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ORIGINAL ARTICLE

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Trends in site-number change of rDNA loci during polyploid evolution in *Sanguisorba* (Rosaceae)

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Abstract To elucidate the evolutionary dynamics of rDNA site number in polyploid plants, we determined 5S and 18S-5.8S-26S rDNA sites for ten species of *Sanguisorba* (2*n*=14, 28, 56) and a single species of each of three outgroup genera, *Agrimonia* (2*n*=28), *Rosa* (2*n*=14), and *Rubus* (2*n*=14) by the fluorescence in situ hybridization (FISH) method. We also estimated phylogenetic relationships among these species using *mat*K chloroplast DNA (cpDNA) sequences, and reconstructed the evolutionary history of rDNA site number based on the maximum parsimony method. The 2*n*=14 and 2*n*=28 plants of all genera except *Rosa* carried two 5S rDNA sites, whereas *Rosa* and 2*n*=56 plants carried four sites. The 2*n*=14 plants had two 18S-5.8S-26S rDNA sites, whereas *Sanguisorba annua* and 2*n*=28 plants had four or six sites. Phylogenetic analysis showed that polyploidization from 2*n*=14 to 2*n*=28 has occurred once or three times in *Sanguisorba* and *Agrimonia*. The 5S rDNA sites duplicated during each ancestral polyploidization were evidently lost after each polyploidization. However, the duplicated 18S-5.8S-26S rDNA sites were all conserved after each polyploidization. Thus, the duplicated 5S rDNA sites tend to have been eliminated, whereas those of 18S-5.8S-26S rDNA tend to have been conserved in *Sanguisorba*. In the most parsimonious hypothesis, 2*n*=14 in *S. annua* is a secondary, putatively dysploid state, reduced from 2*n*=28.

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Introduction

During the last decade, the site number of ribosomal RNA genes (rDNA) of various plant species has been determined by the technique of fluorescence in situ hybridization (FISH) (Bauwens et al. 1991; Griffor et al. 1991; and numerous other studies). These studies revealed that most of the diploid plants examined had two sites (i.e. a single locus) of both 5S and 18S-5.8S-26S rDNA, although some diploids had multiple sites (Fukui et al. 1994b; Ansari et al. 1999; Raina and Mukai 1999; Zhang and Sang 1999). In polyploids derived from such diploids, duplicated rDNA sites corresponding to their duplicated genomes are expected, as has been demonstrated in the colchicine-induced tetraploids or the higher polyploids created by artificial hybridization (Lim et al. 1998).

However, the evolutionary dynamics of the rDNA site number remain unclear. While most natural polyploid plants have multiple (duplicated) sites, sometimes the number of sites detected is different from the number expected based on ploidy level (Adachi et al. 1997; Ansari et al. 1999; Raina and Mukai 1999). These differences may indicate that the evolutionary dynamics of rDNA sites in polyploid plants are complex (Shi et al. 1996), although Dvorák (1990) has suggested that rDNA loci show a general tendency to be eliminated if rendered dispensable by polyploid evolution.

To elucidate the evolutionary dynamics of rDNA site number in polyploid plants, we need to trace the addition and/or deletion of rDNA sites during the history of polyploid evolution. In this paper, we estimate the phylogenetic relationships among ten species of *Sanguisorba* and a single species of each of ten outgroup genera from Rosoideae s.s., *Agrimonia*, *Alchemilla*, *Duchesnea*, *Filipendula*, *Fragaria*, *Kerria*, *Potentilla*, *Rhodotypos*, *Rosa*, and *Rubus*, using *mat*K DNA sequences. We also

Table 1 Species of *Sanguisorba* mainly following the classification of Nordborg (1966), their reported chromosome numbers and their distributions. (C, Central; N, North; E, East; S, South)

Subgenus	Species	Chromosome number	Distribution
Section			
Group			
Dendriopoterium			
Dendriopoterium	*S. menendezii* (Svent.) Nordb.	2*n*=28	Canary Islands
Marcetella	*S. maderensis* (Bornm.) Nordb.	–	Madeira
	S. moquiniana (Webb et Berth.) Nordb.	2*n*=28	Canary Islands
Poteridium	*S. annua* (Nutt. ex Hook) Torr. et Gray[a]	2*n*=14	N. America
Poterium			
Poterium	*S. ancistroides* (Desf.) Ces.	2*n*=28	Spain, Morocco, Algeria
	S. cretica Hayek	2*n*=28	Crete
	S. filiformis (Hook. f.) Hand.-Mazz.[a]	2*n*=28[b]	C. & E. Himalaya
	S. lateriflora (Coss.) A. Br. et Bouche	–	Spain
	S. minor Scop.[a]	2*n*=28, 56	Europe to C. Eurasia & N. Africa
	S. vestita (Pom.) Nordb.	2*n*=56	Morocco & Algeria
Agrimonioides	*S. hybrida* (L.) Nordb.	2*n*=56	Portugal & Spain
Sanguisorba			
Sanguisorba			
albanica	*S. albanica* Andr. et Jáv.	–	Albania
canadensis	*S. alpina* Bunge in Ledeb.	2*n*=28	Mongolia
	S. canadensis L.	2*n*=28, 56	N. America
	S. japonensis (Makino) Kudo[a]	2*n*=28	Japan
	S. stipulata Rafin.[a]	2*n*=28	N.E. Asia
obtusa	*S. albiflora* (Makino) Makino[a]	2*n*=56, 84	Japan
	S. hakusanensis Makino[a]	2*n*=28	Korea & Japan
	S. obtusa Maxim.[a]	2*n*=28	Japan
officinalis	*S. officinalis* L.[a]	2*n*=28, 56	N. Hemisphere
tenuifolia	*S. tenuifolia* Fisch. ex Link[a]	2*n*=56, 84	N.E. Asia
Pterachaenium	*S. diandra* (Hook.) Nordb.	–	Himalaya
	S. dodecandra Mor.	2*n*=56	Italy

[a] Taxa used in this study
[b] Counted for first time in this study

determine the numbers of 5S and 18S-5.8S-26S rDNA sites of *Agrimonia*, *Rosa*, *Rubus*, and *Sanguisorba* using the FISH method. Based on the phylogeny estimated, we reconstruct evolutionary changes in the number of rDNA sites that have occurred during polyploid evolution.

Sanguisorba, belonging to the tribe Sanguisorbeae, occurs in temperate to sub-arctic regions of the Northern Hemisphere. It comprises an annual species with 2*n*=14, *S. annua* Nutt., and 22 perennial species in which 2*n*=28 or 2*n*=56 is prevalent (Table 1). Intraspecific polyploids are often found in 2*n*=28 and 2*n*=56 species (Nordborg 1963, 1966; Mishima et al. 1996, 1998). According to the Rosaceae phylogenies based on the sequence of the *rbc*L gene (Morgan et al. 1994) and ITS (internal transcribed spacer) sequences of 18S-5.8S-26S rDNA (Eriksson et al. 1998), *Filipendula* with 2*n*=14 is the basalmost to Rosoideae s.s., and *Rubus* (2*n*=14) is basally divergent to *Sanguisorba*, *Agrimonia* (2*n*=28, Sanguisorbeae), *Rosa* (2*n*=14, Roseae), and some other genera with 2*n*=14. Thus, *x*=7 is considered to be the ancestral base chromosome number of the clade comprising *Sanguisorba* and related genera, indicating that 2*n*=28 in most species of *Sanguisorba* is of polyploid origin. This genus, therefore, provides a good opportunity for examining evolutionary changes in rDNA sites that have occurred during polyploid evolution. However, the history of polyploidy within *Sanguisorba* has not been fully elucidated because the phylogenetic position of *S. annua* with 2*n*=14 is still unknown (Mishima and Ito 1996; Helfgott et al. 2000).

In the present study, we addressed the following questions: (1) Is *S. annua*, with 2*n*=14, basally divergent to the remaining species of *Sanguisorba*? (2) Does the change in rDNA site number show any specific tendency during polyploidization and subsequent speciation in *Sanguisorba*?

Materials and methods

Plant materials and DNA extraction

Species sampled are listed in Table 2. The *Sanguisorba* materials cover three of four subgenera, three of seven sections, and 10 of 23 species (see Table 1). *Alchemilla*, *Agrimonia*, *Duchesnea*, *Filipendula*, *Fragaria*, *Kerria*, *Potentilla*, *Rhodotypos*, *Rosa*, and *Rubus* were examined to determine the phylogenetic position of *Sanguisorba* in Rosoideae. Plants were cultivated in pots in the nurseries of Tokyo Metropolitan University, Kyushu University, or Nikko Botanical Garden of the University of Tokyo. Voucher

Table 2 Chromosome numbers, collection data, DNA sequence accession numbers, and rDNA signal numbers for taxa examined in this study. Chromosome numbers without notes were re-examined by the authors. Accession numbers are listed for living collections and for sequences analyzed. The number of rDNA sites was detected by the fluorescence in situ hybridization method. A pair of sites (i.e. two signals) is equal to one locus

Taxa	2*n*	Origin	Collection number	GenBank Accession number (*mat*K gene)	Number of rDNA signals	
					5S	18S-5.8S-26S
Alchemilla japonica Nakai et Hara	–[a]	Mt. Hakuba, Nagano Pref., Japan[b]	70-756-1	AB073681	–	–
Agrimonia nipponica Koidz.	28	Shiojiri City, Nagano Pref., Japan	940627	AB073682	2	6
Duchesnea chrysantha (Zoll. et Mor.) Miq.	14[c]	Nikko City, Tochigi Pref., Japan[b]	010615	AB073683	–	–
Filipendula multijuga Maxim	–[d]	Hakuba-mura, Nagano Pref., Japan	010531	AB073684	–	–
Fragaria iinumae Makino	14[e]	Mt. Shibutsu, Gunma Pref., Japan[b]	A1091	AB073685	–	–
Kerria japonica (L.) D.C.	–	Niukawa-mra, Gifu Pref., Japan	010601	AB073686	–	–
Potentilla fragarioides L.	14[f]	Mt. Goshiki, Tochigi Pref., Japan[b]	99-26	AB073687	–	–
Rhodotypos scandens (Thunb.) Makino	–	Nikko Botanical Garden	010615	AB073688	–	–
Rosa multiflora Thumb.	14	Ainoshima, Fukuoka Pref., Japan	515026	AB073700	4	2
Rubus parvifolius L.	14	Fukuoka City, Fukuoka Pref., Japan	315027	AB073699	2	2
Sanguisorba annua	14	Mt. Ashland, Oregon St., USA	97011	AB073689	2	4
filiformis	28	Yungnang, China	010920	AB073690	2	4
hakusanensis	28	Mt. Happo, Nagano Pref., Japan[b]	–[b]	AB073693	2	4
japonensis	28	Hidaka Mts., Hokkaido Pref., Japan[g]	307097	AB073692	2	6
minor	28	Originally from Europe[h]	940401	AB073694	2	4
obtusa	28	Mt. Hayachine, Iwate Pref., Japan[b]	–[b]	AB073695	2	4
officinalis	28	Kuriyama-mura, Tochigi Pref., Japan	310020	AB073696	2	6
stipulata	28	Mt. Shari, Hokkaido Pref., Japan	307143	AB073697	2	4
albiflora	56	Mt. Chokai, Akita Pref., Japan	307093	AB073691	4	8
tenuifolia	56	Ohkuchi City, Kagoshima Pref., Japan	307157	AB073698	4	8

[a] 2*n*=16 is the lowest chromosome number in this genus
[b] Cultivated in Nikko Botanical Garden of University of Tokyo
[c] Naruhashi and Iwatsubo (1991)
[d] 2*n*=14 is the lowest chromosome number in this genus
[e] Iwatsubo and Naruhashi (1989)
[f] Iwatsubo and Naruhashi (1991)
[g] Cultivated in Experimental Field of Hokkaido University
[h] Seeds were germinated using the market product from Sakata Seed

specimens of these plants are deposited in Makino Herbarium of Tokyo Metropolitan University or in the Herbarium of the University of Tokyo. Total DNAs of these plants were extracted from fresh or dried leaves by the cetyltrimethylammonium bromide method (Doyle and Doyle 1987).

Chromosome preparation

Procedures followed the EMA (enzymatic maceration/air-drying) method (Fukui 1996) with minor modifications in enzymatic mixture and treatment time. Root tips were fixed with a 1:1 solution of ethanol and acetic acid without pretreatment with 8-hydroxyquinoline, and stored at –20°C until examination. At least four root tips were examined. After being rinsed in distilled H_2O for 3 h, the root tips were immersed in an enzymatic mixture [4.7% Pectolyase Y-23 (Seishin Corporation), 0.2% Cellulase Onozuka R10 (Yakult), 1 mM EDTA, pH 4.2], subjected to decompression treatment (Centrifugal Vaporizer, EYELA) at room temperature for 10–20 min to improve the penetration of enzyme mixture into cells, and then macerated at 37°C for 40–70 min. After being rinsed again in distilled H_2O for 1 min, the root tips were broken up into free cells on a glass slide using forceps under several drops of 1:1 ethanol and acetic acid, and these were then air-dried.

Probe DNA and labeling for FISH

For 5S rDNA detection, the 5S rDNA coding region was amplified and directly labeled by PCR (polymerase chain reaction) using primers designed by Fukui et al. (1994a). For 18S-5.8S-26S rDNA detection, the 5.8S rDNA coding region and flanking ITSs were amplified and directly labeled by PCR using the primers ITS4 and ITS5 (White et al. 1990), and a part of the 28S rDNA coding region of *Oryza sativa* ssp. *japonica* (Nipponbare) was amplified and labeled using the primers 28v and 28x (Palumbi 1996). Polymerase chain reaction amplification was performed with DNA polymerase, Ex Taq (Takara), using 70% substitution of biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim) for dTTP, under a thermal cycling program of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with a final step of 10 min at 72°C.

Fluorescence in situ hybridization and image observation

Chromosome samples on a glass slide were treated with 100 µg/ml RNase A (Sigma) in 2×SSC at 37°C for 60 min. (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.) They were completely dehydrated through a 70%, 95%, and 99% ethanol series for 10 min each at room temperature and then air-dried.

Hybridization mixture (20 µl) containing 100 ng of labeled probe DNA in 50% (v/v) formamide, 10% (w/v) dextran sulfate, and 2×SSC, was placed on a glass slide. The preparation on the glass slide was mounted with a coverslip sealed by Paper Bond (Kokuyo), placed on a flat plate of a thermal cycler (Zymoreactor II, Atto), and then kept at the programmed temperature of 70°C for 6 min and 37°C for 16 h.

Signals were visualized by Avidin-FITC (fluorescein isothiocyanate) or anti-digoxigenin-FITC (Boehringer Mannheim), following the detection and washing procedures of Fukui et al. (1994b) and Raina and Mukai (1999), respectively. Chromosomes were counter-stained with propidium iodide and fluorescent signals were antifaded with VectaShield (Vector Laboratories). The numbers of 5S and 18S-5.8S-26S rDNA sites were determined for chromosomes at prometaphase, metaphase, or interphase.

A highly sensitive cooled CCD camera (PXL 1400, Photometrics Quantix) was mounted on a fluorescence microscope (AX70, Olympus, or Axioplan, Zeiss) with B- and G-light excitation filters and the fluorescent images were directly frozen in the image

frame memories of IPlab Spectrum. All the B- and G-light excitation images were color-joined by IPlab Spectrum, stored as Photoshop (Adobe) images, and the processed images were printed out. We determined the number of signals in each species based on examining more than five cells by microscopy and based on at least three color-joined images.

Some slides were re-used for hybridization using the other rDNA probe. After the fluorescent signals had completely faded, the probe DNAs hybridized with chromosome samples on a glass slide were denatured in 50% (v/v) formamide with 2×SSC at 90°C for 2 min, and washed out with cold 2×SSC for 10 min. The chromosome samples were dehydrated through a 70%/95%/99% cold-ethanol series for 10 min each, and air-dried.

Phylogenetic analysis of *mat*K sequences

To determine the phylogenetic position of *Sanguisorba* in Rosoideae s.s., a plastid gene, *mat*K, was sequenced in *Alchemilla japonica*, *Agrimonia nipponica*, *Duchesnea chrysantha*, *Filipendula multijuga*, *Fragaria iinumae*, *Kerria japonica*, *Potentilla fragarioides*, *Rhodotypos scandens*, *S. annua*, and *S. filiformis*. A part of the plastid region was amplified using a primer set of *trn*K-3914F and *trn*K-2R (Johnson and Soltis 1995) by PCR and cloned in the pGEM-T vector (Promega) according to the manufacturer's procedure. The clone with insert was purified by Wizard *Plus* SV Minipreps DNA Purification System (Promega). Then 300 ng of purified clone was sequenced using the ABI Prism Big-Dye Terminator Cycle Sequencing Kit and subsequently visualized using automated sequencer, Prism-377 (ABI). Primers used for sequencing were M13-21 (5′-TGTAAA ACGACGGCCAGT-3′), Reverse (5′-AGCGGATAACAATTTCACACAGG-3′), newF (5′-AAATGTGTAGAAGAAGGCAGTATATTG-3′), and newR (5′-TTTTTTGAAGATCCGCTATAATAATG-3′). The *mat*K gene region was used for phylogenetic reconstruction. The sequences of *Agrimonia pilosa* var. *japonica* (AB012001, Matsumoto et al. 1998), *Rosa multiflora* (AB011991, Matsumoto et al. 1998) and *Rubus trifidus* (AB039323, Wu et al., unpublished) were also examined. The sequence data were analyzed with PAUP ver. 4.0b7 (Swofford 2001). The most parsimonious tree was searched with the Branch and Bound option. The tree was rooted using *Filipendula*. A bootstrap analysis (Felsenstein 1985) was conducted with 1000 replicates under the general search option. To determine the phylogeny of *Sanguisorba* species, partial *mat*K sequences were amplified and sequenced using a primer set of *mat*K-AF and *mat*K-MR and another primer set of *mat*K-MF and *mat*K-8R (Ooi et al. 1995; Endo, unpublished) in *Sanguisorba*. Based on the above result, *A. nipponica*, *R. multiflora*, and *R. parviforius* were chosen as outgroups. The sequence data were analyzed with PAUP ver. 4.0b7 (Swofford 2001). The Branch and Bound search was carried out and the tree was rooted using *R. multiflora* and *R. parviforius*. A bootstrap analysis (Felsenstein 1985) was conducted with 1000 replicates under the general search option.

Tracing of cytological characters

The ancestral character states for chromosome number and the number of rDNA sites at each node of the phylogenetic tree were estimated using MacClade ver. 3.0 (Maddison and Maddison 1992).

Results

Number of rDNA sites

Observations at all mitotic stages gave identical numbers of 5S and 18S-5.8S-26S rDNA sites in each species. Digital micrographs of the FISH images are shown in Fig. 1a–f for 5S rDNA and Fig. 1g–m for 18S-5.8S-26S

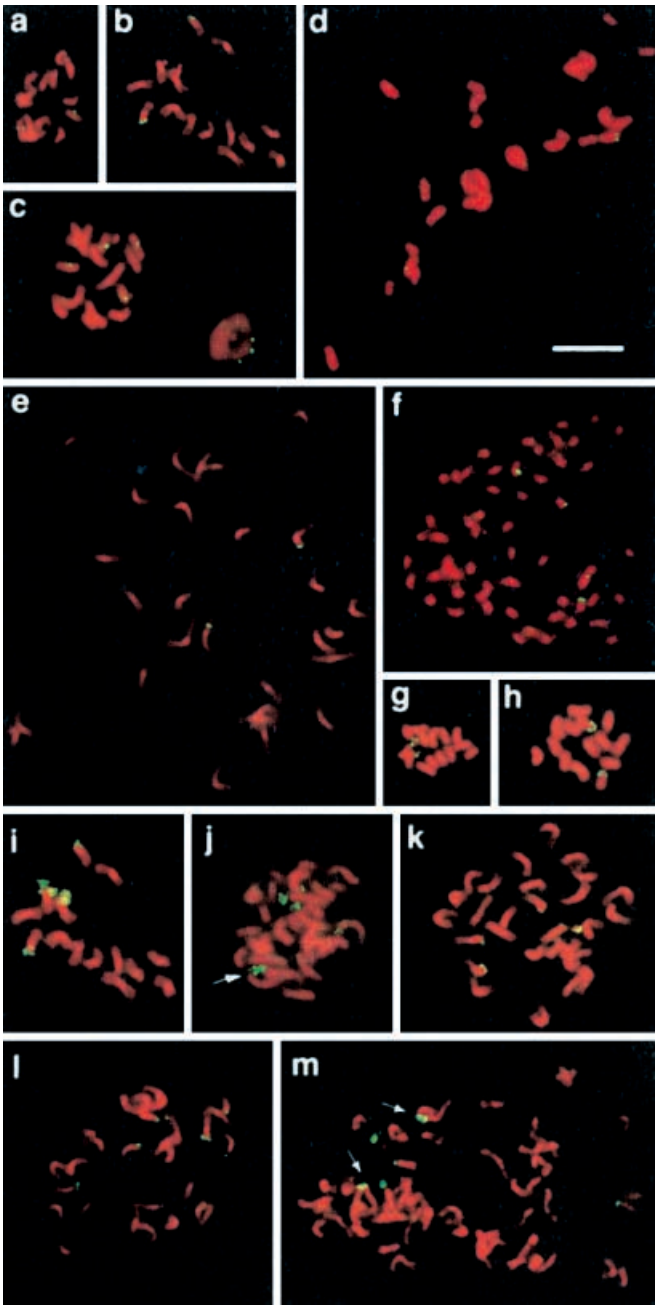

Fig. 1 Fluorescence in situ hybridization (FISH) images for 5S rDNA (**a–f**) and for 18S-5.8S-26S rDNA (**g–m**). *Yellow spots* are signals of in situ hybridization with biotin-labeled rDNA probe. *Rubus parvifolius* chromosomes, with 2*n*=14, in situ hybridized with 5S rDNA probe (**a**) and 18S-5.8S-26S rDNA probe (**g**). *Sanguisorba annua* chromosomes, with 2*n*=14, in situ hybridized with 5S rDNA probe (**b**) and 18S-5.8S-26S rDNA probe (**i**). *Rosa multiflora* chromosomes, with 2*n*=14, in situ hybridized with 5S rDNA probe (**c**) and 18S-5.8S-26S rDNA probe (**h**). *Agrimonia nipponica*, with 2*n*=28, in situ hybridized with 5S rDNA probe (**d**) and 18S-5.8S-26S rDNA probe (**j**, *arrow* indicates closely associated two signals). 2*n*=28 chromosomes of *Sanguisorba*, represented by *S. japonensis*, in situ hybridized with 5S rDNA probe (**e**); 2*n*=56 chromosomes of *Sanguisorba*, in situ hybridized with 5S rDNA probe, represented by *N. tenuifolia* (**f**) and 18S-5.8S-26S rDNA probe, represented by *S. albiflora* (**m**, *arrow* indicates closely associated two signals). 2*n*=28 chromosomes of *Sanguisorba* in situ hybridized with 18S-5.8S-26S rDNA probe, represented by *S. hakusanensis* (**k**) and *S. japonensis* (**l**). *Bar* represents 10 μm

rDNA. The FISH images of *Sanguisorba* species that are not represented in Fig. 1 are available in supplemental figures on the web (http://neco.biology.kyushu-u.ac.jp/~mishima/rDNA/rDNA.html). Table 2 summarizes the results obtained.

5S rDNA

Two signals were detected in *R. parvifolius* and *S. annua*, both of which have 2*n*=14 (Fig. 1a, b), whereas *R. multiflora* (2*n*=14) showed four signals, i.e., two extra sites in addition to the two sites expected for a diploid (Fig. 1c). All 2*n*=28 plants of *Sanguisorba* and *A. nipponica* showed two 5S rDNA sites (Fig. 1d, e), in contrast to the expectation of four sites for tetraploids. *Sanguisorba albiflora* and *S. tenuifolia*, both with 2*n*=56, showed four signals (Fig. 1f) rather than the eight sites expected for octaploids. 5S rDNA signals were detected at an interstitial position in *Rubus* (Fig. 1a), *Rosa* (Fig. 1b), and *Agrimonia* (Fig. 1c), and at a subtelomeric position in *Sanguisorba* (represented in Fig. 1e), except for *S. annua*, in which the 5S rDNA signal occupied a telomeric position (Fig. 1b).

18S-5.8S-26S rDNA

Two signals were detected in *Rubus* and *Rosa* with 2*n*=14, as was expected for the diploid condition (Fig. 1g, h). However, *S. annua*, with 2*n*=14, showed four signals (Fig. 1i). In 2*n*=28 plants of *S. hakusanensis*, *S. stipulata*, *S. obtusa*, *S. filiformis*, and *S. minor*, four signals were detected (Fig. 1k), as was expected based on their tetraploid condition. However, 2*n*=28 plants of *S. officinalis*, *S. japonensis*, and *A. nipponica* showed six signals (Fig. 1j, h), indicating the addition of one extra site per haploid genome. *Sanguisorba albiflora* and *S. tenuifolia*, with 2*n*=56, showed eight signals (Fig. 1m), as expected based on their octaploid condition. The 18S-5.8S-26S rDNA signals occupied a telomeric position on chromosomes of all species examined (Fig. 1g–m).

Phylogenetic relationships

Phylogenetic position of Sanguisorba *in Rosoideae s.s*

The complete *mat*K coding region sequenced had 1503–1521 nucleotides. Of the 1530 aligned characters 233 were parsimony informative. Equal-weighted phylogenetic analysis of *mat*K sequences for two *Sanguisorba* species and 11 outgroup species resulted in one most parsimonious tree (Fig. 2). The tree length was 685, the consistency index (CI) was 0.853, and the retention index (RI) was 0.752. *Agrimonia*, in which there is no diploid (2*n*=14) species, is sister to *Sanguisorba*, and the basal branch of these two genera is supported by a bootstrap value of 100%.

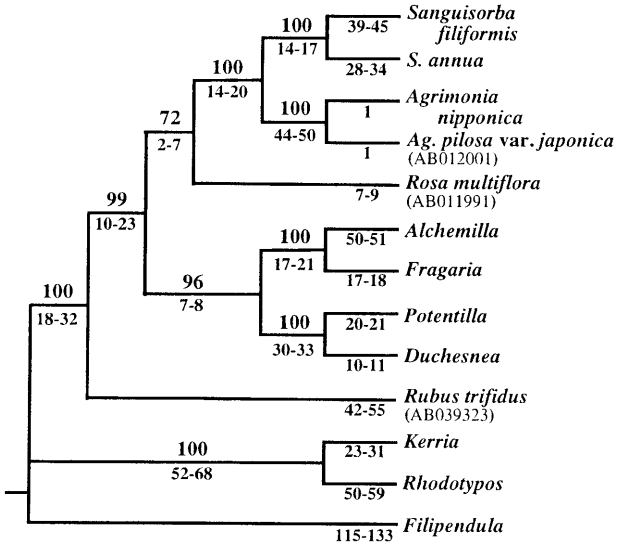

Fig. 2 Most parsimonious tree based on *mat*K sequences for Rosoideae s.s. Bootstrap percentages, based on 1000 replicate analyses, are shown above nodes. Possible length from minimum to maximum is shown below each node

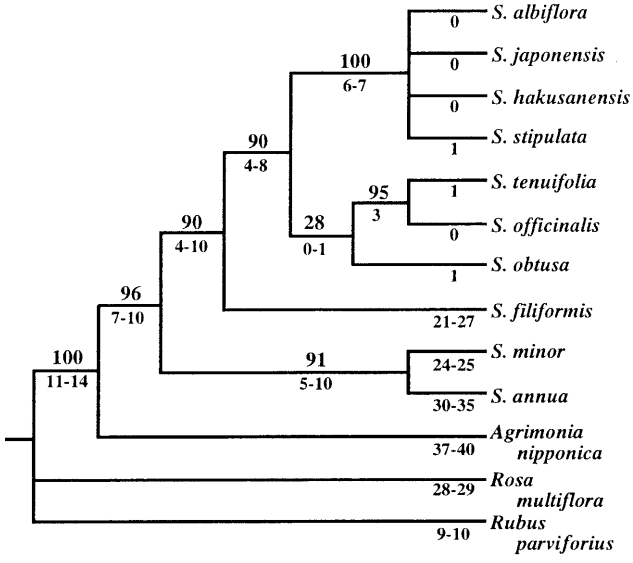

Fig. 3 One of two most parsimonious trees based on *mat*K sequences for *Sanguisorba* and the three outgroup genera. Bootstrap percentages, based on 1000 replicate analyses, are shown above nodes. Possible length from minimum to maximum is shown below each node

Phylogenetic relationships of species in Sanguisorba

The partial *mat*K coding region sequenced had 1187 nucleotides, of which 58 nucleotide sites were parsimony informative. Equal-weighted phylogenetic analysis of *mat*K sequences determined for ten *Sanguisorba* species and three outgroup species resulted in two most parsimonious trees. One of the trees obtained is shown in Fig. 3. The only unstable branch is that of *S. obtusa* with minimum length of 0 and maximum length of 1; the branch is

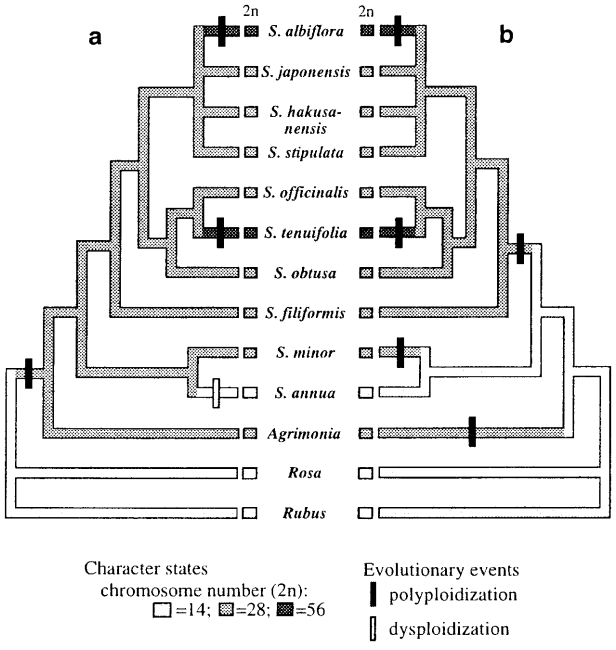

Fig. 4a, b Possible state assignments of chromosome numbers on one of the most parsimonious trees based on *mat*K sequence data for *Sanguisorba* and related taxa. The two hypotheses of polyploidization events are shown. **a** The single polyploidization hypothesis. Ancestral state of *Sanguisorba* is 2*n*=28. **b** The triple polyploidization hypothesis. Ancestral state of *Sanguisorba* is 2*n*=14. One additional evolutionary change is needed for the triple polyploidization hypothesis

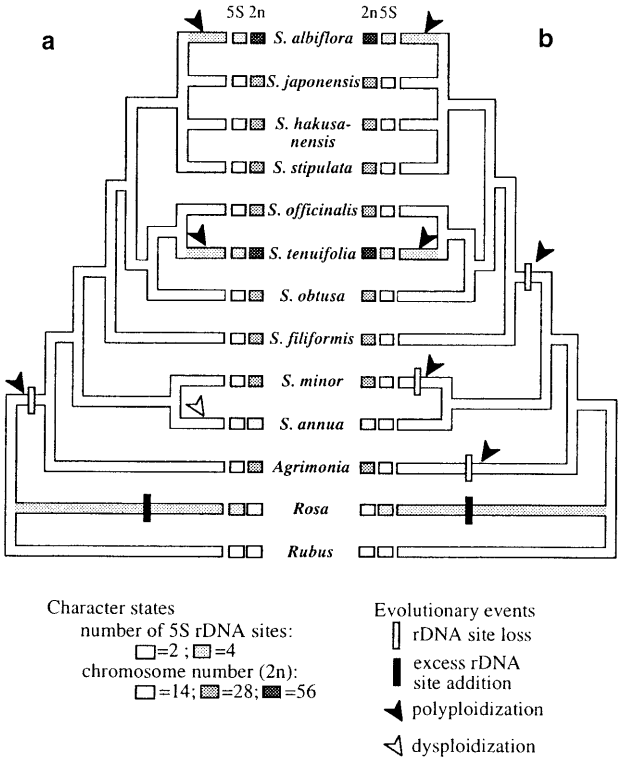

Fig. 5a, b Possible state assignments of 5S rDNA site number on one of the most parsimonious trees based on *mat*K sequence data for *Sanguisorba* and related taxa. The loss or addition of sites unaccompanied by polyploidization is marked as an evolutionary event. **a** Evolutionary events are plotted on the tree corresponding to the single polyploidization hypothesis (Fig. 4a). **b** Evolutionary events are plotted on the tree corresponding to the triple polyploidization hypothesis (Fig. 4b). The single polyploidization hypothesis requires fewer changes in site number than the triple polyploidization hypothesis

collapsed when we choose the parsimony option "collapse branches if minimum length is zero". The difference between the trees, however, had no influence on character trace in the number of chromosomes or of rDNA site. The tree length was 214, the CI was 0.888, and the RI was 0.798.

Among 2*n*=28 species, *S. minor* is sister to the only 2*n*=14 species, *S. annua*. The other polyploid plants form a monophyletic group that is sister to the clade of *S. minor* and *S. annua*. The tree topology agreed with that of Mishima and Ito (1996) based on PCR-RFLPs (restriction fragment length polymorphisms) of chloroplast DNA, except for the positions of *S. albiflora* and *S. obtusa*.

Optimization of character states on the tree

Polyploidization

In the most parsimonious reconstruction of chromosome number evolution, polyploidization from 2*n*=14 to 2*n*=28 may have occurred once in the common ancestor of *Agrimonia* and *Sanguisorba* (Fig. 4a). We call this the single polyploidization hypothesis. In this hypothesis, further polyploidization events from 2*n*=28 to 2*n*=56 occurred independently in *S. tenuifolia* and *S. albiflora*, and dysploidization from 2*n*=28 to 2*n*=14 occurred in *S. annua*. A different hypothesis permits one additional change in chromosome number (Fig. 4b). We call it the triple polyploidization hypothesis. In this hypothesis, polyploidization from 2*n*=14 to 2*n*=28 occurred three times independently in *Agrimonia*, *S. minor*, and the common ancestor of the other *Sanguisorba* species with 2*n*=28. Further polyploidization from 2*n*=28 to 2*n*=56 occurred in *S. tenuifolia* and *S. albiflora*, as in the first hypothesis.

Number of 5S rDNA sites

In the most parsimonious reconstruction, the ancestral number of 5S rDNA sites is two, and the four-site state of 5S rDNA originated from the ancestral state three times, in *Rosa*, *S. tenuifolia*, and *S. albiflora* (Fig. 5). The two hypotheses for the evolutionary history of polyploidization presented in Fig. 4 result in two different hypotheses for evolutionary changes in 5S rDNA sites. Based on the single polyploidization hypothesis (Fig. 4a), the duplicated 5S rDNA sites were lost in a common ancestral tetraploid of *Agrimonia* and *Sanguisorba* (Fig. 5a). When we follow the triple polyploidizat-

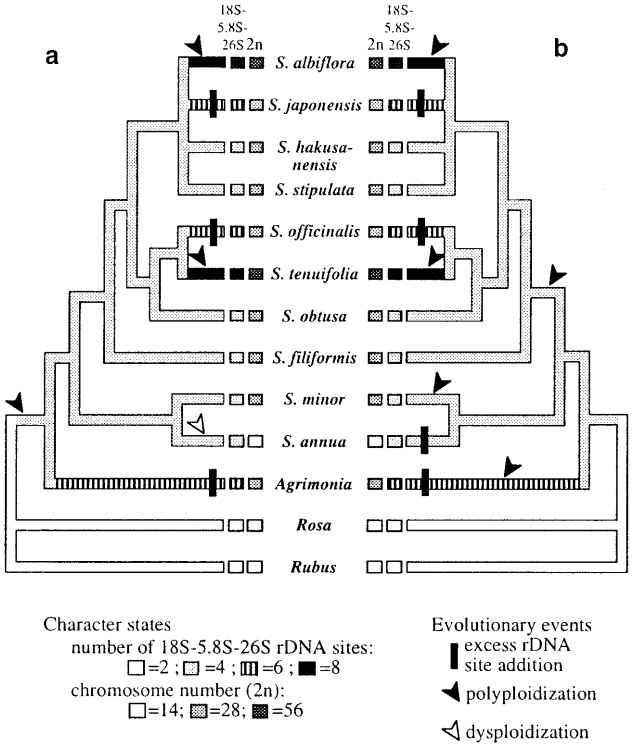

Fig. 6a, b Possible state assignments of 18S-5.8S-26S rDNA site number on one of the most parsimonious trees based on *mat*K sequence data for *Sanguisorba* and related taxa. The loss or addition of sites unaccompanied by polyploidization is marked as an evolutionary event. **a** Evolutionary events are plotted on the tree corresponding to the single polyploidization hypothesis (Fig. 4a). **b** Evolutionary events are plotted on the tree corresponding to the triple polyploidization hypothesis (Fig. 4b). The single polyploidization hypothesis requires fewer changes in site number than the triple polyploidization hypothesis

ion hypothesis (Fig. 4b), the duplicated 5S rDNA sites were lost three times independently in *Agrimonia*, *S. minor*, and the common ancestor of the other *Sanguisorba* species with 2*n*=28 (Fig. 5b). In both hypotheses, four sites evolved in *S. tenuifolia* and *S. albiflora*, corresponding to polyploidization from 2*n*=28 to 2*n*=56, while evolution of four sites in *Rosa* occurred in a homoploid state.

Number of 18S-5.8S-26S rDNA sites

In the common ancestor of *Sanguisorba* and *Agrimonia*, the number of 18S-5.8S-26S rDNA sites increased from the ancestral two-site state to the four-site state (Fig. 6). In *S. japonensis* and *S. officinalis*, both with 2*n*=28, six sites evolved independently. Further duplications from four sites to eight sites coincided with polyploidization in *S. tenuifolia* and *S. albiflora*. As for 5S rDNA, two hypotheses can account for evolutionary changes in 18S-5.8S-26S rDNA sites. Under the single polyploidization hypothesis (Fig. 4a), the 18S-5.8S-26S rDNA sites were duplicated once in the common ancestral tetraploid of *Agrimonia* and *Sanguisorba*, and these sites were conserved after dysploidization in *S. annua* (Fig. 6a). Under the triple polyploidization hypothesis (Fig. 4b), the 18S-5.8S-26S rDNA sites were duplicated four times independently; three duplications in *Agrimonia*, *S. minor*, and the common ancestor of the other *Sanguisorba* species with 2*n*=28 were accompanied by polyploidization, while the fourth duplication in *S. annua* occurred in the homoploid state (Fig. 6b). In both hypotheses, further site addition in the homoploid state occurred in 2*n*=28 plants of *S. japonensis* and *S. officinalis* (Fig. 6).

Discussion

Evolutionary trends in rDNA site number change in *Sanguisorba*

The evolutionary tendency for change in site number differs between 5S and 18S-5.8S-26S rDNA in *Sanguisorba*. The 5S rDNA sites duplicated by ancestral polyploidization were lost once in the single polyploidization hypothesis (Fig. 5a), or three times in the triple polyploidization hypothesis (Fig. 5b). In both hypotheses, there was no extra site addition of 5S rDNA except for *Rosa*, and all site duplications were associated with terminal polyploidization. Unlike 5S rDNA sites, 18S-5.8S-26S rDNA sites duplicated by polyploidization were all conserved after each polyploidization event in both hypotheses. Moreover, there were further additions of 18S-5.8S-26S rDNA sites in both hypotheses (Fig. 6). These results indicate that the duplicated and/or added sites of 5S rDNA tend to be eliminated, whereas those of 18S-5.8S-26S rDNA tend to be conserved. Therefore, the tendency for elimination of redundant sites in rDNA families suggested by Dvorák (1990) holds only in specific cases, for example, for 5S rDNA sites in *Sanguisorba*.

The same phenomena, elimination of 5S rDNA sites and conservation of 18S-5.8S-26S rDNA sites, can be estimated for another Rosaceae species, *Malus ×domestica* (2*n*=34). It is a member of the monophyletic Maloideae, with an ancient origin by auto- or allo-polyploidization (Grant 1971; Morgan et al. 1994; Evans et al. 2000). *Malus ×domestica* has two 5S rDNA sites (Schuster et al. 1997); at least four 5S rDNA sites could be expected if this species was polyploid in origin. In contrast, eight 18S-5.8S-26S rDNA sites were detected (Schuster et al. 1997). Thus, the tendency for site elimination in 5S rDNA and site conservation in 18S-5.8S-26S rDNA revealed in this study may be common to polyploids in Rosaceae.

Ploidy evolution in *Sanguisorba*

As a result of phylogenetic reconstruction, two hypotheses are proposed for polyploid evolution in *Sanguisorba*: the single polyploidization hypothesis (Fig. 3a), and the triple polyploidization hypothesis (Fig. 3b). Both hypotheses support the premise that the 2*n*=28 species of

Sanguisorba are of polyploid origin. However, the two hypotheses suggest remarkably different scenarios for the origin in *S. annua* (2*n*=14); this state represents the ancestral diploid condition in the triple polyploidization hypothesis, but is a secondary state reduced from 2*n*=28 in the single polyploidization hypothesis.

Two lines of evidence support the single polyploidization hypothesis. First, the single polyploidization hypothesis requires consistently fewer evolutionary changes than the triple polyploidization hypothesis; four (single polyploidization hypothesis) compared with five (triple polyploidization hypothesis) changes in chromosome number (Figs. 4), two (single polyploidization hypothesis) compared with four (triple polyploidization hypothesis) changes in 5S rDNA site number (Fig. 5), and three (single polyploidization hypothesis) compared with four (triple polyploidization hypothesis) in 18S-5.8S-26S rDNA site number (Fig. 6). Second, *S. minor*, with 2*n*=28, and *S. annua*, with 2*n*=14, form a clade and share the same number of 5S and 18S-5.8S-26S rDNA sites (two and four, respectively). These site numbers are ancestral states in *Sanguisorba*. If *S. annua* is an ancestral diploid and *S. minor* is a derived tetraploid, *S. minor* would be expected to have four 5S rDNA sites and eight 18S-5.8S-26S rDNA sites. This expectation does not agree with the observation in *S. minor* of two 5S sites and four 18S-5.8S-26S sites.

Chromosome number reduction to exactly half of the putative base chromosome number for *Sanguisorba* (*x*=14) in *S. annua* can be explained by a series of dysploidization events through some states of intermediate chromosome numbers. Such dysploid reduction does not always result in a reduction of rDNA sites, as shown in *Arabidopsis* (The Arabidopsis genome initiative 2000) and *Brachyscome* (Adachi et al. 1997). *S. annua* shows 5S rDNA signals at terminal positions on chromosome arms (Fig. 1b), while all of the other species show 5S rDNA signals at subtelomeric positions (Fig. 1a, c–e). The unique position of 5S rDNA sites in *S. annua* may be a consequence of chromosome rearrangements, although further cytological study is needed.

Dysploid reduction is a common phenomenon in flowering plants and is well known in the Campanulaceae (Stace and James 1996), Compositae (Baldwin 1993; Cerbah et al. 1998; Watanabe et al. 1999), Gentianaceae (Yuan and Kupfer 1997), Iridaceae (Goldblatt and Takei 1997), and Rutaceae (Stace et al. 1993). In Rosaceae, dysploid reduction from *n*=17 to *n*=15 is known in *Vauquelinia* (Morgan et al. 1994), although this is the only known example in the family. Reductions in chromosome number to exactly or almost half or less than half of the ancestral chromosome number have been reported in some genera: 2*n*=8 to 2*n*=4 in *Haplopappus* (Ikeda 1987), 2*n*=26 to 2*n*=14 in *Gentiana* (Yuan and Kupfer 1997), 2*n*=36 to 2*n*=14 in Rutaceae (Stace et al. 1993), 2*n*=18 to 2*n*=4 in *Brachyscome* (Watanabe et al. 1999), 2*n*=24 to 2*n*=8 in *Stevia* (Watanabe et al. 2001), and so on.

Furthermore, drastic dysploid reduction is known to be associated with the change from perennial to annual (reviewed in Grant 1971; Watanabe et al. 1999). Notably, *S. annua* is the only annual species in *Sanguisorba*. Thus, dysploid reduction to 2*n*=14 in *S. annua* may be associated with the evolution of the annual habit.

The overall evidence mentioned above supports the hypothesis that a single polyploidization occurred in the common ancestor of *Agrimonia* and *Sanguisorba*, and that 2*n*=14 in *S. annua* is a result of dysploid reduction.

Triple polyploidization, however, cannot be ruled out by the evidence that is available at present. Ancestral 2*n*=14 species might be extinct both in *Sanguisorba* and *Agrimonia*, and the existence of four sites of the 18S-5.8S-26S rDNA in *S. annua* might be explained by gene duplication and translocation in the diploid level. Further karyological evidence will be needed in order to determine which hypothesis best explains the ploidy evolution of *Sanguisorba*.

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