Fluorescent banding pattern in chromosomes of
Tsuga forrestii and T. sieboldii, Pinaceae

Masahiro Hizume

Faculty of Education, Ehime University, Matsuyama 790-8577, Japan

Author for correspondence: hizume@ed.ehime-u.ac.jp
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ABSTRACT: In two East Asian species of Tsuga, T. forrestii and T. sieboldii their somatic chromosomes were investigated by fluorescent banding technique using DNA-base specifically binding fluorochromes of chromomycin A3 (CMA) and 4',6-diamidino-2-phenylindole (DAPI). Their chromosome numbers were commonly 2n=24. Their karyotypes were very similar to each other and consisted of eight pairs of long metacentric chromosomes and four pairs of short chromosomes. The two pairs of the short chromosomes were submetacentric, one pair was metacentric chromosomes, and the shortest chromosome pair was submeta-subtelocentric. After CMA-staining six CMA-bands appeared at interstitial region of each one arm of six long metacentric chromosomes. Positive DAPI-band was not observed and DAPI-negative regions appeared coincident with all CMA-bands. Centromeric regions of DAPI-stained chromosomes were darker than chromosome arms. Karyotypes and their fluorescent banding patterns of the two Tsuga species were very similar to each other. The fluorescent banded karyotypes of the Tsuga species are compared with those of other Pinaceae genera reported.

KEYWORDS: Chromosome, CMA, DAPI, Fluorescent banding, Karyotype, Pinaceae, Tsuga

Tsuga is one of the 11 genera of the Pinaceae. The Pinaceae consists of two to four subfamilies and the most of taxonomic treatments pleas Tsuga into the subfamily Abietoideae. Tsuga consists of nine species, one subspecies and three varieties. They grow in Northern hemisphere and restricted to regions with oceanic to subcontinental climate in North America and East Asia, where precipitation is available during their growing season (Farjon 1990) and the species are divided into two groups (Melchior and Werdermann 1954). Tsuga longibracteata was separated and established into monotypic genus of Nothotsuga (Hu 1951).

Since Sax and Sax (1933) reported the chromosome number and the simple idiomorph of T. caroliniana, many more karyotypes in eight species of Tsuga such as T. chinensis var. formosana (Kuo et al. 1972), T. canadensis, T. caroliniana, T. chinensis, T. diversifolia, T. heterophylla and T. sieboldii (Hizume 1988), T. chinensis var. tchekiangensis (Li 1988), T. longibracteata (Li 1991) and T. mertensiana (Li et al. 2000) have been reported. The karyotypes of Tsuga species are basically similar to each other in having four pairs of shorter chromosomes and some differences in number of secondary constrictions and their location in their karyotype.

The appearance of secondary construction tends frequently to be different among the reports depending on observation condition. Conventional karyotype of Tsuga was similar to those of Picea, Cedrus, Abies and Keteleeria.

In the Pinaceae, especially the genus Pinus, the fluorescent CMA- and DAPI-banding patterns supply very useful information for chromosome identification and comparative karyotype analysis among the species (Hizume et al. 1983, 1989a, 1990; Doudrick et al. 1995). Application of the fluorescent banding technique on chromosomes of other Pinaceae genera up to the present was in Larix (Hizume et al. 1988; Hizume and Tanaka 1990; Hizume et al. 1993a, 1994, 1998), Picea (Hizume et al. 1989b, 1991; Hizume and Kuzukawa 1995), Abies (Shibata et al. 2004; Puizina et al. 2008), Keteleeria (Hizume et al. 1993b), Pseudotsuga (Hizume and Akiyama 1992; Hizume and Kondo 1992), Cedrus (Dagher-Kharrat et al. 2001) and Pseudolarix (Hizume 2015). The comparative study on the fluorescent banding patterns of these genera is expected to supply information revealing intraspecific, interspecific and intergeneric relationships in the Pinaceae. Fluorescent banding technique has not yet applied to chromosomes of the species of Cathaya, Nothotsuga and Tsuga.

The present report aims to reveal fluorescent banded karyotype of Tsuga species. The fluorescent banded karyotypes of Tsuga species were compared and discussed with those of other genera of the Pinaceae.

MATERIALS AND METHODS

Seeds of Tsuga forrestii Downie and T. sieboldii Carr. were collected in natural forests in Yunshaping, Lijiang, Yunnan Province, the People’s Republic of China and in Odamiyama, Uchiko-cho, Kita-Gun, Ehime Prefecture, Japan, respectively. Their seeds were sown and germinated on wet filter paper in petri dishes at 20°C for 7-10 days. The primary root-tips were collected and treated in 0.05% colchicine for 8 h. Then, those root-tips were fixed in mixture of ethanol, acetic acid and chloroform (volume ratio=2:1:1) and stored in the fixative in a freezer. Fixed root-tips were washed in water after removing fixative with 70% ethanol. Then, root-tips were transferred in 45% acetic acid for 5 min, and treated with 45% acetic acid at 60°C for 10 min. Then, meristematic tissue was dissected from the root-tip and put on a glass slides. An aliquot of
45% acetic acid was dropped on the tissue and cover slip was put on it. Meristematic cells were squashed to spread. The preparation was put on a dry ice for a few minutes and then, ripped off cover slip. The preparation was air-dried overnight. Sequential fluorescent banding technique with CMA and DAPI were described early by Kondo and Hizume (1982). The dried preparation immersed into McIlvaine buffer pH 7.0 for 30 min. The slide glass was treated with 0.1 mg/mL distamycin A for 10 min, then washed with the buffer containing 5 mM MgSO₄ for 10 min and stained with 0.1 mg/mL CMA in the buffer for 10 min. After washing with the buffer for 10 min the preparation was mounted with non-fluorescence glycerin and stored in a refrigerator at 4°C overnight or more. After storage the CMA-stained preparation was observed under a fluorescence microscope equipped with B filter block. Then preparation was dipped in distilled water until drop off the cover glass. Then the preparations were treated with acetic-alcohol (3:1) to remove CMA and glycerin, rinsed briefly with distilled water and then air-dried. The preparation was put in the buffer without MgSO₄ for 10 min, treated with 0.1 mg/mL actinomycin D for 10 min and the wash again with the buffer for 10 min. The preparation was stained with 0.1 μg/mL DAPI for 5 min then washed with the buffer for 5 min. After mounting with the buffer-glycerin (v/v=1:1) mixture the same chromosomes observed CMA fluorescence were observed under the fluorescence microscope with UV filter block. Fluorescence images of same chromosomes stained CMA and DAPI were taken on the film (TMAX, Kodak) and developed with double diluted D-76.

RESULTS AND DISCUSSION

Tsuga forrestii and T. sieboldii had the somatic chromosome number of 2n=24 supporting previous count in T. sieboldii (Hizume 1988) and same chromosome number of other species of Tsuga reported. Karyotypes of these two species were consisted of eight pairs of long

Fig. 1. Fluorescent banded chromosomes of Tsuga forrestii. A: CMA staining, B: DAPI staining. Bar=10μm.
metacentric chromosomes and four somewhat short pairs of chromosomes. Two short pairs were metacentric to submetacentric chromosomes, the 11th pair of chromosomes were metacentric and the shortest pair of chromosomes were submeta-subtelocentric. These karyotypes were also similar to the description of conventional karyotypes reported in eight species of Tsuga (Kuo et al. 1972; Hizume 1988; Li 1988, 1991; Li et al. 2000). Tsuga karyotypes in the previous reports showed some species had a few secondary constrictions at the interstitial zone of the long metacentric chromosomes and the other species did not show any secondary constriction, and then, exact number or location of secondary constrictions were unclear caused by different conditions in conventional chromosome preparation. The karyotype of T. longibracteata had species-specific, elongated centromere at the pair of the shortest chromosomes (Li 1991). Tsuga longibracteata described by Cheng (1932) was proposed for a new genus, Nothotsuga longibracteata by Hu (1951) and Page (1988). This might indicate generic differentiation in karyotype level between Tsuga and Nothotsuga. In order to confirm this speculation,
analyses on chromosomes of these species by fluorescent banding and in situ hybridization using rDNA or other probes are desired to reveal the phylogenetic relationships.

If fluorescent banding technique with DNA base specific fluorochromes was applied to chromosomes of the two Tsuga species, bright CMA-bands were observed at the interstitial region of six long metacentric chromosomes in both species (Figs. 1A and 2A). Bright DAPI-band was not observed at all and the region of CMA-band showed DAPI-negative (Figs. 1B and 2B). Chromosome arms without CMA-band region displayed homogeneous fluorescence of CMA and DAPI. The centromeric regions showed homogeneous CMA fluorescence and several chromosomes showed dull DAPI fluorescence. The fluorescent feature of centromeric regions might suggest that the centromeric region of chromosomes of T. forrestii and T. sieboldii have less affinity to DAPI than chromosome arms or contained specific and somewhat more GC-rich DNA sequence than that of chromosome arms.

The fluorescent banding pattern of two Tsuga species was compared with those of the genera of the Pinaceae reported. The banding pattern of Tsuga chromosomes was different from those of Pinus with many interstitial DAPI-bands (Hizume et al. 1983, 1989b, 1990; Doudrick et al. 1995), Larix and Cedrus with many proximal DAPI-bands (Larix: Hizume et al. 1988, 1993b, 1998; Hizume and Tanaka 1990; Cedrus: Dagher-Kharrat et al. 2001), Picea with a pair of chromosomes with two CMA-bands located very close to each other (Hizume and Kuzukawa 1995; Hizume et al. 1989b, 1991), Larix and Pseudotsuga which have weak CMA-band composed of 5S rDNA at the terminal interstitial region of one long pair of the metacentric chromosome (Hizume et al. 1996; Amarasinghe et al. 1998), and Pseudolarix with many proximal DAPI-bands and CMA-bands (Hizume 2015). Fluorescent banding pattern of Tsuga seemed similar to those of Keteleeria (Hizume et al. 1993b) and Abies (Roth et al. 1997; Shibata et al. 2004).

The family Pinaceae, the subfamily Abietoideae consists of Abies, Cedrus, Keteleeria, Nothotsuga, Pseudolarix and Tsuga. Pseudolarix has a peculiar karyotype (2n=44) consists of four sub-meta-subtelocentric chromosomes and 40 telocentric chromosomes (Sax and Sax 1933; Mergen 1961; Hizume 1988; Li 1994). The karyotype of Pseudolarix was sometimes discussed its origin and proposed to be generated by centromeric fission in 20 chromosomes of Larix (Mergen 1961; Gustafsson and Mergen 1964) or Tsuga (Li 1995). Most of the genera of the subfamily were reported their fluorescent banding patterns except Tsuga and Nothotsuga. Recently, Hizume (2015) reported the fluorescent banding pattern of Pseudolarix chromosomes with all 40 telocentric chromosomes which had the proximal DAPI-bands and ten CMA-bands at near centromeric region, and compared it with those of genera reported in the Abietoideae. The most possible relative of Pseudolarix was suggested that Cedrus had centromeric DAPI-bands on most of the chromosomes. In this report on the chromosomes of two Tsuga species, the fluorescent banding karyotypes had six interstitial CMA-bands and no centromeric DAPI-band (Figs. 1 and 2). The banding pattern of Tsuga species suggested that Tsuga was not considered to be relative of Pseudolarix in location and number of CMA-bands and no appearance of any DAPI-band. In point of view of centromeric DAPI-bands Cedrus would probable candidate of close relative of Pseudolarix, but CMA-band pattern was different distinctly from that of Pseudolarix and Cedrus. In the closely related genera Larix and Pseudotsuga had nearly the same bimodal karyotype (Hizume et al. 1988; Hizume and Akiyama 1992; Hizume and Kondo 1992) and putting into the same clade in molecular phylogenetic trees (Chaw et al. 1997; Gerndt et al. 2001), in Larix AT-rich repetitive sequence (Hizume et al. 2002) might amplify very quickly and form proximal DAPI-bands of most of the chromosomes since differentiation of these genera have started chromosome differentiation, while on the other hand, Pseudotsuga has not occurred this phenomenon at all. CMA-bands containing 45S rDNA repeats frequently appear or disappear by deletion, transposition or translocation in plant chromosomes. These changes of CMA- and/or DAPI-bands should be recognized to occur in chromosomes of species, genera in the Pinaceae. The fluorescent banding patterns with CMA and DAPI of all genera of the subfamily Abietoideae excepting Nothotsuga seem difficult to indicate the genus that is the most closely related with Pseudolarix. To reveal karyotype differentiation in the Pinaceae, in addition to fluorescent banding karyotype, many more analyses of the repetitive DNA located on CMA-band, DAPI-band and C-band in their sequence and distribution on chromosomes of species and/or genera are expected. Recently, genome projects progress in several conifers species of Pinus, Picea, Pseudotsuga and Larix and are constructing saturated genome maps. Comparative study on genome maps of species of the Pinaceae reveals synteny among species or genera in the Pinaceae (Krutovsky et al. 2004; Jermstad et al. 2011; Pavy et al. 2012) and integration between genome map and karyotype by molecular cytogenetic techniques will display dynamic feature of genomic and karyotype differentiation in the Pinaceae.

**Literature Cited**


Pavy, N., Pelgas, B., Laroche, J., Rigault, P., Isabel, N. and


