Characterization of chromosome complement in *Tridactylina kirilowii* (Turcz. ex DC.) Schultz-Bip. by aceto-orcein, CMA, DAPI and FISH

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**ABSTRACT.** *Tridactylina kirilowii* (Turcz. ex DC.) Schultz-Bip., the Anthemideae Cass., Asteraceae displayed the chromosome number of 2n=18 for the first time. The chromosomes of the species at mitotic metaphase were characterized by chromosome size, centromeric position, distribution of the G-C and A-T heterochromatin-rich regions by aceto-orcein, CMA, DAPI, and FISH. The smallest chromosome at mitotic metaphase was approximately 4.1±0.5 µm, while the largest chromosome was 6.9±0.7 µm. The total length of the chromosome complement at mitotic metaphase was 97.5±7.9 µm. The chromosome complement at mitotic metaphase showed 16 median-centromeric chromosomes and two submedian-centromeric chromosomes. The CMA stained the whole chromosomes, and showed four brighter bands on the terminal positions of four chromosomes and were convenient with the four sat-chromosomes. DAPI stained the whole set of the chromosomes at mitotic metaphase, while CMA-DAPI positively, brightly stained some heterochromatin regions in the chromosomes at mitotic prometaphase. Applying FISH using the digoxigenin labeled pTa71 probe of 45S rDNA to sets of the mitotic chromosomes showed four red colored signals that were located at the terminal region displayed after hybridization of the probe with four median chromosomes at mitotic metaphase.

**KEYWORDS:** Chromosome, CMA, DAPI, FISH, *Tridactylina kirilowii*

*Tridactylina* (Turcz ex DC.) Schultz-Bip. is monotypic with *T. kirilowii* (Turcz. ex DC.) Schultz-Bip. in the Asteraceae and endemic to Mts. Yaklonoy, east of Lake Baykal, in Buryat Republic, East Siberia, Russian Federation. The species is closely related to *Chrysanthemum* L. (Tzvelev in Flora of USSR in Komarov 1961) but is distinct by an annual habit. Its leaves are alternate and a few lobed, and its capitula are laxly corymbose and radiate. It has involucral bracts with dark brown margins and receptacle flat to convex, epaleate. Its ray florets are neuter and its limb is yellow. The species is similar for example in foliage to some species of *Chrysanthemum* and *Arctanthemum*. The subtribe Artemisiinae included the probable, more plesiomorphic relatives of the *Artemisia* group, which in previous classifications were hidden within a broad concept of *Chrysanthemum* (Bremer and Humphries 1993). These relatives include the radiate genera *Brachanthemum*, *Chrysanthemum*, *Arctanthemum* and *Tridactylina* with solitary or laxly corymbose capitula, and the disciform *Ajania* and *Phaeostigma* with densely corymbose capitula. These genera have rather thin-walled and more or less faintly ribbed cypselas always without a pappus (Bremer and Humphries 1993).

An appreciation of the types of changes in chromosomes/ genomes that have occurred during species evolution, has a direct impact on the basic and applied botany and to the aims of plant researchers and breeders in understanding genome evolution and genetics makes this area worthy of further investigation (Heslop-Harrison, 2000).

Several genera and species in *Chrysanthemum sensu lato* distributed to China, Japan, Korean Peninsula, Russia and Mediterranean regions have been subjected to intensive chromosomal studies as early as the starting of the 20th century (e.g., Tahara 1915; Shimotomai 1933; Takemoto 1939; Dowrick 1952, 1953; Tanaka et al. 1987; Tanaka et al. 1989; Nakata et al. 1991; Kondo et al. 1992; Kondo and Tanaka 1996; Kondo and Abd El-Twab 2002; Abd El-Twab and Kondo 2003a). Those species were seemed to be closely related to each other and may make readily cross-hybrids. They performed a polyploid series, which plays an important role in chromosome evolution and would even perform introgressive hybridization in the nature (Kondo and Tanaka 1996). Studies on origin and chromosome constitutions of the native species of *Chrysanthemum* may contribute to satisfactory phylogenetic and taxonomic treatment of species relationships and breeding cultivars (Kondo and Tanaka 1996).

Fluorescent banding methods such as chromomycin A₁ (CMA) and 4-6-diamidino-2-phenylindole (DAPI) to determine the G-C (Guanine-Cytosine) and A-T (Adenine-Thymine) rich segments respectively along the chromosomes of various plants could provide more details and critical chromosomal data for phylogenetic relationships of species (Summer 1990; Kondo et al. 1995; Hizume et al. 1997). Therefore, the fluorochrome banding techniques have been employed to some chromosome complements of Japanese and Chinese species of

Fluorescence in situ hybridization (FISH) is another valuable method for studying the chromosomal distribution of DNA sequences and copy numbers at different sites, and to follow evolutionary changes in their physical organization in the genome (Harrison and Heslop-Harrison 1995, Abd El-Twab and Kondo 2003a). FISH makes chromosomes of plant species providing the information of molecular characters of nucleolar organizing region (NORs) (Heslop-Harrison et al. 1991, Abd El-Twab and Kondo 2003a). NOR loci are chromosome regions most intensively studied, in terms of both their structure and function. Molecular cytogenetic evidences indicate that 45S rDNA is located on NOR-bearing chromosomes (Kondo et al. 1996; Ørgaard and Heslop-Harrison 1994; Abd El-Twab and Kondo 2003a). Analyses of NOR distribution and organization in rDNA carrying chromosomes are important to understand the events of divergence and hybridization of species (Abd El-Twab and Kondo 2003a). FISH as Molecular cytogenetic approach to elucidate the species relationships among the Japanese Chrysanthemum species studied were reported the 45S rDNA as important molecular cytogenetic marker (Kondo et al. 1996). Thus, the 45S rDNA probe has applied intensively to mark NOR’s of satellites for identification of sat-chromosomes among the chromosome complements of several artificial hybrid plants (Kondo et al. 1995; Honda et al. 1997; Abd El-Twab and Kondo 1999, 2001, 2003a, 2004a, 2006a). Also the FISH 45S rDNA probe was used to study the genomic DNA interaction in the chromosome complements of several artificial hybrid plants (Kondo et al. 1999a; Kondo and Abd El-Twab 2002; Abd El-Twab and Kondo 2003b, 2004b, 2006b, 2008).

Based on Bremer and Humphries (1993), Tridactylina was considered a sister genus to Chrysanthemum (formerly Dendranthema) and Arctanthemum. Thus, Tridactylina is considered within the concept of Chrysanthemum sensu lato. The present research was conducted in order to identify the chromosome number and characterization of the chromosome complement of Tridactylina by aceto-orcein, CMA, DAPI and FISH.

**MATERIALS AND METHODS**

**Plant materials** Tridactylina kiriwii (Turcz. ex DC.) Schultz-Bip. was collected in Mts. Yablonoy, east of Lake Baycal, in Buryat Republic, East Siberia, Russian Federation, and cultivated in the Laboratory of Plant Genetics and Breeding Science, Department of Agriculture, Faculty of Agriculture, Tokyo University of Agriculture, Funako 1737, Atsugi City, Kanagawa Prefecture, Japan.

**Orcein staining** Somatic chromosomes of Tridactylina kiriwii were observed by the modified squash method of Tanaka (1959). Root tips were pretreated in 0.002 M 8-hydroxyquinoline at about 18°C for 2 h and fixed in 45% acetic acid for 10 min. Then, they were macerated in a mixture of 1 N HCl and 45% acetic acid (2:1) for 15 sec at 60°C, stained in 2% aceto-orcein for 20-30 min and squashed. Microphotographs were taken on CCD camera (Nikon: DS-Ri1) using Nikon Eclips 80i light microscope.

**Chromosome preparations** for CMA, DAPI and FISH Following Kondo et al. (1996) growing root tips were collected and pretreated in 0.002 M 8-hydroxyquinolin at 18°C for 1.5 h. They were fixed in the 1:3 of glacial acetic acid and ethanol at 4°C for 2 h. Fixed roots were excised and washed in distilled water many times to remove the fixative. Five to ten tips 2-5 mm long each from root tips were placed in 1.5 ml microcentrifuge tube containing the enzymic buffer of 5% cellulase (Yakult), 2% pectolyase Y-23 (Kikkoman). They were incubated at 37°C for 15 min, and then, their soft meristematic tissues were washed in distilled water to remove the enzymic solution and were squashed in 45% acetic acid. The cover-slip was removed by the dry-ice freezing method and the preparation was dried at room temperature.

**CMA fluorescent staining** The procedure of fluorescent staining with distamycin A (DMA) and chromomycin A3 (CMA) procedure followed Hizume et al. (1989) and Kondo et al. (1995) with slight modification: Air-dried slides were used for sequential DMA-CMA staining: The slides were preincubated in McIlvaine buffer (pH 7.0) for 30 min and treated with 0.2 mg/ml DMA (Sigma) in McIlvaine buffer for 10 min in a humid chamber; and after the coverslips were removed by pouring ion-free water, the slides were rinsed in the buffer containing 5mM MgSO₄ for 10 min. The slides were, then, stained with 0.1 mg/ml CMA (sigma) for 10 min and then, slides were rinsed in the buffer supplemented with 5mM MgSO₄ for 10 min. Finally slides were mounted in non-fluorescent glycerol and incubated at 4°C overnight to stabilize the fluorochromes for microscopic observation.

**DAPI fluorescent staining** Sequentially fluorescent staining with actinomycin D (AMD) and 4-6-diamidino-2-phenylindole (DAPI) followed Hizume et al. (1989) and Kondo et al. (1995). After DMA-CMA, staining and observation, the slides were destained in 45% acetic acid at room temperature for 45 min and air dried for 1 h. The slides were preincubated in the McIlvaine buffer for 30 min and treated with 0.25 mg/ml actinomycin D for 15 min and rinsed in the McIlvaine buffer for 10 min. They were stained with 0.2 mg/ml DAPI for 10 min. They were rinsed in the McIlvaine buffer for 10 min and were mounted in 1:1 buffer-glycerol mixture for microscopic observation.

**45S rDNA probe** The probe of pTa71 (45S rDNA) consisted of a 9 kb Eco RI fragment of rDNA derived from Triticum aestivum L. (Gerlach and Bedbrook 1979),
which was cloned into pUC19 plasmid. It was comprised of the coding sequence for the 18S, 5.8S and 26S genes and the non-transcribed spacer sequences.

**Labeling of the probe** The probe was labeled with digoxigenin (DIG)-dUTP by Dig DNA labeling kit (Boehringer Mannheim) labeling of the probe was carried out according to the manufacturer's protocol.

The procedure of FISH and detection of the probe followed the methods described by Abd El-Twab and Kondo (1999). FISH red-color was visualized by rhodamin after the hybridization with the digoxigin labeled probe and blue-color by the counter stain of DAPI.

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**RESULTS AND DISCUSSION**

The chromosome number of 2n=18 was counted in *Tridactylina kirilowii* for the first time. The chromosome characterization of *Tridactylina kirilowii* was studied and investigated the chromosome sizes, distribution of centromeric position, distribution of the G-C and A-T heterochromatin-rich regions by aceto orcein (Fig. 1 A-C and G), CMA (Fig. 1E and 2A), DAPI (Fig.1F and 2B) and FISH (Fig. 3). The smallest chromosome was approximately 4.1±0.5 µm and the largest chromosome was 6.9±0.7 µm, while the total length of the chromosome complement was 97.5±7.9 µm. The chromosome complement of the species consisted of 16 median-centromeric chromosomes and two submedian-centromeric chromosomes. It showed four bright CMA-bands at the terminal position of four chromosomes and was convenient with the four sat chromosomes. DAPI band was not clear on the metaphase chromosomes (Fig. 1F), while some heterochromatin CMA-DAPI bright-banded regions were seen on the prometaphase chromosomes (Fig. 2A-C).

FISH using the digoxigenin labeled pTa71 of 45S rDNA probe on the mitotic chromosomes showed four red colored signals that were obtained after hybridization of the probe with four median-centromeric chromosomes at mitotic metaphase (Fig. 3B and C). The interphase nucleus showed four signals and some times more than four signals (Fig. 3A), which might be a reason of NOR region extension due to gene activation of rDNA transcription in

![Fig. 1. Mitotic chromosomes of *Tridactylina kirilowii* stained with aceto-orcein (A-D and G), CMA (E) and DAPI (F). Bar=10 µm.](image-url)
Fig. 2. Prometaphase chromosomes of *Tridactylina kirilowii* stained with CMA (A), DAPI (B) and superimposed image of A and B (C) showing the bright regions of the heterochromatin after CMA and DAPI. Bar=10 µm.

Fig. 3. FISH signals of the 45S rDNA sites hybridized with digoxiginin labeled pTa71 probe on mitotic metaphase chromosomes in *Tridactylina kirilowii* (four red-signals). A. Interphase. B. Metaphase. C. Four enlarged metaphase chromosomes (from B) have the four red signals of the 45S rDNA sites. Red-color was visualized by rhodamin after the hybridization with the digoxiginin labeled probe and blue-color by the counter stain of DAPI. Bar=10 µm.
the interphase nucleoli. 

*Chrysanthemum* taxa usually shows variation of satellites in visible numbers per each chromosome complement at mitotic metaphases according to the orcein staining, probably due to either too much condensation of satellite by 8-hydroxyquinoline and buried satellite in staining, probably due to either too much condensation of satellite at mitotic metaphases according to the orcein staining method, but showed uniformly, exactly four sat-chromosomes by FISH using the pTa71 probe. 

Shimotoi (1933) suggested that only two or three diploid species might involve with establishment of the polyploid series of *Chrysanthemum* due to interspecific cross fertility as well as morphological and ecological evidences. *Chrysanthemum lavandulifolium* is distributed in China, Taiwan and Japan while *C. horaimontanum* is restricted to Taiwan (Kondo et al. 1999b). *Chrysanthemum boreale* is widely distributed in East Asia while *C. japonicum* is restricted to Japan. The presence or absence of the DAPI bands in several *Chrysanthemum* species have been studied (Khuang et al. 1995; Kondo et al. 1995). Among the diploid species reported by Khuang (1997) only *C. japonicum* showed clear DAPI bands that were terminals on the long arm of five chromosomes and the centromeric region on four chromosomes, while in *C. boreale* and *C. lavandulifolium* the whole chromosomes showed strong DAPI staining except the satellite regions that were negative staining. In the present study the whole metaphase chromosomes stained strongly with DAPI staining except that satellite regions that did not stained. The prometaphase chromosomes showed some positive CMA-DAPI staining bright regions that were not clear in the metaphase chromosome. The dark regions stained by aceto-orcein of the prometaphase chromosomes showed similar brightly stained regions by CMA-DAPI, those regions were considered heterochromatin G-C and A-T rich regions. Therefore, the CMA and DAPI staining could be much powerful tool if the prometaphase chromosomes were used to analyze in *Chrysanthemum sensu lato*. 

FISH hybridization signals showed that the physical distribution of the 45S rDNA sites or signals at the terminal-located, NOR regions on the sat-chromosomes. It was suggested that *C. lavandulifolium* might be more primitive than *C. boreale* (Tanaka et al. 1989), that was supported by the FISH data since *C. lavandulifolium* had eight FISH signals of the 45S rDNA (Khuang et al. 1997) while *C. boreale* had six signals (Honda et al. 1997). *Tridactylina kirilowii* showed 22.2% of frequency of the signal number/chromosome complement, that was similar to *C. japonicum* showed, while among the diploid species of *Chrysanthemum* *C. lavandulifolium* had the highest frequency of 44.4% and *C. horaimontanum* had the lowest frequency of 11.1% (Abd El-Twab and Kondo 2003a).

Comparing *Tridactylina kirilowii* with *C. japonicum*, *C. boreale*, *C. lavandulifolium* and *C. horaimontanum* (Tanaka 1959a, b; Khuang 1997; Kondo et al. 1999b) regarding their chromosome complements, the former species showed only a pair of submedian-centromeric chromosomes and the other eight pairs of chromosomes were commonly median-centromeric chromosomes, that was the most primitive karyotype among the diploid *Chrysanthemum sensu lato* according to the theory. With respect to the DAPI staining on the whole metaphase chromosomes, the chromosome complement of *Tridactylina kirilowii* was quite similar to the complement of *C. boreale* and *C. lavandulifolium* (Khuang 1997).

Chromosome mapping in well-diversed species showed that rDNA was largely confined to regions that were defined cytologically as heterochromatin (Lohe and Roberts 1988). In the present study the sites of the 45S rDNA were located at CMA-heterochromatin of G-C rich region. An especially striking property of satellite DNA was its apparent liability in evolution, both in a qualitative and quantitative sense. (Lohe and Roberts 1988). Physical distribution of FISH hybridization signals of 45S rDNA sites exhibited that *Tridactylina kirilowii* had four signals on four median-centromeric chromosomes.

Among the diploid taxa of *Chrysanthemum sensu lato* by using FGISH (Abd El-Twab 2002) and nrDNA molecular data (Masuda et al. 2009) *C. boreale*, *C. japonicum*, *C. horaimontanum* and *C. lavandulifolium* were seemed very closely related to each other. In contrast, the nrDNA molecular data suggested that *Tridactylina kirilowii* and diploid taxa of *Chrysanthemum sensu stricto* were distantly related to each other. Thus, it is necessary to make artificial cross-hybridization between *Tridactylina kirilowii* and the diploid taxa of *Chrysanthemum sensu stricto* to analyze their hybrid chromosomes by FISH and GISH for clarification and justification of their genetic relationships and concepts.

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