筑波実験植物園研報　24：63-69, 2005.
Ann. Tsukuba Bot. Gard.

Distribution Patterns of 45S Ribosomal DNA Sites on Somatic Chromosomes of three Subspecies of *Inula britannica* (Asteraceae) in Japan and Russia

Yukiko SAITO[1,2], Goro KOKUBUGATA[1,2], Katsuhiko KONDO[3], Irina V. TATARENKO[4] and Pavel V. KULIKOV[5]

齊藤由紀子[1,2]・國府方吾郎[1,2]・近藤勝彦[3]・Irina V. Tatarenko[4]・Pavel V. Kulikov[5]：

日本とロシアに産するオグルマ属３亜種（キク科）における
染色体上45S ribosomal DNA部位の比較

The genus *Inula* (tribe Inuleae, Asteraceae) is consisted of about 100 species, and is primarily distributed in the Eurasian Continent and Africa, extending to Japan (Kitamura 1981). *Inula britannica* L. is one of common species between Japan and the Eurasian Continent, and *I. britannica* subsp. *britannica* is widely distributed in the Eurasian Continent, while two subspecies of subsp. *japonica* (Thunb.) Kitam. and subsp. *linariifolia* (Turcz.) Kitam. are endemic to Japan (Kitamura 1937, Koyama 1995). *Inula britannica* subsp. *linariifolia* is taxonomically distinguished from the other two subspecies by having longer and linear leaves, and subsp. *japonica* is distinguished from subsp. *britannica* by having larger heads (Kitamura 1937). Previously three chromosome numbers of $2n$ = 16, 24 and 32 were reported on infraspecific taxa of *I. britannica,* and suggested the basic chromosome number of x = 8 was supposed for this genus (Okabe 1937, Löve and Löve 1961, Arano 1962, Nishikawa 1980, 1984, Probatova and Sokolovskaia 1990, Kokubugata and Koyama 1999).

Recently the fluorescence *in situ* hybridization (FISH) method has been applied to various wild taxa of Asteraceae, and has been confirmed as one of powerful methods to analyze chromosomal evolutions (e. g., Kondo *et al.* 1996, Kokubugata and Koyama 1999, Kokubugata and Matsumoto 1999, Saito *et al.* 2003). The specific aim of the present study is to compare chromosome number and karyotype by the orcein standard squash method and distribution patterns of 45S ribosomal DNA (rDNA) sites on somatic chromosomes in the three subspecies of *I. britannica* subsp. *britannica,* subsp. *japonica* and subsp. *linariifolia* by the FISH method.

Materials and methods

Orcein standard squash method

[1] Graduate School of Agriculture, Ibaraki University, Ami, Ibaraki 300-0393. 〒300-0393 茨城県阿見町 茨城大学大学院農学研究科.

[2] Tsukuba Botanical Garden, National Science Museum, Tsukuba, 305-0005. 〒300-0005 国立科学博物館 筑波研究資料センター 筑波実験植物園.

[3] Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526. 〒739-8526 広島県東広島市．広島大学大学院理学研究科付属植物遺伝子保管実験施設.

[4] Research and Education Center of Population Biology, Moscow State Pedagogical University, Kibalchich St. 5, kor 6, Moscow, Russia.

[5] Botanical Garden, Ural Division, Russian Academy of Sciences, 8 Marta St., 202, Ekatherinburg 620144, Russia.

Table 1. Plant materials and chromosomal characters in three subspecies of *Inula britannica*

Subspecies	Locality*	2n	No. satellite	No. rDNA
subsp. *britannica*	RUSSIA, Orenburg: close to Boevaya Gora village, Mt. Boevaya Gora (*G. Kokubugata 2602*)	16	2	2
subsp. *japonica*	Cultivated in Tsukuba Botanical Garden (*G. Kokubugata 2022*)	24	3	3
subsp. *linariifolia*	JAPAN, Honshu: Tanuma, Aso, Tochigi (*G. Kokubugata 2021*)	24	2	3

* Voucher specimens are deposited in TNS.

Three subspecies of *Inula britannica* were collected and brought in the Tsukuba Botanical Garden, National Science Museum, Tokyo (Table 1). Root tips were pretreated in 2mM 8-hydroxyquinoline at 20℃ for 4h, and fixed in acetic ethanol (1 : 3) at 4℃ for at least 2h. They were macerated in 45% acetic acid at 60℃ for 10 sec, and stained in 2% aceto-orcein at 20℃ for 2h, then squashed under a cover slip. This aceto-orcein squash method resulted in well-spread chromosomes that were suitable for the present study.

PCR amplification and DNA labeling

Total DNA isolated from *Aster ageratoides* Turcz. (*G. Kokubugata 2810*, TNS) by the DNeasy Plant Mini Kit (QIAGEN Cat. No. 69104) was used as a template for polymerase chain reaction (PCR) as FISH probes. The PCR amplification of part of the 45S ribosomal DNA (rDNA) array was performed using primer NS1 (5' -GTA GTC ATA TGC TTG TCT-3') and NS4 (5' -CTT CCG TCA ATT CCT TTA AG-3') designed by White *et al.* (1990). These were located at the 5' end of 18S (NS1) and 2/3 downstream in 18S (NS4), respectively, resulting in an 18S rDNA fragment of approximately 1150bp. The PCR profile for 30 cycles of DNA amplification was 1 min at 94℃, 1 min at 56℃ and 1 min at 72℃. The amplified DNA fragments were labeled with digoxigenin- (DIG) dUTP by the Nick Translation Kit following the manufacturer's protocol (Roche, Cat. No. 976776). The labeled probe was dissolved in 50% formamide and 10% dextran sulfate (w/v) in 2×SSC, and adjusted to a final DNA-concentration of 5 μg/ml. This hybridization mixture was denatured at 75℃ for 10 min before being immediately chilled in ice-cold water for 10 min.

FISH using rDNA probes

Fixed root tips were digested in a mixture of 2% cellulase "Onozuka" RS (Yakult) and 1% pectolyase "Y-23" (Seisin) dissolved in distilled water (w/v ; pH 4.5) at 36℃ for 20 min, and then washed in distilled water for 10 min. A single digested root tip was placed on a glass slide, the meristematic apex dissected and squashed in 45% acetic acid under a glass cover slip. The cover slip was removed from the slide by the dry-ice method. The air-dried root meristem preparation was treated with 0.1% RNase (w/v) in 2×SSC at 37℃ for 1h. After a wash in 2×SSC at room temperature for 10 min, it was submerged in 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS) at room temperature for 5 min, and then rinsed in distilled water at room temperature for 10 min. The preparation was then dehydrated through an ethanol

series (75%, 80% and 100% ethanol at room temperature for 3 min each), and dried at 36℃ for 30 min. The preparation was covered with 10 μl of the hybridization mixture, and mounted with a silicone-coated cover slip before being sealed with rubber solution. Then, the preparation was denatured at 80℃ for 10 min and placed in a humid chamber at 37℃ overnight for DNA hybridization to occur. Following the hybridization, the slide was rinsed in 4×SSC at 40℃ for 10 min twice. The hybridization signals on the chromosomes were detected with 20 μg/ml Anti-digoxigenin-rhodamine, Fab-fragments (Roche, Cat. No. 1207750) in 1% bovine serum albumin dissolved in 4×SSC at 37℃ for 1h. The slide was rinsed in 4× SSC at room temperature for 10 min twice in a dark box, and received 100 μl of 4,6-diamidino-2-phenylindole (DAPI; Sigma Cat. No. D9542) for counter-staining at 4℃ for 30 min before being mounted with a coverslip. The hybridization signals fluoresced red while the non-hybridized region fluoresced blue when visualized using a double band excitation filter (Zeiss, filter set No. 23).

Description of chromosomes

Chromosomes at mitotic metaphase were classified by arm ratio (R = long arm length / short arm length ratio) following Levan *et al.* (1964). Median- (R = 1.0 to 1.7), submedian- (R = 1.8 to 3.0), subterminal- (R = 3.1 to 7.0) and terminal-(R = 7.1 to ∞) centromeric position were designated and symbolized as "**m**", "**sm**", "**st**" and "**t**", respectively.

Results and Discussion

Orcein standard squash method

Plant of *Inula britannica* subsp. *britannica* showed the chromosome number of 2n = 16 (Fig. 1A), while that of *I. britannica* subsp. *japonica* and subsp. *linariifolia* showed the chromosome number of 2n = 24 (Figs. 1B and C). The present chromosome number of 2n = 16 in *I. britannica* subsp. *britannica* was corresponded with Probatova and Sokolovskaia (1990); that in *I. britannica* subsp. *japonica* of 2n = 24 were corresponded Okabe (1937), Arano (1962) and Nishikawa (1980; as *I. britannica* var. *chinensis* Regel) and that in *I. britannica* subsp. *linariifolia* was corresponded with Kokubugata and Koyama (1999).

The chromosome compliment of the plant of *I. britannica* subsp. *britannica* was consisted of ten **m** and six **sm** chromosomes with a satellite on the terminal region of the short arm of two **sm** chromosomes (Fig. 1A, arrows); that of *I. britannica* subsp. *japonica* was consisted of fifteen **m** and nine **sm** chromosomes with a single satellite located on the terminal region of the short arm of three **sm** chromosomes (Fig. 1B, arrows); and that of subsp. *linariifolia* was consisted of fifteen **m** and nine **sm** chromosomes with a single satellite located on the terminal region of the short arm of two **sm** chromosomes (Fig. 1C, arrows)

FISH method

Plant of *I. britannica* subsp. *britannica* with 2n = 16 exhibited an rDNA site on the satellite of the two **sm** chromosomes (Fig. 2A, arrows); that of *I. britannica* subsp. *japonica* with 2n = 24 exhibited a rDNA site on the satellite of the three **sm** chromosomes (Fig. 2B, arrows); and that of *I. britannica* subsp. *linariifolia* with 2n = 24 exhibited a rDNA site on the satellite of the two **sm** chromosomes (Fig. 2C, arrows) and the terminal region of a **sm** chromosome (Fig. 2C, arrowhead).

Cytological conclusion

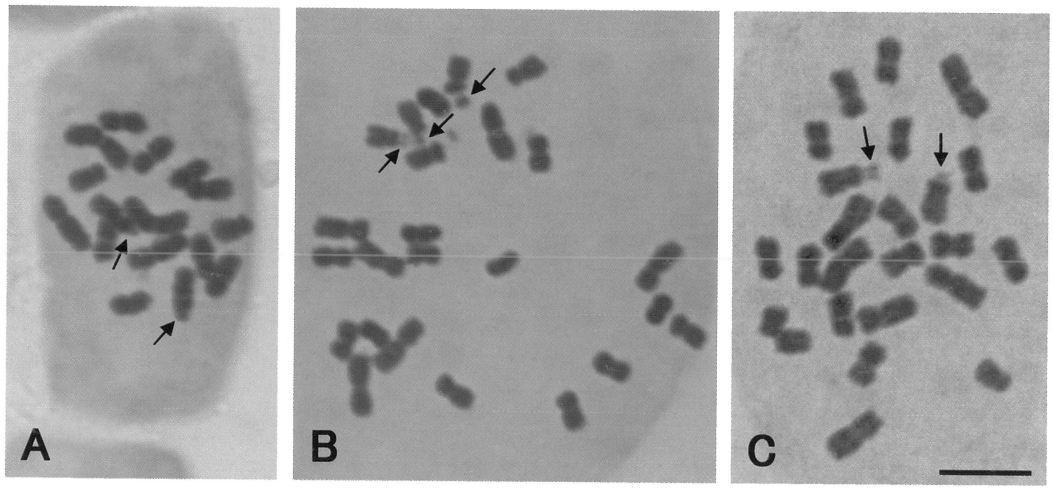

Fig. 1. Orcein-stained chromosome at mitotic metaphase of three subspecies of *Inula britannica,* **A**. subsp. *britannica*, **B**. subsp. *japonica*, **C**. subsp. *linariifolia*. Arrows show the satellites. Bar shows 10 μ m.

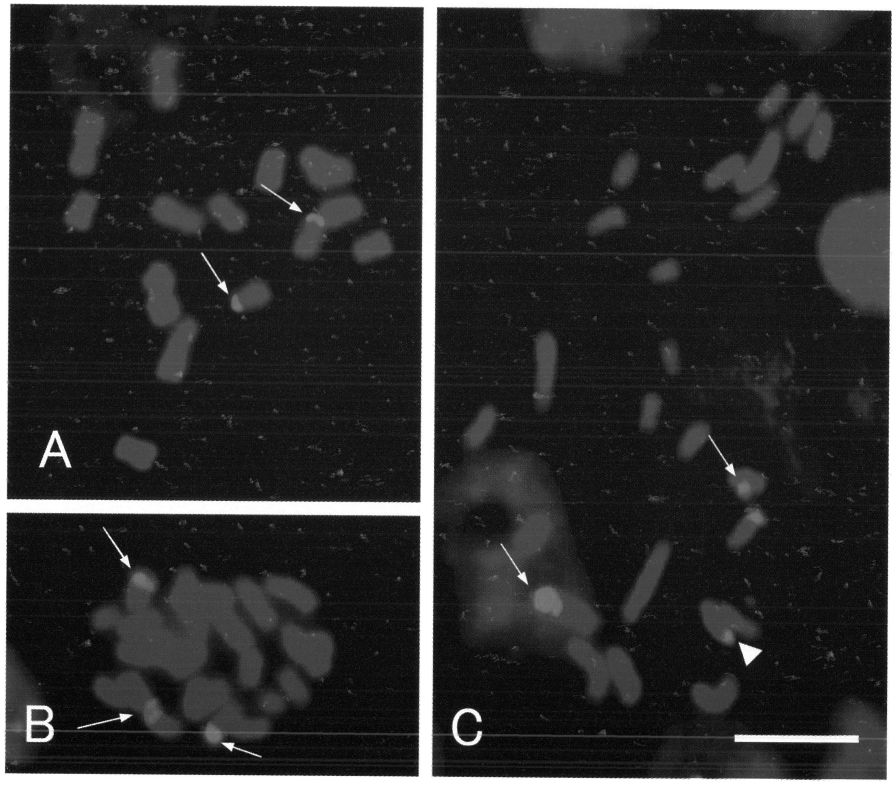

Fig. 2. FISH-detected chromosome at mitotic metaphase of three subspecies of *Inula britannica.* **A**. subsp. *britannica*, **B**. subsp. *japonica*, **C**. subsp. *linariifolia*. Arrows show rDNA sites on satellites; arrowhead shows rDNA site on the terminal. Bar shows 10 μ m.

Previous cytological studies suggested the basic chromosome number of x = 8 for *Inula* (Okabe 1937, Arano 1962, Kokubugata and Koyama 1999). The existence of two rDNA sites in the plant of *I. britannica* subsp. *japonica* with 2n = 16 and that of three rDNA sites in the plant of *I. britannica* subsp. *britannica* and *linariifolia* with 2n = 24 supposes the basic chromosome number of x = 8 for the genus *Inula* agreeing with Okabe (1937), Arano (1962) and Kokubugata and Koyama (1999).

Although *I. britannica* subsp. *japonica* and subsp. *linariifolia* commonly had the chromosome number of 2n = 24 as the triploid cytotype, the later subspecies had two satellites in a complement. Kokubugata and Koyama (1999) also reported the same result in a different accession of *I. britannica* subsp. *linariifolia* from that investigated in the present study (*G. Kokubugata 2021*). The two satellites in the triploid cytotype (2n = 24) in *I. britannica* subsp. *linariifolia* may be due to one of the following possible factors:

1) *artificial factor* : one of the three satellites might easily break off from the short arm during the standard squash method or the enzyme-maceration in FISH protocol. However, this could be unlikely because we also confirmed two satellites in additional two metaphase complements of *I. britannica* subsp. *linariifolia* (data no shown).

2) *Structural factors*: one of the three rDNA-coded satellites might have been lost or decreased during previous cell cycles except for a small rDNA-coded fragment on the terminal of the short arm. Variation in copy numbers of rDNA genes have been demonstrated in FISH by employing different staining techniques ($AgNO_3$, CMA and FISH) (De Souza *et al.* 2004).

3) *Functional variation*: 3a) *hybrid origin*: the plant of *I britannica* subsp. *linariifolia* might be an undetected hybrid between diploid (2x) and tetraploid (4x) parents with differently sized rDNA sites. However, this must be unlikely as there is no morphological evidence pointing the plant (*G. Kokubugata 2021*) to be a hybrid origin; 3b) *differential amphiplasty*: one of three nucleolar organizer regions (NOR) sites might be more condensed than the others, which might be linked to differential transcription of rDNA genes (*i.e.*, the selective inactivation of rDNA sites). It was termed 'amphiplasty' when all chromosomes are affected, and 'differential amphiplasty' when only particular chromosomes are affected (Viegas *et al.* 2002). Navashin (1934) first observed differential amphiplasty in a certain *Crepis* species where one parental set of ribosomal DNA (rDNA) clusters were affected. Although the amphiplasty has been mainly observed and studied in hybrids, rDNA heteromorphy has also been reported in species of diverse genera, such as *Galtonia* (Liliaceae; Forrest and Jong 2004) and *Glycine* (Leguminosae; Singh *et al.* 2001). At the present stage, it is difficult to ascertain which mechanism is responsible for the present phenomenon for *I. britannica* subsp. *linariifolia*. Further cytological studies must be necessary for investigating the phenomenon by the silver staining method to distinguish active and inactive NORs.

Summary

Somatic chromosomes of three subspecies of *Inula britannica* (Asteraceae) were observed by the orcein standard squash method and fluorescent *in situ* hybridization (FISH) method using 18S ribosomal DNA (rDNA) probes. The plant of *Inula britannica* subsp. *britannica* showed the somatic chromosome number of 2n = 16 and the karyotype consisted of 10 **m** and 6 **sm** chromosomes, while that of *I. britannica* subsp. *japonica* and subsp. *linariifolia* showed the chromosome number of 2n = 24 and the karyotype consisted of 15 **m** and 9 **sm** chromosomes. The three subspecies of *I. britannica* observed had same basic chromosome number of x = 8, similar karyotype and distribution pattern of rDNA sites in the haploid karyotype without a small fragment in subsp. *linariifolia*. Previously lack of satellite excepting a small rDNA fragment in

another triploid plant of subsp. *linariifolia* has been also reported (Kokubugata and Koyama 1999). The present cytotaxonomic study suggests that the three subspecies might be phylogenetically close to each other.

摘　要

キク科*Inula britannica* の３亜種，subsp. *britannica*，subsp. *japonica* (オグルマ)，subsp. *linariifolia* (ホソバオグルマ)，の体細胞分裂中期染色体をアセトオルセイン染色法と18S ribosomal DNA (rDNA) をプローブとした蛍光 *in situ* hybridization法を用いて観察した。*I. britannica* subsp. *britannica*は2n = 16で10個の中部動原体型染色体，６個の次中部動原体型染色体から構成されていた。オグルマとホソバオグルマは2n = 24で15個の中部動原体型染色体，９個の次中部動原体型染色体から構成されていた。ホソバオグルマでは過去に報告された別個体同様に付随体の欠失が確認された。本研究において，３亜種の基本数はx = 8で，ハプロイドにおける核型とrDNA分布パターンが類似していることが示され，これら３亜種が系統学的に近縁であることが示唆された。

Acknowledgements

We are grateful to Dr. V. P. Verkholat, Dr. A. Gontcharov, Prof. H. Ogura, Dr. T. Funamoto, Dr. Y. Hoshi for helping in the field trips in Russia. This study was in part founded by the Grant-in-Aid for the Scientific Programs (A; No. 14255014: Representative to K. Kondo and No. 15201050: Representative to T. Konishi) of Japanese Society for the Promotion of Science.

References

Arano, H., 1962. Cytotaxonomic studies in subfam. Carduoideae of Japanese Compositae, V. Karyotype analysis and its karyological considerations in some genera. La Kromosomo **53-54**: 1794-1810 (In Japanese with English abstract).

De Souza, L., A.C. Swarca and A.L. Dias, 2004. Analysis of the nucleolus organizer region in 5 species of the genus *Pimelodus* (Siluriformes, Pimelodidae), using $AgNO_3$, CMA, and FISH with the 18S rDNA probe. Caryologia **57**:144-150.

Forrest, L.L. and K. Jong, 2004. Karyotype asymmetry in *Galtonia* and *Pseudogaltonia* (Hyacinthaceae). Edinburgh J. Bot. **60**: 569-579.

Kitamura, S. 1937. Compositae Japonicae. I. Mem. Coll. Sci. Kyoto Imperial Univ., Ser. B **13**: 337-357.

————, 1981. Compositae (Asteraceae). *In:* Satake, Y., J. Ohwi, S. Kitamura, S. Watari and T. Togashi (eds.) Wild Flowers of Japan. Herbaceous Plants III, Heibonsha, Tokyo. pp. 156-235.

Kokubugata, G. and H. Koyama, 1999. A cytotaxonomic study of two species of *Inula* (Asteraceae) by fluorescence *in situ* hybridization using ribosomal DNA probe. Acta Phytotax. Geobot. **50**: 27-33.

———— and S. Matsumoto, 1999. A chromosomal comparison of three species of *Crepidiastrum*, section Suffruticosae (Asteraceae) by fluorescent *in situ* hybridization using ribosomal DNA probe. Chrom. Sci. **3**: 105-109.

Kondo, K., Y. Honda and R. Tanaka, 1996. Chromosome marking in *Dendranthema japonicum* var. *wakasaense* and its closely related species by fluorescence *in situ* hybridization using rDNA probe pTa71. La Kromosomo **II-81**: 2875-2791.

Koyama, H., 1995. Genus *Inula*. *In* : Iwatsuki, K., T. Yamazaki, D.E. Boufford and H. Ohba (eds). Flora of Japan III. Kodansha, Tokyo. pp. 101-102.

Levan, A., K. Fredga and A. A. Sandberg, 1964. Nomenclature for centromeric position on chromosome. Hereditas **52**: 201-220.

Löve, A. and D. Löve, 1961. Chromosome Numbers of Central and Northwest European Plant Species. Opera Botanica, Lund. p. 581.

Navashin, M., 1934. Chromosomal alterations caused by hybridization and their bearing upon certain general genetic problems. Cytologia **5**: 169-203.

Nishikawa, T., 1980. Chromosome counts of flowering plants of Hokkaido (3). J. Hokkaido Univ. Educ. Sect. II B **31**:

19-23 (in Japanese with English abstract).

————, 1984. Chromosomes counts of flowering plants of Hokkaido (7). J. Hokkaido Univ. Educ. Sect. II B **35**: 31-42 (in Japanese with English abstract).

Okabe, S. 1937. Triploidie in *Saxifraga stolonifera* Meerb. und *Inula britannica* subsp. *japonica* Kitam. Cytologia **Fuji jubilaei volumen**: 527-534.

Probatova, N.S. and A.P. Sokolovskaia, 1990. Chromosome numbers in some representatives of the families Asclepiadaceae, Asteraceae, Boraginaceae, Chenopodiaceae, Lamiaceae, Oleaceae, Onagraceae, Scrophulariaceae, Solanaceae, Urticaceae from the Soviet Far East. Bot. Zhurn. **75**: 1619-1622.

Saito, Y., G. Kokubugata, T. Katsuyama, W. Marubashi and T. Iwashina, 2003. Cytological comparisons of somatic chromosomes in × *Crepidiastrixeris denticulato-platyphylla* and speculation of its parental species (Asteraceae). Chrom. Sci. **7**: 43-48.

Singh, R.J., H.H. Kim and T. Hymowitz, 2001. Distribution of rDNA loci in the genus *Glycine* Willd. Theor. Appl. Genet. **103**: 212-218.

Viegas, W., N. Neves, M. Silva, A. Caperta and L. Morais-Cecílio, 2002. Nucleolar dominance: a 'David and Goliath' chromatin imprinting process. Curr. Genomics **3**: 563-576.

White, T. J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direst sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M.A., Gelfand, J.J. Sninsky and J.T. White (eds), PCR Protocals: A Guide to Methods and Applications. Academic Press, San Diego. pp. 315-322.