.

POINTS TO NOTE

1. For some species, both strands of sequences confirmed polymorphic sites, i.e. more than one clear base calling, or in some rare cases indels sites. In the case of they were interpretable and able to give a consensus sequence,

this consensus sequence was thus used in later phylogenetic studies.

2. In some cases, it was not possible to obtain an interpretable good sequence, for instance some products of *trn*L, this problem could be solved by applying stricter conditions or a cloning technique.

A1.8 PHYLOGENETIC ANALYSIS

Phylogenetic trees were generated using PAUP* Version 4.0b4 (Swofford, 1998), run on a Power Macintosh 6400/200 or G4 with character states unordered. The branch-and-bound search option, which guarantees to find the shortest tree or trees, was selected for the analyses that contain less than 20 species, with MULPARS and furthest addition sequence options. Heurustic serach is an alternative option in searching the best trees for a large data matrix. Although it does not guaruntee to find the best trees, it is fast and efficient to recover the nearest true trees. Different addition sequence and different branch swapping are employed in the analyses helping to find the best trees (usually tree bisection-reconnection (TBR) branch swapping and random taxon addition sequence were used). Then successive weighting searches were applied, using Rescaled Consistency index (RC, mean value) (Swofford, 1993) until the tree length of resulting tree remains unchanged.

Descriptive statistics reflecting the amount of phylogenetic signal in the parsimony analyses were given by consistency index (CI) (Kluge and Farris, 1969), retention index (RI) (Farris, 1989), and resulting rescaled consistency index (RC) (Swofford, 1993). Additionally, the g_1 statistics (Hillis and Huelsenbeck, 1992) were obtained by calculating the tree-length distribution of 10000 random trees using RANDOM TREES under PAUP* to assess the amount of phylogenetic signal in the data set, in comparison to random noise. The strength of individual clades of the trees were evaluated by using bootstrap value (Felsenstein, 1985) and decay index (Bremer, 1988; Donoghue *et al.*, 1992). The bootstrap values were performed in PAUP*, set to branch-and-bound search option and 1000 replicates. The decay indices were obtained by comparing the strict consensus of all equal-length trees up

to four steps longer than the shortest tree, using branch-and-bound search option.

For all analyses of sequence data, gaps (indels) were treated as missing data, i.e. do not affect the analysis in any way (Soltis and Kuzoff, 1995; Susanna *et al.*, 1995; Downie and KatzDownie, 1996). Indels were scored as a separate presence/absence character and added to the sequence data matrix (Oxelman and Liden, 1995; Wojciechowski *et al.*, 1993). To investigate the effect of these additional data, a separate analysis without indels scored as characters was undertaken. Character-state changes were weighted equally, except for some analyses in which character-state weighting parsimony was implemented: transversions were weighted over transitions by the observed ratio, e.g. 1.7 in ITS1 and ITS2 of the *Roscoea* study.

APPENDIX TWO: A MATRIX OF ITS SEQUENCES OF THE HEDYCHIEAE (CHAPTER TWO)

```
[                          10        20        30        40        50        60    ]
[                          .         .         .         .         .         .     ]
                       ITS1
Alpinia                TTGTTGAG--AGTGCATT-----GAATGATGGATGGTTGCGAATGTGTCAACGTGCCCC---TTT    [55]
Pleuranthodium         TTGTTGAG--AGAGCACA-----GAATGATGGATGGTTGTGAATGTGTCAACGTGTCCC---TTT    [55]
Renealmia              TTGTTGAGGGAGAGCATT-----GAATGATGGATGGTTGTGAATGTGTTAACGTGCCCC---TT-    [56]
Boesenbergia.aurantiaca TTGTTGAG--AGAGCACA-----GAATGATGGATGGTTGTGAACGTGTGAATGCGCCCC---TTT   [55]
B.basispicata          TTGTTGAG--AGAGCATA-----GAATGATGGATGGTTGTGAACCTGTGAATGCGTCCC---TTT    [55]
B.cordata              ??????????????????????????ATGATGGATGGTTGTGAACGTGTGAATGCGCCGC---TTT   [62]
B.gelatinosa           TTGTTGAG--AGAGCATA-----GAATGATGGATGGTTGTGAACGTGTGAATGCGTCCC---TTT    [55]
B.longiflora           TTGTTGAG--AGAGCATA-----AAATGATGGATGGTTGTGAACGTGTGAATGTGTCCC---TTT    [55]
B.aff.longiflora       TTGTTGAG--AGAGCATA-----AAATGATGGATGGTTGTGAACGTGTGAATGTGTCCC---TTT    [55]
Camptandra.ovata       ??????????????????????GATTGATGGATAATTGTGAATGTGTGAACGTGCCCC---TTT    [62]
C.parvula              TTGTTGAG--AGAGCATA-----GATAGATGGATGATTGTGAATGTGTGAATGTGGCCC---TTT    [55]
Caulokaempferia        TTGTTGAG--AGAGCATA-----GAATGACGGATGATTGTGAACGTGTGAATGCGCCCC---TTT    [55]
Cautleya.spicata       TTGTTGAG--AGAGCATA-----GAATGATGGATGGTTGTGAATGTGTAAATGTGCCCC---TTT    [55]
Cornukaempferia        TTGTTGAG--AGAGCATA-----GAATGACGGATGGTTGTGAACGCGTGAATGTGTCCC---TTT    [55]
Curcuma.alismatifolia  TTGTTGAG--AGAGCATA---TAGAATGACGGATGAATGTGAACGTGTGAACGTGACCC---TTT    [57]
C.amada                TTGTTGAG--AGAGCATAGCATRGAATGATGGATGATTGCGAACGTGTGAACGTGACCC---TTT    [60]
C.ecomata              TTGTTGAG--AGAGCATA-----GAATGATGGATGATTGTGAATGTGTGAACGCGACCC---TTT    [55]
C.harmandii            TTGTTGAG--AGAGCATA---TAGAATGATGGATGAATGTGAATGTGTGAACGTGACCC---TTT    [57]
C.parviflora           TTGTTGAG--AGAGCATA---TAGAATGACGGATGAATGTGAATGTGTGAACGTGACCC---TTT    [57]
C.rubescens            TTGTTGAG--AGAGCATA-TATAGAATGATGGATGATTGTGAACGTGTGAACGCGACCC---TTT    [59]
Distichochlamys        TTGTTGAG--AGAGCATA-----CAATGACGGATGGTTGTGAATGTGTGAATGCGTCTC---TTT    [55]
Haniffia               TTGTTGAGAGAGAGCATA-----GAATGATGGATGATTGTGAATGTGTGAACGTGCCCC---TTT    [57]
Hedychium.coccineum    TTGTTGAG--AGAGCACA-----AGACGATGGATGGTTGTGAATGTGTGAACGCGCCCC---TTT    [55]
H.gardnerianum         TTGTTGAG--AGAGCACA-----AGACGATGGATGGTTGTGAATGTGTGAACGCGCCCC---TTT    [55]
H.x raffillii          TTGTTGAG--AGAGCAYA-----AGACGATGGATGGTTGTGAATGTGTGAACGCGCCCC---TTT    [55]
H.villosum             TTGTTGAG--AGAGCATA-----AGACGATGGATGATTGCGAATGTGTGAACGCGCCCC---TTT    [55]
H.sp.                  TTGTCGAG--AGAGCATA-----AGACGATGGATGGTTGTGAACGTGTGAACGCGCCCC---TTT    [55]
Hitchenia              TTGTTGAG--AGAGCATAGAAT-GATGGATGGATGATTGTGAATGTGTGAACGTGACCC---TTT    [59]
Kaempferia.angustifolia TTGTTGAG--AGAGCATC-----GAATGACGGATGTTTGTGAACGTGTGAATGCTTCCT---CCT   [55]
K.elegans              TTGTTGAG--AGAGCACAACACAGAATGACGGATGGT-GCGAACGTGTGAATGTGTCCCT--TTC    [60]
K.rotunda              TTGTTGAG--AGAGCACG-----GACCGATGGATGGTTGTGAATGTGTGAATGTGTCCC---TTC    [55]
Paracautleya           TTGTTGAG--AGAGCATAGAAT-GATGGA----TGATTGTGAATGTGTGAACGTGACCC---TTT    [55]
Pommereschea           TTGTTGAG--AGAGCACA-----GAATGACGAATGTTTGTGAATGTGTGAATGCGCCCCT--TTC    [56]
Pyrgophyllum           TTGTTGAG--AGAGTATA-----GAATGATGGATGATTGTGAATGTGTGAGCGTGCTCC---TTT    [55]
Roscoea.bhutanica      TTGTTGAG--AGAGCATA-----GAATGACGGATGGTTGTGAATGTGTGAATGTGCCCC---TTT    [55]
R.humeana              TTGTTGAG--AGAGCACA-----GAATGACGGATGGTTGTGAATGTGTGAATGTGCCCC---TTT    [55]
Rhynchanthus           TTGTTGAG--AGAGCATA-----GAATGATGGATGGTTGTGAATGTGTGAATGTGCCCC---TTT    [55]
Scaphochlamys.kunstleri TTGTTGAG--AGAACATAACACAAAATGACGGATGGTTGCGAATGTGTGAATGTGTCCCT--TTT   [61]
S.lanceolata           TTGTTGAGASAKAAAATT----AAATGGACGGTTGTTTTTGATTGTTTGAWTCCTYCCC---TTC    [58]
Smithatris             TTGTTGAG--AGAGCATA-----GAATGATGGATGATTGTGAACGTGTGAACGTGACCC---TTT    [55]
Stahlianthus           TTGTTGAG--AGAGCATA---TAGAATGATGGACGAATGTGAATGTGTGAACGTGACCC---TTT    [57]
Zingiber               TTGTTGAG--AGAGCATA---TAGAATGACGGATGGCTGCGAACGTGTGAATGTGTCCCCCCTTT    [60]
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[                                70        80        90        100       110       120       130]
[    .                           .         .         .         .         .         .         .]

Alpinia                          CCTTGCCC----------------------------------------CATGTTGCT----GGGC    [76]
Pleuranthodium                   TCTCGTCC----------------------------------------CATGTCAGT----GGGC    [76]
Renealmia                        CCTTGCCC----------------------------------------CATGTTGGT----GTGC    [77]
Boesenbergia.aurantiaca          CCTTGCCCCCCGAT----------------------------------GTCGTCGGC----GGGC    [82]
B.basispicata                    CCTTGCCC----------------------------------------CATGTTGGT----GGGC    [76]
B.cordata                        CCTWGGACCC--------------------------------------CATGTCGGC----GGGC    [85]
B.gelatinosa                     CCTTGACC----------------------------------------CATGTTTGT----GGGC    [76]
B.longiflora                     CGTTGCCC----------------------------------------CATGTTGGT----GGGC    [76]
B.aff.longiflora                 CCTTGCCC----------------------------------------CATGTTGGT----GGGC    [76]
Camptandra.ovata                 CCTTGGCC----------------------------------------CATGTTGGT----TGGG    [83]
C.parvula                        CCATGCCC----------------------------------------CATGTTGGT----TGGG    [76]
Caulokaempferia                  CCTTGCCCCC--------------------------------------CATGTCGGC----GGGC    [78]
Cautleya.spicata                 CCTTTCCC----------------------------------------CATGTTGGT----GGGC    [76]
Cornukaempferia                  CCTCGCCCGCCC------------------------------------CGTGTCGGC----GGGC    [80]
Curcuma.alismatifolia            CTTTAGCC----------------------------------------CATGTTGGT----GGGC    [78]
C.amada                          CGTC-GCCCATCC-----------------------------------CATGTTGGT----GGGC    [85]
C.ecomata                        CGTTAGCC----------------------------------------CACGTTGGT----GGGC    [76]
C.harmandii                      CTTTAGCC----------------------------------------CATGTTGGT----GGGC    [78]
C.parviflora                     CTTTAGCC----------------------------------------CATGTTGGT----GGGC    [78]
C.rubescens                      CGTCAGCCCAT-------------------------------------CAACTTGGT----GGGC    [83]
Distichochlamys                  CCTTGCCCCCAACA----------------------------------TATGTTGGT----GGGT    [82]
Haniffia                         CCTTGCCCGCCC------------------------------------CATGTTGGC----GGGC    [82]
Hedychium.coccineum              CCTCGCCCCGCC------------------------------------CATGTCGGC----GGGC    [80]
H.gardnerianum                   CCTCGCCCCGCC------------------------------------CATGTCGGC----GGGC    [80]
H.x raffillii                    CCTCGCCCCGCC------------------------------------CATGTCGGC----GGGC    [80]
H.villosum                       CCTCGCCCCACC------------------------------------CATGTCGGC----GGGC    [80]
H.sp.                            CCTTGCCCCACC------------------------------------CATGTCGGC---GGGGC    [81]
Hitchenia                        C-TCAGCC----------------------------------------CACGTTGGT----GGGC    [79]
Kaempferia.angustifolia          CCTCGCCCCCAC------------------------------------TRCCTCGGC----GGGC    [80]
K.elegans                        CTTTGCCCCTCGACGTGCATCGTGCATGCATATTGCATATTGCATATGCATGTCGGC----GGGC    [121]
K.rotunda                        CTTCCCCCCC--------------------------------------CACGTAGGC----GGGC    [78]
Paracautleya                     CGTCAGCC----------------------------------------CATGTTAGT----GGGC    [76]
Pommereschea                     CTC-GCCCCTCA------------------------------------TTGGG----GGGC    [76]
Pyrgophyllum                     CCTTGCCC----------------------------------------AATGTTGGT----GGGC    [76]
Roscoea.bhutanica                CCTT-CCC----------------------------------------CATCTCGGT----GGGC    [75]
R.humeana                        CCTT-CCC----------------------------------------CATCTCGGT----GGGC    [75]
Rhynchanthus                     CCTCGCCCCC--------------------------------------CATGTCGGT----GGGC    [78]
Scaphochlamys.kunstleri          CTC-GCCCCGCG------------------------------------TTGGC----GGGC    [81]
S.lanceolata                     CYTCCCCCCCCCC-----------------------------------CWSSYGGSC----GGGG    [84]
Smithatris                       CGTTAGCC----------------------------------------CACGTTGGT----GGGC    [76]
Stahlianthus                     CTTTAGCC----------------------------------------CATGTTGGT----GGGC    [78]
Zingiber                         CCTCGCCTCCACCAACCAACACCCA----------------------TGTGTCGGTTGGCGGGC    [102]
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[                              140        150        160        170        180        190    ]
[                              .          .          .          .          .          .      ]

Alpinia                 AATT-GATCGTAG-CTCGGTGCGATCGGCACCAAGGAATAA-TAA----------ACTGAGAAGC  [128]
Pleuranthodium          GTTT-GACCCTAC-TTCGGTGCGATCGGCACCAAGGAATAA-TGA----------ACTCAGAAGG  [128]
Renealmia               AATT-GACCGTAG-CTCGGTGCGATCGGCACCAAGGAACAA-TGA----------ACTTAGAAGC  [129]
Boesenbergia.aurantiaca -ATT-GACCGTAG-CTCGGTGCGATCCGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [133]
B.basispicata           -ATT-GACCGTAG-CTCGGTGCGATCCGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [127]
B.cordata               -ATC-GACCGWWG-CTCGGTGCGATCCGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [136]
B.gelatinosa            -ATT-GACCGTAG-CTCAGTGCGATCCGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [127]
B.longiflora            -ATT-GACTAGAG-CTCGGTGCGATCCGCACTAAGGAACAA-TAA----------ACTCGGAAGC  [127]
B.aff.longiflora        -ATT-GACTAGAG-CTCGGTGCGATCAGCACTAAGGAACAA-TAA----------ACTCGGAAGC  [127]
Camptandra.ovata        GATT-GACCATAV-CTCAGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGGAGC  [135]
C.parvula               GATTTGACCATAAGCTCAGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [130]
Caulokaempferia         -ATT-GACCGTAG-CTCGGTGCGATCCGCACTAAGGAACAA-CGA----------ACTCGGAAGC  [129]
Cautleya.spicata        GATT-GACCGTAG-CTCAGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [128]
Cornukaempferia         GATC-GACCGTAG-CTCAGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [132]
Curcuma.alismatifolia   GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTTGAAAGC  [130]
C.amada                 GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-CGA----------ACTTGGAAGC  [137]
C.ecomata               GATT-GAC-;TA----CGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [123]
C.harmandii             GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ATTTGAAAGC  [130]
C.parviflora            GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTTGAAAGC  [130]
C.rubescens             GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-CGA----------ACTTGGAAGC  [135]
Distichochlamys         GATT-GACCATAG-CTCGGTGCGATCAGCACTAAGGAACAA-TAAGGAACAATGAACTCGGAAGC  [144]
Haniffia                AATT-GACCGCAG-CCCGGTGCGATCGGCACTAAGGAATAA-TGA----------ACTCGGAAGC  [134]
Hedychium.coccineum     GATT-GACCGTAG-CCGGGTGCGATCGGCACCAAGGAACAA-TGA----------ACTCGGAAGC  [132]
H.gardnerianum          GATT-GACCGTAG-CCGGGTGCGATCGGCACCAAGGAACAA-TGA----------ACTCGGAAGC  [132]
H.x raffillii           GATT-GACCGTAG-CCGGGTGCGATCGGCACCAAGGAACAA-TGA----------ACTCGGAAGC  [132]
H.villosum              GATT-GACCGTAG-CCGGGTGCGATCGGCACCAAGGAACAA-TGA----------ACTCGGAAGC  [132]
H.sp.                   GATT-GACCGTAG-CCGGGTGCGATCGGCACCAAGGAACAA-TGA----------ACTCGGAAGC  [133]
Hitchenia               GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTTGGAAGC  [131]
Kaempferia.angustifolia GATT-GACCATCA-ATCGCTGCGACCGGCACTMAGGAACAA-TGA----------ATTCAGAMGC  [132]
K.elegans               GATT-GACCGTAG-CTCGGTGCGATCAGCACTAAGGAACAA-TGAATG------AACTCGGAAGC  [177]
K.rotunda               GGTT-GACCGTCG-STCRGTCCGTTCGGCAYTAAGKAACAA-TSA----------ATTMCGAMGC  [130]
Paracautleya            GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTTGGAAGC  [128]
Pommereschea            GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [128]
Pyrgophyllum            GATT-GAACGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGAAAGC  [128]
Roscoea.bhutanica       GATT-GACCGTAG-CTCAGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [127]
R.humeana               GATT-GACCGTAG-CTCAGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [127]
Rhynchanthus            GATT-GACTGTAT-CTCGGTGCGATCGGCACTAAGGAAAAA-TGA----------ACTCGGGAGC  [130]
Scaphochlamys.kunstleri GAAT-GACCGTAG-CTCGGTGCGATCGGCACTAASGAACAA-TGA----------ACTCGGAAGC  [133]
S.lanceolata            GATT-G-CCSTTGGTTGGTTCCGTCCGCCATTAGGGACCAT-TGA----------ATTCGGARCC  [136]
Smithatris              GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTCGGAAGC  [128]
Stahlianthus            GATT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAA-TGA----------ACTTGAAAGC  [130]
Zingiber                GGTT-GACCGTAG-CTCGGTGCGATCGGCACTAAGGAACAAATGA----------ACTCGGAAGC  [155]
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[                    200       210       220       230       240       250       260]
[                    .         .         .         .         .         .         .]

Alpinia              AAAGGGCCCC--TCGGTGTGTGCGGGG--AGCCCAATGCGTCGGAGAAGCCTCGAAA-TCAAA---  [185]
Pleuranthodium       AGAGGGCCC--TCGGCGTGCGTGGGG--AGCCCAATGCTTCGGAGACAGCTCGAAAATCAAA---  [186]
Renealmia            AGAGGGCCC--TCGATGTGCGCGGGG--AGCCCAATGCGGAGGAGATGCCACGGAA-TCGAA---  [186]
Boesenbergia.aurantiaca AGACGGCCCC-CCGGCGTGCGCGAGGG-AGCCCGATGCATCGGAGATTCCTCGGAA-TCAAA---  [192]
B.basispicata        AGAGGGCCCC-TTGGCATGCAC-AGGT-AGCCCAATGCGTCAGAGATTCCTCGGAA-TCAAA---  [185]
B.cordata            AGACGGCTCC-CCGGCGTGCGCGAGGG-AGCCCGATGCGTCGGAGATTCCTCGGAG-TCAAA---  [195]
B.gelatinosa         GGAGGGCCCC-TTGTCGTGCAC-AGGG-AGCCCAACGCGTCGAGGATTGCTCGGAA-TCAAA---  [185]
B.longiflora         AGAGGRCCCC-TTGGCRTGCAC-AGGG-AGCCCAATGCRTCGGAGATTCCTCGGAA-TCAAA---  [185]
B.aff.longiflora     AGARGGCCCC-TTGGCGTGCAC-AGGG-AGCCCAATGCGTCGGAGATTCCTCGGAA-TCAAA---  [185]
Camptandra.ovata     AAAGGGCCCC-TTGGCGTGCGCGGGG--AGCCCATTRCWTCAAASATTCCTCGKAA-TCAAA---  [193]
C.parvula            AGAGGACCCC-TCGGCGTGCGCGGGG--AGCCCAATGCATCGGAGATTCCTCGTAA-TCAAA---  [188]
Caulokaempferia      AGACGGGCCC---------CGC-AAGGGAGCCCGATGCGTCGGAGATTCCTCGGAA-TCAAA---  [180]
Cautleya.spicata     AGAGGGCCCC-TTGGCGTGCGCGGGGG-AGCCCAATGCGTCGGAGATTTTTCGAAA-TCAAA---  [187]
Cornukaempferia      GGAGGGCTCC-TCGGCGTGCGC-AGGG-AGCCCAATGCGTCGGAGATTCCTCGGAA-TCAAAA--  [191]
Curcuma.alismatifolia AGAGGGCCCC-TTGGCGTGAGCGGGG--AGCACAATGCGTCGAAGATTCTTCGGAA-TCAAA---  [188]
C.amada              AGAGGGCCCCCTTAGCGTGAGCGGGG--AGCCCAATGCGTCGGAGATTCTTCGGAA-TCAAA---  [196]
C.ecomata            AGAGGGCCCC-TTGCTGTGAGCGGGG--AGCCCAATGCATCGAAGATTCCTCGGAA-TCAAA---  [181]
C.harmandii          AGAGGGCCCC-TTGGCGTGAGCGGGG--AGCACAATGCGTCGAAGATTCTTCGGAA-TCAAA---  [188]
C.parviflora         AGAGGGCCCC-TTGGCGTGAGCGGGG--AGCACAATGCRTCGAAGATTCTTCGGAA-TCAAA---  [188]
C.rubescens          AGAAGGCCCCCTTAGCGTGAGCGGGG--AGCCCAATGCGTCGGAGATTCTTCGGAA-TCAAA---  [194]
Distichochlamys      AAAGGGCCCC-TTGGCGTGCAC-AGGG-AGCCCAATGCGTCGGAGATTCCTCGGAA-CCAAA---  [202]
Haniffia             AGACGGCCCC-TTGGCGTGCGC-AGGG-AGCCCAATGCGTCGGAGATTCCTCGGAA-TCAAA---  [192]
Hedychium.coccineum  AGAGGGCCCC-TCGACGTGCGCGGGGGGAGCCCAATGCGTCGGAGACTCCTCGAAA-TCAAA---  [192]
H.gardnerianum       AGAGGGCCCC-TCGACGTGCGCGGGGGGAGCCCAATGCGTCGGAGACTCCTCGAAA-TCAAA---  [192]
H.x raffillii        AGAGGGCCCC-TCGACGTGCGCGGGGGGAGCCCAATGCGTCGGAGACTCCTCGAAA-TCAAA---  [192]
H.villosum           AGAGGGCCCC-TCGACGTGCGCGGGGG-AGCCCAATGCGTCGGAGATTCCTCGGAA-TCAAA---  [191]
H.sp.                AGAGGGCCCC-TCGACGTGCGCGGGGG-AGCCCAATGCGTCGGAGATTCCTCGGAA-TCAAA---  [192]
Hitchenia            AGAGGGCCCC-TCAGCGTGAGCGGGG--AGCCCAATGCGTCGGAGATTCTTCGGAA-TCAAA---  [189]
Kaempferia.angustifolia AGACGGCCCS-CYCCCTTCCGC-AGGC-AGSCCMCTGCATCAGTGATTCCTCCGAA-TCAATCAT  [193]
K.elegans            AGAGGGCTCC-TTGGCGTGCAC-AGGG-GGCCCAATGCGTCGGAGATTCCTCGGGAATCAATCAA  [239]
K.rotunda            AGASGGCCCC-CYGGCGTGCTC-AGGG-AGCCCAATGCGTCGGAGATTSWTCGGAA-TCAATCAA  [191]
Paracautleya         AGAGGGCCCC-TTAGCGTGAGCGGGG--AGCACAATGCGTCGGAGATTCTTCGGAA-TCAAA---  [186]
Pommereschea         AGAAGGCCCC-TTGCCGTGCGCGGGG--AGCCTAATGCGTCGGAGATTCCTCGGAA-TCAAA---  [186]
Pyrgophyllum         AGAGGGTCCC-TTGGCGTGCCCGGGG--AGCCAAAAGCATCGTAGATTCCTCGGAA-TCAAAA--  [187]
Roscoea.bhutanica    AGAGGGCCCC-TTGGCGTGCCCGGGG--AGCCCAATGCGTCGGAGATTTCTCGAAA-TCAAA---  [185]
R.humeana            AGAGGGCCCC-TCGGCGTGCGCGGGGGGAGCCCGATGCGTCGGAGATATCTCGAAA-TCAAA---  [187]
Rhynchanthus         AGAGGGCCCC-TCGGCGTGCGCGGGG--AGCCTAATGCATCGGAGATTCCTCGGAA-TCAAA---  [188]
Scaphochlamys.kunstleri AAAGGKTCCC-TTGGCGTGCGC-AASG-AGCCCGATGCGTCGGAGATTCCTCGGAA-TCAAA---  [191]
S.lanceolata         AARGGTTCCC-TTGGCTTGCCC-ARGGGGACCCCATKSCTYCGGGGTTCCTTCGGA-TCCAAA--  [196]
Smithatris           AGAGGGCCCC-TCGCCGTGAGCGGGG--AGCACAATGCGTCGAAGATTCTTCGGAA-TCAAA---  [186]
Stahlianthus         AGAGGGCCCC-TTGGCGTGAGCGGGG--AGCACAATGCGTCAAAGATTCTTCGGAA-TCAAA---  [188]
Zingiber             GGAGGGCCCC-TTGGCGTGCAC-AGGGGAGCCCAATGCGTCGGAGATTCCTCGGAA-TCAAA---  [214]
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[                              270        280        290        300        310        320   ]
[                              .          .          .          .          .          .     ]
                          5.8S
Alpinia                  --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [248]
Pleuranthodium           --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [249]
Renealmia                --TGACTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [249]
Boesenbergia.aurantiaca  --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [255]
B.basispicata            --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [248]
B.cordata                --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAAAACGTAGTGAAATGCGATACT  [258]
B.gelatinosa             --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [248]
B.longiflora             --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [248]
B.aff.longiflora         --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [248]
Camptandra.ovata         --TGACTCTCGACAATGGATATCTCGGCTCCTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [256]
C.parvula                --TGACTCTCGGCAATGGATATCTCGGCTCCTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [251]
Caulokaempferia          --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [243]
Cautleya.spicata         --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [250]
Cornukaempferia          --CGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [254]
Curcuma.alismatifolia    --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [251]
C.amada                  --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAA-TGCGATACT  [258]
C.ecomata                --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [244]
C.harmandii              --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [251]
C.parviflora             --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [251]
C.rubescens              --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [257]
Distichochlamys          --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [265]
Haniffia                 --TGACTCCCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [255]
Hedychium.coccineum      --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [255]
H.gardnerianum           --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [255]
H.x raffillii            --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [255]
H.villosum               --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [254]
H.sp.                    --TGACTCTCGGCAATGGATATCTCGGCTCYTGYATCGATGAAGAACGTAGTGAAATGCGATACT  [255]
Hitchenia                --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [252]
Kaempferia.angustifolia  -ATGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [257]
K.elegans                AATGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [304]
K.rotunda                -ATGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [255]
Paracautleya             --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [249]
Pommereschea             --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [249]
Pyrgophyllum             --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [250]
Roscoea.bhutanica        --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [248]
R.humeana                --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [250]
Rhynchanthus             --TGACTYTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [251]
Scaphochlamys.kunstleri  --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGMAATGCGATACT  [254]
S.lanceolata             --TGA??????????????????????????????????????????????????????????  [259]
Smithatris               --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [249]
Stahlianthus             --TGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [251]
Zingiber                 --CGACTCTCGGCAATGGATATCTCGGCTCTTGCATCGATGAAGAACGTAGTGAAATGCGATACT  [277]
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[                          330        340        350        360        370        380        390]
[                           .          .          .          .          .          .          .]

Alpinia                    TGGTGTGAATTGCAGAATCTCGTGAATCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [313]
Pleuranthodium             TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [314]
Renealmia                  TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [314]
Boesenbergia.aurantiaca    TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGGAGCCTTG   [320]
B.basispicata              TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [313]
B.cordata                  TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [323]
B.gelatinosa               TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTCGTGCCCGAGGCCTTG   [313]
B.longiflora               TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTC   [313]
B.aff.longiflora           TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [313]
Camptandra.ovata           TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [321]
C.parvula                  TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [316]
Caulokaempferia            TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [308]
Cautleya.spicata           TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [315]
Cornukaempferia            TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [319]
Curcuma.alismatifolia      TGGTGTGAATTGCAGAATCTCGCGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [316]
C.amada                    -GGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [322]
C.ecomata                  TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [309]
C.harmandii                TGGTGTGAATTGCAGAATCTCGCGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [316]
C.parviflora               TGGTGTGAATTGCAGAATCTCGCGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [316]
C.rubescens                TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [322]
Distichochlamys            TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [330]
Haniffia                   TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [320]
Hedychium.coccineum        TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [320]
H.gardnerianum             TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [320]
H.x raffillii              TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [320]
H.villosum                 TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [319]
H.sp.                      TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [320]
Hitchenia                  TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [317]
Kaempferia.angustifolia    -GGTGTCAATTGCAGAATCTC-TGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTC   [320]
K.elegans                  TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTC   [369]
K.rotunda                  TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTC   [320]
Paracautleya               TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [314]
Pommereschea               TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [314]
Pyrgophyllum               TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCTCGAGGCCTTG   [315]
Roscoea.bhutanica          TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [313]
R.humeana                  TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [315]
Rhynchanthus               TGGTGTGAATTGCAGAATCTCGTGAACCATTGAGTCTTTGAACGCAAGTTGTGCCTGAGGCCTTG   [316]
Scaphochlamys.kunstleri    TGGTGTGAATTGC??????????????????????????????????????????????????   [319]
S.lanceolata               ????????????????????????????????GTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [324]
Smithatris                 TGGTGTGAATTGCAGAATCTCGCGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [314]
Stahlianthus               TGGTGTGAATTGCAGAATCTCGCGAACCATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [316]
Zingiber                   TGGTGTGAATTGCAGAATCTCGTGAA-CATTGAGTCTTTGAACGCAAGTTGTGCCCGAGGCCTTG   [341]
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[                          400        410        420        430        440        450    ]
[                           .          .          .          .          .          .     ]
                                                  ITS2
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Alpinia                  TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCCTTTGCTCCTTG--CTTT-----G   [371]
Pleuranthodium           TGGCCGA-GGCACGCCTGCTTGGGCGTCATTGCATCGTCGCTTTTGCTCCCTG--CCTT-----G   [371]
Renealmia                CGGTCGAGGGCACGCCTGCTTGGGCGTCATCGCATCGTCGCCTTTGCTCCTCT--CTTT-----G   [372]
Boesenbergia.aurantiaca  TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCCTTCGCTCCATG--CGTG-----G   [378]
B.basispicata            TGGTCGAGGGCACGCCTGCTTGGGTGTCATGGCATCGTCGCCTTTGCTGCATG--CATT-----G   [371]
B.cordata                TGACCGAGGGCACGCCTGCTTGGGCGTCATGTCATCGTCGCCTTCGCWCCATG--CRTG-----G   [381]
B.gelatinosa             TGGTCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCCTTGGCTCCATG--CGTT-----G   [371]
B.longiflora             TGGTCGAGGGCACGCCTGCTTGGGCGCCATGGCATCGTCGCCTTTGCTCCAAG--CGTT-----G   [371]
B.aff.longiflora         TGGTCGAGGGCACGCCTGCTTGGGCGCCATGGCATCGTCGCCTTTACTCCGAG--CGTT-----G   [371]
Camptandra.ovata         TGGCCGAGGGCACGCCTGCTTGGGTGTCATGGCATCGTCGCTTTTGCACSATG--CGGT-----G   [379]
C.parvula                TGGTCGAGGGCACGCCTGCTTGGGTGTCATGGCATCGTCGCTTTTGCACCAGC--TGGCCT---G   [376]
Caulokaempferia          TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCCTTCGCTCCATG--CGTG-----G   [366]
Cautleya.spicata         TGGCCGAGGGCACGCCTGCTTGGGCGTCATGACATCGTCGCTTTTGCTCCATG--CGTT-----A   [373]
Cornukaempferia          TGGCCGAGGGCACGCCTGCTTGGGGGTCATGGCATCATCGCCTTTGCGCCATC--CATTTGT-CG   [381]
Curcuma.alismatifolia    TGGTCGAGGGCACGCCTGCTTGGGTGTCATGACATTGTCGCTTATGCCCCATG--CTTT-----G   [374]
C.amada                  TGGTCGAGGGCACGCCTGCTTGGGTGTCATGACATCGTCGCTTTTGCTCCATG--CTTC-----G   [380]
C.ecomata                TGGTCGAGGGCACGCCTGCTTGGGTGTCATGGCATTGTCGCTTTTGCTCCATG--CTTC-----G   [367]
C.harmandii              TGGTCGAGGGCACGCCTGCTTGGGCGTCATGACATCGTCGCTTATGCTCCATG--CTTC-----G   [374]
C.parviflora             TGGTCGAGGGCACGCCTGCTTGGGTGTCATGACATTGTCGCTTATGCCYCATG--CTTT-----G   [374]
C.rubescens              TGGTCGAGGGCACGCCTGCTTGGGTGTCATGACATCGTCGCTTTTGCTCCATG--CTTC-----G   [380]
Distichochlamys          TGGTCGAGGGCACGCCTGCTTGGGTGTCATGGCATCGTCGCCTTTGCTCCATG--CGTC-----G   [388]
Haniffia                 TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCACCGTCGCTCTCGCTCCATG--CATT-----C   [378]
Hedychium.coccineum      TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCTTTCGCTCCACG--CATT-----G   [378]
H.gardnerianum           TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCTTTCGCTCCACG--CATT-----G   [378]
H.x raffillii            TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCTTTCGCTCCACG--CATT-----G   [378]
H.villosum               TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCTTTCGCTCCACG--CGTT-----G   [377]
H.sp.                    TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCTTTCGCTCCACG--CGTT-----G   [378]
Hitchenia                TGGTCGAGGGCACGCCTGCTTGGGTGTCATGACATTGTCGCTTTTGCTCCATG--CTTC-----G   [375]
Kaempferia.angustifolia  TGGCCGAGGGCACGCCTGCTTGGGAGTCATGGCACCGCCGCCTCTGCTCCATG--CAAT-----A   [378]
K.elegans                TGGCCGAGGGCACGCCTGCTTGGGAGTCATGGCATTGCCGCCTCCGCTCCACG--CGATAT---G   [429]
K.rotunda                TGGCCGAGGGCACGCCTGCTTGGGAGTCATGGCATTGCCGCCTTTGCACCACCACCATGTAATGA   [385]
Paracautleya             TGGTCGAGGGCACGCCTGCTTGGGTGTCATGACATTGTCGCTTTTGCTCCATG--CTTT-----G   [372]
Pommereschea             TGGTCGAGG-CACGCCTGCTTGGGCGTCATGACATCGTCACGTTTGCTCCACG--CATT-----G   [371]
Pyrgophyllum             TGGTCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCTTTTGCTCCATG--CTTT-----G   [373]
Roscoea.bhutanica        TGGCCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCTTTTGCTCCATG--CGTT-----G   [371]
R.humeana                TGGCCGAGGGCACGCCTGCTTGGGCGTCATGACATCGTCGCTTTTGCTCCATG--CGTT-----G   [373]
Rhynchanthus             TGGTCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCTTTTGCTCCATG--CGTT-----G   [374]
Scaphochlamys.kunstleri  ????????????????????????????????????ATCGTCGCCTTCGCTCCATG--CATGCGTT-G   [381]
S.lanceolata             TGGTCGAGGGCACGCCTGCTTGGGCGTCATGGCATCGTCGCCTTTGCTCCATG--CATGCATGCG   [387]
Smithatris               TGGTCAAGGGCACGCCTGCTTGGGTGTCATGGCATCGTCGCTTTTGCTCCATG--CTTT-----T   [372]
Stahlianthus             TGGTCGAGGGCACGCCTGCTTGGGTGTCATGACATCGTCGCTTATGCTCCATG--CTTT-----G   [374]
Zingiber                 TGGCCGAGGGCACGCCTGCTTGGGTGTCATGGCATCGCCGCCTCTGCTCCATG--CCCT-----G   [399]
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[                              460        470        480        490        500        510        520]
[                              .          .          .          .          .          .          .]

Alpinia                        CTGCTGGTGCTAAGTGCGGAAATTGGCCTCGTGTGCC----CTCGGGCGAGGGCACAGTCGGTTG  [432]
Pleuranthodium                 CTG---GCGGCAAGCGCGGAAATTGGCCTCGTGTGCC----CTCGGG------CATAGTCGGTCG  [423]
Renealmia                      TTG---GTGTCAAGTGCGAAAATTGGCCTCGTGTGCC----CTCGGG------CACAGTCGGCTG  [424]
Boesenbergia.aurantiaca        TTG---GCG-TGAGCGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CACAGTCGGTCG  [429]
B.basispicata                  TTG------CTGAGTGCGAAAATTGACCCCGTGTGCC----CTCGGG------CACAGTCGGTCG  [420]
B.cordata                      TTG---GCG-TGAGCGCTGAAATWGGCCCCGTGTGCC----CTCGAG----GGCACAGTCGGTCG  [434]
B.gelatinosa                   TTG---GTGCTGAGCGCGGAGATTGACCCCGTGTGCC----CTTAGG------CACAGTCGGTCG  [423]
B.longiflora                   TTG---GTGCTGAGTGCGAAAATTGACCCCGTGTGCC----CTCGGG------CATAGTCGGTTG  [423]
B.aff.longiflora               TTG---GTGTTGAGTGCGAAAATTGACCCCGTGTGCC----CTCGRG------CATAGTCGGTTG  [423]
Camptandra.ovata               TTG---GTGTCGAGCGCGGAAATTGGCCCCGTGTGCC----CTTGGG------CACAGTCGGCTG  [431]
C.parvula                      TTG---GTGTCGAGTGCGGAAATTGGCCCCGTGTGCC----CTTGGG------CACAGTCGGTTG  [428]
Caulokaempferia                TTG---GCG-TGAGCGCGGAAATTGGCCCCGTGTGCC----CTAGGG------CACAGTCGGTCG  [417]
Cautleya.spicata               TTG---GCATCGAGCGCGGAAATTGGCCTCGTGTGTC----CTCGGG------CACAGTCGGTTG  [425]
Cornukaempferia                TTG---GCGCCGAGCGCGGAAGTTGGCCTCGTGTGCC----CTCGGG------CACAGTCGGTCA  [433]
Curcuma.alismatifolia          TTG---GCATCGAGTGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CATAGTCGGTCG  [426]
C.amada                        TCG---GCATTGAGCGCGGAAGTTGGCCCCGTGTGCC----CTCGGG------CACAGTCGGTCG  [432]
C.ecomata                      TYA---GCATTGAGCGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CACAGTCGGTCG  [419]
C.harmandii                    TTG---GCATTGAGTGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CATAGTCGGTCG  [426]
C.parviflora                   TTG---GCATTGAGTGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CATAGTCGGTCG  [426]
C.rubescens                    TCG---GCATTGAGCGCGGAAGWTGGCCCCGTGTGCC----CTCKGG------CACAGTCGGTCG  [432]
Distichochlamys                TTGCTGGTGCCGAGTGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CATAGTCGGTCG  [443]
Haniffia                       GTG---GTGTTGAGCGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CACACTCGGTTG  [430]
Hedychium.coccineum            TTG---GCG--GCGAGCGGAAATTGGCCCCGTGTGTC----CTCGGG------CACAGTCGGTCG  [428]
H.gardnerianum                 TTG---GYG--GCGAGCGGAAATTGGCCCCGTGTGTC----CTCGGG------CACAGTCGGTCG  [428]
H.x raffillii                  TTG---GTG--GCGAGCGGAAATTGGCCCCGTGTGTC----CTCGGG------CACAGTCGGTCG  [428]
H.villosum                     TTG---GTG--GCGAGCGGAAATTGGCCCCGTGTGTC----CTCGGG------CACAGTCGGTCG  [427]
H.sp.                          TTG---GTGGGGCGAGCGGAAATTGGCCCCGTGTGTC----CTCGGG------CACAGTCGGTCG  [430]
Hitchenia                      TTG---GCATTGAGCGCGGAAGTTGGCCCCGTGTGCCTGCCCTCGGG------CACAGTCGGTCG  [431]
Kaempferia.angustifolia        TTG---GTGACGAGCGCGTAAATTGGCCCCGTGTGYC----CTCGGG------CACAGTCTGCTG  [430]
K.elegans                      CTG---GTGCTGAGCGCGTAGATTGGCCCCGTGCGCC----CTCGGG------CACAGTCGGCCG  [481]
K.rotunda                      ATGCTGGTGCCGAGCGCGTAAATTGGCCCCGTGTGCC----CTCGGG------CACAGTCGGTTG  [440]
Paracautleya                   TTG---GCATKGAGCGCGGAAATTGACCCCGTGTGCC----CTCGGG------CACAGTCGGTCG  [424]
Pommereschea                   TTG---GTGTCAAGCGCGGAAATTGGCCCCGTGTGTC----CTCGGG------CACAGTCGGTTG  [423]
Pyrgophyllum                   CTG---GCGTCGATCGCGGAAATTGGCCTCGTGTGCC----CTCAGG------CACAGTCGGTTG  [425]
Roscoea.bhutanica              CTG---GTGTCGAGCGCGGAAATTGGCCTCGTGTGTC----CTCGGG------CACAGTCGGTTG  [423]
R.humeana                      CTG---GTGTCAAGCGCGGAAATTGGCCTCGTGTGTC----CTCGGG------CACAGTCGGTTG  [425]
Rhynchanthus                   TTG----CGTCGAGCGCGGAAATTGGCCCCGTGTGTC----ATCGGG------CACAGTCGGCTG  [425]
Scaphochlamys.kunstleri        CTGGTGGTGCCTAGTGCGGAAATTGGCCCCRTGTGCC----CTCGGG------CACAGTCGGCCG  [436]
S.lanceolata                   CTGCTGGTGCCGAGTGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CACAGTCGGTCG  [442]
Smithatris                     TTG---GCATTGAGCGCGGAAATTGGCCTCGTGTGCC----CTCGAG------CACAGTCGGTCG  [424]
Stahlianthus                   TTG---GCATTGAGTGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CACAGTCGGTCG  [426]
Zingiber                       TCA---TGGCCGAGCGCGGAAATTGGCCCCGTGTGCC----CTCGGG------CACAGTCGGCCG  [451]
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[                                530       540       550       560       570       580    ]
[                                .         .         .         .         .         .      ]

Alpinia                  AAGAGTGGGTAGTCG---GTAGACGTCGGGCRCGATGGGTGTTGGTCACTCTATGCGTGAATCGA  [494]
Pleuranthodium           AAGAGCGGGTAGTCG---ACAATCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAATTGA  [485]
Renealmia                AAGAGTGGGTAGTCG---GCAGTCGTCGGGCGCGATGGGTGTTGGTCGCCCTGTGCGTGAATTGA  [486]
Boesenbergia.aurantiaca  AAGAGCGGGCAGTCG---GCAGACGTCGGGCACGATGGGCGTTGGTCGCCGTGAGCGGGAACAGA  [491]
B.basispicata            AAGAGTGGGTAGCCG---GCAATCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGGGAACAGA  [482]
B.cordata                AAGAGCGGGTAGTCG---GCAATCGTCGGGCACGATGGGCGTTGGTCGCCGCCAGCGGGAACAGA  [496]
B.gelatinosa             AAGGGTGGGTAGTCG---GCAATCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGGGAACAGA  [485]
B.longiflora             AAGAGCGGGTAGTCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGGGAACAKA  [485]
B.aff.longiflora         AAGAGTGGGTAGTCG---GCAGTCGTCGGTCACGATGGGTGTTGGTCGCCGTGAGCGGGAACAGA  [485]
Camptandra.ovata         AAGAGTGGGCAGACG---GCAGTAGTCGGGCACGATGGGTGTTGGTCGCTGTGAGCGGGAATCGA  [493]
C.parvula                AAGAGTGGGTCGGCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGGGAATCGA  [490]
Caulokaempferia          AAGAGCGGGCAGTCG---GCAATCGTCGGGCACGATGGGCGTTGGTCGCCGTGAGCGGGAACAGA  [479]
Cautleya.spicata         AAGAGTGGGTAGTCC---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGA  [487]
Cornukaempferia          AAGAGCGGGTAGTCGTCGGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCATGAGCGGGAACAGA  [498]
Curcuma.alismatifolia    AAGAGTGGGTACTCG---GCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGA  [488]
C.amada                  AAGAGTGGGTAGTCG---GTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGA  [494]
C.ecomata                AAGAGTGGGTAGTCG---GTATTCGTCGAGCACGATGGATGTTGGTCGTCGCGAACGGGAACTGA  [481]
C.harmandii              AAGAGTGGGTACTCG---GCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGA  [488]
C.parviflora             AAGAGTGGGTACTCG---GCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGA  [488]
C.rubescens              AAGAGTGGGTAKTCG---GTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGA  [494]
Distichochlamys          AAGAGTGGGAAGTCG---ACAATCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGGGAACAGA  [505]
Haniffia                 AAGAGTGGGTAGTCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGGGAACAGA  [492]
Hedychium.coccineum      AAGAGTGGGTAGTCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGCGAGCGGGAACAGA  [490]
H.gardnerianum           AAGAGTGGGTAGTCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGCGAGCGGGAACAGA  [490]
H.x raffillii            AAGAGTGGGTAGTCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGCGAGCGGGAACAGA  [490]
H.villosum               AAGAGCGGGTAGTCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGCGAGCGGGAACAGA  [489]
H.sp.                    AAGAGTGGGTAGTCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGCGAGCGGGAACAGA  [492]
Hitchenia                AAGAGTGGGTA-TCG---GTA------GAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGA  [486]
Kaempferia.angustifolia  AAGAGCGGGTATTCG---GCAATCGTCTGGCGCAACAGGTGTTGGTCGCCGCGGGCGGGAACAGA  [492]
K.elegans                AAGAGCGGGCAGTCG---CCGGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGA  [543]
K.rotunda                AAGAGCGGGCAGTCG---CCAATCGTCAGGCACGATGGGTGTTGGTCGCGGTGAGCGGGAACAGA  [502]
Paracautleya             AAGAGTGGGTAGTCG---GTAATCGTCGAGCACGATGGACGTTGGTCGTCGCAAGCGAGAACTGA  [486]
Pommereschea             AAGAGTGGGATGTCG---GCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGGGAACAGA  [485]
Pyrgophyllum             AAGAGTGGGTAGTCG---GCAGCCGTCGGGCATGATGGGTGTTGGTCGCCGTTAGCGGGAACTGA  [487]
Roscoea.bhutanica        AAGAGCGGGTAGTCC---GAAGTCGTCGGCCACGACGGGTGTTGGTCGCCGTGAGCGAGAACAGA  [485]
R.humeana                AAGAGTGGGTAGTCC---GCAGTCGCCGGGCACGACGGGTGTTGGTCGCCTTGAGCGAGAACAGA  [487]
Rhynchanthus             AAGAGTGGATAGTCG---ACAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGGGAACAGA  [487]
Scaphochlamys.kunstleri  AAGAGCGGGAAGTCG---GCAATCGTCKGGCACGATGGATGTTGGTCGCCGTGAGCGGGAACAGA  [498]
S.lanceolata             AAGAGCGGGAAGTCR---GCAATCGTCGGGCGCGATGGATGTTGGTCGCCGTGAGCGGGAACAGA  [504]
Smithatris               AAGAGTGGGTAGTCG---GTAGTCGTCGAGCACGACGGATGTTGGTCGCCATGAGCGGGAACTGA  [486]
Stahlianthus             AAGAGTGGGCACTCG---GCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGA  [488]
Zingiber                 AAGAGCGGGTAGTCT---GCAGTCGTCGGGCACGACGGGCGTTGGTCGCCGTGAGCGGGAACCGA  [513]
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[                                590       600       610       620       630       640       650]
[                                .         .         .•        .         .         .         .]

Alpinia                          ACATCGT--CCCCG-TCGT---ACTGGGATG---AGTCCTCAAG----AGACCTTG---TGTG--   [541]
Pleuranthodium                   ACGTTGT--CCCCG-TCGT---GTCGGGATG---AGTCCTCAAG----AGACCCTA---TGTG--   [532]
Renealmia                        ACGTTGT--CCCCG-TCGT---GTTGGGATG---AGTCCTCAAG----------------TG--   [523]
Boesenbergia.aurantiaca          ACATCGT--CCTCG-TCGT---TTCGGGACG---AGCCCTCAAG--AGAGACCCTG---TGCG--   [540]
B.basispicata                    ACATCAC--CCCCGATCAT---TTCTGGACG---AGTCCTCAAG----AGACCCTG---TGTG--   [530]
B.cordata                        ACATCGT--CCTCG-TCGT---TTCGGGACG---ARCCCTCAAG----AGAACCTG---TGCG--   [543]
B.gelatinosa                     ACATCAC--CCCCS-TCGT---TTTTGGGTK---AATCCTCAAG----AAACCCKG---TWTG--   [532]
B.longiflora                     ACATCGG--CCTCG-TCAT---TTTTGGACG---AGTCCTCAAG----AGACCCTG---TTTG--   [532]
B.aff.longiflora                 ACATCGG--CCTCG-TYGT---TTTTRGACA---AGTCCTCAAG----AGACCTTA---TTTG--   [532]
Camptandra.ovata                 ACGTCGT--CCTCG-TCGT---GTAGGGATG---AGTCCTCAAG----AGACCCTG---TCAG--   [540]
C.parvula                        ATATCGT--CCCCG-TCGT---GTTGGGATG---AGTCCTCAAG----AGACCCTG---TCTG--   [537]
Caulokaempferia                  ACATCGT--CCTCG-TCGT---TTTGGGATG---AGCCCTGGAT--AGAGACCCTG---TGCG--   [528]
Cautleya.spicata                 ACGTCGT--CCCCG-TCGT---TTTGGGAAT----GTCCTCAAG----AGACCCTG---TGTG--   [533]
Cornukaempferia                  ACGTCGT--CCTCG-TCGG---TTCGGGACT---AGTCCTCAAG----AGACCCTG---TGCG--   [545]
Curcuma.alismatifolia            ACGTCGT--CCTCG-TCAT---TTTGGGATG---AGTCCTCAAG----AGACCCTA---CGTG--   [535]
C.amada                          ACGTCGTGTCCTCG-TCGT---TTTGGGATG---AGTCCTCCAG----AGACCCTG---TGTG-A   [544]
C.ecomata                        ACGTCGT--CCTCG-TCGT---TTCGGGATG---AGTCCTCAAG----AGACCCTG---TGTG--   [528]
C.harmandii                      ACGTCGT--CCTCG-TCAT---TTTGGGATG---AGTCCTCAAG----AGACCCTA---TGTG--   [535]
C.parviflora                     ACGTCGT--CCTCG-TCAT---TTTGGGATG---AGTCCTCAAG----AGACCCTA---TGTG--   [535]
C.rubescens                      ACGTCGT--CTTCG-TCRT---TTTGGGATG---AGTCCTCAATC---AGACCCTK---TKTG-A   [543]
Distichochlamys                  ACGTCGT--CCTCG-TCGT---TTGGAGATG---AGTTCTCAAG----AGACCCTG---TGTG--   [552]
Haniffia                         ACGTCGT--CCCTG-TCGT---TTTGGGATG---AGCCCCCAAA---GAGACCCTA---TTTG--   [540]
Hedychium.coccineum              ACGTCGT--CCCCG-TCGT---CTCGGGATG---AGTCCTCAAG----AGACCCTG---TGCG--   [537]
H.gardnerianum                   ACGTCGT--CCCCG-TCGT---CTCGGGATG---AGTCCTCAAG----AGACCCTG---TGCG--   [537]
H.x raffillii                    ACGTCGT--CCCCG-TCGT---CTCGGGATG---AGTCCTCAAG----AGACCCTG---TGCG--   [537]
H.villosum                       ACGTCGT--CCCCG-TCGT---CTCGGGATG---AGTCCTCAAG----AGACCCTG---TGCG--   [536]
H.sp.                            ACGTCGT--CCCCG-TCGT---CTCGGGACG---AGTCCTCAAG----AGACCCTG---TGTG--   [539]
Hitchenia                        ACGTCGC--C-TCG-TCGT---TTTGGGATG---AG-CCTCAATCAAGAGACCCTG---TGTG-A   [536]
Kaempferia.angustifolia          ACGTCTC--CCCCGTCTGT----TTGGGACA---AGCCCTCAATCA-GAGACACTC---TGTG--   [542]
K.elegans                        ACATCGT--CCCCG-TCGTTTCCGGATGACGATGAGCCCTCGTCAA-GAGACCCTGCTGTGTGTG   [604]
K.rotunda                        ACATCKT--CCCCG-TCGT---ATTGGGATGA-GTGTCCCCAAG----AGACCCTG---TGTG-A   [552]
Paracautleya                     ACGTCGT--CCTCG-TCGT---TTTGGGATG---AGTCCTCAAAG---AGACCTTG---TGTG-A   [535]
Pommereschea                     ACGTCGT--ACCCA-A-GT---TGTGGGATG---ATTCCTCAAG----AGACCCTT---TGTG--   [531]
Pyrgophyllum                     ACGTTGT--CCCCG-TCGT---GCTGGGATG---AGACCTCAAG----AGACCCTG---TGTG--   [534]
Roscoea.bhutanica                ACGTCGT--CCCCG-TCGT---TTTAGGATT-----TCCTCAAG----AGACCCCG---TGTG--   [530]
R.humeana                        ACGTCGT--CCCCG-TCGC---TTTAGGATT----GTCCTCAAG----AGACCCCG---TGTG--   [533]
Rhynchanthus                     ACGTCGT--ACCCT-TCAT---TGTGGGATG---ATTCCTCAAG----AGACCTTG---TGTG--   [534]
Scaphochlamys.kunstleri          ACGTCGT--CCTCG-TCGT---TTTGAGACGATGAGTCCTCCTCAAAGAGACCCTG---TGCG--   [552]
S.lanceolata                     ACGTCGT--CCTCG-TCGT---TTTGAAACG---AGTCTC-AAG----AGACCCTG---CTTG-G   [551]
Smithatris                       ACGTCGT--CCTCG-TCGT---TTCGGAACG---AGTCCTCAAG----AGACCCTG---TGTG--   [533]
Stahlianthus                     ACGTCGT--CCTCG-TCAT---TTTGGGATG---AGTCCTCAAG----AGGCCCTA---TGTATG   [537]
Zingiber                         ACGTCGT--CCCCG-TCGTCATTTTCGGACG---AACCCTCAAG----AGACCCTG---CGTG--   [563]
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[                          660        670        680        690        700        710    ]
[                          .          .          .          .          .          .      ]

Alpinia                  --ATTGCAGCATCGCATGAAAG--------TGCCGTG----TTCATCATA-----TTGTGGC---  [584]
Pleuranthodium           --AATGCGGCATCACGTGAAAG--------TGCCGTG----TCCATCTGA-----TTGTGGC---  [575]
Renealmia                --ATTGCGGCGTCGCGTGAAAG--------TGCCGTG----TTCGTCATA-----TTGTGGC---  [566]
Boesenbergia.aurantiaca  --ATCGCGGCATCGGACGAAAG--------TGCCGTGT--GTCCATCTAC-----TTGTGGC---  [585]
B.basispicata            --ATTGTGGCATCGGGTGAAAG--------TGCCGTG----CCCATCAAC-----TTGTGGC---  [573]
B.cordata                --ATTGCGGCATCGGACAAAAG--------TGCCGTG----TCCATCTAA-----TT????????  [589]
B.gelatinosa             --ATTGKGGAATCGGGTKTTAA--------TGCCGTG----SCCAACAAC-----TTGTGGC---  [575]
B.longiflora             --ATTGTGGCATTAGGTCAAAG--------TGYCATG----TCCATCAAC-----TTGTGGC---  [575]
B.aff.longiflora         --AWTGTGGCATCAGGTCAAAG--------TGCCATG----TCCATCAAC-----TTGTGGC---  [575]
Camptandra.ovata         --ATAGCCGAGTCGGGCGGAAG--------TTCCGTG----AGCATCATA-----TT????????  [586]
C.parvula                --GTTGCGGAGTCGGGTGAAAG--------TGCCGTA----TGCATCATA-----TTGTGGC---  [580]
Caulokaempferia          --ATTGCGGCATCGGACGAAAG--------TGCCGTG----CCCATCTAC-----TTGTGGC---  [571]
Cautleya.spicata         --ATTGTGATGTCGTGTGAAAG--------TGCCGTG----TCCATCAAA-----TTGTGGC---  [576]
Cornukaempferia          --ATTGCGGCGTCGGGCGAAAG--------CGCGGCG----TCCATCAAA-----CTGTGGC---  [588]
Curcuma.alismatifolia    --ATTGCAGAGTCGGATGAAAG--------CGCTGTG----TCAATCATCAT---TCGCGGC---  [580]
C.amada                  TGATTGCGGAGTCGCGTGAAAG--------CGCCGCG----TCAATCAT------TTGCGGC---  [588]
C.ecomata                --ATTGCGGAGTCGGTTGAAAG--------TGCCGTG----TCAATCAT------TTGTGGC---  [570]
C.harmandii              --ATTGCAGAGTCGGATGAAAG--------CGCTGTG----TCAATCATCAT---TTGCGGC---  [580]
C.parviflora             --ATTGCAGAGTCGGACGAAAG--------CGSTGTG----TCAATCATCAT---TTGCGGC---  [580]
C.rubescens              TGATTGCGGAGTCKCGTGAAAG--------CGCCGCG----TCAATCAT------TTGCGGC---  [587]
Distichochlamys          --TTTGTGGCATCGGGCGAAAG--------TGCCGTG----TCCATCAAC-----TTGTGGC---  [595]
Haniffia                 --ATTGTGGCGTCGGGTGAAAG--------TGCCGTG----TCCATGAAC-----TTGTGGC---  [583]
Hedychium.coccineum      --AATGCGGCGTCGGCCGAAAG--------TGCCGCG----CCCATCAAA-----TTGTGGC---  [580]
H.gardnerianum           --AATGCGGCGTCGGCCGAAAG--------YGCCGCG----CCCATCAAA-----TTGTGGC---  [580]
H.x raffillii            --AATGCGGCGTCGGCCGAAAG--------YGCCGCG----CCCATCAAA-----TTGTGGC---  [580]
H.villosum               --AATGCGGCGTCGGGCGAAAG--------CGCCGCG----CCCATCAAA-----TTGTGGC---  [579]
H.sp.                    --AATGCGGCGTCGGCCGAAAG--------TGCCGCG----CCCATCAAA-----TTGTGGC---  [582]
Hitchenia                TGATCGCGGAGCCGCGTGAAAG--------CGCCGCG----TCAATCAT------TTGCGGC---  [580]
Kaempferia.angustifolia  TGWGTGTTGTGTCGGGTGAGTG--------TGGCGCA-CC??????????????????????????  [598]
K.elegans                TGATCGTGGCGTCGTGAGCTAAAAAGTGC-CTCGGCGTCCGTCCATCACATCAACTTGTGGC---  [665]
K.rotunda                TTGTGGCGGCGTCCGGCGAAAA--------TGCCGCG-CCGTCCATCAAC-----TTGTGGC---  [600]
Paracautleya             TGATTGCGGAGTCGCGTGAAAG--------TGCCGTG----TCAATCAT------TTGCGGC---  [579]
Pommereschea             --ATTGTGGCATCGAGCGAAAG--------CACCGTG----TCCATCAAA-----TTGTGGC---  [574]
Pyrgophyllum             --ATTGTGGAGTCGGGTGAAAG--------TGCCGTG----TCCATCAAA-----TTGTGGC---  [577]
Roscoea.bhutanica        --ATTGTGATGTGGTGTGAAAG--------TGCCGTG----TCCATCAAA-----TTGTGGC---  [573]
R.humeana                --ATTGTGACGTCGTGCGAAAG--------TGCCGTG----TCCATCAAA-----TTGTGGC---  [576]
Rhynchanthus             --ATTGTGGCATCGGGTGAAAG--------TGCCGTG----CCCATCAAA-----TTGTGGC---  [577]
Scaphochlamys.kunstleri  --ATTGCGGCGTCGGACGAAAG--------TGCCGTGTCCGTCAACTAAC-----TTGTGGC---  [599]
S.lanceolata             TGATTGCGGCGTCGGGCGAAAG--------TGCCGTGTCCGTCAACTAAC-----TTGTGGC---  [600]
Smithatris               --ATTGCGGAGTCGGACGAAAG--------TGCCGTG----TCAATCAT------TTGTGGC---  [575]
Stahlianthus             TGATTGCAGAGTCGGATCAAAG--------CGCTGTG----TCAATCATCAT---TTGCGGCGGC  [587]
Zingiber                 --ATTGCGGCACCGGGCGAAAGAAAGAAAGCGCCGTGTCCGTCAATCAAC-----TTGTGGC---  [618]
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[                              720]
[                               .  ]

Alpinia                    CCCAAGT   [591]
Pleuranthodium             CCCAAGT   [582]
Renealmia                  CCCAAGT   [573]
Boesenbergia.aurantiaca    CCCAAGT   [592]
B.basispicata              CCCAAGT   [580]
B.cordata                  ???????   [596]
B.gelatinosa               CCCAAGT   [582]
B.longiflora               CCCAAGT   [582]
B.aff.longiflora           CCCAAGT   [582]
Camptandra.ovata           ???????   [593]
C.parvula                  CCCAAGT   [587]
Caulokaempferia            CCCAAGT   [578]
Cautleya.spicata           CCCAAGT   [583]
Cornukaempferia            CCCAAGT   [595]
Curcuma.alismatifolia      CCCAAGT   [587]
C.amada                    CCCAAGT   [595]
C.ecomata                  CCCAAGT   [577]
C.harmandii                CCCAAGT   [587]
C.parviflora               CCCAAGT   [587]
C.rubescens                CCCAAGT   [594]
Distichochlamys            CCCAAGT   [602]
Haniffia                   CCCAATC   [590]
Hedychium.coccineum        CCCAAGT   [587]
H.gardnerianum             CCCAAGT   [587]
H.x raffillii              CCCAAGT   [587]
H.villosum                 CCCAAGT   [586]
H.sp.                      CCCAAGT   [589]
Hitchenia                  CCCAAGT   [587]
Kaempferia.angustifolia    ???????   [605]
K.elegans                  CCCAAGT   [672]
K.rotunda                  CCCAAGT   [607]
Paracautleya               CCCAAGT   [586]
Pommereschea               CCCAATC   [581]
Pyrgophyllum               CCCAAGT   [584]
Roscoea.bhutanica          CCCAAGT   [580]
R.humeana                  CCCAAGT   [583]
Rhynchanthus               CCCAAGT   [584]
Scaphochlamys.kunstleri    CCCAAGT   [606]
S.lanceolata               CCCAAGT   [607]
Smithatris                 CCCAAGT   [582]
Stahlianthus               CCCAAGT   [594]
Zingiber                   CCCAAGT   [625]
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APPENDIX THREE: A MATRIX OF *trn*L-F SEQUENCES OF THE HEDYCHIEAE (CHAPTER TWO)

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[                                10        20        30        40        50        60        70        80        90]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGTAATCCTGAGCCAAATCCTTAGTTTGCTAAACCTTAGTTTTATCAAA  [90]
Renealmia                        TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAAGGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAAACTAAGGTTTATCAAA  [90]
Pleuranthodium                   TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTGAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAAACTAAGGTTTATCAAA  [90]
Boesenbergia.aurantiaca          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
B.basispicata                    TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Camptandra.parvula               TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAAC--------TATCAAA  [82]
Caulokaempferia                  TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Cautleya                         TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAAACTAAGGTTTATCAAA  [90]
Cornukaempferia                  TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTAAGGTTTATCAAA  [90]
Curcuma.alismatifolia            TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTGAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
C.amada                          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Distichochlamys                  TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Hedychium.gardnerianum           TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
H.sp.                            TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Kaempferia.angustifolia          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTCAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAAACTAAGGTTTATCAAA  [81]
K.elegans                        TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTAAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
K.rotunda                        TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTAAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTTATAAACCTTAGTTTTATCAAA  [90]
Paracaulteya                     TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Pyrgophyllum                     TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Roscoea.bhutanica                TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTT--------------TATCAAA  [75]
R.humeana                        TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTT--------------TATCAAA  [75]
Scaphochlamys.kunstleri          TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
S.lanceolata                     TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Smithatris                       TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Stahlianthus                     TGGTAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
Zingiber                         TGATAACTTCCAAATTCAGAGAAACCCTGGAATTTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACCTTAGTTTTATCAAA  [90]
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[                                100       110       120       130       140       150       160       170       180]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                          ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
Renealmia                        ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
Pleuranthodium                   ----------CTATAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
Boesenbergia.aurantiaca          ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAACGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
B.basispicata                    ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAACGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
Camptandra.parvula               ----------CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [159]
Caulokaempferia                  ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAACGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
Cautleya                         ----------CTAGAATAAAAAAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [168]
Cornukaempferia                  ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
Curcuma.alismatifolia            ----------CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TCG   [167]
C.amada                          ----------CTAGAAAAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TCG   [166]
Distichochlamys                  ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAATTGACTACGTTTCG--TTG   [166]
Hedychium.gardnerianum           ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGACTGAAGATGACTACGTGTCG--TTG   [166]
H.sp.                            ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
Kaempferia.angustifolia          ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGGTGACTACGTTTCGCGTTG   [159]
K.elegans                        ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
K.rotunda                        ----------CTAGAATAAAAAAA---GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGGTGACTACGTTTCG--TTG   [165]
Paracaulteya                     ----------CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TCG   [167]
Pyrgophyllum                     ----------CTAGAATAAAAAAA---GGATAGGTGCAGAGACTCGATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [165]
Roscoea.bhutanica                ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [151]
R.humeana                        ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [151]
Scaphochlamys.kunstleri          ----------CTAGAAT-----------GATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [157]
S.lanceolata                     ----------CTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [166]
Smithatris                       ----------CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TCG   [167]
Stahlianthus                     ----------CTAGAATAAAAAAAAA-GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TCG   [167]
Zingiber                         TTTTATCAAACTAGAATAAAAAAAA--GGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTCG--TTG   [176]
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[                                190       200       210       220       230       240       250       260       270]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                  GTAGTTGGAATCCGTCTATCAAAATTATAGAAAGGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Renealmia                GTAGTTGGAATCCGTCTATCAAAATTACAGAAAGGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Pleuranthodium           GTAGTTGGAATCCGTCTATCAAAATTACAGAAAATATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Boesenbergia.aurantiaca  GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
B.basispicata            GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Camptandra.parvula       GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [249]
Caulokaempferia          GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGGCATATCAAATCAAACGA  [256]
Cautleya                 GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [258]
Cornukaempferia          GTAGTTGGAATCCGTCTATCAAAATTAAAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Curcuma.alismatifolia    GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [257]
C.amada                  GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Distichochlamys          GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Hedychium.gardnerianum   GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
H.sp.                    GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Kaempferia.angustifolia  GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATATTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [249]
K.elegans                GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATATTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
K.rotunda                GTAGTTGGAATCCGTTTATCAAAATTACAGAAAAGATATTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [255]
Paracaulteya             GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [257]
Pyrgophyllum             GTAGTTGGAATCCATCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [255]
Roscoea.bhutanica        GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [241]
R.humeana                GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [241]
Scaphochlamys.kunstleri  GTAGTTGGAATCCGTATATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [247]
S.lanceolata             GTAGTTGGAATCCGTATATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [256]
Smithatris               GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [257]
Stahlianthus             GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [257]
Zingiber                 GTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGTTCCTATATACCTAATACATACGTATACATACTGACATATCAAATCAAACGA  [266]
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[                                280       290       300       310       320       330       340       350       360]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                          TTAATCATGACCCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGCAAAAT-----------  [309]
Renealmia                        TTAATCATGACCCGAATTTATT-----------ATATTAATTTATTATATTATATGTATAATTATAATATGCAAAAT-----------  [322]
Pleuranthodium                   TTAATCATGACTCGAATCCATT-----------ATATTA--------TATA-----GATAATTATAATATGAAAAAT-----------  [309]
Boesenbergia.aurantiaca          TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [309]
B.basispicata                    TTWATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [309]
Camptandra.parvula               TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [302]
Caulokaempferia                  TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAATT----------C  [311]
Cautleya                         TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [311]
Cornukaempferia                  TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [309]
Curcuma.alismatifolia            TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [310]
C.amada                          TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [309]
Distichochlamys                  TTAATCACGACTCGAATCCATT-----------ATATTA--------TATGATATGGATAATTATAATATGAAAAAT-----------  [314]
Hedychium.gardnerianum           TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [309]
H.sp.                            TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [309]
Kaempferia.angustifolia          TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [302]
K.elegans                        TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [309]
K.rotunda                        TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [308]
Paracaulteya                     TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [310]
Pyrgophyllum                     TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATTAAAAAT-----------  [308]
Roscoea.bhutanica                TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [294]
R.humeana                        TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATTAAAAAT-----------  [294]
Scaphochlamys.kunstleri          TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [300]
S.lanceolata                     TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [309]
Smithatris                       TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [310]
Stahlianthus                     TTAATCATGACTCGAATCCATT-----------ATATTA--------TATG-----GATAATTATAATATGAAAAAT-----------  [310]
Zingiber                         TTAATCATGACTCGAATCCATTCTCGAATCCATTATATTA--------TATG-----AATAATTATAATAT--AAAATTATATGAATAAT  [341]
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[                                370       380       390       400       410       420       430       440       450]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                          --------------TCAGAATTAGAGTTATTTT--ATTGTGCC---AATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [379]
Renealmia                        --------------TCAGAATTAGAGTTATTGTGC----------CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [387]
Pleuranthodium                   --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATT-----ATTA   [377]
Boesenbergia.aurantiaca          --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [382]
B.basispicata                    --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [382]
Camptandra.parvula               --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [375]
Caulokaempferia                  AGAATTAGAAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [399]
Cautleya                         --------------TCAGAATTAGAGTTATTGTGAATCCAATC--CAATGGAAGTCGAAAGAAGAATTGAATATTCAATTCAATTATTA   [384]
Cornukaempferia                  --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGATTTAATAATTGAATATTCAATT-----ATTA   [377]
Curcuma.alismatifolia            --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CGATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTACTA   [383]
C.amada                          --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CGATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [382]
Distichochlamys                  --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATT-----ATTA   [382]
Hedychium.gardnerianum           --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CGATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [382]
H.sp.                            --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CGATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [382]
Kaempferia.angustifolia          --------------TCAGAATTAGAGTTATTATGAATCCAGTC--CAATGGAAGTTGAAAGGAAAATTGAATATTCAATTCAATTATTA   [375]
K.elegans                        --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGGAGAATTGAATATTCAATTCAATTATTA   [382]
K.rotunda                        --------------TAAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGGAGAATTGAATATTCAATTCAATTATTA   [381]
Paracaulteya                     --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CGATGGAAGTTGAAAGAAGAATTGAATATTAAATTCAATTATTA   [383]
Pyrgophyllum                     --------------TCAGAATTAGAGTTATTGTGAATCCAGTCTCCAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [383]
Roscoea.bhutanica                --------------TAAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [367]
R.humeana                        --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATT-----ATTA   [362]
Scaphochlamys.kunstleri          --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [373]
S.lanceolata                     --------------TAAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [382]
Smithatris                       --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CGATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTACTA   [383]
Stahlianthus                     --------------TCAGAATTAGAGTTATTGTGAATCCAGTC--CGATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTACTA   [383]
Zingiber                         TATAATATAAAAAATTCAGAATTAGAGTTATTGTGAATCCAGTC--CAATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAATTATTA   [429]
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[                                460       470       480       490       500       510       520       530       540]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                  AATCATTCATTCCATAATTTGATAGATCTTTTGAAAAACAGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [469]
Renealmia                AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACAGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [477]
Pleuranthodium           AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACGGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [467]
Boesenbergia.aurantiaca  AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [472]
B.basispicata            AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAATAGAGAGTCCCATTCTACATGTCAATACC [472]
Camptandra.parvula       AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [465]
Caulokaempferia          AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [489]
Cautleya                 AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [474]
Cornukaempferia          AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [467]
Curcuma.alismatifolia    AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [473]
C.amada                  AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [472]
Distichochlamys          AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACT [472]
Hedychium.gardnerianum   AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [472]
H.sp.                    AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [472]
Kaempferia.angustifolia  AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [465]
K.elegans                AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [472]
K.rotunda                AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [471]
Paracaulteya             AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [473]
Pyrgophyllum             AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [473]
Roscoea.bhutanica        AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [457]
R.humeana                AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [452]
Scaphochlamys.kunstleri  AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [463]
S.lanceolata             AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [472]
Smithatris               AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [473]
Stahlianthus             AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [473]
Zingiber                 AATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTACATGTCAATACC [519]
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[                    550        560        570        580        590        600        610        620        630]
[                    .          .          .          .          .          .          .          .          .]
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Alpinia	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTCGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTGAT	[559]
Renealmia	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTGAT	[567]
Pleuranthodium	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTCGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTGAT	[557]
Boesenbergia.aurantiaca	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[562]
B.basispicata	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[562]
Camptandra.parvula	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCMATAAAAAGGGAAT	[555]
Caulokaempferia	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[579]
Cautleya	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[564]
Cornukaempferia	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTA????????????????????	[538]
Curcuma.alismatifolia	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTCGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[563]
C.amada	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[562]
Distichochlamys	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[562]
Hedychium.gardnerianum	CACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[562]
H.sp.	GACAACAATGA??ATAAAAAGGTAAT	[496]
Kaempferia.angustifolia	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[555]
K.elegans	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[562]
K.rotunda	GACAACAATGAAATTTAT??????????????????????????????GTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[529]
Paracaulteya	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[563]
Pyrgophyllum	GACAACAATGAAATTTTTAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTTTATCCCCAATAAAAAGGGCAT	[563]
Roscoea.bhutanica	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[547]
R.humeana	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[542]
Scaphochlamys.kunstleri	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[553]
S.lanceolata	GACAACAACGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[562]
Smithatris	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[563]
Stahlianthus	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[563]
Zingiber	GACAACAATGAAATTTATAGTAAGAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAAGGTAAT	[609]

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[                                640       650       660       670       680       690       700       710       720]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                          TTTACTTCCTAAATATT------TATTCTCC-TTTTTTT---CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [632]
Renealmia                        TTTAGTTCCTAAATATT------TATCCTCC-TTTTTTT---CATCAGCGATTCAGTTCAAACAAAATTAA-------CTCACT------   [634]
Pleuranthodium                   TTTACTTCCTAAATATT------TATCCTCC-TTTTTTT---CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [630]
Boesenbergia.aurantiaca          TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTTTCATCAGCGATTCAGTTCAAACAAAATTCAAAATTCACTATCTTTCTCA   [645]
B.basispicata                    TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTT-CATGAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [637]
Camptandra.parvula               TTTAMCTCCCAAAAAWT------TATCCTCCCTTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [631]
Caulokaempferia                  TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [654]
Cautleya                         TTTACTTCCTAAATATTTATATTTATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [645]
Cornukaempferia                  ??????TCCTAAATATT------TATCCTCC-TTTTTTTTTTCATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [608]
Curcuma.alismatifolia            TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTTTCATCAGCGATTCAGTTCAAACAAAATTCA-------GTATCTTTCTCA   [639]
C.amada                          TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [637]
Distichochlamys                  TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [637]
Hedychium.gardnerianum           TTTACTTCCTAAATCTAAATATTTATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [643]
H.sp.                            TTTACTTCCTAAATCTAAATATTTATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [577]
Kaempferia.angustifolia          TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTTTCATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [631]
K.elegans                        TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTT--CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [636]
K.rotunda                        TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [604]
Paracaulteya                     TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [638]
Pyrgophyllum                     TTTACTTCCTAAATATT------TATCCTCCCTTTTTTTTTTCATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [640]
Roscoea.bhutanica                TTTACTTCCTAAATATT------TATCCTCC-TTTTTCTTTTCATCCGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [623]
R.humeana                        TTTACTTCCTAAATATT------TATCCTCC-TTTTTCTTTTCATCCGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [618]
Scaphochlamys.kunstleri          TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTT--CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [627]
S.lanceolata                     TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTT--CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [636]
Smithatris                       TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTT--CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [637]
Stahlianthus                     TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTTTCATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [639]
Zingiber                         TTTACTTCCTAAATATT------TATCCTCC-TTTTTTTTT-CATCAGCGATTCAGTTCAAACAAAATTCA-------CTATCTTTCTCA   [684]
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[                                730       740       750       760       770       780       790       800       810]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                          TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [721]
Renealmia                        --CACTCCAATTTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [721]
Pleuranthodium                   TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [719]
Boesenbergia.aurantiaca          TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [734]
B.basispicata                    TTCACTCCACTTTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAAAT  [727]
Camptandra.parvula               TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACGAA-T  [720]
Caulokaempferia                  TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [743]
Cautleya                         TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [734]
Cornukaempferia                  TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTCAAATTCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [697]
Curcuma.alismatifolia            TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCGTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [728]
C.amada                          TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [726]
Distichochlamys                  TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [726]
Hedychium.gardnerianum           TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTTGATAGATACAATACCTCTACAAA-T  [732]
H.sp.                            TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTTGATAGATACAATACCTCTACAAA-T  [666]
Kaempferia.angustifolia          TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [720]
K.elegans                        TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [725]
K.rotunda                        TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [693]
Paracaulteya                     TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [727]
Pyrgophyllum                     TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCGTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [729]
Roscoea.bhutanica                TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCCTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [712]
R.humeana                        TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCCTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [707]
Scaphochlamys.kunstleri          TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGAATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [716]
S.lanceolata                     TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [725]
Smithatris                       TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [726]
Stahlianthus                     TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTCTACAAA-T  [728]
Zingiber                         TTCACTCCACTCTTTCACAACACAAATGTATCCGAACTAAAATCCTTGGATCTTATCCTAATTTCGATAGATATAATACCTCTACAAA-T  [773]
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[                                820       830       840       850       860       870       880       890       900]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                          AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATACTTACGCTTACTAGTCAAATTTTTGACTACTT   [811]
Renealmia                        AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCACATCATTATCCTTACGCTTACTAGTAAAATTTTTTACTACTT   [811]
Pleuranthodium                   AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTAAAATTTTTGACTACTT   [809]
Boesenbergia.aurantiaca          AAACATATATAGGCAAATAATCTTTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [824]
B.basispicata                    AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [817]
Camptandra.parvula               AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [810]
Caulokaempferia                  AAACATATATAGGCAAATAATCTTTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [833]
Cautleya                         AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [824]
Cornukaempferia                  AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [787]
Curcuma.alismatifolia            AAACATATATGGGCAAATAATCTCCATTATTGAATCATTCACAGTCCGTATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [818]
C.amada                          AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCGTATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [816]
Distichochlamys                  AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCYTACGCCTACTAG??????????????????   [798]
Hedychium.gardnerianum           AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [822]
H.sp.                            AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [756]
Kaempferia.angustifolia          AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTGGTTAAATTTTTTACTACTT   [810]
K.elegans                        AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTATTTAAATTTTTTACTACTT   [815]
K.rotunda                        AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTGGTTAAATTTTTTACTACTT   [783]
Paracaulteya                     AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCGTATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [817]
Pyrgophyllum                     AAG----------------TCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [802]
Roscoea.bhutanica                AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [802]
R.humeana                        AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [797]
Scaphochlamys.kunstleri          AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [806]
S.lanceolata                     AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [815]
Smithatris                       AAACATATATGGGCAAATAATCTCTATTATTGAATCATCCACAGTCCGTATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [816]
Stahlianthus                     AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCGTATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [818]
Zingiber                         AAACATATATGGGCAAATAATCTCTATTATTGAATCATTCACAGTCCATATCATTATCCTTACGCTTACTAGTTAAATTTTTTACTACTT   [863]
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[                                910       920       930       940       950       960       970       980       990]
[                                .         .         .         .         .         .         .         .         .]

Alpinia                          TTT---AGTCCCTTTAATTGACATAGACACAAACACTACAACTACACCAGTATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [896]
Renealmia                        TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGTATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [890]
Pleuranthodium                   TTT---AGTACCTTTAATTGACATAGACACAAACACTACA------TCAGTATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [888]
Boesenbergia.aurantiaca          TTT---AGTCCCTTTAATTGACATAGACATAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [903]
B.basispicata                    TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [896]
Camptandra.parvula               TTTTTTAGTCCCTTTAATTGACATAGACACAAACACTACC------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCA-TT--GGT   [891]
Caulokaempferia                  TTT---AGTCCCTTTAATTGACATAGACATAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [912]
Cautleya                         TTT---AGTCCCTTTAATTGACATAGACACAAACACGACA------CCGGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT-GGGT   [904]
Cornukaempferia                  TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [866]
Curcuma.alismatifolia            TTT---AGTCCCTTTAATTGACATAGACACAAACATTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGA---GG?   [895]
C.amada                          TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [895]
Distichochlamys                  ??????????????????????????????????????????????????????????????????????????????????????   [798]
Hedychium.gardnerianum           TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [901]
H.sp.                            TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [835]
Kaempferia.angustifolia          T--------------------ATAGACACAAATACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [871]
K.elegans                        TTT---AGTCCCTTTAATTGACATAGACACAAATACTACA------CCAGGATGATGCATGGGAAATAGTCGGGATAGCTCAGTT-GGGT   [895]
K.rotunda                        T--------------------ATAGACACAAATACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [844]
Paracaulteya                     TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [896]
Pyrgophyllum                     TTT---AGTCCCTTTAATTGACATATACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCT??????????   [873]
Roscoea.bhutanica                TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTTTTGGT   [883]
R.humeana                        TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [876]
Scaphochlamys.kunstleri          TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [885]
S.lanceolata                     TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATAGTCGGGATAGC-CAGTT--GGT   [893]
Smithatris                       TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [895]
Stahlianthus                     TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGGGAAATGGTCGGGATAGCTCAGTT--GGT   [897]
Zingiber                         TTT---AGTCCCTTTAATTGACATAGACACAAACACTACA------CCAGGATGATGCATGAGAAATGGTCGGGATAGCTCAGTT--GGT   [942]
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[                                1000      ]
[                                .         ]

Alpinia                          AGAGCAGAGGACTGAAAA   [914]
Renealmia                        AGAGCAGAGGACTGAAAA   [908]
Pleuranthodium                   AGAGCAGAGGACTGAAAA   [906]
Boesenbergia.aurantiaca          AGAGCAGAGGACTGAAAA   [921]
B.basispicata                    AGAGCAGAGGACTGAAAA   [914]
Camptandra.parvula               AGAGCAGAGGACTGAAAA   [909]
Caulokaempferia                  AGAGCAGAGGACTGAAAA   [930]
Cautleya                         AGAGCAGAGGAA??????   [916]
Cornukaempferia                  AGAGCAGAGGACTGAAAA   [884]
Curcuma.alismatifolia            ????????????????????[895]
C.amada                          AGAGCAGAGGACTGAAAA   [913]
Distichochlamys                  ????????????????????[798]
Hedychium.gardnerianum           AGAGC????????????????[906]
H.sp.                            AGAGCAGAGGACTGAAAA   [853]
Kaempferia.angustifolia          AGAGCAGAGGACTGAAAA   [889]
K.elegans                        AGAGCAGAGGACTGAAAA   [913]
K.rotunda                        AGAGCAGAGGACTGAAAA   [862]
Paracaulteya                     AGAGCAG??????????????[903]
Pyrgophyllum                     ????????????????????[873]
Roscoea.bhutanica                AGAGCAGAGGACTGAAAA   [901]
R.humeana                        AGAGCAGAGGACTGAAAA   [894]
Scaphochlamys.kunstleri          AGAGCAGAGGACTGAAAA   [903]
S.lanceolata                     AGAGCAGAGGACTGAAAA   [911]
Smithatris                       AGAGCAGAGGACTGAAAA   [913]
Stahlianthus                     AGAGCAGAGGACTGAAAA   [915]
Zingiber                         AGAGCAGAGGACTGAAAA   [960]
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APPENDIX FOUR: LOCALITIES OF *ROSCOEA* SPECIMENS

Latitude	Longitude	Species	Collector(s)	Number
27 50 N	101 15 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Rock	16009
30 27 N	078 05 E	*Roscoea purpurea* Sm.	Thomson	1341
27 20 N	100 05 E	*Roscoea schneideriana* (Loes.) Cowley	Rock	4726a
27 20 N	100 05 E	*Roscoea cautleoides* Gagnep.	Forrest	5969
25 34 N	091 53 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Clarke	38491C
27 20 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Rock	3475
27 09 N	100 12 E	*Roscoea cautleoides* Gagnep.	Rock	24831
27 09 N	100 12 E	*Roscoea tibetica* Batalin	KEYSE	136
31 06 N	077 10 E	*Roscoea purpurea* Sm.	Brown, Countess of Dalhousie	-
27 50 N	100 40 E	*Roscoea scillifolia* (Gagnep.) Cowley	Handel-Mazzetti	3166
27 27 N	087 57 E	*Roscoea auriculata* K.Schum.	Williams	967
27 33 N	090 42 E	*Roscoea bhutanica* Ngamriab.	Grierson & Long	1826
31 06 N	077 10 E	*Roscoea purpurea* Sm.	Gamble	4663A
27 44 N	088 33 E	*Roscoea auriculata* K.Schum.	Younghusband	-
27 20 N	100 05 E	*Roscoea cautleoides* Gagnep.	Forrest	2687
27 09 N	100 12 E	*Roscoea cautleoides* Gagnep.	Rock	24930
27 02 N	088 16 E	*Roscoea alpina* Royle	Hara, Kanai, Kurosawa, Murata & Togashi	6183
28 26 N	084 55 E	*Roscoea capitata* Sm.	Gardner	847
25 34 N	091 53 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Tessier-Yandell	280
-	-	*Roscoea purpurea* Sm.	Reid	-
29 19 N	082 22 E	*Roscoea nepalensis* Cowley	Polunin, Sykes & Williams	362
28 13 N	085 27 E	*Roscoea alpina* Royle	Schilling & Sayers	418
26 10 N	103 02 E	*Roscoea tibetica* Batalin	Maire	-
27 37 N	087 53 E	*Roscoea auriculata* K.Schum.	KEKE	291
31 06 N	077 10 E	*Roscoea alpina* Royle	-	-
27 09 N	100 12 E	*Roscoea tibetica* Batalin	KEYSE	572
27 09 N	100 12 E	*Roscoea tibetica* Batalin	KEYSE	518
27 09 N	100 12 E	*Roscoea tibetica* Batalin	KEYSE	518
29 50 N	082 08 E	*Roscoea alpina* Royle	Bailey	
27 33 08 N	088 40 05 E	*Roscoea auriculata* K.Schum.	Long & Noltie	115
31 11 N	077 38 E	*Roscoea alpina* Royle	Maclagan	723
-	-	*Roscoea purpurea* Sm.	Brown, Countess of Dalhousie	-

27 12 N	100 10 E	*Roscoea cautleoides* Gagnep.	Forrest	2070
25 40 N	100 11 E	*Roscoea cautleoides* Gagnep.	Forrest	4809
25 49 N	098 40 E	*Roscoea tibetica* Batalin	Gamble	1639
25 42 N	100 11 E	*Roscoea cautleoides* Gagnep.	Bartholomew, Boufford, Li, Ma, Nicolson, Ying & Yu	998
27 09 N	100 12 E	*Roscoea cautleoides* Gagnep.	Chamberlain, Grey-Wilson, Li Y., McBeath, Schilling, Xu T. & Yuan H.	542
27 55 N	101 30 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Gamble	4376
27 09 N	100 12 E	*Roscoea humeana* Balf.f. & W.W.Sm.	KEYSE	361
-	-	*Roscoea purpurea* Sm.	Watt	-
27 50 N	101 15 E	*Roscoea tibetica* Batalin	Gamble	4349
25 40 N	100 11 E	*Roscoea forrestii* Cowley	Forrest	11726
27 20 N	100 05 E	*Roscoea schneideriana* (Loes.) Cowley	Rock	4726
27 50 N	100 40 E	*Roscoea forrestii* Cowley	Kingdon-Ward	4104
27 35 N	086 32 E	*Roscoea auriculata* K.Schum.	Stainton	7174
27 27 N	086 09 E	*Roscoea alpina* Royle	Dhwoj	490
23 22 N	103 24 E	*Roscoea debilis* var. *debilis* Gagnep.	Henry	11102A
30 27 N	078 05 E	*Roscoea purpurea* Sm.	Anderson	-
25 42 N	100 11 E	*Roscoea cautleoides* Gagnep.	Delavay	231
21 14 N	093 55 E	*Roscoea australis* Cowley	Kingdon-Ward	22124
30 42 N	077 51 E	*Roscoea purpurea* Sm.	Chatterjee	-
26 42 N	100 45 E	*Roscoea cautleoides* Gagnep.	Gamble	3923
27 28 N	088 53 E	*Roscoea bhutanica* Ngamriab.	Dungboo	56
25 43 N	100 02 E	*Roscoea tibetica* Batalin	Bartholomew, Boufford, Li, Ma, Nicolson, Ying & Yu	107
21 14 N	093 55 E	*Roscoea australis* Cowley	Kingdon-Ward	22380
27 30 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	5930
28 40 N	098 15 E	*Roscoea tibetica* Batalin	Forrest	19236
26 19 N	098 21 E	*Roscoea tibetica* Batalin	Kingdon-Ward	3199
-	-	*Roscoea bhutanica* Ngamriab.	Gould	356
27 34 N	086 26 E	*Roscoea auriculata* K.Schum.	Dhwoj	4
27 25 N	101 33 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Handel-Mazzetti	2491
-	-	*Roscoea tibetica* Batalin	-	1276
-	-	*Roscoea alpina* Royle	Reid	-
27 40 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	10218
27 45 N	089 10 E	*Roscoea auriculata* K.Schum.	Dungboo	9
27 33 59 N	100 01 64 E	*Roscoea tibetica* Batalin	ACE	251

27 37 N	091 30 E	*Roscoea purpurea* Sm.	Lyon	9054
31 06 N	077 10 E	*Roscoea alpina* Royle	Lace	975
-	-	*Roscoea debilis* var. *limprichtii* (Loes.) Cowley	Limpricht	855
		Roscoea capitata Sm.	Bailey	242?
27 28 N	088 53 E	*Roscoea bhutanica* Ngamriab.	King's collector	454
-	-	*Roscoea purpurea* Sm.	Drummond	26414
27 50 N	100 40 E	*Roscoea tibetica* Batalin	Schneider	1625
25 55 N	100 30 E	*Roscoea forrestii* Cowley	McLaren	B105
28 35 N	099 00 E	*Roscoea tibetica* Batalin	Gamble	152
31 16 N	077 27 E	*Roscoea alpina* Royle	Watt	7910
28 21 N	096 37 E	*Roscoea wardii* Cowley	Kingdon-Ward	8382
25 02 N	098 28 E	*Roscoea debilis* var. *debilis* Gagnep.	Howell	333
27 09 N	100 12 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Cribb	C41
28 14 N	084 59 E	*Roscoea ganeshensis* Cowley & W.J.Baker	Baker, Burkitt, Miller & Shrestha	34
27 22 48 N	100 05 50 E	*Roscoea tibetica* Batalin	ACE	353
32 34 N	076 08 E	*Roscoea alpina* Royle	Lace	1724
27 28 N	088 53 E	*Roscoea alpina* Royle	Cooper	195
27 23 N	088 05 E	*Roscoea alpina* Royle	Rohmoo	793
27 35 N	086 32 E	*Roscoea tumjensis* Cowley	McCosh	65
27 09 N	099 24 E	*Roscoea tibetica* Batalin	Alden, Alexander, Long, McBeath, Noltie & Watson	1684
25 20 N	098 35 E	*Roscoea debilis* var. *debilis* Gagnep.	Forrest	8456
26 55 N	100 10 E	*Roscoea schneideriana* (Loes.) Cowley	Handel-Mazzetti	4152
25 40 N	100 11 E	*Roscoea forrestii* Cowley	Forrest	11726
27 20 N	100 05 E	*Roscoea scillifolia* (Gagnep.) Cowley	Forrest	6513
27 35 N	100 05 E	*Roscoea scillifolia* (Gagnep.) Cowley	Forrest	6354
30 27 N	078 05 E	*Roscoea purpurea* Sm.	Anderson	-
26 10 N	103 02 E	*Roscoea tibetica* Batalin	Maire	490
27 00 N	104 56 E	*Roscoea schneideriana* (Loes.) Cowley	Maire	267
27 44 N	088 33 E	*Roscoea auriculata* K.Schum.	King's collector	60
25 40 N	100 11 E	*Roscoea tibetica* Batalin	Forrest	7041
27 45 N	099 30 E	*Roscoea schneideriana* (Loes.) Cowley	Forrest	10655
31 06 N	077 10 E	*Roscoea purpurea* Sm.	Drummond	26413
31 06 N	077 10 E	*Roscoea alpina* Royle	Gamble	4585A
-	-	*Roscoea schneideriana* (Loes.) Cowley	Bonati	3462
31 13 N	077 24 E	*Roscoea alpina* Royle	Sherriff	7312
25 35 N	091 38 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Griffith	5736
25 35 N	091 38 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Mann	347
-	-	*Roscoea purpurea* Sm.	Wallich	6528A
26 55 N	100 10 E	*Roscoea cautleoides* Gagnep.	Rock	4102
26 55 N	100 10 E	*Roscoea humeana* Balf.f.	Handel-Mazzetti	4154

		& W.W.Sm.		
24 47 N	103 16 E	*Roscoea debilis* var. *debilis* Gagnep.	Ducloux	688
30 27 N	078 05 E	*Roscoea alpina* Royle	Drummond	22734
27 00 N	104 56 E	*Roscoea tibetica* Batalin	Maire	-
28 02 93 N	099 45 42 E	*Roscoea tibetica* Batalin	ACE	484
29 17 N	082 10 E	*Roscoea nepalensis* Cowley	Polunin, Sykes & Williams	4391
27 12 N	100 10 E	*Roscoea cautleoides* Gagnep.	Forrest	2178
27 50 N	100 40 E	*Roscoea cautleoides* Gagnep.	Gamble	5269
29 22 N	082 12 E	*Roscoea nepalensis* Cowley	Polunin, Sykes & Williams	4381
31 06 N	077 10 E	*Roscoea alpina* Royle	-	328
27 40 N	091 12 E	*Roscoea purpurea* Sm.	Ludlow & Sherriff	309
31 06 N	077 10 E	*Roscoea alpina* Royle	Parmanand	364
29 17 N	082 10 E	*Roscoea nepalensis* Cowley	Polunin, Sykes & Williams	4391
27 30 N	099 45 E	*Roscoea cautleoides* Gagnep.	Gamble	236
26 42 N	100 45 E	*Roscoea tibetica* Batalin	Handel-Mazzetti	3351
28 03 N	097 35 E	*Roscoea wardii* Cowley	Kingdon-Ward	6885
27 09 N	100 12 E	*Roscoea tibetica* Batalin	KEYSE	136
28 09 N	085 24 E	*Roscoea capitata* Sm.	Halliwell	34
28 36 N	083 39 E	*Roscoea purpurea* Sm.	Stainton, Sykes & Williams	1596
29 22 N	082 24 E	*Roscoea alpina* Royle	Polunin, Sykes & Williams	159
27 44 N	088 33 E	*Roscoea auriculata* K.Schum.	Cave	111/47
27 22 N	092 04 E	*Roscoea purpurea* Sm.	Kingdon-Ward	13755
27 27 N	089 39 E	*Roscoea bhutanica* Ngamriab.	Gould	912
27 29 N	088 54 E	*Roscoea alpina* Royle	Gould	2937
27 44 N	088 33 E	*Roscoea auriculata* K.Schum.	Hooker	-
31 06 N	077 10 E	*Roscoea alpina* Royle	Lace	975
27 50 N	101 15 E	*Roscoea tibetica* Batalin	Rock	5486
27 20 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Rock	4549
31 06 N	077 13 E	*Roscoea purpurea* Sm.	Maclagan	437
27 20 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Rock	3344
27 40 N	100 48 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	21437
28 05 N	085 20 E	*Roscoea capitata* Sm.	Kanai, Hara & Ohba	723600
28 38 N	083 37 E	*Roscoea nepalensis* Cowley	Stainton, Sykes & Williams	1628
27 29 N	089 38 E	*Roscoea bhutanica* Ngamriab.	Grierson & Long	116
27 30 N	089 37 E	*Roscoea bhutanica* Ngamriab.	Gould	925
-	-	*Roscoea alpina* Royle	Gamble	26988
27 36 N	088 39 E	*Roscoea auriculata* K.Schum.	King's collector	53
27 20 N	100 05 E	*Roscoea tibetica* Batalin	Forrest	5988
27 45 N	099 30 E	*Roscoea tibetica* Batalin	Forrest	10638
27 00 N	104 56 E	*Roscoea schneideriana*	Maire	-

		(Loes.) Cowley		
25 34 N	091 53 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Kingdon-Ward	18682
27 35 N	085 26 E	*Roscoea purpurea* Sm.	Schilling	609
27 40 N	100 48 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	21447
27 20 N	100 05 E	*Roscoea schneideriana* (Loes.) Cowley	Rock	4888
27 33 N	087 47 E	*Roscoea purpurea* Sm.	Stainton	1198
21 22 N	093 59 E	*Roscoea australis* Cowley	Kingdon-Ward	22292
25 42 N	100 11 E	*Roscoea cautleoides* Gagnep.	Orleans	
26 55 N	100 10 E	*Roscoea cautleoides* Gagnep.	Forrest	258
27 25 22 N	099 56 28 E	*Roscoea debilis* Gagnep.	Alden, Alexander, Long, McBeath, Noltie & Watson	1540
30 27 N	078 05 E	*Roscoea alpina* Royle	King	-
27 20 N	100 05 E	*Roscoea tibetica* Batalin	Rock	4617
25 40 N	100 11 E	*Roscoea tibetica* Batalin	Forrest	4808
23 22 N	103 24 E	*Roscoea debilis* var. *debilis* Gagnep.	Henry	11102B
27 09 N	100 12 E	*Roscoea humeana* Balf.f. & W.W.Sm.	KEYSE	44
-	-	*Roscoea alpina* Royle	Watt	3362
25 04 N	102 41 E	*Roscoea praecox* K.Schum.	Cavalerie	4763
27 09 N	088 05 E	*Roscoea alpina* Royle	King's collector	57
27 26 N	092 08 E	*Roscoea purpurea* Sm.	Kingdon-Ward	11529
28 40 N	082 57 E	*Roscoea purpurea* Sm.	Stainton, Sykes & Williams	3372
23 22 N	103 24 E	*Roscoea praecox* K.Schum.	Henry	11117
26 55 N	100 10 E	*Roscoea tibetica* Batalin	Handel-Mazzetti	4153
-	-	*Roscoea praecox* K.Schum.	Gregory & Gregory	-
28 17 N	083 49 E	*Roscoea purpurea* Sm.	Grey-Wilson & Phillips	274
26 40 N	099 40 E	*Roscoea tibetica* Batalin	Forrest	23229
22 39 N	093 37 E	*Roscoea australis* Cowley	Venning	10
31 06 N	077 10 E	*Roscoea alpina* Royle	Parmanand	398
28 26 N	084 55 E	*Roscoea tumjensis* Cowley	Gardner	525
31 06 N	077 10 E	*Roscoea alpina* Royle	Schlich	-
27 34 N	086 26 E	*Roscoea auriculata* K.Schum.	Dhwoj	4
25 42 N	100 11 E	*Roscoea cautleoides* Gagnep.	Delavay	92
27 20 N	100 05 E	*Roscoea tibetica* Batalin	Rock	4393
27 25 N	088 10 E	*Roscoea auriculata* K.Schum.	King's collector	63
28 23 N	083 38 E	*Roscoea purpurea* Sm.	Kanai, Hara & Ohba	723603
30 27 N	078 05 E	*Roscoea purpurea* Sm.	Anderson	-
22 30 N	093 30 E	*Roscoea australis* Cowley	-	85
25 04 N	102 41 E	*Roscoea praecox* K.Schum.	Schoch	179
28 38 N	083 37 E	*Roscoea alpina* Royle	Stainton, Sykes & Williams	958
27 41 N	088 45 E	*Roscoea auriculata* K.Schum.	Ribu & Rhomoo	5520
26 55 N	099 50 E	*Roscoea tibetica* Batalin	Rock	25147
28 20 N	097 40 E	*Roscoea wardii* Cowley	Kingdon-Ward	9682
27 36 N	089 38 E	*Roscoea bhutanica* Ngamriab.	Ludlow, Sherriff & Hicks	16377

-	-	*Roscoea purpurea* Sm.	Cleghorn	-
28 12 N	085 05 E	*Roscoea capitata* Sm.	Stainton	3833
28 30 N	097 05 E	*Roscoea wardii* Cowley	Kingdon-Ward	10476
28 08 N	096 58 E	*Roscoea wardii* Cowley	Kingdon-Ward	19623
26 16 N	100 00 E	*Roscoea schneideriana* (Loes.) Cowley	Gregory & Gregory	-
31 16 N	077 27 E	*Roscoea alpina* Royle	Watt	7910
27 20 N	100 05 E	*Roscoea tibetica* Batalin	Rock	4709
28 30 N	097 05 E	*Roscoea bhutanica* Ngamriab.	Ludlow & Sherriff	2275
-	-	*Roscoea purpurea* Sm.	Buchanan-Hamilton	-
27 43 N	091 30 E	*Roscoea purpurea* Sm.	Ludlow, Sherriff & Hicks	20845
27 34 N	089 40 E	*Roscoea bhutanica* Ngamriab.	Cooper	3252
25 28 N	091 46 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Koelz	33255
-	-	*Roscoea purpurea* Sm.	Watt	5770
27 59 N	086 56 E	*Roscoea auriculata* K.Schum.	Wollaston	281
27 25 N	101 33 E	*Roscoea cautleoides* Gagnep.	Handel-Mazzetti	2253
27 20 N	100 05 E	*Roscoea tibetica* Batalin	Forrest	5815
23 22 N	103 24 E	*Roscoea praecox* K.Schum.	Hancock	170
-	-	*Roscoea alpina* Royle	Watt	3362
-	-	*Roscoea alpina* Royle	Watt	-
30 27 N	078 05 E	*Roscoea purpurea* Sm.	Anderson	-
29 06 N	082 54 E	*Roscoea alpina* Royle	Polunin, Sykes & Williams	2266
29 12 N	079 25 E	*Roscoea purpurea* Sm.	Duthie	24985
30 28 N	078 06 E	*Roscoea purpurea* Sm.	Huggins	2
32 23 N	077 15 E	*Roscoea alpina* Royle	Drummond	23185
25 18 N	091 42 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Clarke	17590A
29 04 N	082 21 E	*Roscoea purpurea* Sm.	Polunin, Sykes & Williams	436
29 03 N	082 44 E	*Roscoea purpurea* Sm.	Polunin, Sykes & Williams	2500
28 16 N	083 49 E	*Roscoea alpina* Royle	Barclay & Synge	2416
27 40 N	085 12 E	*Roscoea purpurea* Sm.	Codrington	238
28 39 N	083 12 E	*Roscoea alpina* Royle	Stainton, Sykes & Williams	3021
27 35 N	085 26 E	*Roscoea purpurea* Sm.	Hara	723602
27 43 N	085 19 E	*Roscoea alpina* Royle	Bailey	-
29 47 N	081 17 E	*Roscoea purpurea* Sm.	Tyson	101
31 06 N	077 10 E	*Roscoea alpina* Royle	Lace	975
28 17 N	097 10 E	*Roscoea wardii* Cowley	Kingdon-Ward	7112
-	-	*Roscoea purpurea* Sm.	Wallich	-
-	-	*Roscoea alpina* Royle	Madden	-
27 29 N	089 34 E	*Roscoea bhutanica* Ngamriab.	Cooper	2526
25 35 N	091 38 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Hooker	-
-	-	*Roscoea scillifolia* (Gagnep.) Cowley	Delavay	3283
-	-	*Roscoea brandisii* (King ex Baker) K.Schum.	Brandis	-
29 47 N	082 01 E	*Roscoea alpina* Royle	Polunin, Sykes & Williams	4340

27 23 N	088 13 E	*Roscoea auriculata* K.Schum.	Long, McBeath, Noltie & Watson	131
28 30 N	083 28 E	*Roscoea alpina* Royle	Stainton, Sykes & Williams	2836
25 40 N	100 11 E	*Roscoea tibetica* Batalin	Forrest	4807
30 27 N	078 05 E	*Roscoea alpina* Royle	Haines	2301
27 30 N	089 38 E	*Roscoea bhutanica* Ngamriab.	Cooper	1512
31 06 N	077 10 E	*Roscoea alpina* Royle	-	-
31 06 N	077 10 E	*Roscoea alpina* Royle	-	-
27 50 N	101 15 E	*Roscoea tibetica* Batalin	Gamble	4286
33 40 N	073 08 E	*Roscoea alpina* Royle	Fleming	-
24 35 N	099 54 E	*Roscoea tibetica* Batalin	Yu	16596
26 14 N	102 56 E	*Roscoea praecox* K.Schum.	McLaren	V 47 A
27 33 N	087 51 E	*Roscoea purpurea* Sm.	KEKE	258
28 11 N	085 22 E	*Roscoea capitata* Sm.	Polunin	691
25 35 N	091 38 E	*Roscoea brandisii* (King ex Baker) K.Schum.	-	-
21 14 N	093 55 E	*Roscoea australis* Cowley	Cooper	6009
26 55 N	100 10 E	*Roscoea schneideriana* (Loes.) Cowley	Schneider	1770
25 34 N	091 53 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Clarke	44607A
-	-	*Roscoea auriculata* K.Schum.	Bailey	-
27 20 N	100 05 E	*Roscoea tibetica* Batalin	Rock	4589
28 28 N	085 00 E	*Roscoea tumjensis* Cowley	Gardner	790
27 28 N	088 53 E	*Roscoea alpina* Royle	Dungboo	58
27 37 N	101 05 E	*Roscoea tibetica* Batalin	Handel-Mazzetti	2966
27 35 N	085 22 E	*Roscoea purpurea* Sm.	Bailey	-
26 55 N	100 10 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Handel-Mazzetti	4145
27 13 N	089 33 E	*Roscoea bhutanica* Ngamriab.	Cooper	1300
31 06 N	077 10 E	*Roscoea purpurea* Sm.	Gamble	4663E
28 05 N	089 41 E	*Roscoea alpina* Royle	Ludlow, Sherriff & Hicks	16439
31 06 N	077 10 E	*Roscoea alpina* Royle	Jacquemont	1024
27 39 N	091 09 E	*Roscoea purpurea* Sm.	Cooper	4182
27 09 N	100 12 E	*Roscoea tibetica* Batalin	KEYSE	470
26 55 N	100 10 E	*Roscoea schneideriana* (Loes.) Cowley	Schneider	2264
-	-	*Roscoea purpurea* Sm.	Wallich	6528A
31 06 N	077 10 E	*Roscoea purpurea* Sm.	Lace	2153
27 12 N	100 05 E	*Roscoea tibetica* Batalin	Forrest	2396
27 27 N	089 39 E	*Roscoea bhutanica* Ngamriab.	Gould	251
27 20 N	100 05 E	*Roscoea schneideriana* (Loes.) Cowley	Forrest	6407
27 22 48 N	100 05 50 E	*Roscoea tibetica* Batalin	ACE	346
25 55 N	100 30 E	*Roscoea forrestii* Cowley	McLaren	B106
28 26 N	084 55 E	*Roscoea tumjensis* Cowley	Gardner	790
27 00 N	104 56 E	*Roscoea tibetica* Batalin	Maire	235
27 20 N	100 05 E	*Roscoea cautleoides* Gagnep.	Forrest	5890
26 55 N	100 10 E	*Roscoea cautleoides* Gagnep.	Rock	11443
25 40 N	100 11 E	*Roscoea debilis* var. *debilis* Gagnep.	Forrest	6917

26 55 N	100 10 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Chamberlain, Grey-Wilson, Li Y., McBeath, Schilling Xu T. & Yuan H.	SBL 000000181
-	-	*Roscoea cautleoides* Gagnep.	Forrest	30047
25 40 N	100 11 E	*Roscoea debilis* var. *debilis* Gagnep.	Forrest	6917
27 12 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	2347
26 55 N	100 10 E	*Roscoea cautleoides* Gagnep.	Rock	5069
-	-	*Roscoea tibetica* Batalin	-	928
27 43 N	085 19 E	*Roscoea capitata* Sm.	Wallich	6529
27 28 N	088 53 E	*Roscoea alpina* Royle	Dungboo	6
27 20 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Rock	4549
27 37 55 N	100 02 16 E	*Roscoea tibetica* Batalin	ACE	236
28 42 N	082 52 E	*Roscoea nepalensis* Cowley	Stainton, Sykes & Williams	3328
27 27 N	089 39 E	*Roscoea bhutanica* Ngamriab.	Gould	912
24 47 N	103 16 E	*Roscoea debilis* var. *debilis* Gagnep.	Ducloux	1257
26 40 N	099 40 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	21527
27 40 N	101 30 E	*Roscoea cautleoides* Gagnep.	Schneider	1232
28 15 N	101 20 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Rock	23852
27 40 N	101 30 E	*Roscoea cautleoides* Gagnep.	Schneider	1200
25 20 N	098 35 E	*Roscoea debilis* var. *debilis* Gagnep.	Forrest	8456
27 45 N	099 30 E	*Roscoea schneideriana* (Loes.) Cowley	Forrest	10945
27 40 N	099 10 E	*Roscoea schneideriana* (Loes.) Cowley	Forrest	12910
27 28 N	088 53 E	*Roscoea tibetica* Batalin	Dungboo	-
27 45 N	099 30 E	*Roscoea scillifolia* (Gagnep.) Cowley	Forrest	10657
27 30 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	6092
27 50 N	099 36 E	*Roscoea tibetica* Batalin	CLD-90	483
27 09 N	100 12 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Chamberlain, Grey-Wilson, Li Y., McBeath, Schilling, Xu T. & Yuan H.	SBL 000000612
27 50 N	099 36 E	*Roscoea tibetica* Batalin	CLD-90	282
27 20 N	100 05 E	*Roscoea forrestii* Cowley	McLaren	105B
-	-	*Roscoea auriculata* K.Schum.	Hara, Kanai, Kurosawa, Murata & Togashi	6300493
27 20 N	100 05 E	*Roscoea cautleoides* Gagnep.	CLD-90	687
27 55 N	101 30 E	*Roscoea cautleoides* Gagnep.	Gamble	4355
-	-	*Roscoea cautleoides* Gagnep.	Maire	472

26 40 N	099 40 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	21527
25 18 N	091 42 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Clarke	17590B
27 09 N	100 12 E	*Roscoea cautleoides* Gagnep.	-	173
25 40 N	100 11 E	*Roscoea cautleoides* Gagnep.	McLaren	128
27 48 19 N	099 54 31 E	*Roscoea tibetica* Batalin	ACE	406b
26 55 N	100 10 E	*Roscoea cautleoides* Gagnep.	Rock	3351
25 40 N	100 11 E	*Roscoea cautleoides* Gagnep.	Forrest	7050
28 14 N	101 15 E	*Roscoea schneideriana* (Loes.) Cowley	Rock	17811
27 09 N	100 12 E	*Roscoea tibetica* Batalin	KEYSE	572
21 14 N	093 55 E	*Roscoea australis* Cowley	Kingdon-Ward	22124
27 28 N	088 53 E	*Roscoea bhutanica* Ngamriab.	Dungboo	4244
26 55 N	100 10 E	*Roscoea cautleoides* Gagnep.	Rock	3441
25 02 N	098 28 E	*Roscoea debilis* var. *debilis* Gagnep.	Howell	44
27 40 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	10239
27 33 59 N	100 01 64 E	*Roscoea humeana* Balf.f. & W.W.Sm.	ACE	250
23 22 N	103 24 E	*Roscoea debilis* var. *debilis* Gagnep.	Henry	11102C
27 20 N	100 05 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Rock	3793
27 20 N	100 05 E	*Roscoea scillifolia* (Gagnep.) Cowley	Rock	4759
26 42 N	100 45 E	*Roscoea cautleoides* Gagnep.	Gamble	3922
-	-	*Roscoea purpurea* Sm.	Wallich	6528A
27 09 N	100 12 E	*Roscoea cautleoides* Gagnep.	-	43
27 20 N	100 05 E	*Roscoea scillifolia* (Gagnep.) Cowley	Rock	4759
26 35 N	102 15 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Schneider	1192
27 00 N	104 56 E	*Roscoea schneideriana* (Loes.) Cowley	Maire	-
28 13 N	085 27 E	*Roscoea capitata* Sm.	Kanai & Shakya	671948
25 40 N	100 45 E	*Roscoea cautleoides* Gagnep.	ACE	981
27 43 N	085 19 E	*Roscoea capitata* Sm.	Wallich	6529
27 09 N	100 12 E	*Roscoea tibetica* Batalin	KEYSE	470
27 50 N	101 15 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Rock	16008
28 18 N	083 46 E	*Roscoea alpina* Royle	Stainton, Sykes & Williams	5382
-	-	*Roscoea purpurea* Sm.	Reid	-
-	-	*Roscoea alpina* Royle	Clarke	28272
25 28 N	091 46 E	*Roscoea brandisii* (King ex Baker) K.Schum.	Hooker	1452
26 55 N	100 10 E	*Roscoea cautleoides* Gagnep.	Rock	3330
27 20 N	100 05 E	*Roscoea cautleoides*	Forrest	6387

		Gagnep.		
27 28 N	087 15 E	*Roscoea purpurea* Sm.	Long, McBeath, McKean, Rae & Bhattarai	149
27 43 N	085 19 E	*Roscoea capitata* Sm.	Wallich	6529
31 06 N	077 10 E	*Roscoea purpurea* Sm.	-	-
28 53 N	082 59 E	*Roscoea alpina* Royle	Polunin, Sykes & Williams	2460
27 41 N	088 45 E	*Roscoea auriculata* K.Schum.	Cooper	378
31 11 N	077 38 E	*Roscoea alpina* Royle	Maclagan	723
28 09 N	085 24 E	*Roscoea purpurea* Sm.	Halliwell	35
27 44 N	088 33 E	*Roscoea auriculata* K.Schum.	Younghusband	-
28 10 N	085 34 E	*Roscoea purpurea* Sm.	Polunin	1949
26 14 N	102 56 E	*Roscoea praecox* K.Schum.	McLaren	V 47 A
25 42 N	100 11 E	*Roscoea tibetica* Batalin	McLaren	B67
23 22 N	103 24 E	*Roscoea debilis* var. *debilis* Gagnep.	Henry	11102
25 42 N	100 11 E	*Roscoea forrestii* Cowley	Gebauer	-
27 42 N	087 47 E	*Roscoea auriculata* K.Schum.	Stainton	1067
27 36 N	088 39 E	*Roscoea auriculata* K.Schum.	-	-
27 20 N	100 05 E	*Roscoea scillifolia* (Gagnep.) Cowley	Rock	4448
25 40 N	100 11 E	*Roscoea tibetica* Batalin	Forrest	4806
25 04 N	102 41 E	*Roscoea praecox* K.Schum.	Maire	467
25 04 N	102 41 E	*Roscoea praecox* K.Schum.	Maire	467
-	-	*Roscoea alpina* Royle	Bailey	-
27 30 N	099 45 E	*Roscoea cautleoides* Gagnep.	Gamble	237
-	-	*Roscoea alpina* Royle	Reid	-
28 05 N	085 20 E	*Roscoea capitata* Sm.	Kanai, Hara & Ohba	721776
27 40 N	085 12 E	*Roscoea purpurea* Sm.	Codrington	236
27 40 N	100 48 E	*Roscoea humeana* Balf.f. & W.W.Sm.	Forrest	21437
27 22 15 N	099 57 58 E	*Roscoea tibetica* Batalin	Alden, Alexander, Long, McBeath, Noltie & Watson	1506

APPENDIX FIVE: ADDITIONAL IDENTIFICATION KEYS OF *ROSCOEA* (CHAPTER FIVE)

1. A KEY FROM FLORA OF CHINA

(Wu and Larsen, 2000)

1a. Corolla tube 1.6-4(-5) cm; labellum usually less than 2.5 cm

2a. Plants usually less than 15 cm when mature

3a. Central lobe of corolla orbicular; labellum not reflexed *R. alpina*

3b. Central lobe of corolla oblong; labellum slightly to conspicuously reflexed

4a. Leaves obscurely to densely hairy especially when young; bracts elliptic; lateral staminodes oblong, 1-1.3 cm *R. tibetica*

4b. Leaves glabrous; bracts tubular; lateral staminodes narrowly obovate-cuneate, ca. 1.4 cm *R. kunmingensis*

2b. Plants usually more than 15 cm tall when mature

5a. Leaves appearing after anthesis, 3-6 cm wide; bracts much shorter than calyx *R. humeana*

5b. Leaves appearing before anthesis, 1-2.8 cm wide; bracts longer than calyx

6a. Lateral staminodes elliptic to obliquely obovate, 1-1.4 cm; labellum 1.3-2 x 0.8-1.2 cm, with white lines at throat *R. scillifolia*

6b. Lateral staminodes obliquely spathulate, ca. 2 cm; labellum ca. 2.5 x 1.4 cm, without white lines at throat *R. capitata*

1b. Corolla tube (3-)4-12.5 cm; labellum usually more than 2.5 cm

7a. Bracts obtuse or truncate at apex

8a. Leaves glaucous abaxially *R. wardii*

8b. Leaves not glaucous abaxially

9a. Leaves distinctly narrowed, petiolelike between sheath and blade; ligule prominent *R. debilis*

9b. Leaves not narrowed and petiolelike between sheath and blade; ligule obscure *R. forrestii*

7b. Bracts acute at apex

10a. Leaves auriculate *R. auriculata*

10b. Leaves not auriculate

11a. Leaves absent at anthesis *R. praecox*

11b. Leaves present at anthesis

12a. Leaves forming a rosette at apex of pseudostem; labellum not reflexed, lobes usually emarginate; stigma hooked *R. schneideriana*

12b. Leaves not forming a rosette at apex of pseudostem; labellum reflexed, lobes usually not emarginate; stigma not hooked *R. cautleoides*

2. A KEY BY TONG SHAO-QUAN

(Tong, 1992)

The original version is in Chinese. It was translated into English by Mr. Chun-Neng Wang, a fellow Ph.D. student from Taiwan.

1a. Leaf auriculate *R. auriculata*

1b. Leaf not auriculate

2a. Bract short, 2-4 mm, white, transparent

3a. Leaf abaxial white grey, calyx bilobed, labellum lobes with three white lines *R. wardii*

3b. Leaf abaxial green, calyx three teeth, labellum with no lines *R. tibetica*

2b. Bract long, 0.7-7 cm, not white, not transparent

4a. Leaf on top rosette, labellum no claw, appendages ball, stigma balled *R. schneideriana*

4b. Leaf not rosette, labellum claw

5a. Leaf petiolate *R. debilis*

5b. Leaf not petiolate

6a. Bract tubular

7a. Bract long, 2.6-5 cm

8a. Ligule triangular, bracts shorter than calyx, corolla tube longer than calyx ca. 1.5 cm, dorsal petal obovate *R. cautleoides*

8b. Ligule semicircular, bracts longer than calyx, corolla tube shorter than calyx, dorsal petal elliptic *R. scillifolia*

7a. Bract short, 7 mm *R. kunmingensis*

6b. Bract not tubular

9a. Dorsal petal circular *R. alpina*

9b. Dorsal petal not

10a. Inflorescence capitulate, dorsal petal 2 cm, corolla tube shorter than calyx *R. capitata*

10b. Inflorescence not, dorsal petal 3.5-4 cm, corolla tube longer than calyx

11a. Labellum as long as dorsal petal *R. praecox*

11b. Labellum not

12a. L. shorter than DP, bracts shorter than calyx, staminodes 2 times shorter than DP *R. humeana*

12b. L. longer than DP, bracts equal to longer than calyx staminodes half the length of DP *R. forrestii*