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Physical mapping of ribosomal RNA genes in the genus *Artemisia* L. (Asteraceae)

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Abstract — The fluorescence *in situ* hybridization (FISH) using 5S and 18S rDNA probes was applied to four species of the genus *Artemisia.* In all four *Artemisia* species, 5S and 18S rDNA were located in the terminal position. Moreover, all 18S rDNA sites were the same number and collocated with 5S rDNA. The collocation of the 5S and 18S rDNA might be a common feature of chromosome evolution in the genus *Artemisia*. In section *Dracunculus* have eight and nine rDNA sites while section *Artemisia* has four rDNA sites. Despite the polyploidy had been observed, the number of rDNA sites were relatively constant within the section *Dracunculus* and *Artemisia* respectively.

Key words: *Artemisia*, *Dracunculus*, FISH, polyploidization, rDNA.

INTRODUCTION

The genus *Artemisia* L. (Asteraceae) is the largest genus in the tribe Anthemideae (Asteraceae), and is consisted of more than 200 to 500 species, according to various authors (MCARTHUR 1978; MABBERLEY 1990; LING 1991a;b; BREMER and HUMPHRIES 1993; VALLÉS and SILJAK-YAKOVLEV 1997; TORRELL *et al.* 1999; TORRELL and VALLÉS 2001). This genus is widely distributed in the Northern Hemisphere, but is rather rare in the Southern Hemisphere (MCARTHUR and SANDERSON 1999; TORRELL and VALLÉS 2001; KREITSCHITZ and VALLÉS 2003). Many chromosome numbers have been reported in the genus (MATSUURA and SUTO 1935; SAKAI 1935; SHIMOTOMAI 1946; SUZUKA 1950; 1952; MASUMORI 1961; ARANO 1962; 1964; KAWATANI and OHNO 1964; SHARMA and SARKAR 1968; BOLKOHOVSKIKH *et al.* 1969; ROUSI 1969; BAKSHI and KAUL 1984; MALAKHOVA 1990; MCARTHUR and SANDERSON 1999; HOSHI *et al.* 2003). The most common basic chromosome number of the genus is x = 9, with ploidy levels up to dodecaploid (SHIMOTOMAI 1946; SUZUKA 1952; KAWATANI and OHNO 1964; MALAKHOVA 1990). In the genus *Artemisia,* polyploidy has played a major role in the karyological evolution, however, their relationships and phylogeny are unclear (ARANO 1962; KAWATANI and OHNO 1964).

One of the most important roles in the evolution of higher plants is polyploidy, which have been estimated to be 70% of all angiosperm species (STEBBINS 1971; PAMELA and DOUGLAS 2000). Thus, the study of chromosome numbers has provided essential information to examine phylogenetic relationships and speciation in higher plant. On the other hand, since the inter- and intraspecific similarity of chromosome conformation of this genus, it is difficult to clarify and justify the systematic relationships using karyotypes (MASUMORI 1961; ARANO 1962; 1963; 1965; 1970; HOSHI *et al.* 2003).

Fluorescence *in situ* hybridization (FISH) with rDNA is a useful marker for chromosome identification, which provides the phylogenetic information of closely related taxa (*e.g.,* ANSARI *et al.* 1999; SINGH 2001). In plant, FISH for ribosomal RNA genes have been widely used for karyotyping (LEITCH and HESLOP-HARRISON 1993; MATOBA *et al.* 2001; RAINA *et al.* 2001; NAVRÁTILOVÁ *et al.* 2003), and for studying genome organization and chromosome evolution within many genera (ALI *et al.* 2000; ANAMTHAWAT-JÓNSSON and BÖDVARSÓTTIR 2001; HASTEROK *et al.* 2001; RAINA *et al.* 2001; RAN *et al.* 2001; SINGH *et al.* 2001; LI and ZHANG 2002). Recently, TORRELL *et al.* (2001; 2003) have reported the number of 5S

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and18S rDNA loci in some species of *Artemisia*, but they did not refer on the Eurasian species.

In this study, we investigated the first time the 5S and 18S rDNA on the chromosomes in four species of the genus *Artemisia.* The aim of the present study was to investigate the number and loci of 5S rDNA and 18S rDNA using FISH, and consider the mechanism of chromosomal evolution in the genus *Artemisia*. Moreover, we investigated whether distribution of 5S and 18S rDNA played a role in phylogenetic relationships among taxa in the genus *Artemisia*.

MATERIALS AND METHODS

Plant materials and chromosome preparation - Materials for the present study were collected in Japan and Russia, and were cultivated in the Department of Plant Science, Kyushu Tokai University (Table 1). The seedlings were pretreated with 2 mM 8-hydroxyquinoline at room temperature for 3 h before they were fixed in ethanol-acetic acid (3:1) at 4°C for 24 h. Chromosomal preparations were prepared by the modified method of LEITCH and HESLOP-HARRISON (1993). Fixed seedlings were washed three times in distilled water and their root-tips were cut into 2-3 mm long. Ten to thirty root-tips were placed in a microcentrifuge tube containing 500 µl of enzyme solution (1% Cellulase Onozuka-RS (Yakult Honsha) and 0.5% Pectolyase Y-23 (Kikkoman), pH 4.2) and incubated at 37°C for 1 h. They were washed three times with distilled water. Ten to 20 µl of the fixative was dropped onto root-tip and air-dried.

PCR amplification of 5S and 18S ribosomal DNA - Total genomic DNA was extracted according to the method of SHAW (1988) from young growing leaves of *A. annua*. PCR amplification of 5S and 18S rDNA followed the procedure of HIZUME (1993) and SOGIN (1990), respectively. The sequences of the primers were as follows: 5'-CGGTGCATTAATGCTGGTAT-3' and 5'-CCATCAGAACTCCGCAGTTA-3' for the repeating units in 5S rRNA gene clusters, and 5'-AACCTGGTTGATCCTGCCAGT-3' and 5'-TGATCCTTCTGCAGGTTCACCTAC-3' for the 18S rRNA coding regions. PCR reaction mixture (total 50 µl) contained 60 ng of template DNA, 200 pmol of each primer, 0.1 mM of each dNTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 1 unit of Taq polymerase (TOYOBO). Amplification was performed in a T1 Thermocycler (Biometra) programmed for one cycle of 4 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 60 sec at 72°C, and 1 cycle of 5 min at 72°C.

Fluorescence in situ *hybridization (FISH)* - The 5S and 18S rDNA probes amplified by PCR were labeled by means of random primer DNA labeling with digoxgenin-dUTP (Takara) and nick translation with biotin-14-dATP (invitorogen), respectively, following the supplier's instructions. Labeled DNA probes were denatured at 95°C for 10 min. Hybridization mixtures contained 50% formamide, 10% dextran sulfate and each probe at a concentration of 4 ng/µl in 2xSSC. 25 µl of hybridization mixture was put on a chromosomal preparation and covered with a cover slip and sealed with rubber gum. The slides were denatured at 80°C for 3 min on a hot plate and then incubated overnight at 37°C in a humid chamber. After overnight incubation, coverslips were floated off in 2 x SSC and slides were rinsed in 2 x SSC at 42°C for 10 min, 0.2 x SSC at 42°C for 10

Table 1 — Six taxa of *Artemisia* in Eurasia studied.

Taxon	Location	Chromosome numbers		Reference
		Present count (2n)	Previous count (2n)	
Sect. Dracunculus				
A. capillaris	Japan: Amakusa-city, Kumamoto Pref. Japan: Yamaguchi Pref.	18	16, 18, 27	Mendelak and Schweizer, 1986; Qiao *et al.*, 1990: Kawano *et al.*, 1995
A. japonica	Japan: Ooita-gun, Ooita Pref. Japan: Minamiaso-gun, Kumamoto Pref. Russia: Vladivostok	36 36 36	18, 36	Volkova and Boyko, 1985; Volkova and Boyko, 1986; Nishikawa, 1984; Hoshi *et al.* 2003
Sect. Artemisia				
A. annua	Japan: Nagasaki Pref.	18	18	Volkova and Boyko, 1986
A. stolonifera	Japan: Ooita-gun, Ooita Pref. Japan: Minamiaso-gun, Kumamoto Pref. Russia: Vladivostok	36 36 36	36	Volkova and Boyko, 1985; Hoshi *et al.* 2003

min, and 2 x SSC/0.2% Tween 20 at room temperature for 10 min twice. Slides were blocked with 5% (w/v) bovine serum albumen in 2 x SSC/0.2 % Tween 20 for 10 min at room temperature. Signal was detected with 100 µl of 5 µg/ml anti-digoxigenin-rhodamine (Roche) and 2 µg/ml avidin-FITC (Roche) in 2 x SSC in each slide for 1 h at 37°C in a humid chamber. Slides were washed in 2 x SSC/0.2 % Tween 20 for 10 min twice, 2 x SSC at room temperature. The slides were then mounted in Vectashield Mounting Medium (Vector Lab.) containing 500 ng/ml of 4',6-diamidino-2-phenylinodole (DAPI).

RESULTS

Results showed that the basic chromosome number of all taxa was x = 9 (Table 1). Two species are diploid ($2n$ = 18) and the two remaining were tetraploid ($2n$ = 36). These chromosome numbers were confirmed previous reports (Table 1). Chromosomes were numbered in decreasing order of length.

Figure 1 showed the results of FISH of the taxa studied. FISH with biotin labeled 18S rDNA (detected with avidin-FITC) and digoxigenin labeled 5S rDNA (detected with anti-DIG-rhodamine) revealed chromosome specific signal patterns. In all four *Artemisia* species studied, 5S and 18S rDNA were located in the terminal position (Fig. 1). Moreover, all 18S rDNA sites are the same number and collocated with 5S rDNA (Table 2, Fig. 1C, F, I and L). Intraspecific rDNAs variations was not observed (Table 2).

Diploid *A. capillaris* had nine rDNA sites on the median centromeric chromosomes, and seven of them were major and the remaining two were minor (Table2, Fig. 1A, B and C). Two of the hybridization signals were minor, each at end of short arms of chromosome 2 and 3. Two major sites were located at the terminal positions of the long arms of chromosome 13 and 14. The other five were located on the short arms of chromosome 1, 9, 10, 11, and 12.

Eight rDNA signals were observed in the chromosomes of *A. japonica*. Six sites are located at the end of short arms of median centromeric chromosome 7, 8, 15, 16, 28 and 29, and the other two signals are at the end of short arms of submedian centromeric chromosome 22 and 23 (Table 2, Fig. 1D, E and F).

In *A. annua*, four sites were found in the chromosomes (Table 2, Fig. 1G, H and I). Two sites were located at the end of short arms of submedian centromeric chromosome 14 and 15, and the other two ones were at the end of short arm subterminal centromeric chromosome 11 and 12.

Four rDNA signals were observed on the chromosomes of *A. stolonifera* (Table 2, Fig. J, K and L). Two sites were located at the end of short arms of submedian centromeric chromosome 31 and 32, and the other two ones were at the end of short arms of chromosome 35, 36.

DISCUSSION

Several investigations have reported the chromosome number, and karyotypes have been applied to identify intra- and interspecific variation and to evaluate the phylogenetic relationships among several species of the genus *Artemisia* (Masumori 1961; Arano 1962; 1963; 1964; 1965; 1970; Kawatani and Ohno 1964). However, the inter- and intraspecific chromosome is difficult to distinguish by their length and centromere position because of similar chromosome size, and

Table 2 — Number of 18S and 5S rDNA sites.

Taxon	Location	2n	Ploidy level	18S rDNA		5S rDNA		Collocated	
				No.	%	No.	%	No.	%
Sect. Dracunculus									
A. capillaris	Japan: Amakusa-city, Kumamoto Pref.	18	2x	9	50,0	9	50,0	9	50,0
	Japan: Yamaguchi Pref.	18	2x	9	50,0	9	50,0	9	50,0
A. japonica	Japan: Ooita-gun, Ooita Pref.	36	4x	8	22,2	8	22,2	8	22,2
	Japan: Minamiaso-gun, Kumamoto Pref.	36	4x	8	22,2	8	22,2	8	22,2
	Russia: Vladivostok	36	4x	8	22,2	8	22,2	8	22,2
Sect. Artemisia									
A. annua	Japan: Nagasaki Pref.	18	2x	4	22,2	4	22,2	4	22,2
A. stolonifera	Japan: Ooita-gun, Ooita Pref.	36	4x	4	11,1	4	11,1	4	11,1
	Japan: Minamiaso-gun, Kumamoto Pref.	36	4x	4	11,1	4	11,1	4	11,1
	Russia: Vladivostok	36	4x	4	11,1	4	11,1	4	11,1

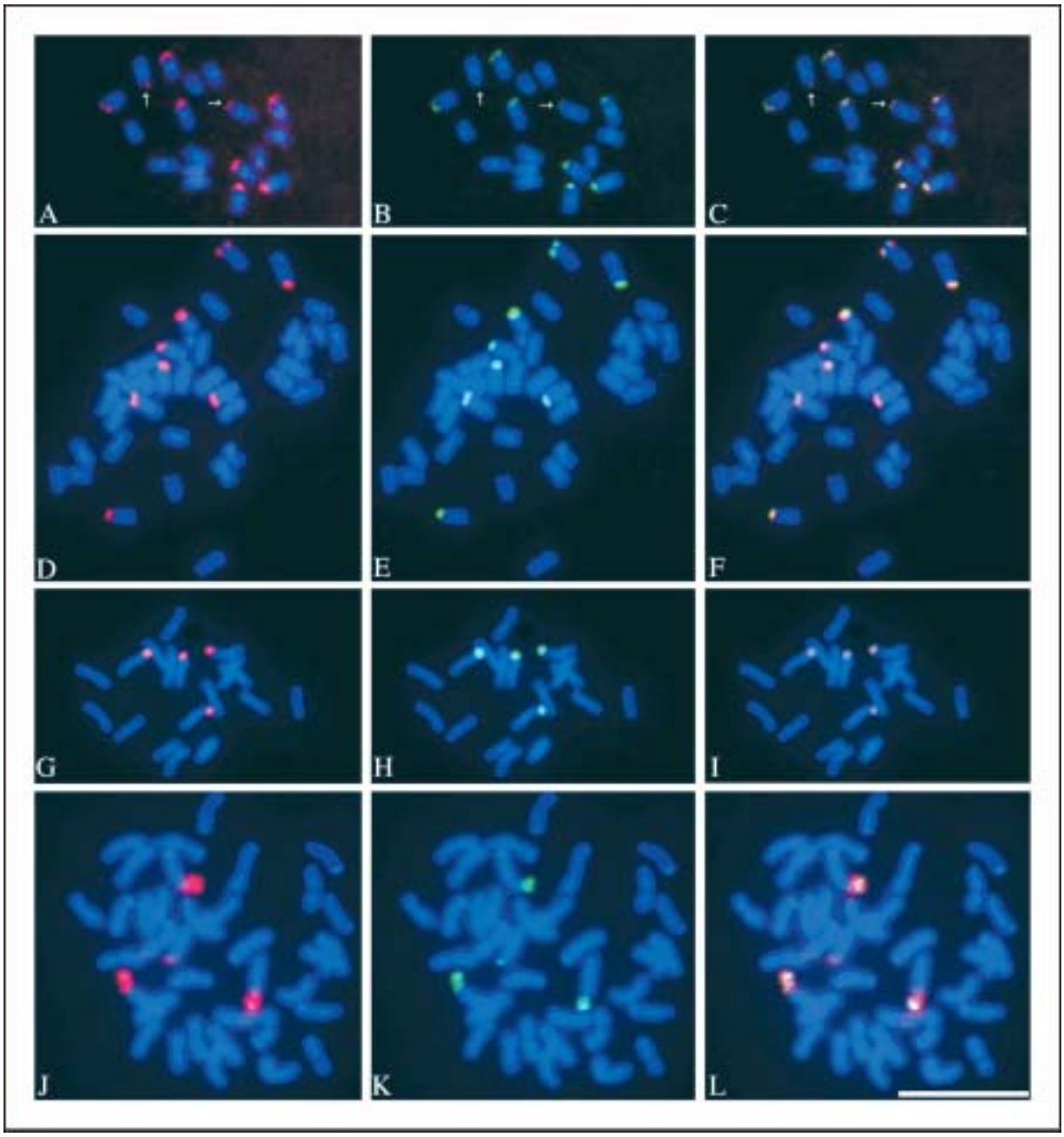

Fig. 1 — Somatic metaphase chromosomes of *Artemisia capillaris* (A, B and C), *A. japonica* (D, E and F), *A. annua* (G, H and I) and *A. stolonifera* (J, K and L). Localization of 5S rDNA in red when digoxigenin-labelled (A, D, G and J), 18S rDNA in green when biotin-labelled (B, E, H and K), and simultaneous detection of 5S and 18S rDNA (C, F, I and L). Chromosomes were counter stained with DAPI. Arrows indicate minor rDNA loci. Scale bar represents 10 μm for A to L.

identification by karyotypes is sometime unreliable (Hoshi *et al.* 2003).

Our FISH results showed that all four *Artemisia* species, 5S and 18S rDNA were located in the terminal position (Fig. 1). Moreover, all 18S rDNA sites were the same number and collocated with 5S rDNA (Table 2, Fig. 1). Intraspecific rDNAs variations were not observed (Table 2). In higher eukaryotes, generally 5S ribosomal DNA was unlinked to the 18S to 25S ribosomal DNA (Ansari *et al.* 1999). In Asteraceae, the independently distribution of 5S and 18S rDNA loci on chromosomes had been observed in several species and its relatives (Matoba *et al.* 2001; 2005; Ruas *et al.* 2005). Recently, Torrell *et al.* (2003) had reported the number and loci of 18S rDNA in

some *Artemisia* species, and 5S rDNA sites were the same or a lower number and collocated with 18S rDNA. The collocation of the 5S and 18S rDNA might be a common feature of chromosome evolution in the genus *Artemisia*.

Our FISH results showed that there were nine, eight, four and four rDNA sites on the chromosomes of *A. capillaris*, *A. japonica*, *A. annua* and *A. stolonifera* respectively. In *A. capillaris*, nine hybridization sites were observed, and seven of them were major and the remaining two were minor (Fig. 1A, B and C). Fluorescence *in situ* hybridization was not a fully quantitative technique, but the intensity of signals provided approximate information concerning the copy number of gene (Appels *et al.* 1980; Maluszynska and Heslop-Harrison 1991). The existence of two minor rDNA loci could be presumed that the translocation of rDNA from one chromosome bearing rDNA loci to another chromosome. A similar phenomenon was reported in other species (Gu and Xiao 2003; Zhang and Sang 1999; Hanson *et al*. 1996), and especially the telomeric location of rDNA loci was likely to cause this phenomenon (Zhang and Sang 1999; Hanson *et al*. 1996). Perhaps, the minor sites of the chromosome 2 and 3 in *A. capillaris* were considered that translocation occurs from chromosome 2 to chromosome 3.

In some genera, the number of rDNA loci correlate with the process of polyploidization (Calderini *et al*. 1996). Polyploidy, up to 12x ($2n$ = 108), have been recognized as common phenomenon in the genus *Artemisia* (Kawatani and Ohno 1964; Sharma and Sarkar 1968; Bolkhovskikh *et al*. 1969; Rousi 1969; Bakshi and Kaul 1984; Volkova and Boyko 1985; Malakhova 1990; McArthur and Sanderson 1999; Hoshi *et al*. 2003). Nine and four rDNAs sites were distributed in the chromosomes of diploid *A. capillaris* and *A. annua* respectively (Fig. 1C and I). On the chromosomes of tetraploid *A. japonica* and *A. stolonifera*, there were eight and four rDNAs sites respectively. The low loci numbers of rDNA site found in tetraploid *A. stolonifera* seem to contradict the hypothesis of the polyploidization. On the contrary, the highest numbers of rDNA sites found in diploid species *A. capillaris* (Fig. 1C). Thus, the results of this study indicated that the number of rDNA sites did not reflect the polyploid complex of the genus *Artemisia*.

According to Watson *et al*. (2002), the internal transcribed spacers (ITS) of nuclear ribosomal DNA phylogeny suggested an early divergence of subg. *Dracunculus* from the remaining *Artemisia* subgenera. In diploid species, *A. capillaris* (sect. *Dracunculus*) has nine rDNA sites while *A. annua* (sect. *Artemisia*) has four rDNA sites. Possible explanation for this phenomenon is loss of some rDNA or fusion with other rDNA sites in the evolutionary process (Thomas *et al.* 1997; Snowdon *et al*. 1997). In either event, there has been decrease in number of rDNA sites during evolutionary process of the genus *Artemisia*.

In section *Dracunculus* have eight and nine rDNA sites while section *Artemisia* has four rDNA sites. Despite the polyploidy was observed, the number of rDNA sites were relatively constant within the section *Dracunculus* and *Artemisia* respectively. After polyploidization, elimination of rDNA might be occured. For example, it has been known that the rDNA sites were eliminated from additional genome (Calderini *et al*. 1996; Thomas *et al*. 1997). Thus, it seems that the number of rDNA loci did rely on the polyploidization, and the number was kept at the section level. In view of this feature, we proposed that there were distinct differentiation process of rDNA between sect. *Dracunculus* and sect. *Artemisia*.

In this study, we demonstrated that the number and loci of 5S and 18S rDNA were associated with the evolutionary process of the genus *Artemisia*. ITS data indicated that some "*Absinthium*" species had associated or been embedded within "*Artemisia*" (Torrell *et al*. 1999; Watson *et al.* 2002). Further research will be needed to obtain a more obvious view of the relationships among sect. *Artemisia*, sect. *Dracunculus* and sect. *Absinthium*.

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