PHYLOGENY AND DISJUNCTION IN *ROSCOEA* (*ZINGIBERACEAE*)

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A phylogenetic study of *Roscoea* (*Zingiberaceae*), a high-altitude genus of an otherwise tropical 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 436bp (ITS1, 203bp; ITS2, 233bp). 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.

Keywords. Biogeography, China, cladistics, ITS, the Himalaya.

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 & Baker, 1996). It occurs along the Himalaya from the west (Kashmir), to the east (south-western China), between 1200 and 4880m (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

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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 & Katz-Downie, 1996), *Gesneriaceae* (Möller & Cronk, 1997a, 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 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.

MATERIALS AND METHODS

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. 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 1). These species represent all major areas of distribution of the genus (Fig. 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 those others from central Nepal, i.e. *Roscoea capitata* and *Roscoea ganeshensis*. On the other hand, *Roscoea forrestii* is one of the species that only occurs in south-central 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. These 15 species comprise most (83%) of the genus (total 18 species).

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

TABLE 1. Accessions of *Curcuma*, *Cautleya* and *Roscoea* examined for ITS1 and ITS2 sequence variation. [a] Number as shown in Fig. 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	Nepal, Tibet, Bhutan, India (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 brandisii* (Baker) K. Schum.	India (Meghalaya)	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* Royle	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 wardiii* Cowley	India, Tibet, Burma (Yunnan)	RBGE 1987 1608	AF192254	AF192255

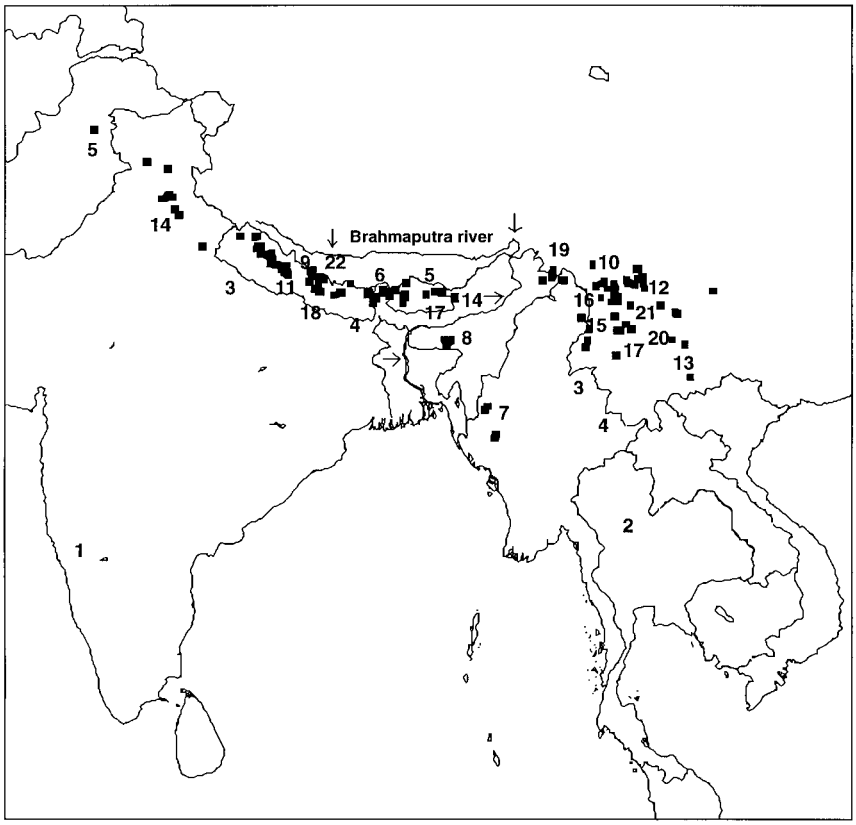

FIG. 1. Simplified geographical distribution of *Roscoea* species described to date (number 5 to 19 referring to the species listed in Table 1; number 20 = *Roscoea debilis*, number 21 = *Roscoea forrestii*, number 22 = *Roscoea nepalensis*). The position of the number is an indication of the species (note. two species, i.e. *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.

robust form of *C. gracilis* while *C. robusta* is possibly 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 outgroups 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.

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 the 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 & Doyle, 1987) as a detergent gives lower levels of enzyme inhibition than other methods (Scott & Bendich, 1994). The modified protocol is as follows.

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

The solution was mixed gently 4 or 5 times and centrifuged for 2min at 13,000rpm. The aqueous (upper) phase was removed to a clean tube and re-extracted with 200µl wet chloroform. Again this was mixed gently to obtain a momentary single phase and centrifuged for 2min at 13,000rpm. In another clean tube with the aqueous phase, 600µl cold (−20°C) propan-2-ol was added and the contents were mixed gently to precipitate the nucleic acids. After 10–15min at room temperature, the pellet of nucleic acids was precipitated by centrifuging for 2min at 13,000rpm. The supernatant was removed and 1ml of wash buffer (76% ethanol, 10mM ammonium acetate) was added. The tube was left for at least 30min to remove the 2x CTAB from the pellet. The supernatant was then aspirated as much as possible after the tube was centrifuged for 2min at 13,000rpm. Next, the pellet was dried completely by using an incubator drying oven for 10min at 50°C. Lastly the pellet was dissolved in 30–50µl of sterile distilled water to obtain DNA concentration between 10–30ng/µl and stored at −20°C until required.

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 & 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 = 50µl) contained (in order of addition) 32.5µl of sterile distilled water, 5.0µl of 10x Dynazyme™ reaction buffer (1x: 10mM Tris-HCl, pH 8.8 at 25°C, 1.5mM $MgCl_2$, 50mM KCl, 0.1% Triton X-100; Finnzymes Oy, Espoo, Finland), 1.0µl of a mix of each dNTP at 10mM (final concentration 200µM) (Sigma Chemicals, Poole, Dorset, UK), 5.0µl of each primer at 10µM (final concentration 1µM) (Oswel DNA Service, Southampton, UK), a 1.0µl aliquot of unquantified total genomic (template) DNA and 0.5µl (1U) of Dynazyme II thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland). PCR amplification of the ITS region was carried out in 0.2ml microcentrifuge tubes in a Perkin Elmer thermal cycler. Each PCR reaction cycle proceeded as follows: 1, 1min at 94°C to denature the double-stranded template DNA; 2, 2min at 55°C to anneal primers to single-stranded template DNA; and 3, 1min at 72°C to extend primers. The first cycle was preceded by an initial denaturation step of 3min 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 700bp. 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 analysed 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 20µl in volume and contained (in order of addition) 6µl of sterile distilled water, 8µl of Reaction Mix, 1µl of primer at 3.2µM and 5µl 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 & 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.

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 number AF186195-AF186213, see Table 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 was determined by inspection, and transition/transversion ratio using MacClade Version 3.0.1 (Maddison & Maddison, 1992). Sequence divergences among taxa were calculated using the DISTANCE MATRIX option in PAUP Version 3.1.1 (Swofford, 1993), based on unambiguously alignable regions (Fig. 2).

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 MulTrees 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 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 & Farris, 1969), retention index (RI: Farris, 1989), and the resulting rescaled consistency index (RC: Swofford, 1993). Additionally, the g_1 statistics (Hillis & Huelsenbeck, 1992) were obtained by calculating the tree-length distribution of 10,000 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 & Kuzoff, 1995; Susanna *et al.*, 1995; Downie & Katz-Downie, 1996). Indels were scored as a separate presence/absence character and added to the sequence data matrix (Wojciechowski *et al.*, 1993; Oxelman & Lidén, 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.

Mapping the distribution area of Roscoea

Two hundred and eighty eight 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

(a)

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                                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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FIG. 2A

(b)

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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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FIG. 2B

(c)

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                    190       200       210       220       230       240       250       260       270
alignment           .         .         .         .         .         .         .         .         .
gaps                .         .         .         .         .         .         .         .         .

Curcuma amada       GGAGATTCTTCGGAATCAAATGAATCGTCGCTTTTGCTCCATGCTTCGTCGGCATTGAGCGCGGAAGTTGGCCCCGTGTGCCCTCGGGCA [267]
Curcuma parviflora  GAAGATTCTTCGGAATCAAATGAATTGTCGCTTTATGCTTCATGCTTTGTTGGCATTGAGTGCGGAAATTGGCCCCGTGTGCCCTCGGGCA [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          GGAGATTTGTCGAAATCAAATGAATCGTCGCTTTTGCTCCATGCGTTGCTGGTGCCGAGCGCGGAAATTGGCCTCGTGTGTCCTCGACA [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]
```

FIG. 2C

(d)

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                          280        290        300        310        320        330        340        350        360
alignment                 .          .          .          .          .          .          .          .          .
gaps                      .          .          .          .          .          .          .          9          .

Curcuma amada             CAGTCGGTCGAAGAGTGGGTAGTCGGTAATCGTCGAGCACGATGGACGTTGGTCGTCGCGAGCGAGAACTGAACGTCGTGTCCTCGTCGT   [357]
Curcuma parviflora        TAGTCGGTCGAAGAGTGGGTACTCGGCAATCGTCGAGCACGATGGGCGTTGGTCGTCGCAAGCGAGAACTGAACGTCGT--CCTCGTCAT   [346]
Cautleya gracilis         CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [345]
Cautleya spicata          CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGTCGGGCACGATGGGTGTTGGTCGCCGTGAGCGAGAACAGAACGTCGT--CCCCGTCGT   [345]
R.cautleoides             CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGCCGGGCACGACGGGTGTTGGTCGCCTTGAGCGAGAACAGAACGTCGT--CCCCGTCGC   [345]
R.wardii                  CAGTCGGTTGAAGAGTGGGTAGTCCGCAGTCGCCGGGCACGACGGGTGTTGGTCGCCTTGAGCGAGAACAGAACGTCGT--CCCCGTCGC   [345]
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]
```

FIG. 2D

(e)

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                    370        380        390        400        410        420        430
alignment           11         .          .   1      .          .          .          1.1
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]
```

FIG. 2E. 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; hyphens 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.

database system by Richard Pankhurst and Martin Pullan, RBGE: www.rbge.org.uk/research/pandora.home) at RBGE. The latitude–longitude format data in PANDORA were then exported and modified for use with MapPad (a freeware program by John Keltner, NOAA Paleoclimatology Program: http://www.ngdc.noaa.gov/paleo/softlib.html. The simplified distribution map of *Roscoea* is shown in Fig. 1 (including the outgroups).

RESULTS

Sequence analysis

Alignment of internal transcribed spacer sequences of the 19 taxa analysed resulted in a 436bp long data matrix (Fig. 2); its characteristics (including G+C content) are given in Table 2. The number of unresolved bases ranged from 0 to 5bp per sequence.

The lengths of ITS1 and ITS2 were, on average, 189.7 and 225.1bp. Alignment of all taxa required the insertion of 14 gaps of 1 to 5bp length, 8 in ITS1 and 6 in ITS2

TABLE 2. 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 10,000 random trees)	–1.022	–1.663	–1.509
Average number of steps per character	0.448	0.446	0.447

of which 5 and 2, respectively, were potentially informative. The lengths of aligned ITS1 and ITS2 regions were 203 and 233bp 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 2).

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 analysed (Table 2). 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.

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 (Fig. 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 value of 0.559 calculated from 60 phylogenetic studies for 19 taxa (Sanderson & 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 = 100, 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: 1, a Chinese clade comprising eight species from China and Burma, separated by two character changes (BS = 67, DI = 2); 2, a Himalayan clade with seven species from the Himalaya, separated by four character changes (one indel) (BS = 59, DI = 1) (Fig. 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 the 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 poten-

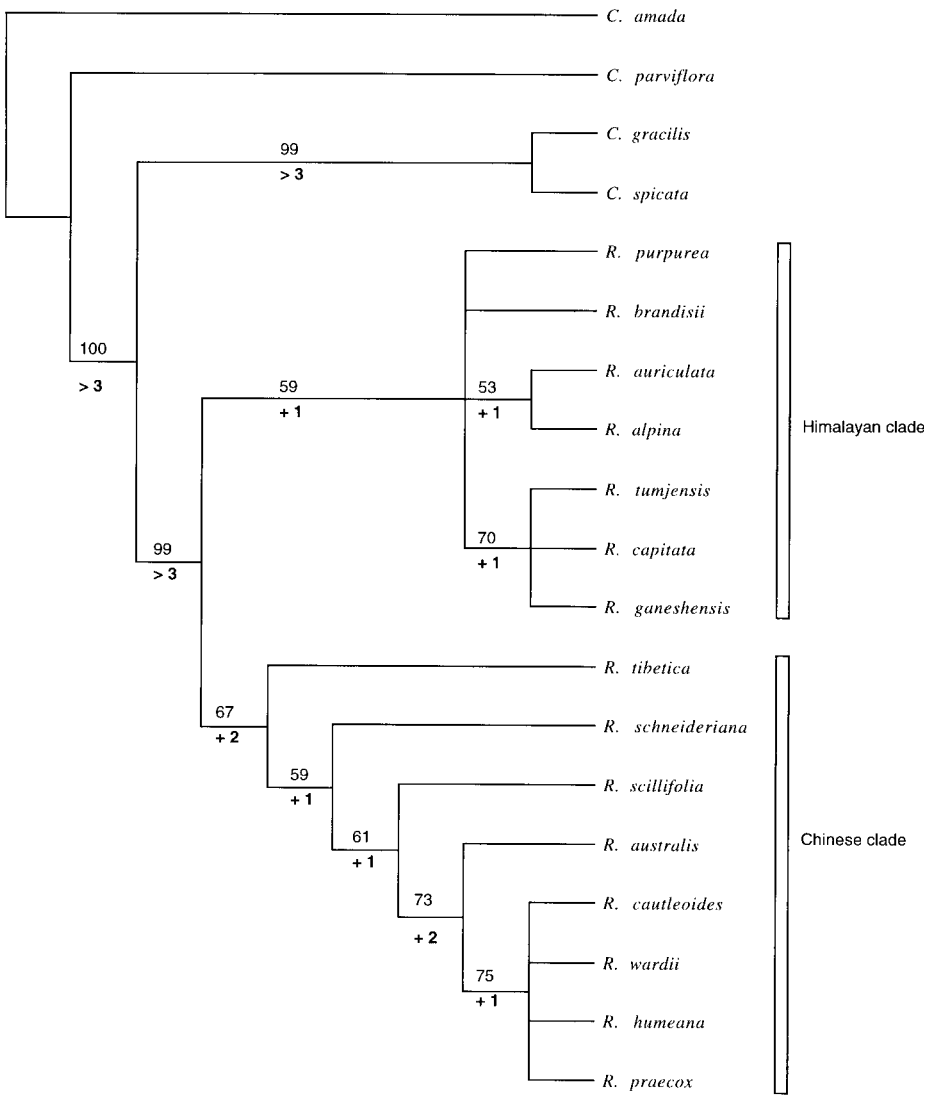

FIG. 3. Strict consensus tree based on five most parsimonious trees for 15 *Roscoea*, two *Cautleya* and two *Curcuma* taxa of 213 steps length based on parsimony analysis of the combined ITS1 and ITS2 sequence data plus the coded indels. Upper numbers are bootstrap values of 1000 replicates. Lower (boldface) numbers are decay indices (CI = 0.812; RI = 0.793; RC = 0.644).

tially 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 reanalysing the data (coded indels excluded) in a parsimony analysis gave a single most parsimonious tree (Fig. 4).

DISCUSSION

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 & 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 & 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 c.52 to 45.8Ma 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 thus are conserved to some extent (Liu & Schardl, 1994; Van Nues *et al*., 1994). It is interesting to find that speciation (as in the *Saintpaulia ionantha* complex of Möller & Cronk, 1997b and in the *Roscoea cautleoides* complex in this study) has been able to outstrip variation in the comparatively fast-evolving ITS region. Möller & 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.

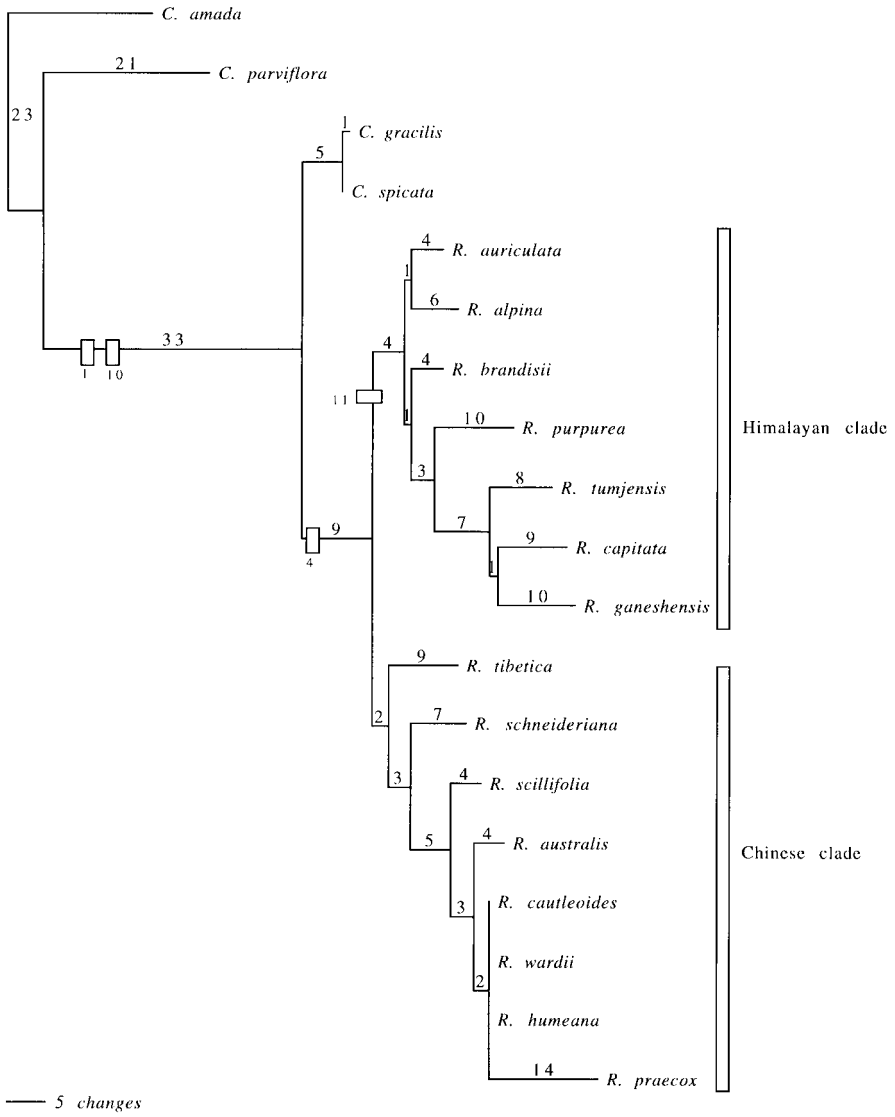

FIG. 4. The single most parsimonious tree obtained from the weighting of transitions and tranversions (one of the five trees found in the unweighted search). Numbers above branches indicate number of character changes shared amongst taxa (branch length, from unweighted analysis), including autapomorphic changes. Bars and numbers associated indicate the indels and their positions in the sequences (see Fig. 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 the other ten genera of Hedychieae (Searle & Hedderson, in press). 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 4880m and *Cautleya* spp. (Kumar, 1994; Wu & Larsen, in press) between 900 to 3100m above sea level. 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 (c.3 × 1cm), fusiform, fascicled tuber roots, whereas in *Cautleya*, the tuber roots are cylindrical. 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.

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 that it is also divided into two distinct groups. Seven species from China and one species from Burma form the first group (Chinese clade) (Fig. 3), while the rest (seven species from the Himalaya) form the second group (Himalayan clade). These two groups are supported by morphological characters as shown in Table 3. 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 south-central China, but is also recorded from high-altitude sites of 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 (including all provinces in north-eastern India; level 3 botanical region, Hollis & Brummit, 1992), as shown in Fig. 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 & 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 & Hedderson, in press). 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

TABLE 3. The distinguishing characters between the two groups of *Roscoea* spp.

Chinese group	Himalayan group
1. Sheathing leaf number 3–5	Sheathing leaf number 0–2
2. Leaf number 0–4	Leaf number >4
3. Leaf base not auriculate, except *R. australis*, *R. tibetica*	Leaf base auriculate, except *R. capitata*
4. Leaves almost forming rosette, except *R. cautleoides*	Leaves not forming rosette
5. Corolla tube length <6cm, except *R. humeana*, *R. australis*	Corolla tube length >6cm
6. Appendage tip obtuse (*R. australis*, *R. scillifolia*: obtuse-pointed, *R. schneideriana*: globular)	Appendage tip pointed
7. Epigynous gland length <5mm	Epigynous gland length >5mm, 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. cautleoides*	Ratio labellum length/dorsal petal length >1

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 occurred 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 & 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.

The Brahmaputra gap

The distribution of *Roscoea* (Fig. 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 north-eastern

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 north-eastern India, may be genuine along with other examples of Indian disjunctions (Rao, 1994), i.e *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).

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 south-eastern Tibet) and the western area (Bhutan and in nearby Tibet) (Fig. 5). There are two possible explanations for this: 1, that *R. tibetica* is a genuine transgressor which crosses the phytogeographical

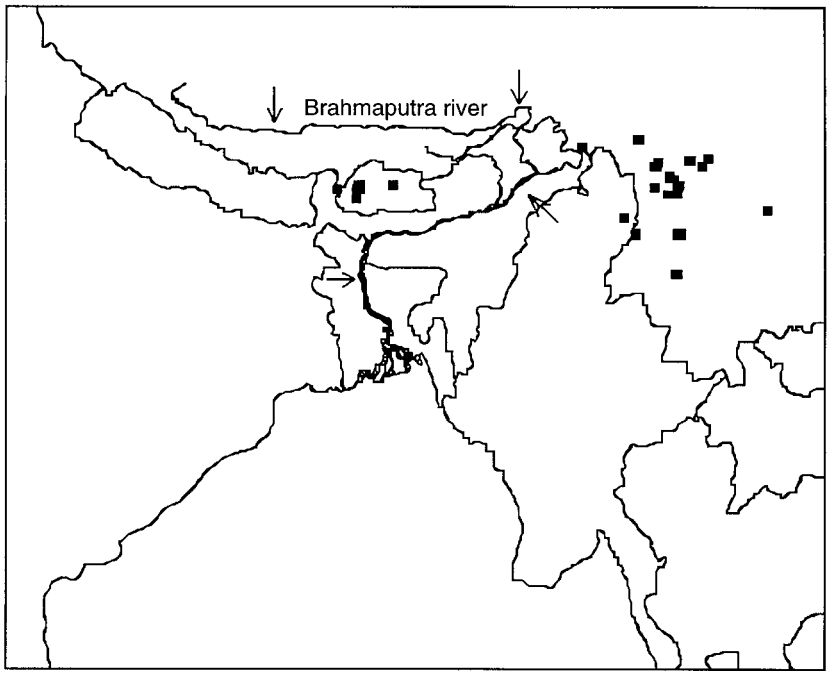

FIG. 5. Distribution map of *Roscoea tibetica* showing the discontinuity between Chinese and Bhutanese populations. Arrows show the course of the Brahmaputra river.

TABLE 4. The distinguishing characters between 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

boundary; or 2, the Bhutanese populations of *R. tibetica* represent a separate species, possibly more closely allied to Himalayan ones.

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). Further studies of Bhutanese *Roscoea tibetica* remain a priority.

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REFERENCES

ARDIYANI, M. (1997). The classification of *Curcuma* L.: a morphological and molecular study. MSc thesis, University of Edinburgh.

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

BALDWIN, B. G., SANDERSON, M. J., PORTER, J. M. *et al.* (1995). The ITS

region of nuclear ribosomal DNA: a valuable source of evidence on angiosperms phylogeny. *Ann. Missouri Bot. Gard.* 82: 247–277.
BREMER, K. (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* 42: 795–803.
COWLEY, J. (1982). A revision of *Roscoea* (Zingiberaceae). *Kew Bull.* 36: 747–777.
COWLEY, J. & BAKER, W. (1996). *Roscoea ganeshensis*. *Kew Mag.* 13: 8–13.
DONOGHUE, M. J., OLMSTEAD, R. G., SMITH, J. F. & PALMER, J. D. (1992). Phylogenetic relationships of Dipsacales based on *rbc*L sequences. *Ann. Missouri Bot. Gard.* 79: 333–345.
DOWNIE, S. R. & KATZ-DOWNIE, D. S. (1996). A molecular phylogeny of Apiaceae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *Amer. J. Bot.* 83: 234–251.
DOYLE, J. J. & DOYLE, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull. Bot. Soc. Amer.* 19: 11–15.
FARRIS, J. S. (1989). The retention index and homoplasy excess. *Syst. Zool.* 38: 406–407.
FELSENSTEIN, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.
HILLIS, D. M. & HUELSENBECK, J. P. (1992). Signal, noise, and reliability in molecular phylogenetic analysis. *J. Heredity* 83: 189–195.
HOLLIS, S. & BRUMMITT, R. K. (1992). *World Geographical Scheme for Recording Plant Distribution. Plant Taxonomic Database Standards no. 2, version 1.0*. Pittsburgh: Carnegie Mellon University.
JAIN, S. K. & PRAKASH, V. (1995). Zingiberaceae in India: Phytogeography and Endemism. *Rheedea* 5: 154–169.
KIM, S. C., CRAWFORD, D. J., FRANCISCO ORTEGA, J. & SANTOS GUERRA, A. (1996). A common origin for woody *Sonchus* and five related genera in the Macaronesian islands-molecular evidence for extensive radiation. *Proc. Natl. Acad. Sci. USA* 93: 7743–7748.
KLUGE, A. G. & FARRIS, J. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst. Zool.* 18: 1–32.
KUMAR, S. (1994). The genus *Cautleya* Royle (Zingiberaceae) in India. *J. Indian Bot. Soc.* 73: 195–197.
LARSEN, K. (1980). Annotated key to the genera of Zingiberaceae of Thailand. *Nat. Hist. Bull. Siam Soc*. 28: 151–169.
LARSEN, K., LOCK, J. M., MAAS, H. & MAAS, P. J. M. (1998). Zingiberaceae. In: KUBITZKI, K. (ed.) *Families and genera of vascular plants*, Vol. IV, pp. 474–495. Berlin: Springer.
LIU, J.-S. & SCHARDL, C. L. (1994). A conserved sequence in internal transcribed spacer 1 of plant nuclear rDNA genes. *Plant Molec. Biol.* 26: 775–778.
MADDISON, W. P. & MADDISON, D. R. (1992). *MacClade*, version 3.01. Sunderland, MA: Sinauer.
MÖLLER, M. & CRONK, Q. C. B. (1997a). Origin and relationship of *Saintpaulia* (Gesneriaceae) based on ribosomal DNA internal transcribed spacers (ITS) sequences. *Amer. J. Bot*. 84: 956–965.
MÖLLER, M. & CRONK, Q. C. B. (1997b). Phylogeny and disjunct distribution: evolution of *Saintpaulia* (Gesneriaceae). *Proc. Roy. Soc. London* 264: 1827–1836.
OXELMAN, B. & LIDÉN, M. (1995). Generic boundaries in the tribe Sileneae (Caryophyllaceae) as inferred from nuclear rDNA sequences. *Taxon* 44: 525–542.
RANGSIRUJI, A. (1999). A study of the infrageneric classification of *Alpinia* Roxb. (Zingiberaceae) using molecular data. PhD thesis, University of Edinburgh.

RAO, R. R. (1994). *Biodiversity in India (Floristic Aspects)*. Dehra Dun, India: Bishen Singh Mahendra Pal Singh.

ROWLEY, D. B. (1998). Minimum age of initiation of collision between India and Asia north of Everest based on the subsidence history of the Zhepure Mountain section. *J. Geol.* 106: 229–235.

SANDERSON, M. J. & DONOGHUE, M. J. (1989). Patterns of variation in levels of homoplasy. *Evolution* 43: 1781–1795.

SCOTT, O. R. & BENDICH, A. J. (1994). Extraction of total cellular DNA from plants algae and fungi. In: *Plant Molecular Biology Manual*, D1: pp. 1–8. Belgium: Kluwer Academic Publishers.

SEARLE, R. J. & HEDDERSON, T. A. (in press). A preliminary phylogeny of the *Hedychieae* (Zingiberaceae) based on internal transcribed spacer sequences of the nuclear-encoded rRNA cistron. In: WILSON, K. L. & MORRISON, D. A. (eds) *Systematics and Evolution of Monocots, Proceesdings of the Second International Conference on the Comparative Biology of the Monocots, Sydney, September 1998*. Melbourne: CSIRO.

SMITH, R. M. (1994). Zingiberaceae. In: NOLTIE, H. J. (ed.) *Flora of Bhutan*, Vol. 3, Part 1, pp. 182–209. Edinburgh: Royal Botanic Garden Edinburgh.

SMITINAND, T., SHIMIZU, T., KOYAMA, H. & FUKUOKA, N. (1970). Contributions to the Flora of Southeast Asia, I. Taxonomy and phytogeography of some temperate species in Thailand. *South E. Asian Stud.* 8: 171–186.

SOLTIS, D. E. & KUZOFF, R. K. (1995). Discordance between nuclear and chloroplast phylogenies in the *Heuchera* group (Saxifragaceae). *Evolution* 49: 727–742.

SPEARING, J. K. (1977). A note on closed leaf-sheaths in Zingiberaceae-Zingiberoideae. *Notes Roy. Bot. Gard. Edinburgh* 35: 217–220.

SUSANNA, A., JACAS, N. G., SOLTIS, D. E. & SOLTIS, P. S. (1995). Phylogenetic relationships in tribe Cardueae (Asteraceae) based on ITS sequences. *Amer. J. Bot.* 82: 1056–1068.

SWOFFORD, D. L. (1993). *PAUP: phylogenetic analysis using parsimony, version 3.1.1.* Champaign, IL: Illinois Natural History Survey.

VAN NUES, R. W., RIENTES, J. M. J., VAN DE SANDE, C. A. F. M. *et al.* (1994). Separate structural elements within internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S ribosomal RNA. *Nucl. Acids Res.* 22: 912–919.

WEN, J., SHI, S., JANSEN, R. K. & ZIMMER, E. A. (1998). Phylogeny and biogeography of *Aralia* sect. *Aralia* (Araliaceae). *Amer. J. Bot.* 85: 866–875.

WOJCIECHOWSKI, M. F., SANDERSON, M. J., BALDWIN, B. G. & DONOGHUE, M. J. (1993). Monophyly of aneuploid *Astragalus* (Fabaceae): evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *Amer. J. Bot.* 80: 711–722.

WU, T.-L. & LARSEN, K. (in press). Zingiberaceae. In: *Flora of China*, Vol. XXIV.

YOKOTA, Y., KAWATA, T., IIDA, Y., KATO, A. & TANIFUJI, S. (1989). Nucleotide sequences of the 5.8S rDNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. *J. Molec. Evol.* 29: 294–301.

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