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ORIGINAL ARTICLE

Multiple hybridization origin of *Ranunculus cantoniensis* (4*x*): evidence from *trn*L-F and ITS sequences and fluorescent in situ hybridization (FISH)

Liang Liao · Lingling Xu · Daming Zhang · Liang Fang · Huisheng Deng · Junwei Shi · Tongjian Li

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Abstract ITS sequences of *Ranunculus cantoniensis* apparently an allotetraploid were polymorphic at ten nucleotide sites. ITS-based phylogeny of the complex and its allied species showed that ITS clones of the tetraploid were clustered with *R. silerifolius* var. *silerifolius*, *R. chinensis*, *R. silerifolius* var. *dolicathus* and *R. trigonus*. Chloroplast *trn*L-F phylogeny showed that the complex is a natural group, in which the tetraploid shared the same clade with *R. silerifolius* var. *dolicathus* and *R. silerifolius* var. *silerifolius*, whose genetic distances were zero. rDNA FISH showed that the longest rDNA-chromosome of the tetraploid was similar to that of *R. silerifolius* var. *dolicathus* exclusively. Combining *trn*L-F, ITS and FISH data, it is suggested that the most probable parents of the tetraploid were *R. silerifolius* var. *silerifolius*, *R. chinensis* and *R. silerifolius* var. *dolicathus*, among them *R. silerifolius* var. *silerifolius* donated most. Evidences from DNA sequences and chromosome FISH indicated that the tetraploid was most probably a homoploid hybrid. Thus, a scenario of the tetraploid formation is proposed: the tetraploid was synthesized by two rounds of hybridization. The first round was between two pairs of diploids, forming two tetraploids. The second round was between the two primary tetraploids, producing the allotetraploid, *R. cantoniensis*, eventually.

L. Liao (✉) · L. Xu · D. Zhang · L. Fang · H. Deng · J. Shi · T. Li
College of Life Science, Jiujiang University,
East Xunyang Road 320, 332000 Jiujiang, Jiangxi, China
e-mail: LiaoL58@yahoo.com.cn

D. Zhang
State Key Laboratory of Systematic and Evolutionary Botany,
Institute of Botany, Chinese Academy of Sciences,
100093 Beijing, China

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Introduction

The *Ranunculus cantoniensis* DC. complex was first defined by Tamura (1978) and confirmed later by Okada (1984) based on Japanese flora, including *R. chinensis* Bunge. (2*x*), *R. silerifolius* Lévl. var. *silerifolius* (2*x*) and *R. cantoniensis* (4*x*). Liao et al. (1995), Liao and Xu (1997) revised the definition based on the complete distribution and chromosome basic number ($x = 8$), including all Tamura's species plus *R. silerifolius* Lévl. var. *dolicathus* L. Liao (2*x*), and further considered *R. trigonus* Hand.-Mazz (2*x*) as allied species of the complex. Geographically the members of the complex are parapatric (Fig. 1). Four or five cytotypes were found in *R. silerifolius* var. *silerifolius* (Fujishima and Kurita 1974; Okada and Tamura 1977). The variation in cytotypes of *R. cantoniensis* (4*x*) was also found, which was assumed to origin from hybridization between *R. chinensis* and *R. silerifolius* var. *silerifolius*, and later confirmed by artificial hybridization experiment (Okada 1981, 1984). Cytogenetical studies on the successive generations of offspring from the above tetraploid hybrid ($2n = 32$) showed that aneuploids ($2n = 30$ to 35) occurred frequently (Okada 1989). Takahashi (2003) localized 45S and 5S rDNA on four cytotypes of *R. silerifolius* var. *silerifolius*. However, no variation of cytotypes was found in the Chinese populations of *R. silerifolius* var. *silerifolius, R. silerifolius* var. *dolicathus* and *R. cantoniensis* (Liao et al. 1995; Liao and Xu 1997). Our preliminary study showed that the ITS sequence was heterogeneous in *R. cantoniensis* in direct sequencing PCR products, whereas the other species of the complex showed homogeneous. Therefore the problems arise: was

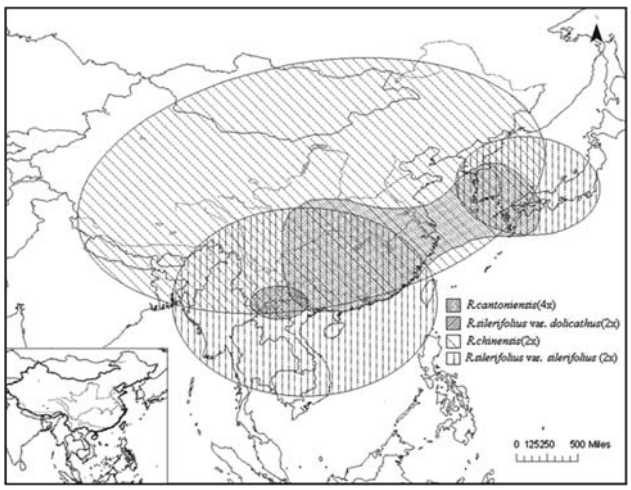

Fig. 1 Distribution of *Ranunculus silerifolius* var. *dolicathus*, *R. silerifolius* var. *silerifolius*, *R. chinensis* and *R. cantoniensis* (drawn according to Wang 1995a, b; Liao and Xu 1997)

mainland tetraploid *R. cantoniensis* formed and evolved along the same phylogenetic pathway as that in Japan? How many diploid parents were involved in the polyploidy formation?

Polyploidization plays a significant role in plant evolution (Wendel 2000; Barton 2001; Adams and Wendel 2005; Gompert et al. 2006; Chapman and Burke 2007). Speciation via homoploid hybrids occurred quite often but few, particularly the polyploid examples, were well documented (Rieseberg 1997; Ferguson and Sang 2001; Hegarty and Hiscock 2005). The evolution pattern of polyploids was often revealed by karyotype, FISH and sequence data (Hodkinson et al. 2002; Gu and Xiao 2003; Hegarty and Hiscock 2005; Koga et al. 2007). However, few studies have applied FISH and sequence data together so far to reveal the origin of polyploidy in Ranunculaceae. This study is focused on the nature and origin of the tetraploid *R. cantoniensis* by both FISH and DNA sequences being inherited maternally or biparentally.

Materials and methods

Sequencing of *trn*L-F and ITS

DNA sequences of chloroplast *trn*L-F and nuclear rDNA's internal transcribed spacers (ITS) of the taxa sampled by this study are listed in Table 1. The DNAs from at least five plants of each species were extracted by dried leaf CTAB method following Doyle and Doyle (1987). The *trn*L-F sequences of the samples were amplified with the primers c and f from Taberlet et al. (1991). Primer 1 and primer 4 of ITS were from White et al. (1990). The purified PCR products were sequenced by the dideoxy chain termination method with an ABI PRISM^{TM} Bigdye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin Elmer, Norfolk, CT, USA). For ITS sequences of *R. cantoinensis*, the PCR products were cloned by using DNA CLONING VECTOR KIT (Bio Basic Inc.), and then 12 clones were selected for sequencing. Sequences were aligned using Clustal X (Thompson et al. 1997). Maximum-likelihood (ML) phylogenetic trees for each data set were estimated by using PAUP* 4.0b10 (Swofford 2002) with tree-bisection-recombination branch-swapping in each case, with the best-fit model of nucleotide substitution for each data set determined using MODELTEST (Posada and Crandall 1998). The selected likelihood model of ITS was K81+G obtained by the Akaike information criterion and that of *trn*L-F was K81uf+I. To assess the support for individual nodes on the phylogenies, we undertook a bootstrap analysis utilizing 1,000 replicates. *R. japonicus* Thunb., a species in ser. *Ranunculus*, was used as outgroup, whereas the other taxa belong to ser. *Repentes*,

Table 1 Plant materials investigated in this study

Taxa	Locality	Voucher	GenBank accession #	
			ITS	*trn*L-F
Ranunculus japonicus Thunb. (2*x*) (outgroup)	Jiujiang, Jiangxi, China	Liao04004262	DQ410728	DQ410744
R. trigonus Hand.-Mazz. (2*x*)	Kunming, Yunnan, China	Fang0505123	DQ410724	DQ410738
R. silerifolius Lévl. var. *silerifolius* (2*x*)	Simao, Yunnan, China	Liao41303	DQ410723	DQ410735
R. silerifolius Lévl. var. *dolicathus* L. Liao (2*x*)	Simao, Yunnan, China	Liao412010	DQ410725	DQ410737
R. chinensis Bunge. (2*x*)	Simao, Yunnan, China	Liao41505	DQ410727	DQ410739
R. shuichengensis L. Liao (2*x*)	Liupanshui, Guizhou, China	Liao42605	DQ410719	DQ410741
R. cantoniensis DC. (4*x*)	Jiujiang, Jiangxi, China	Liao040512	DQ410721	DQ410736
R. diffusus DC. (4*x*)	Kunming, Yunnan, China	Fang0505122		DQ410734
R. vaginatus Hand.-Mazz. (5*x*)	Liupanshui, Guizhou, China	Fang0505121		DQ410732
R. sieboldii Miq. (6*x*, 8*x*)	Liupanshui, Guizhou, China	Fang42602		DQ410733
R. repens L. (2*x*)	Zhongdian, Yunnan, China	Shi080104		EU382995

a series closely related to ser. *Ranunculus* (Wang, 1995) among sect. *Ranunculus*. In the ITS phylogeny of Hörandl et al. (2005), *R. japonicus* was also located outside the *R. cantoniensis* complex.

Fluorescent in situ hybridization (FISH)

Five taxa, i.e. *R. silerifolius* var. *silerifolius*, *R. silerifolius* var. *dolicathus*, *R. chinensis*, *R. cantoniensis* and *R. trigonus*, were analyzed by FISH in this study (Table 1). Root tips were collected from wild populations (three to five individuals for each species), pretreated with 0.1% colchicine aqueous solution at 20°C for 2 h, fixed in ethanol:glacial acetic acid (3:1 v/v) for 24 h, and stored in ethanol (70%) at −20°C until use. The fixed root tips were macerated with a mixture of 1% pectolyase Y-23 (Yakult, Japan) and 2% cellulase R-10 (Yakult, Japan) at 37°C for 25 min. The chromosome spreads were prepared by conventional squash technique. 18S rDNA probes were prepared from *R. chinensis* genomic DNA and amplified through the primers of 1F and 1R (Troitsky et al. 1991). 5S rDNA probes were prepared through the primers of 5SL1 and 5SL2 (Hizume 1993) from *R. chinensis*. The purified PCR products of both 18S and 5S rDNA were labeled by random priming for digoxigenin (DIG)-11-dUTP (Roche). The fluorescent in situ hybridization procedures followed Zhang and Sang (1998, 1999). After hybridization and immune reaction the slides were observed under a BIO-RAD MRC-1024 Laser Scanning Confocal Microscope. Five cells with best chromosome spreading were photographed, measured and karyotypically analyzed for the indices of chromosome lengths and arm-ratio. The chromosomes were classified according to the nomenclature system of Levan et al. (1964).

Results

Sequences of *trn*L-F

The lengths of *trn*L-F sequences range from 817 to 893 bp in 11 taxa, including the *R. cantoniensis* complex and all its allied species (Table 1). As shown in the ML tree based on the *trn*L-F data (Fig. 2), all members of the *R. cantoniensis* complex, *R. cantoniensis*, *R. silerifolius* var. *silerifolius*, *R. silerifolius* var. *dolicathus*, *R. chinensis*, and its allied species *R. trigonus* were grouped into a branch, in which the sequences of *R. cantoniensis*, *R. silerifolius* var. *silerifolius* and *R. silerifolius* var. *dolicathus* were all identical, and the pairwise genetic distances among these three taxa were zero (Table 2), indicating that the complex was a natural group, while *R. trigonus* was the sister group to the complex.

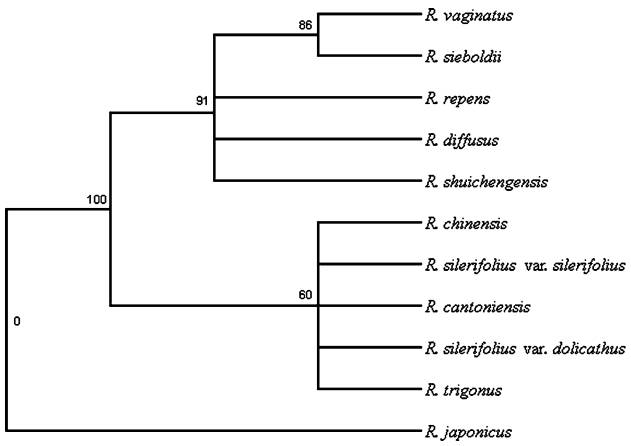

Fig. 2 Maximum-likelihood tree based on the plastid *trn*L-F data. Bootstrap values > 50(%) are indicated above the branches

Sequences of ITS

The lengths of ITS sequences were quite uniform among all diploids of the *R. cantoniensis* complex and its allied species, e.g. 170–171 bp in ITS1, 162 bp in 5.8S and 206–207 bp in ITS2. The total length ranged from 536 to 540 bp, where 15 sites were parsimony informative out of 54 variable sites, among them five were located at the 5.8S region, but none was informative.

All the taxa except *R. cantoniensis* showed homogeneous ITS sequences by direct sequencing PCR products. But in *R. cantoniensis*, ten nucleotide sites showed heterogeneous or polymorphic, demonstrating that the PCR products consisted of different sequences, and needed cloning. Further cloning to the PCR products of *R. cantoniensis* showed that the ten heterogeneous sites distributed in ITS1 (six sites) and ITS2 (four sites). Totally five different sequences were detected from 12 clones sequenced, one of them (Clone 7) was exactly the same as that of *R. chinensis*.

As shown in the strict consensus tree (Fig. 3I, II), ITS clones of *R. cantoniensis* assembled into two clades with 92% bootstrap support. One clade included nine clones of

Table 2 Pairwise genetic distance calculated from *trn*L-F sequences in *Ranunculus cantoniensis* complex and its allied species

Taxa	1	2	3	4	5	6
R. silerifolius var. *silerifolius*	–					
R. cantoniensis	0.00000	–				
R. silerifolius var. *dolicathus*	0.00000	0.00000	–			
R. chinensis	0.00231	0.00231	0.00231	–		
R. trigonus	0.00233	0.00231	0.00231	0.00463	–	
R. japonicus	0.01934	0.01934	0.01934	0.02056	0.01935	–

R. cantoniensis, *R. silerifolius* var. *silerifolius*, *R. silerifolius* var. *dolicathus* and *R. trigonus*, named as genome A, in which Clone 1, 4, 5, 6, 8, 10, 11 and 12 were grouped together with *R. silerifolius* var. *silerifolius*, and hence named as genome A_1, and Clone 2 was grouped with *R. silerifolius* var. *dolicathus* and *R. trigonus*, named as genome A_2. Another clade including Clone 3, 7 and 9 of *R. cantoniensis* and *R. chinensis* was named as genome B. It was obvious that the *R. cantoniensis* genome has complicated genome composition, containing A_1, A_2 and B, in which majority (8/12) of clones belong to A_1, while 3/12 of clones belong to B and 1/12 belongs to A_2. Therefore, the diploids, *R. silerifolius* var. *silerifolius* and *R. chinensis,* had most probably participated in the formation of the tetraploid, *R. cantoniensis*, approving Okada's (1984) view. Furthermore, *R. silerifolius* var. *dolicathus* or *R. trigonus* may also play a role as genomic donor in synthesizing the tetraploid.

Fluorescent in situ hybridization (FISH)

18S rDNA FISH signals of all five taxa examined in this work were localized at the secondary constriction of the short arm of NOR-chromosome (Figs. 4, 5). 5S rDNA FISH signals were all localized at the interstitial region of the short arm of chromosomes. The chromosomes bearing 18S signals were #3, #5 or #7 (orders according to the nomenclature system of Levan et al. 1964) in different taxa of the four diploids, respectively, while the chromosomes with 5S signals were #2, #5 or #6 (Figs. 4, 5). Both *R. silerifolius* var. *dolicathus* and the tetraploid *R. cantoniensis* shared the longest chromosomes with 5S rDNA loci (#2′ and #5′ chromosomes, respectively), and these two chromosomes might have common origin. In *R. silerifolius* var. *silerifolius* the chromosomes with rDNA loci were similar to that reported by Takahashi (2003), except 5S-chromosome.

Interestingly in *R. silerifolius* var. *dolicathus* (diploid) and the tetraploid, *R. cantoniensis*, the homologous chromosomes with 18S or 5S loci showed length asymmetry (Figs. 4, 5). The unequal homologues were generally due to the chromosomes in heterozygosity, in other words, each of the homologues was obtained from different parents.

Discussion

Hybridization, followed by genic recombination, introgression and even brought to speciation, is one of the major mechanisms in plant evolution (Hegarty and Hiscock 2005; Mallet 2007). Speciation through homoploid hybrids with the potential to combine traits for improving ecological

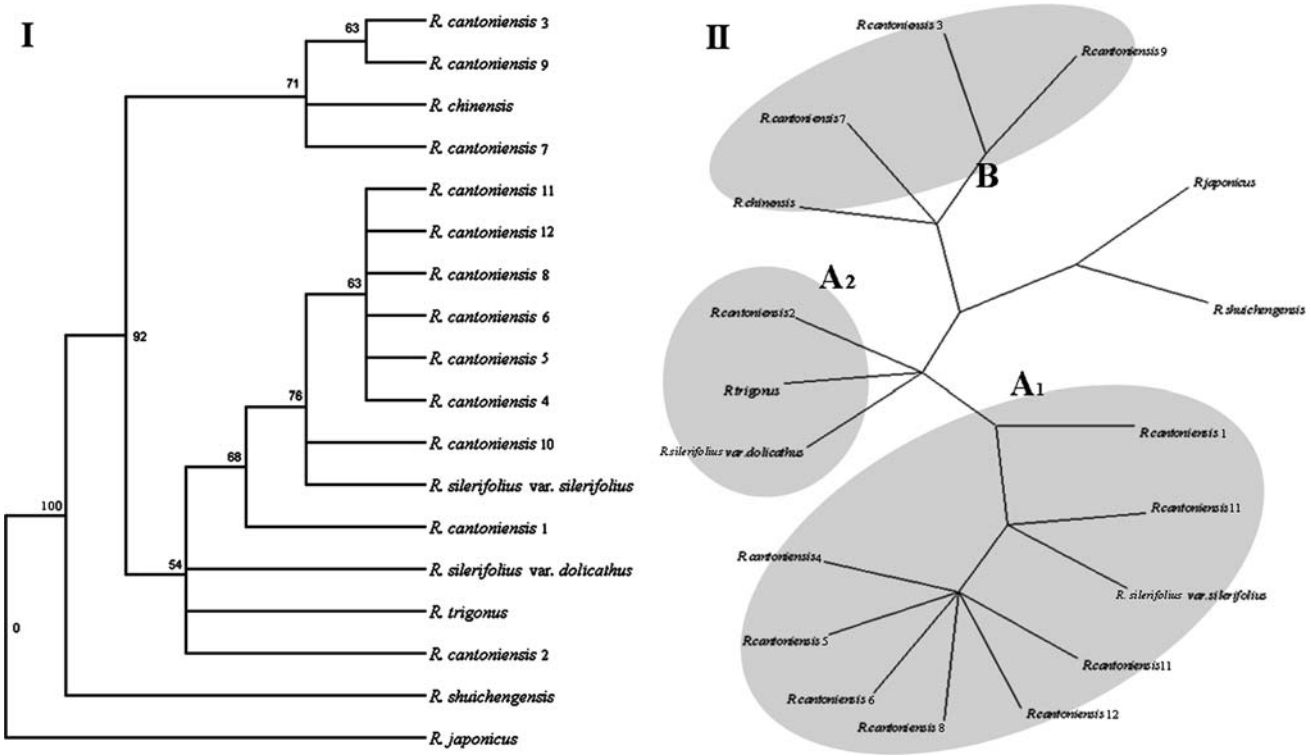

Fig. 3 Maximum Likelihood tree based on nuclear ITS data of the *Ranunculus cantoniensis* complex and its allied species. Bootstrap values > 50% are indicated above the branches

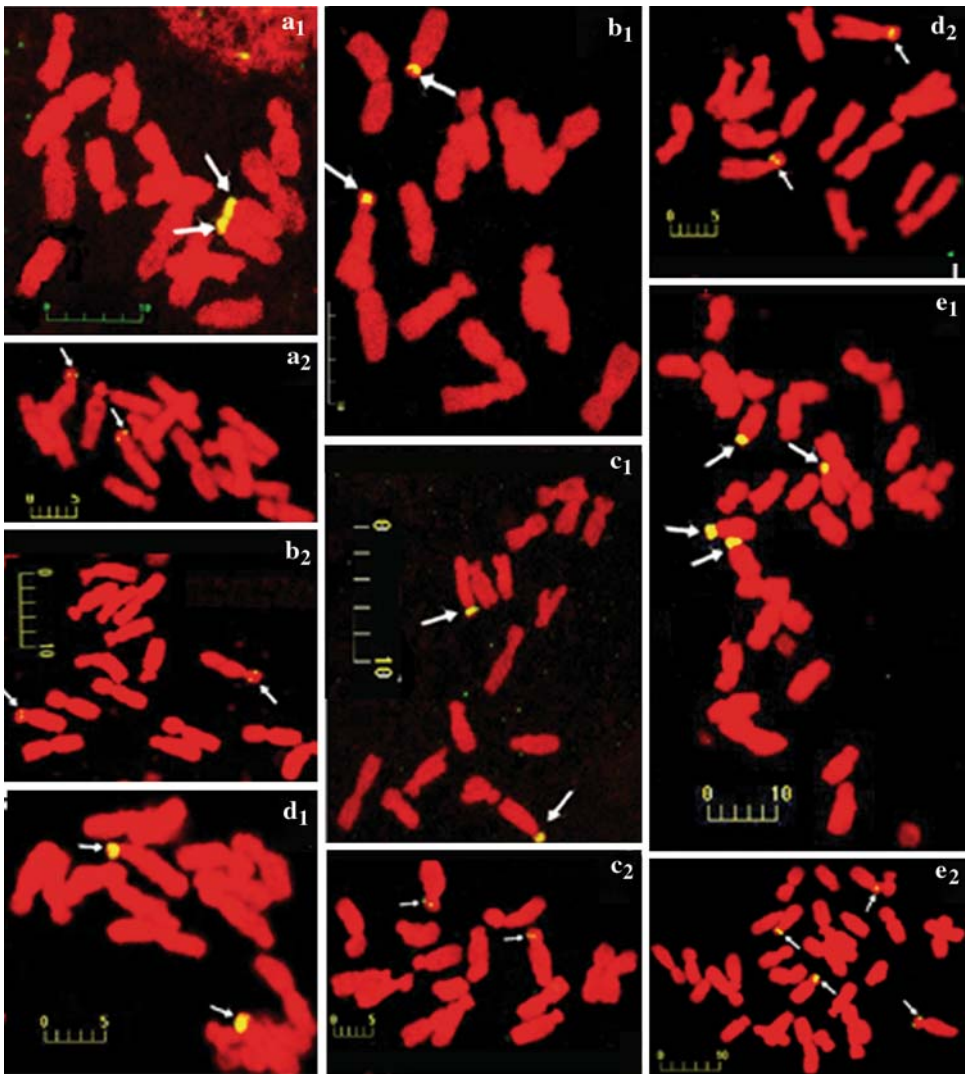

Fig. 4 FISH localization of 18S and 5S rDNA on chromosomes of the *Ranunculus cantoniensis* complex and its allied species. a_1, b_1, c_1, d_1 and e_1 show 18S localization (*arrowheads*). a_2, b_2, c_2, d_2 and e_2 show 5S localization (*arrowheads*). a_1, a_2 *R. silerifolius* var. *silerifolius* (2*x*). b_1, b_2 *R. silerifolius* var. *dolicathus* (2*x*). c_1, c_2 *R. chinensis* (2*x*). d_1, d_2 *R. trigonus* (2*x*). e_1, e_2 *R. cantoniensis* (4*x*). The scale of each *bar* is equal to 1 μm in all images

adaptation (Rieseberg 1997; Gross and Rieseberg 2005) happened frequently but most documented cases were limited to diploids (Rieseberg 1997; Hegarty and Hiscock 2005) with few examples in polyploids (Ferguson and Sang 2001), although most of the flowering plants are polyploids (Adams and Wendel 2005). One of the difficulties lies on how to identify unambiguously the homoploid hybrids and their parents (Ferguson and Sang 2001). In the present study, a widely distributed tetraploid, *R. cantoniensis*, is found with complicated or polymorphic genetic components, or in other words, the tetraploid may actually be an allotetraploid. In the ITS sequence-based phylogeny (Fig. 3), the 12 ITS clones of the tetraploid were all grouped with its closest diploid relatives, i.e. *R. silerifolius* var. *silerifolius*, *R. chinensis*, *R. silerifolius* var. *dolicathus* and *R. trigonus*. A problem arises that theoretically an allotetraploid would have two parental donors but why in the present case the tetraploid could have so many possible donors? In the phylogeny inferred from maternally inherited *trn*L-F sequences (Fig. 2), *R. silerifolius* var. *silerifolius*, *R. silerifolius* var. *dolicathus*, *R. chinensis* and *R. trigonus* are merged with the tetraploid in a clade, in which the sequences of *R. cantoniensis*, *R. silerifolius* var. *silerifolius* and *R. silerifolius* var. *dolicathus* were all identical. Therefore, both *R. silerifolius* var. *silerifolius* and *R. silerifolius* var. *dolicathus* could be the most possible candidates of the maternal donor of the tetraploid. In FISH-localized 5S-chromosomes, the tetraploid has a longest rDNA-bearing chromosome (#5'), while among the closest relatives, *R. silerifolius* var. *dolicathus* is the only diploid that bears a chromosome (#2′) with almost the same length and 5S pattern as that of the tetraploid (Figs. 4, 5). Thus, both DNA sequence-based phylogeny and FISH-localized rDNA pattern suggest *R. silerifolius* var. *dolicathus* to be one of the tetraploid parents. Among the four diploids mentioned above, *R. silerifolius* var. *silerifolius* is most possibly involved in the formation of the tetraploid, since it is the only diploid that shares the same clade with majority

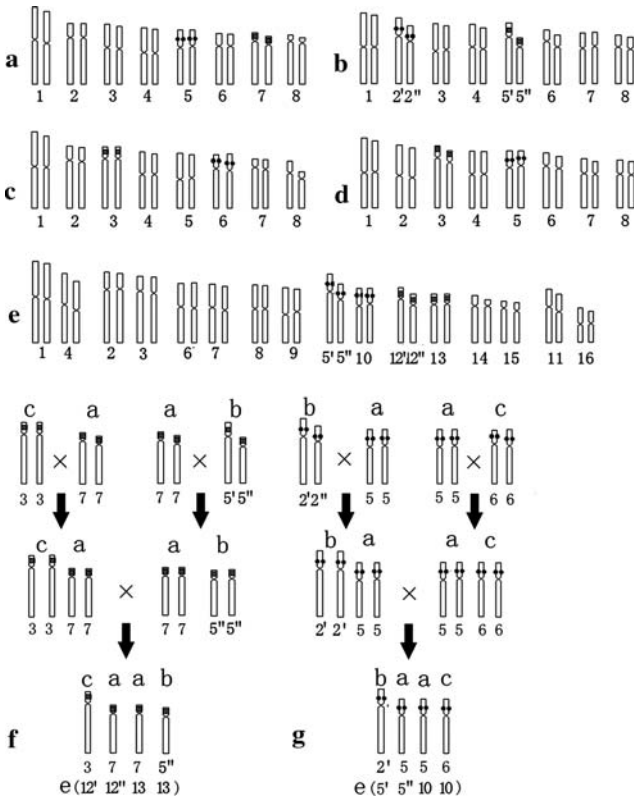

Fig. 5 Idiograms of the *Ranunculus cantoniensis* complex and its allied species showing the physical locations of 18S (*gray squares*) and 5S rDNA (*dark dots*). **a** *R. silerifolius* var. *silerifolius* (2*x*), 18S loci at #7 chromosomes and 5S at #5. **b** *R. silerifolius* var. *dolicathus* (2*x*), a pair of chromosomes with 18S loci but one long (#5′) and another short (#5″), a pair of chromosomes with 5S loci, one long (#2′) and another short (#2″). **c** *R. chinensis* (2*x*), 18S loci at #3 chromosomes and 5S at #6. **d** *R. trigonus* (2*x*), 18S loci at #3 chromosomes and 5S at #5. **e** *R. cantoniensis* (4*x*), four 18S-chromosomes, three shorter (#12″ and two #13) and one longer (#12′), and four chromosomes with 5S loci: three shorter (#5″ and two #10) and one longer (#5′). **f** A possible scenario of the tetraploid formation based on 18S chromosomes with referring to ITS phylogeny, in which two rounds of hybridization make the final tetraploid a homoploid hybrid. The first round of hybridization between two pairs of diploids form two tetraploids, while the second round between the two primary tetraploids produce a tetraploid with a 18S-chromosome pattern similar to the present tetraploid, *R. cantoniensis*. **g**: The same scenario as in **f** but based on 5S-chromosome data

(8/12) of ITS clones of the tetraploid (Fig. 3). The possibility of the diploids above being the parents of the tetraploid is also supported by their parapatric distribution (Fig. 1). However, rDNA FISH results (Figs. 4, 5) have showed weak, if not none, similarity between *R. trigonus* and the tetraploid in the rDNA patterns (Figs. 4, 5).

Therefore, all data above suggest that the tetraploid, *R. cantoniensis*, is a homoploid hybrid of allotetraploid with three possible diploid parents, *R. silerifolius* var. *silerifolius*, *R. chinensis* and *R. silerifolius* var. *dolicathus*, among them *R. silerifolius* var. *silerifolius* donates most. A possible scenario is thus proposed (Fig. 5f, g), in which two rounds of hybridization is suggested. The first round of hybridization is between two pairs of diploids, *R. silerifolius* var. *silerifolius* × *R. silerifolius* var. *dolicathus* and *R. silerifolius* var. *silerifolius* × *R. chinensis*, forming two tetraploids, with genomic constitution of $A_1A_1A_2$ A_2 and A_1A_1BB, respectively. The latter tetraploid (A_1A_1BB), *R. silerifolius* var. *silerifolius* × *R. chinensis*, was once reported and confirmed by Okada (1984) through artificial hybridization experiment. The second round of hybridization is between the two primary tetraploids of the first round, producing an allotetraploid with genomic components covering all the three diploids. However, the contributions of the three diploids are not equal, because *R. silerifolius* var. *silerifolius* participates in the first hybridization twice. As showed in ITS phylogeny and rDNA FISH, the allotetraploid contains more elements of *R. silerifolius* var. *silerifolius*, and would have genomic constitution of $A_1A_1A_2B$. However, further research is necessary to survey more tetraploid types in the complex, especially the missing tetraploid parent with genomic component of $A_1A_1A_2$ A_2.

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