

## Karyotypic studies on *Campanumoea* (Campanulaceae)—endemic to China

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**Abstract** Radix Campanumoeae (*C. javanica* Bl.) has been used in Miao herbal medicine to treat neurasthenia and consumptive disease for hundreds of years. Though Radix Campanumoeae shows great potential for utilization in medical studies, this herb crop has not been cultivated industrially in China. Many species in the *Campanumoea* genus are similar in phenotype; therefore, a karyotypic study would prove useful for clearly distinguishing Radix Campanumoeae from related species within the same genus, for germplasm preservation and for breeding Radix Campanumoeae. 10 accessions of four species in this

genus and 5 accessions in 5 relative genera from SCSB were used for karyotype determination. The results showed a karyotype of  $2n = 16 = 2m + 12sm + 1st$  in *Campanumoea*, and a karyotype of  $2n = 18 = 6m + 12sm$  in *Cyclocodon*. Based on the chromosome number and the karyotypic formula, we suggest that the *Campanumoea* genus can be divided into two genera, which is in agreement with results observed from pollen morphology and from homological usage in Chinese herbal medicine. The chromosome length in *C. javanica* subsp. *javanica* and *C. javanica* subsp. *japonica* Makino, ranged from 2.24 to 1.38  $\mu\text{m}$  and 2.04 to 1.31  $\mu\text{m}$ , respectively; and their haploid sets were almost identical (13.15 and 13.16  $\mu\text{m}$ , respectively). This indicates that chromosomal rearrangements occur within chromosomes IV, V and VII without a net gain or loss of genetic material.

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### Introduction

Radix Campanumoeae [*Campanumoea javanica* (Blume) Hook. f.], commonly referred to as TU DANG SHEN in Chinese, belongs to the *Campanumoea* Blume genus of the Campanulaceae family (Wang 2002). The root of Radix Campanumoeae has been

used to nourish one's vitality and as a galactopoietic and haemostatic agent in Chinese traditional medicine (CTM) for hundreds of years (Wang 2002). In Miao herbal medicine, it has been used to treat consumptive disease, internal injury, child nocturia, neurasthenia, and to relieve a cough et al. (Wang 2002). Because Radix Campanumoeae herbs are used as alternatives to ginseng (*Panax ginseng* C. A. Meyer) in several areas of China (they both have a similar active function), they are commonly referred to as counterfeit ginseng in CTM (Dharmananda 2002).

There has been increasing interest in Radix Campanumoeae herbs because of their potential to treat neurasthenia and consumptive disease (Zhang et al. 2005; Li et al. 2008). Recently, Sun et al. reported that Radix Campanumoeae herbs contain lobetyolin (with a content of 0.686–4.035 mg g<sup>-1</sup> (Sun et al. 2009), a substance with protective functions against gastric mucosal injury caused by ethanol (Huo et al. 2005). According to reports from the Chinese health department, nearly 30% of people have various forms of gastritis in China, and 40% of these patients are in serious condition (Hou et al. 2010). Radix Campanumoeae herbs are therefore highly invaluable to pharmacology research for gastric ulcers and other diseases. Additionally, this herb has been used to treat liver and kidney impairment, cold and dampness, osteoporosis, lassitude in loin and legs caused hyperosteogeny et al. (Wang 2002). Although, Radix Campanumoeae had been used for hundreds years within the Miao community, industrial cultivation has yet to be carried out in China. For a long time, this species has been neglected in research, extension and breeding programs, and scientific interest has only emerged in the last decade. Radix Campanumoeae can therefore be considered as a neglected and underutilized crop (Khoshbakht et al. 2007).

Sun and He studied 11 species belonging to *Codonopsis* Wall. ex Roxb. and *Campanumoea* Bl. according to their difference in seed macromorphology and seed coat micromorphology. The results indicate that *Codonopsis micrantha* Chipp and *Campanumoea javanica* Blume, *Campanumoea javanica* Blume. subsp. *japonica* (Makino) Hong are very similar in these characteristics (Sun et al. 2009). Furthermore, many species within the genus are quite similar when only considering morphological and phenotypic characteristics (Hong 1983). As a nearly cosmopolitan angiosperm family, the species of the

*Campanulaceae* family appear to be prone to considerable phenotypic plasticity (Eddie 1997; Eddie and Ingrouille 1999) as well as ontogenetic variation, and this has led to the addition of superfluous species names to the existing literature. For Radix Campanumoeae alone, there are 16 Chinese appellations in various herb directories.

Karyotypic changes involving chromosome structure, such as inversions, translocations and deletions, and chromosome number by aneuploidy/diploidy or polyploidy play an important role in plant evolution and speciation (Stebbins 1971; Lim et al. 2000; Levin 2002; Lysak et al. 2006; Schubert 2007). Karyotype analysis is a useful method for characterizing a plant chromosome. The structure of the chromosome is of vital importance when studying the origin, the evolution and the classification of a particular plant (Yang et al. 2005). Until now, karyotype information about *Campanumoea* Bl. was absent, simply stating  $x = 8$  and  $x = 9$  for *Campanumoea* and *Cyclocodon*, respectively in some reports (Hong 1995). Karotype study in *Campanumoea*, may prove useful in understanding its genetics, relationship with other species within the same genus and researching new substitute plants with similar medical function. Moreover, though there is a great diversity between *Campanumoea* and *Cyclocodon* in morphological characteristics, (particularly in pollen characteristics), many reports classified them into a single genus (Morris and Lammers 1997; Mutthy 1982). Karyotype study could provide utile information to differentiate the two sections of *Campanumoea*, as well as provide further insight into germplasm preservation and breeding in Radix Campanumoeae.

## Materials and methods

Karyotypic analysis was carried out on 10 accessions of *Campanumoea* and 5 accessions from relative genus collections from the Chinese South-West Wild Germplasm Source Bank, Kunming Institute of Botany, Chinese Academic of Science (Kunming, Yunnan) (Table 1).

Seeds were kept at 25°C during germination. Seeds were then soaked with deionized (DI) water in beakers for 15 min before being placed in Petri dishes on two layers of Whatman no. 1 filter paper wetted with DI water. When the seed radicles reached 1–5-mm, we treated the germination seeds with 0.003 M

**Table 1** Seed sources and origins used in experiment

Species	Accession no.	Time of collected	Seed origin	Altitude (m)	Environment
<i>Campanumoea parviflora</i> (Wall.) Benth.	SCSB-B-000566	2007-11-10	Lúchun, Yunnan	1,823	At the edge of monsoon evergreen broad leaved forest
<i>Campanumoea lancifolia</i> (Roxb.) Merr.	SCSB-B-000612	2007-12-9	Malipo, Yunnan	1,219	Among evergreen broad leaved forest
<i>Campanumoea lancifolia</i> (Roxb.) Merr.	SCSB-B-000618	2007-12-10	Maguan, Yunnan	1,553	At roadside of hillside
<i>Campanumoea javanica</i> Bl. subsp. <i>javanica</i>	SCSB-B-000589	2007-11-13	Jingpin, Yunnan	2,183	At the edge of evergreen broad leaved forest
<i>Campanumoea javanica</i> Bl. subsp. <i>javanica</i>	SCSB-A-000172	2006-9-23	Malong, Yunnan	2,406	On the stock of greenbrier in the riverside
<i>Campanumoea javanica</i> Bl. subsp. <i>javanica</i>	SCSB-A-000360	2006-12-7	Fumin, Yunnan	1,770	Among secondary forest on the riverside
<i>Campanumoea javanica</i> Bl. subsp. <i>javanica</i>	SCSB-B-000261	2006-11-20	Jinghong, Yunnan	1,033	At the edge of tropical rain forest
<i>Campanumoea javanica</i> Bl. subsp. <i>javanica</i>	SCSB-A-000303	2006-10-25	Tengchong, Yunnan	1,531	In the bushes of roadside
<i>Campanumoea javanica</i> Bl. subsp. <i>japonica</i> (Makino) Hong	SCSB-HN-0804	2007-9-25	Yizhang, Hunan	1,302	In the bushes
<i>Campanumoea javanica</i> Bl. subsp. <i>japonica</i> (Makino) Hong	SCSB-HN-1179	2007-10-2	Laifeng, Hubei	855	In the bushes
<i>Codonopsis clematidea</i> (Schrenk) C. B. Cl.	SHI-A2007465	2007-9-7	Nilka, Xinjiang	1,620	On grassland
<i>Cyananthus inflatus</i> Hook. f. et Thoms.	SCSB-A-00412	2006-11-5	Jing dong, Yunan	2,450	Weed in field
<i>Platycodon grandiflorus</i> (Jacq.) A. DC.	SCSB-JS0317	2007-10-11	Jurong, Jiangsu	113	Plain
<i>Adenophora tracheliooides</i> Maxim	SCSB-JS0302	2007-10-11	Jurong, Jiangsu	113	Plain
<i>Asyneuma chinense</i> Hong	SCSB-B-00591	2007-11-13	Jingpin, Yunan	2,183	At the edge of evergreen broad leaved forest

8-hydroxy-quinoline solution for 2 h at room temperature, then fixed the seeds in freshly prepared Carnoy's fixative (1 part glacial acetic acid: 3 parts ethanol) and stored them in a refrigerator for at least 24 h before use. Before preparing the chromosome samples, the root tips were hydrolyzed at 60°C in dissociated solution (1 N HCl: 45% acetic acid = 1:1) for 10 s, washed with DI water and dyed with orcein for 30 min. Chromosome samples were prepared using the squash method. For each accession, 10 metaphase events were digitized, using a digital micro-camera (Optronics) attached to an Olympus BX 60 microscope, for counting and measurements. The length of the short or long arm of each chromosome was calculated using Sigma Scan Pro v. 1.0 software. These values were used to

calculate the following: chromosome number (CN), total length of chromosome TL (TL = short arm length + long arm length), haploid set length (HSL = total chromosome length of set), arm ratio (AR = long arm length/short arm length) and relative length of chromosome RL (RL = TL/HSL). Chromosomes were classified on the basis of arm ratio according to Levan et al. (1964).

## Results and discussion

### Chromosome number of *Campanumoea*

The genus *Campanumoea* Bl. consists of five species, and it was likely endemic to eastern Asia. Based on

**Table 2** Karyotype formula and symmetry type of some species in *Campanulaceae* used in this experiment

Species	Accession no	Chromosome number <sup>a</sup>	Karyotype formula	Type of symmetry
<i>Campanumoea parviflora</i>	SCSB-B-000566	2n = 18	2n = 2X = 6 m + 12sm	3B
<i>Campanumoea lancifolia</i>	SCSB-B-000612	–	–	b
<i>Campanumoea lancifolia</i>	SCSB-B-000618	2n = 18	–	b
<i>Campanumoea javanica</i> subsp. <i>javanica</i>	SCSB-B-000589	2n = 16	2n = 2X = 16sm	3A
<i>Campanumoea javanica</i> subsp. <i>javanica</i>	SCSB-A-000360	2n = 16	2n = 2X = 4 m + 12sm	3A
<i>Campanumoea javanica</i> subsp. <i>javanica</i>	SCSB-A-000303	2n = 16	2n = 2X = 2 m + 12sm + 2st	3A
<i>Campanumoea javanica</i> subsp. <i>javanica</i>	SCSB-B-000261	2n = 16	2n = 2X = 2 m + 12sm + 2st	3A
<i>Campanumoea javanica</i> subsp. <i>javanica</i>	SCSB-A-000172	2n = 16	2n = 2X = 8 m + 8sm	2A
<i>Campanumoea javanica</i> subsp. <i>japonica</i>	SCSB-HN-0804	2n = 16	2n = 2X = 4 m + 12sm	3A
<i>Codonopsis clematidea</i>	SHI-A2007465	2n = 16	2n = 2X = 6 m + 10sm	3A
<i>Cyananthus inflatus</i>	SCSB-A-000412	–	–	
<i>Platycodon grandiflorus</i>	SCSB-JS0317	2n = 18	2n = 2X = 2 m + 16sm	3A
<i>Adenophora tracheliooides</i>	SCSB-JS0302	2n = 36	2n = 2X = 18 m + 18sm	2B
<i>Asyneuma chinense</i>	SCSB-B-000591	–	–	

<sup>a</sup> Chromosome number counted based on 10 cells

<sup>b</sup> Symbol in table indicate no result in the experiment

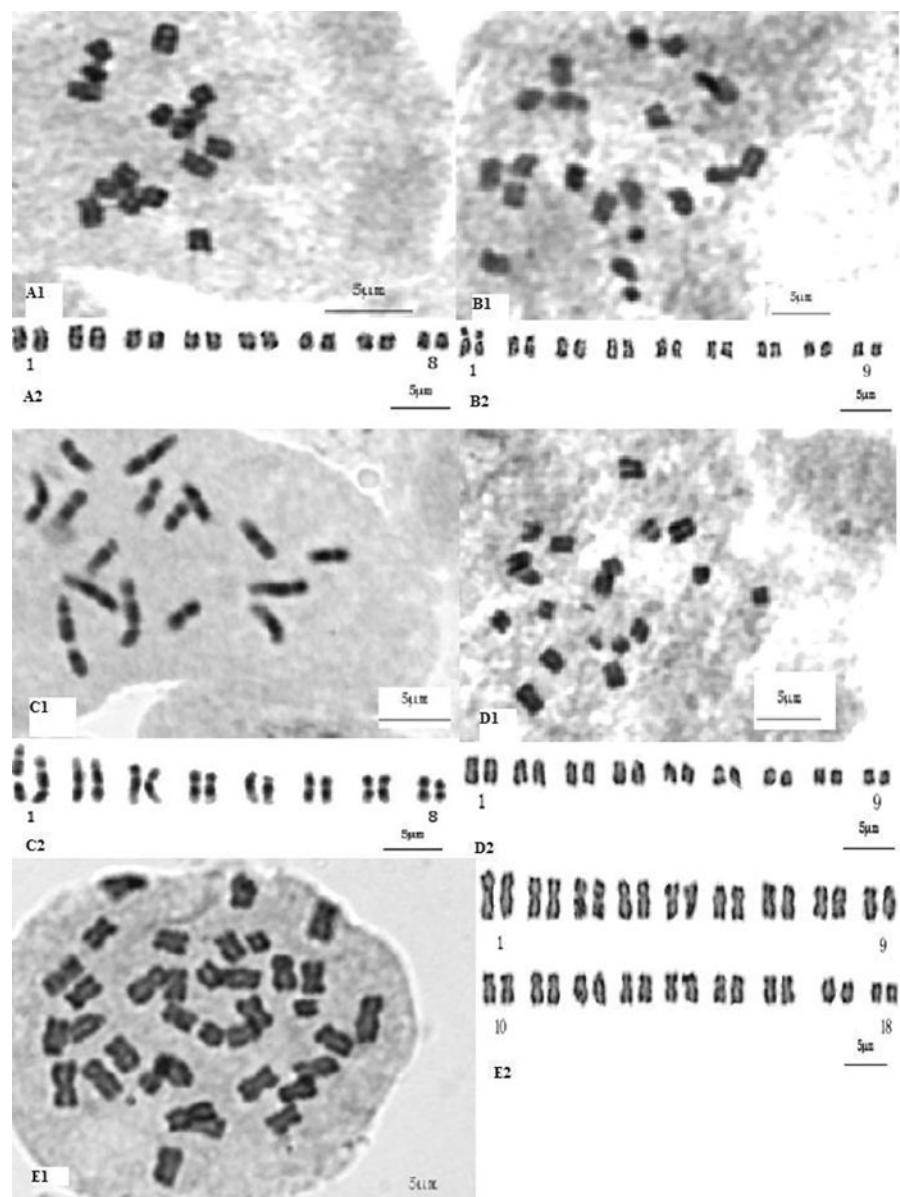
their morphological differences, many authors have divided the *Campanumoea* genus into two sections: *Campanumoea* and *Cyclocodon* (Clarke 1981; Tsoong 1935; Hong 1983; Shimizu 1993). Though the distribution of genus *Campanumoea* Blume extends farther South than the genus *Codonopsis* Wall. to tropical Indian, Indochina, The Philippines, Malaysia, Indonesia and New Guinea (Moeliono and Tuyn 1960), some authors (Moeliono and Tuyn 1960; Van Thuan 1969; Lammers 1992) expand the description of genus *Codonopsis* Wall. to include *Campanumoea*. Murthy (1983) discovered that the pollen of *Campanumoea* was identical to that of which had been reported for *Codonopsis*: suboblate, 5-7-colporate, with spinules ca. 1 µm long. The pollen grains, however, in *Cyclocodon* were oblate spheroidal and 3-colporate, with spinules ca. 3 µm long. Murthy proposed that *Campanumoea* Blume could be grouped with the species of sect. *Campanumoea* transferred into *Codonopsis* Wall., but sect. *Cyclocodon* was preserved as a distinct genus. Species of section *Campanumoea* indeed resemble those of *Codonopsis* Wall., but differ in its baccate fruit. Additionally, the species lack the distinctive foxy odor found in most *Codonopsis*, and the ovary is often 6-locular with 6-lobed stigma. *Campanumoea*, as a single genus, is commonly accepted (Hong 1983).

In the present study, the diploid number was 2n = 18 in *C. parviflora* (Wall.) Benth. and *C. lancifolia* (Roxb.) Merr., and the diploid number was 2n = 16 in *C. javanica* Bl. subsp. *javanica* and *C. javanica* Bl. subsp. *japonica* Makino (Table 2; Fig. 1). The diploid number was recorded as 2n = 16 in *Codonopsis* Wall. according to a number of reports (Rosén 1931; Gadella 1966; Lee et al. 1988; Zhukova 1967; Krasnoborov et al. 1980; Nishikawa 1985; Sui et al. 1985; Jee et al. 1989; Yoo and Lee 1989; Ge and Wang 1990; Tian and Zhao 2007; Zhao and Li 2005), 2n = 16 in *Codonopsis clematidea* (Schrenk) C. B. Cl. was confirmed in the present study (Table 2; Fig. 1). Section *Campanumoea* appears to have the same basic number as in *Codonopsis* as stated in Murthy's proposal.

#### Karyotype of *Campanumoea* and the division of two sections in taxonomy

The karyotype was 2n = 2X = 2m + 12sm + 2st and 2n = 2X = 4m + 12sm in *C. javanica* Bl. subsp. *javanica* and *C. javanica* Bl. subsp. *japonica* Makino, respectively (Table 2). The karyotype was 2n = 2X = 6m + 10sm in *Codonopsis clematidea* (Schrenk) C. B. Cl. (Table 2). Tian et al. reported that the karyotype was 2n = 12 m + 4sm in both *Codonopsis*

**Fig. 1** Metaphase chromosome and Karyograms of *C. javanica* subsp *javanica* and other species in relative genus. A1 Metaphase chromosome of *C. javanica* subsp *javanica* ( $2n = 16$ ), A2 Karyograms of *C. javanica* subsp *javanica*; B1 Metaphase chromosome of *C. parviflora* ( $2n = 18$ ), B2 Karyograms of *C. parviflora*; C1 Metaphase chromosome of *Codonopsis clematidea* ( $2n = 16$ ), C2 Karyograms of *Codonopsis clematidea*; D1 Metaphase chromosome of *Platycodon grandiflorus* ( $2n = 18$ ), D2 Karyograms of *Platycodon grandiflorus*; E1 Metaphase chromosome of *Adenophora trachelioides* ( $2n = 36$ ), E2 Karyograms of *Adenophora trachelioides*. Scale bar = 5  $\mu$ m



*pilosula* Franch. and *Codonopsis lanceolata* (Siebold et Zucc.) Trautv. We found that the karyotype asymmetry in *C. javanica* Bl. was higher than that of *Codonopsis* Wall. as reported in our previous study (Zhang et al. 2010). According to Stebbins's theory, the direction of karyotype evolution was from symmetry to asymmetry in higher plants. Primordial plants were typically symmetrical and asymmetry was more pronounced in special or higher order plants (Stebbins 1971). Accordingly, *C. javanica* Bl. and its allied species would be considered more advanced than those species of *Codonopsis* Wall.

For a more detailed comparison of the karyotype, the average values for chromosome character of *C. javanica* Bl. subsp *javanica*, *C. javanica* Bl. subsp. *japonica* and *Codonopsis clematidea* (Schrenk) C. B. Cl. were re-calculated (Table 3). Because of the polymorphisms in chromosome length and the negative correlation between HSL and biotope altitude we observed (Zhang et al. 2010), three accessions with the same or similar biotope altitude were selected for comparison: SCSB-A-000360, SCSB-B-000612 and SHI-A-2007465 for *C. javanica* Bl. subsp. *javanica*, *C. javanica* Bl. subsp. *japonica*

**Table 3** Average values for chromosome features of *C. javanica* subsp *javanica*, *C. javanica* subsp *japonica* and *Codonopsis clematidea*

CL	<i>C. javanica</i> subsp <i>javanica</i> <sup>a</sup>				<i>C. javanica</i> subsp <i>japonica</i> <sup>a</sup>				<i>Codonopsis clematidea</i>				
	TL (μm)	RL	AR	Type	TL (μm)	RL	AR	Type	TL	RL	AR	Type	New TL (μm) <sup>b</sup>
I	2.24	8.50	1.60	m	2.04	7.58	2.07	sm	5.21	7.84	1.36	m	2.06
II	1.94	7.36	2.17	sm	1.92	7.32	2.50	sm	4.88	7.34	2.18	sm	1.93
III	1.73	6.59	3.96	st	1.75	6.65	2.33	sm	4.42	6.64	1.21	m	1.75
IV	1.53	5.81	2.00	sm	1.65	6.27	1.79	sm	4.22	6.34	2.72	m	1.67
V	1.48	5.62	1.90	sm	1.65	6.27	2.29	sm	4.02	6.04	2.33	sm	1.59
VI	1.43	5.43	2.50	sm	1.46	5.57	2.20	sm	3.61	5.43	2.86	sm	1.43
VII	1.43	5.43	2.50	sm	1.38	5.23	2.75	sm	3.61	5.43	1.70	sm	1.43
VIII	1.38	5.26	2.03	sm	1.31	5.10	1.77	sm	3.28	4.93	1.89	sm	1.30
HSL	13.15				13.16				33.25				18.07

CL chromosome; TL total chromosome length; RL relative length; HSL haploid set length; AR arm ratio; m metacentric; sm submetacentric; st subtelocentric

<sup>a</sup> all of the data was average of accessions used experiment

<sup>b</sup> New data came from the actual data divided by 2.529 (33.25/13.15)

and *Codonopsis clematidea* (Schrenk) C. B. Cl., respectively. From Table 3, we observed that the TL for each chromosome was very similar between *C. javanica* Bl. subsp *javanica* and *C. javanica* Bl. subsp *japonica*, but the TL of *Codonopsis clematidea* varied significantly. This observed difference may have been due to chromosome sampling at various cell stages, the chromosome length dependence during each stage of the cell cycle, and the observation that chromosomes appeared larger at pre-metaphase than at metaphase. The HSL of *Codonopsis clematidea* is 2.529 (33.25/13.15, see Table 3) times that of *C. javanica* Bl. subsp *javanica*. For this reason we re-calculated the TL of *Codonopsis clematidea* by diving each TL by 2.529. The new TL values were 1.93, 1.75, 1.67, 1.59, 1.43, 1.43, 1.30 for chromosomes I, II, III, IV, V, VI, VII, VIII, respectively. The new data were very close to that of *C. javanica* Bl. subsp *javanica*, *C. javanica* Bl. subsp *japonica* (Table 3) and the TL of chromosomes II, III, VI were the same or almost the same in the three species; chromosomes IV, V and VIII were the same or almost the same for *C. javanica* Bl. subsp *japonica* and *Codonopsis clematidea*. Therefore, six chromosomes out of eight were similar in length in species *C. javanica* Bl. subsp *japonica* and *Codonopsis clematidea*, a similar degree of them were greater than that of *C. javanica* Bl. subsp *javanica*, *C. javanica* Bl. subsp *japonica*. The data was also in

accordance with the proposed transfer of *Campanumoea* into the *Codonopsis* genus suggested by (Murthy 1983).

For further comparison of the HSL difference between *C. javanica* and *Codonopsis clematidea*, paired-sample T tests were done using SPSS software (Table 3). The correlation coefficients for *C. javanica* Bl. subsp *javanica*—*C. javanica* Bl. subsp *japonica*, *C. javanica* Bl. subsp *javanica*—*C. clematidea* and *C. javanica* Bl. subsp *japonica*—*C. clematidea* were 0.931, 0.948 and 0.992, respectively. The test showed that three paired samples had a linear dependence relation ( $P < 0.01$ ) and no difference between pairs among the three species ( $P > 0.05$ ). The std. error mean in intra-genus species (*C. javanica* Bl. subsp *javanica*, *C. javanica* Bl. subsp *japonica*) was 0.04062, which is greater or almost as in inter-genus species. The std. error mean in *C. javanica* Bl. subsp *javanica*—*C. clematidea*, *C. javanica* Bl. subsp *japonica*—*C. clematidea* was 0.1000, 0.03381, respectively. These data may not provide detailed information at the gene or molecular level, but it indeed demonstrated the difficulty in distinguishing the three species and did indicate that section *Campanumoea* was very close to *Codonopsis* in chromosome length. Further research using chromosome banding or fluorescent dye staining is needed to discriminate the difference in species formation. Also, the chromosome characteristics of more species

within *Codonopsis* is needed in order to confirm the relation between section *Campanumoea* and genus *Codonopsis*.

A general characteristic of plants is their ability to accumulate a large number of secondary compounds. Many of these secondary compounds play a unique role in the interaction between plants and the environment. Some secondary metabolic pathways are unique to particular plants, whereas others are more broadly distributed (Dong et al. 2001). Tangshenoside I is a metabolite found in *Codonopsis* (Han et al. 1990), as well as in *C. javanica* Bl. subsp *javanica*. The flavonoids which are found in *C. javanica* Bl. have never been found in *Codonopsis* (Zhang et al. 2005). Products synthesized by the early steps of the flavonoid pathway are found in bryophytes and ferns, whereas gymnosperms and angiosperms accumulate additional classes of flavonoids; this probably reflects the recruitment of more genes in flavonoid biosynthesis, as well as the novel functions played by these compounds during evolution (Dong et al. 2001). According to what we have mentioned above, *C. parviflora* and its allied species would be considered more advanced than species of *Codonopsis*. We suggest that it is better to consider section *Campanumoea* as a single genus within the family *Campanulaceae*. This is in agreement with the results from Hong and Pan (1998), which on the basis of pollen morphology, seed coat, and gross morphology, maintained the genus *Cyclocodon* and the genus *Campanumoea* separate.

#### Relationship of *Campanumoea* with other genera

Though small variations existed among accessions in *C. javanica* Bl. subsp. *javanica* and *C. javanica* Bl. subsp. *japonica* Makino, the karyotypic formula for *C. javanica* Bl. subsp. *javanica* was generally  $2n = 2X = 2m + 12sm + 2st$  belonging to 3A in five accessions (Table 2). An exception in the accession (collected no. SCSB-A-000172) was 2A. The exception, we thought could be caused by the wrong arrangement of the centromere of the minor chromosome. For *C. javanica* Bl. subsp. *japonica* Makino, the karyotype was  $2n = 2X = 4m + 12sm$  and belonged to 3A (Table 2). Conversely, the  $2n = 2X = 18 = 6m + 12sm$  karyotype in *C. parviflora* and *C. lancifolia* belonged to 3B (Table 2).

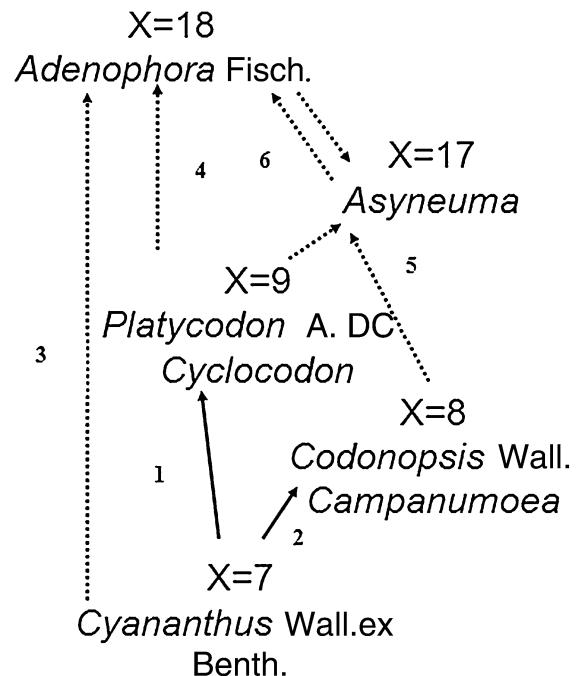
(Hong and Ma. 1991) suggested that the direction of Campanulaceae follows in four ways: the ovary position is from superior, (*Cyananthus* Wall. ex Benth.) to half-inferior, (*Codonopsis* Wall., *Leptocodon*, *Platycodon* (Jacq.) A. DC.) and to inferior (or basically inferior) (*Campanula*, *Asyneuma* Wall., *Adenophora* Fisch., *Homocodon* Hong, *Peracarpa* Wall., *Campanumoea* etc.); the pollen grains are long-colporate, (*Cyananthus*, *Codonopsis*, *Leptocodon*) to short-colporate (*Echinocodon*, *Campanumoea*, etc.) or long-colporate (*Platycodon*), and lastly to porate (*Campanula*, *Asyneuma*, *Adenophora* etc.); the way and position of dehiscence is valved in the superior part and (*Cyananthus*, *Codonopsis*, *Leptocodon*, etc.) poricidal in the inferior part (*Campanula*, *Asyneuma*, *Adenophora*, etc.); the chromosome number is  $x = 7$  (*Cyananthus*) to  $x = 8$  [*Codonopsis*, *Echinocodon*, *Wahlenberia* (p. p.), *Campanula* (p. p.)] or to  $x = 9$  (*Platycodon*, *Wahlenberia* (p. p.), *Campanumoea* etc.); and for the second type of chromosome (such as  $n = 17, 18$ ) they suggested that  $x = 17$  could be from  $x = 8$  or  $x = 9$ .

The basic chromosome number and degree of polyploidy in Campanulaceae was diverse in that it was from  $x = 6, 7$  to  $x = 13, 15, 17$ . The basic chromosome number in the family has been suggested to be  $x = 8$  (Böcher 1964; Contandriopoulos 1984) but Raven (1975) suggested that  $x = 7$  is the ancestral number. An ancestral base chromosome number of  $x = 7$  is supported by the counts for *Cyananthus* (Kumar and Chauhan 1975; Hong and Ma 1991). In our experiment, the basic number was  $x = 8$ , the karyotype was  $2n = 2x = 16$  and the karyotype asymmetry belonged to 3A (such as in *Codonopsis clematidea*). In *Platycodon grandiflorus*, a diploid plant, the basic chromosome number was determined to be  $x = 9$ , and the asymmetry type belonged to 3A. *Adenophora trachelioides* was determined to be diploid, have a chromosome number of  $x = 18$ , a karyotype of  $2n = 2x = 36$ , and an asymmetry type belonging to 2B. In *Cyananthus inflatus*, the chromosome number was  $2n = 28$ , according to IPCN (Index to Plant Chromosome: <http://mobot.mobot.org/W3T/Serchipen.html>); its basic number,  $x = 7$ , suggested it was tetraploid.

On the basis of pollen morphology, seed coat, and gross morphology, Hong and Pan (1998) restored the genus *Cyclocodon*, which was formerly included in

*Campanumoea* along with *C. celebica* Blume and *C. lancifolia* (Roxb.) Merr. They considered *Cyclcodon* to be more closely related to *Platycodon* than to *Campanumoea* s. str. (i.e., *C. javanica* Blume and *C. inflata* (Hook. f.) C. B. Clarke). *Campanumoea* and *Cyclcodon* have baccate fruits but appear to be rather distant from *Canarina*. They divided Campanulaceae into 6 groups. *Cyananthus* and *Codonopsis* belonged to Group 1 (*Cyananthus*); *Platycodon* and *Campanumoea* belonged to Group 2 (*Platycodon*); and *Adenophora* and *Asyneuma* belonged to Group 6 (*Campanula*). Furthermore, they considered that Campanulaceae were more closely related to *Platycodon*. Based on karyotype, the basic number of *Campanumoea* and *Codonopsis* was  $x = 8$ , their karyotype asymmetry type was 3A, and were very similar in their morphological characteristics; the main difference was that the fruit of *Campanumoea* was the berry. *Platycodon* and *Cyclcodon* had the same basic number ( $x = 9$ ), and their karyotype asymmetry was similar.

Our experiment suggests that *Cyananthus*, *Campanumoea* and *Codonopsis* belong to Group 1, *Cyananthus* while *Platycodon* and *Cyclcodon* belong to Group 2. Based on this hypothesis, a possible direction for the Campanulaceae genus is shown in Fig. 2. We believe the ancestor of Campanulaceae to have a basic number of  $x = 7$  as is seen in *Cyananthus* Wall. ex Benth. We predict that from the ancestor, it could form a new species by three possible paths: the first two ways (1st and 2nd path) may occur via chromosome breakage, and form species of basic numbers  $x = 8$  and  $x = 9$ , such as in *Codonopsis* Wall. (including section *Campanumoea* ( $x = 8$ ) and *Platycodon* A. DC., *Cyclcodon* Griff. ( $x = 9$ ); the other possibility (the 3rd path) is via autopolyploidization of  $x = 7$  formed paleopolyploids that gradually formed modern secondary diploids or polyploids. Based on the occurrence of secondary diploids and polyploids, more complex diploids (paleopolyploids) could originate via a 4th path: through diploidization to form  $x = 18$  from a progenitor of  $x = 9$  or from a 5th path: allopolyploidization formed via hybridization of  $x = 8$  and  $x = 9$  progenitors to form  $x = 17$ . Lastly, in a 6th path,  $x = 18$  and  $x = 17$  could be interconverted via chromosomal loss or chromosomal rupture. Between the 4th and 5th path, we preferred the 4th path due to the asymmetry in *Adenophora* ( $x = 18$ ) being at a low level. From the 5th path



**Fig. 2** A evolutionary directions between some genera in Campanulaceae. The primordial ancestor in Campanulaceae was what have basic number  $x = 7$ , such as *Cyananthus* Wall. ex Benth. From the progenitor there were three paths to form secondary species: 1, 2 paths via chromosome breakage, formed group which basic number  $x = 8$ , 9; 3 path via autopolyploidization of  $x = 7$  formed paleopolyploids, gradually formed modern secondary diploid or polyploid. Path 4 from progenitor of  $x = 9$  via diploidization formed  $x = 18$ . Path 5 via allopolyploidization from  $x = 8$  to  $x = 9$  forming  $x = 17$ . Path 6 from  $x = 18$  via lost one chromosome formed  $x = 17$  or one chromosome in  $x = 17$  rupture, formed  $x = 18$ , this path was an inter-conversional path

however, *Adenophora*'s asymmetry could be at higher level and *Asyneuma*'s asymmetry level could be the same or almost the same as *Adenophora*'s.

Certainly, the evolutionary pathway mentioned above was based on chromosome number and karyotype. Many changes in DNA sequence or chromosomal segmentation are possible during evolution, and more detailed information will be needed to determine what happened during the evolutionary course. As mentioned by Manda'kova': karyotype evolution in species with identical chromosome number but belonging to distinct phylogenetic clades is a longstanding question of plant biology, intractable by conventional cytogenetic techniques (Manda'kova' and Lysak 2008). They apply comparative chromosome painting (CCP) to reconstruct karyotype

evolution in eight species with  $x = 7$  ( $2n = 14, 28$ ) chromosomes from six Brassicaceae tribes. Their results showed karyotypic preservation in the tribes *Calepineae*, *Conringieae*, and *Noccaeeae*, whereas karyotypes of *Eutremeae*, *Isatideae*, and *Sisymbrieae* were characterized by an additional translocation. It is therefore better to use whole genome analyses, such as fluorescent in situ hybridization with multiple probes or with a functional gene unique to a particular characteristic or adaption. Recently, more functional genes or higher-diversity genes have been used for the appraisement of speciation (Greenberg et al. 2003; Barbash et al. 2004), analysis of species differences (Doebley et al. 1997; Gompel and Carroll 2003), and adaptive intraspecific variation (Johanson et al. 2000; Kroymann et al. 2003).

#### Chromosomal rearrangement and its involvement during speciation in *Campanumoea*

Karyotypic variation was seen among accessions and the variation could be caused by a growth in altitude as we had reported (Zhang et al. 2010). Two accessions in *Campanumoea*, with similar altitude (SCSB-A-000303 and SCSB-HN-0804, for *C. javanica* subsp. *javanica* and *C. javanica* subsp. *japonica* Makino, respectively) were analyzed for their karyotypic characteristics (Table 3). Their karyotype was quite similar. DNA content was assessed by measuring the HSL and chromosome RL. Average values for haploid set length were 13.15  $\mu\text{m}$  in *C. javanica* subsp. *javanica* and 13.16  $\mu\text{m}$  in *C. javanica* subsp. *japonica*. The largest chromosome (chromosome I) of *C. javanica* subsp. *javanica* represented 17.03% of the total genome and the shortest (chromosome VII) represented 10.49%. For the same chromosomes in *C. javanica* subsp. *japonica*, these values were 15.50 and 9.95%, respectively. This matching was also observed for the other chromosome pairs, most of them demonstrating very close total length with the exception of chromosomes I, IV, V and VII (Table 3). In *C. javanica* subsp. *javanica*, the lengths for chromosomes IV, VI, and VII were 2.24, 1.53, 1.48 and 1.43  $\mu\text{m}$ , respectively; in *C. javanica* subsp. *japonica*, chromosomes IV, VI and VII had lengths of 2.04, 1.65, 1.65 and 1.31  $\mu\text{m}$ , respectively. Considering the results presented above on the HSL and RL, these differences in chromosome morphology can be explained by rearrangement of segments within the

chromosome, without net gain or loss of genetic material. Further cytogenetic investigations including chromosome banding or in situ hybridization of the two species as well as of the other three species of *Campanumoea* would provide the information necessary to appraise the evolutionary and taxonomic relationships within these taxa.

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