A Chromosomal Study of *Roscoea* and *Cautleya* (Zingiberaceae): phylogenetic implications

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ABSTRACT

Chromosome counts of *Roscoea alpina* Royle, *R. auriculata* K. Schum., *R. purpurea* Sm. and *Cautleya spicata* (Sm.) Baker are presented. Two species: *R. auriculata*, *R. purpurea* confirm the widely reported number of 2n = 24. However, both *R. alpina* and *C. spicata* have chromosome number of 2n = 26. *R. auriculata*'s chromosome number is a new count. Chromosome structures of the species studied are metacentric. The chromosome numbers are discussed in light of the phylogeny of the genera.

Key words: Chromosome number - *Roscoea alpina* - *R. auriculata* - *R. purpurea* - *Cautleya spicata* - Phylogeny

INTRODUCTION

Roscoea is a small genus, distributed mainly in temperate regions, with nineteen species in the tropical plant family, Zingiberaceae (1-3). It is found along the Himalaya ridges, from Pakistan in the west to Southwest China in the east. Molecular phylogenetic studies of the tribe Zingibereae (formerly Hedychieae) (4,5) and the genus *Roscoea* (6) revealed that *Cautleya* is a sister group to *Roscoea* and the two genera are monophyletic. There are two species in *Cautleya*, namely *C. gracilis* (Sm.) Dandy and *C. spicata* (5). *Cautleya* can be found at higher elevations than *Roscoea* along the Himalayas. One species, *Cautleya gracilis* is found in Northern Thailand.

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 in the genus was by Sharma and Bhattacharyya (7) on *R. alpina*, a wide spread species along the Himalayas (6). The diploid number of the species was reported to be twenty-four. All the counts, up to the present are summarised in **Table 1**. In all, eight taxa (42%), eighteen lineages are reported. While most of the counts report 2n = 24, the only species *R. purpurea* (five lineages) from the Himalayas is found to have 2n = 26, besides one lineage of 2n = 24 **(Table 1)**. An incident of polyploidy, 2n = 48, was also observed in an unidentified *Roscoea* species with no locality record (8). All the counts in *Cautleya* are presented in **Table 2**. *Cautleya gracilis* has n = 12, 13 while *Cautleya spicata* has n = 13.

A recent study by West & Cowley (9) reported that all four Chinese *Roscoea* species: *R. cautleoides* Gagnep., *R. debilis* Gagnep., *R. schneideriana* (Loes.) Cowley and *R. tibetica* Batalin (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. Most reports agreed that the chromosome number for *R. purpurea* is 2n = 26. However, one publication (43) indicated that it is 2n = 24. Thus one of the aims of this article is to determine if this result is genuine or the result of some error. To find out, the chromosome counts of three Himalayan *Roscoea* species: *R. alpina*, *R. auriculata* and *R. purpurea* have been carried out. In addition, *Cautleya spicata* was also chosen to confirm its reported chromosome number. 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 phylogeny of *Roscoea* and *Cautleya*.

Table 1. A summary of previously reported chromosome counts in *Roscoea*.

Species	Place of origin	Number of chromosomes		Author (s)
1. *Roscoea cautleoides* Gagnep.	China (Sichuan, Yunnan)	2n = 24	-	(8,9,35)
2. *Roscoea debilis* Gagnep.	China (Yunnan)	2n = 24	-	(9)
3. *Roscoea humeana* Balf. f. & W. W. Sm.	China (Sichuan, Yunnan)	2n = 24	-	(8,34)
4. *Roscoea schneideriana* (Loes.) Cowley	China (Sichuan, Yunnan)	2n = 24	-	(9)
5. *Roscoea tibetica* Batalin	Tibet, Bhutan, Myanmar and China (Sichuan, Yunnan)	2n = 24	-	(9,36)
6. *Roscoea alpina* Royle	India, Nepal, Bhutan and China (Xizang)	2n = 24	n = 12	(7,8,42)
7. *Roscoea purpurea* Sm. (syn. *Roscoea procera* Wall.)	India, Nepal and Bhutan	2n = 24	-	(43)
8. *Roscoea purpurea* Sm. (syn. *Roscoea procera* Wall.)	India, Nepal and Bhutan	2n = 26	n = 13	(8,26,29, 30,44)
9. *Roscoea* species	-	2n = 48	-	(8)

Table 2. A summary of previously 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, Nepal, Myanmar and N. Thailand	n = 12	(26)
Cautleya gracilis (Sm.) Dandy (syn. *Cautleya lutea* Royle)	China (Sichuan, Yunnan, Xizang), India, Nepal, Myanmar and N. Thailand	n = 13	(26)
Cautleya spicata (Sm.) Baker	China (Guizhou, Sichuan, Yunnan, Xizang), India and Nepal	n = 13	(26,29, 30)

MATERIALS AND METHODS

Collection and Storage of Root Tips

Root tips of three species of *Roscoea* from the Himalayas, namely *Roscoea alpina*, *R. auriculata* and *R. purpurea* and *Cautleya spicata* were taken from the Royal Botanic Gardens Edinburgh around midday (11.30-12.30 p.m.) (see **Table 3** for plants in this study). This time range has been found to give high numbers of cells of metaphase in Zingiberaceae (10,11). The root tips were then washed with tap water a few times and once with distilled water.

Table 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

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 (12). The root tips were treated in either 1-bromonapthalene (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 good metaphases rather than the dyes used in the staining stage (13). 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 (12) 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.

Hydrolysis and staining

The cell wall was softened using an acid to make the cells easier to squash. The acid used in this study was 5N HCl and the hydrolysis performed for 30 minutes. Additional softening with enzymes can be employed in the later step depending on the schedule used (14). In this study, 4% cellulase and pectinase are used after the staining stage at 60°C for 30 minutes. Feulgen which is a dye made mainly from pararosaniline, is used to stain the chromosomes. The dye is light sensitive and thus the staining is carried out in dark room, for 3 hours. DNA is stained a deep magenta colour while the other cell components remain unstained.

Slide Preparation, Squash and Observation

The root tips were cut off into small pieces and placed on a clean slide, then macerated with 2% acetic-orcein or 2% aceto-carmine using a brass-tapping rod. A number 1 coverslip was placed on the sample and the sample was warmed over a flame. Squashing was done by pressing the slides firmly and suddenly between sheets of blotting paper or filter paper. The edges of the coverslip were sealed immediately with a rubber solution. The material may be heated again over the lamp to increase the intensity of the stain. Slides were observed under a light microscope and made permanent by the quick freeze method using liquid nitrogen (14,15). A block of aluminium was immersed in liquid nitrogen for equilibrating the temperature of the aluminium to that of the liquid nitrogen. The aluminium block 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 both the slide and coverslip were 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 few days.

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* **(Table 3)**. The chromosomes are mainly metacentric, with occasional submetacentrics **(Figure 1-14)**. The size of the chromosomes ranges between 1-2 μm. The pre-treatment of the root tips by MBN is noticed to gives a slightly higher percentage of metaphase cells in the plants studied than OQ. Feulgen stain gives well-stained chromosomes in this study.

DISCUSSION

Timing of root tip collection

There are two times of day which are the best to collect root tips. West and Cowley (9) 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 the Zingiberaceae (10,11) and other families (14). Midday is known to be at a 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 (14). Nonetheless, the midmorning period, 9-10 am, is preferred by Chen (16) and Augsonkitt *et al*. (17). Lim (10) collected root tips at midday for mitotic studies, but at 9-11 a.m. for meiotic studies of flower buds. No systematic study on the relationship of the two periods and the metaphases of root tips of *Roscoea* species is conducted in this study.

Pre-treatment and staining

Literature review shows that researchers in cytotaxonomic studies of Zingiberaceae have used various pre-treatment and staining chemicals. Chen (16) stated that 1-bromonapthalene (MBN) and Paradichlorobenzene (PDB) are better in treating the material of Zingiberaceae plants than other chemicals. West and Cowley (9) 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 researchers of *Curcuma* species (Ardiyani, pers. comm.; Nasir, pers. comm.). Papers on cytological studies of *Zingiber officinale* Roscoe (18,19) and *Curcuma* species (20) show that OQ and PDB are preferred for the pre-treatment. PDB is preferred by Thai researchers in the studies of Zingiberaceae's chromosome (17,21,22). In addition, there is one report of colchicine as the pre-treatment chemical in the study of *Zingiber officinale* (23).

Feulgen has proved so far to be effective in staining the chromosomes of Zingiberaceae. Examples are Lim (10), Newman (11), and West and Cowley (9). Chen (16) also used and recommended a derivative of basic fuchsin, carbolo fuchsin for its convenience and reliability. In other plant families, Jong (24) 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 (14). However, the state of the root tips is observed to be far more important than the stain (13). The healthy state of root tips collected for the study is the main reason for the well-stained chromosomes observed (13). Other dyes, such as Heamatoxyline are found to stain components of the cell as well as the chromosomes in *Curcuma* species, thus will not yield well distinct-coloured chromosomes from the background (Ardiyani, pers. comm.).

The chromosome number

The chromosome number of individuals is sometimes found to be different to the number of species because of factors, such as chromosome fission and misdivision of the paired chromosomes at meiosis. An example is *Crepis tectorum* L. (2n = 8) where in 4000 plants, 10 plants, 4 plants and 4 plants have 2n = 9, 10 and 11, respectively (25). 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. Thus meiotic studies of the species are needed before any statement can be confirmed. Change this, it doesn't make sense. The pairing of the homologous chromosomes during meiosis will allow hs to readily differentiate between 2n = 24 and 2n = 26. It is noticed that n = 12 and n = 13 populations of *Cautleya gracilis* show correlations with some vegetative and floral characters (26). Plants with n = 13 are shorter, possess small-sized leaves and bracts and have lesser 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, 2,250-2,500 m, in comparison to those with n = 12 that occur between 2,000-2,200 m. Flower colour in *Roscoea* populations has not been found to correlate with the chromosome information (9).

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 size is ≤ 2 μm and large size is ≥ 10 μm (27). Most are metacentric in shape with submetacentrics and occasional subacrocentrics (13). West and Cowley (9) found that all chromosomes in *Roscoea* are uniform in shape i.e. metacentric and the total length of the chromosomes range between 1-2 μm. Chromosome sizes of *Roscoea* species in this study are 1-2 μm and conform to that found by West and Cowley (9). 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 Zingiberaceae (28). The smallest chromosomes found in Zingiberaceae are those of *Curcuma*, a genus with the highest basic chromosome number of x = 21 **(Figure 15)**. Chromosome sizes in *Curcuma* range between 0.24-0.99 μm in six species studied by Joseph *et al* (20).

The chromosome and the Phylogeny of the genera

An incident of the chromosome number 2n = 34 of *Cautleya spicata* (7) has had quite an impact on cytotaxonomic interpretation of the family Zingiberaceae as a whole. Apart from three other reports (26,29,30) that all recorded n = 13 for *Cautleya spicata*, Chen (31) 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 have published a series of chromosome counts of Zingiberaceae in six papers (32-37), *Cautleya spicata* was not one of the species counted. Mehra & Sachdeva (26) pointed out that the *Cautleya spicata* count of Sharma & Bhattacharyya (7) 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 (4,5,38,39) have shown that *Cautleya* is a

sister group to *Roscoea* and the basic chromosome number of the clade is x = 12 and 13 **(Figure 15)**. In light of the molecular phylogenetic findings, it also suggests that the basic chromosome number x = 17 appears only in *Hedychium* in the family (33,40) which is confirmed to be a monophyletic group within the tribe (38).

The sister clade of *Roscoea/Cautleya* is a clade of *Pommereschea* and *Rhynchanthus* (5,38,39) **(Figure 15)**. The basic chromosome number of *Pommereschea* is x = 11 or 2n = 22 (41), while that of *Rhynchanthus* is x = 22, 2n = 44 (35). This suggests that the basic chromosome number of the clade of *Pommereschea/Rhynchanthus* is x = 11. In *Roscoea/Cautleya* clade, the basic chromosome number is x = 12 and 13 (see **Tables 1-3**). Within the context of Zingiberaceae 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 addition of chromosomes in the clade of *Roscoea/Cautleya*. The pollen character seems to support the relationships among the genera in particular the type of spine on the surface of the pollen. Pollen in *Pommereschea* and *Rhynchanthus* are spineless whereas pollen of *Roscoea* and *Cautleya* are long-spined (31).

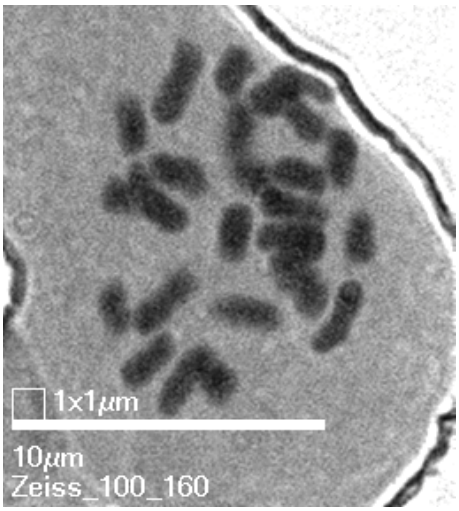

Figure 1. *Roscoea purpurea.*

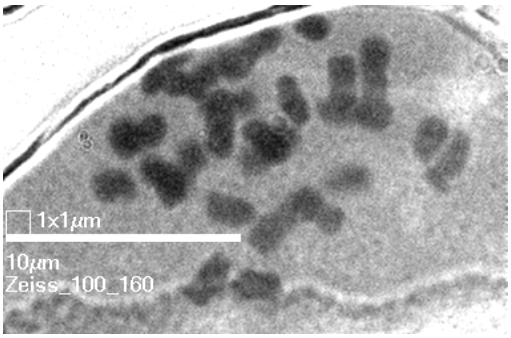

Figure 2. *R. purpurea.*

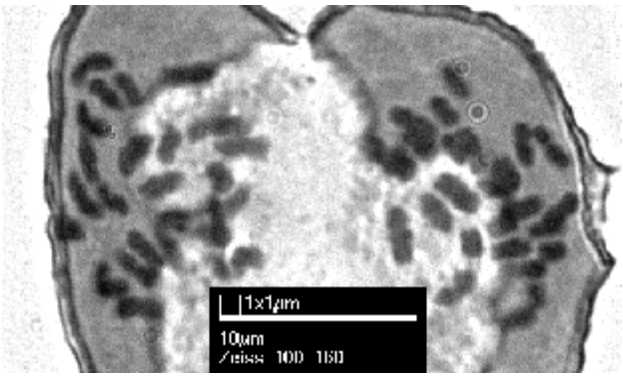

Figure 3. *R. purpurea.*

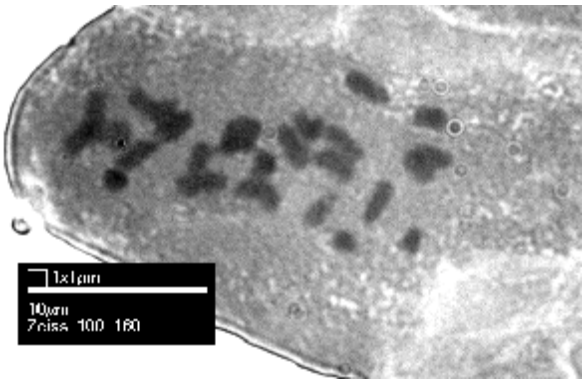

Figure 4. *R. purpurea.*

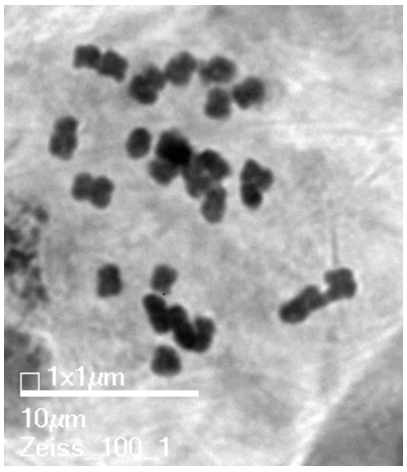

Figure 5. *Roscoea alpina.*

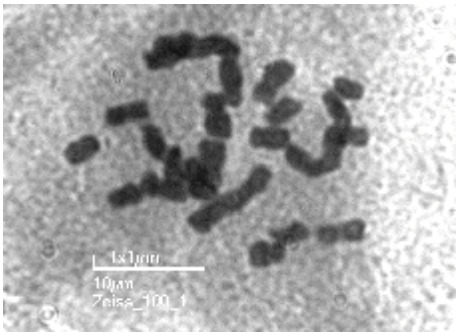

Figure 6. *R. alpina.*

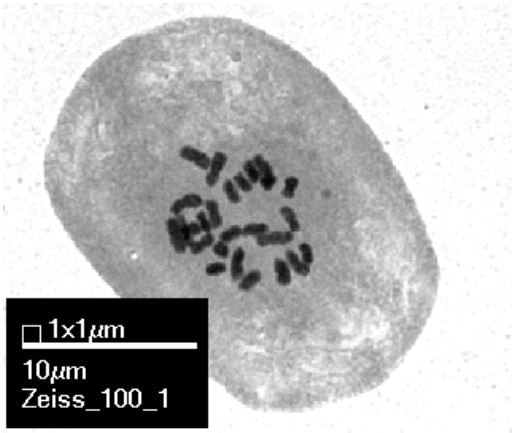

Figure 7. *R. alpina.*

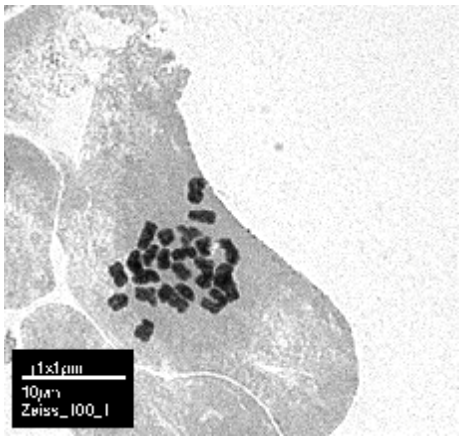

Figure 8. *R. alpina.*

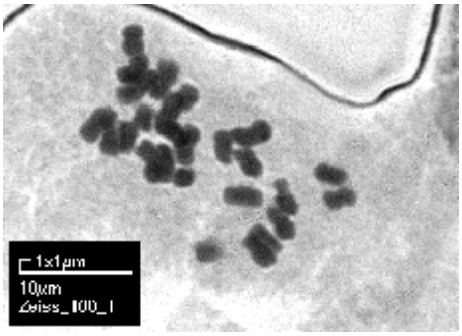

Figure 9. *R. auriculata.*

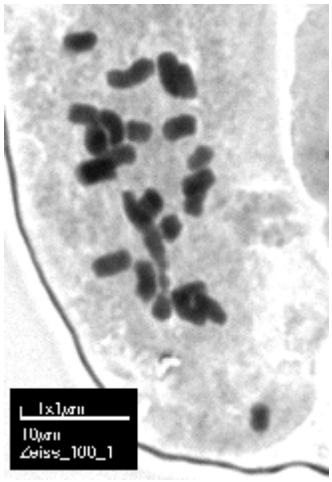

Figure 10. *R. auriculata.*

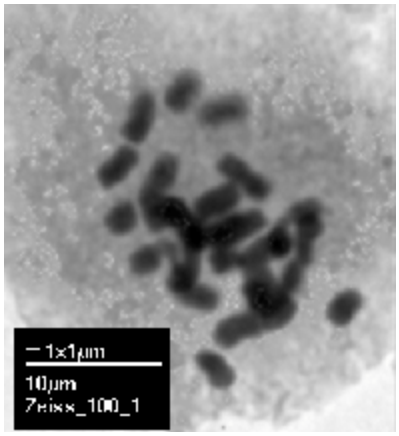

Figure 11. *R. auriculata.*

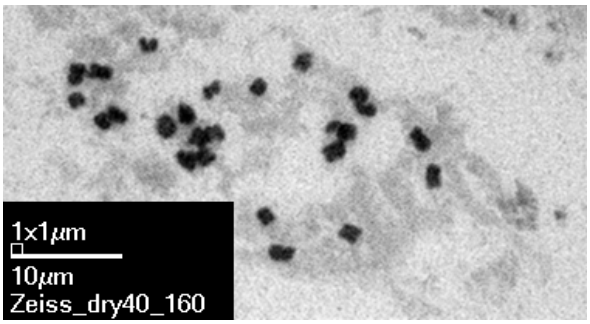

Figure 12. *R. auriculata.*

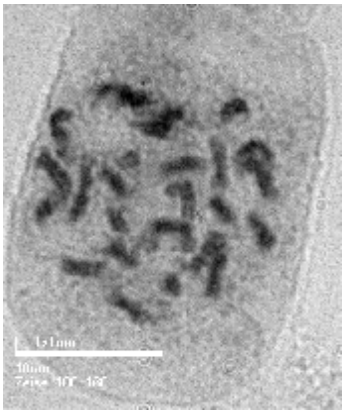

Figure 13. *Cautleya spicata.*

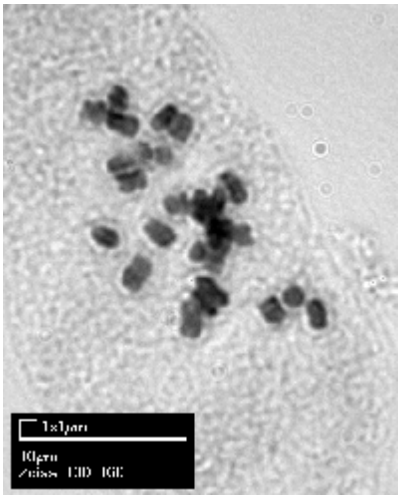

Figure 14. *C. spicata.*

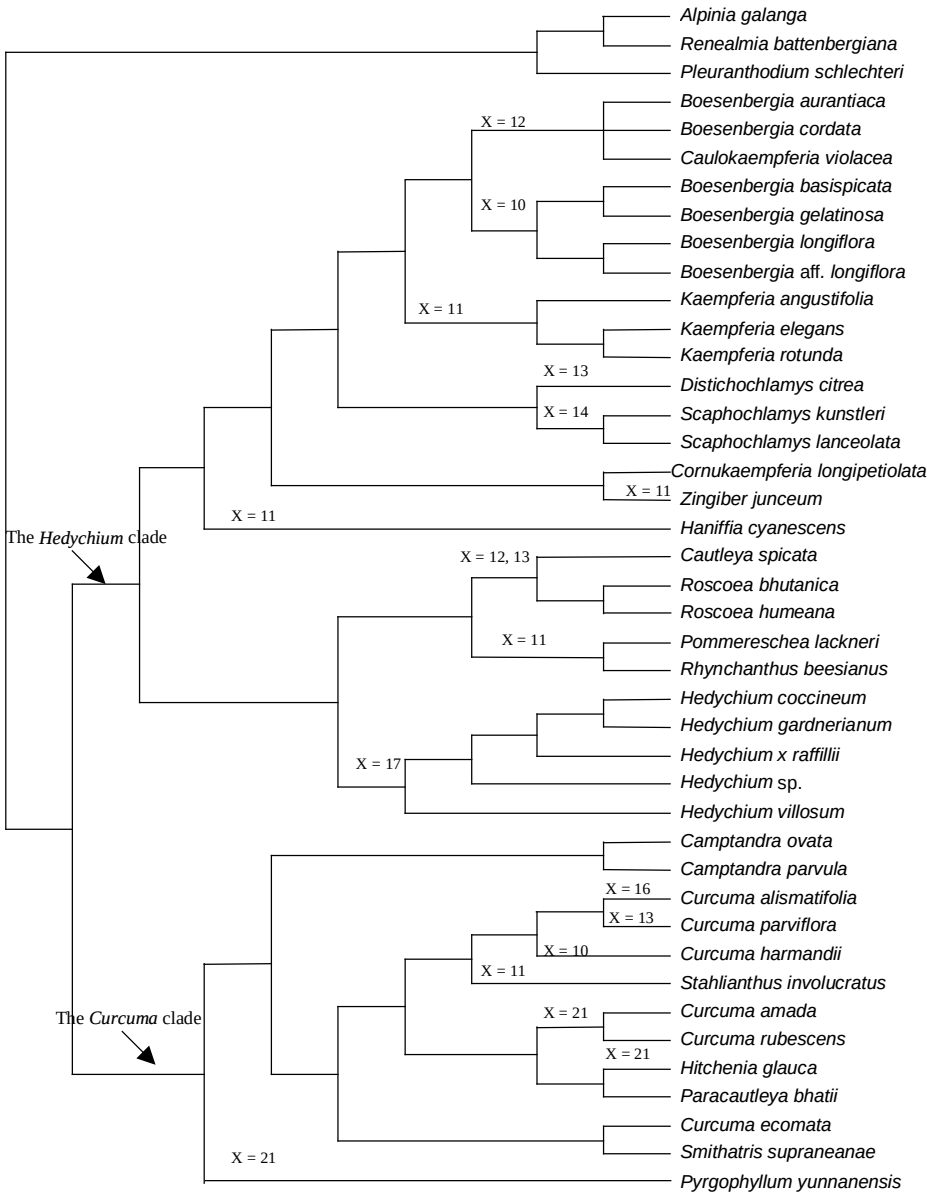

Figure 15. The strict consensus tree of two equally optimal trees resulting from the maximum likelihood analysis of 42 taxa ITS data set (ln-likelihood = 5551.712) (5). The basic chromosome numbers shown are representative, i.e. not known for all species in the tree.

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บทคัดย่อ

ฉัตรชัย งามเรียบสกุล

การศึกษาโครโมโซมของพืชสกุล *Roscea* และ *Cautleya (Zingiberaceae)*: นัยทางประวัติวิวัฒนาการ

เสนอจำนวนโครโมโซมของ *Roscoea alpina* Royle, *R. auriculata* K.Schum., *R. purpurea* Sm. และ *Cautleya spicata* (Sm.) Baker การศึกษาครั้งนี้พบว่า *R. auriculata* และ *R. purpurea* มีค่า 2n = 24 ซึ่งเป็นค่าที่ตรงกับการศึกษาก่อนนี้ แต่พบว่า 2n = 26 ใน *R. alpina* และ *C. spicata* จำนวนโครโมโซมของ *R. auriculata* เป็นค่าที่ไม่มีการรายงานมาก่อน โครงสร้างของโครโมโซมที่ศึกษาเป็นแบบ metacentric วิจารณ์ความสัมพันธ์ระหว่างจำนวนโครโมโซมและประวัติวิวัฒนาการของทั้งสองสกุล

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