Chromosome banding in the genus *Pinus* IV. Fluorescent banding patterns of chromosomes in eight taxa of haploxyline pines

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ABSTRACT: Somatic chromosomes of seven taxa of Asian haploxyline pines, *Pinus armandii* var. *amamiana*, *P. armandii* var. *armandii*, *P. parviflora*, *P. pumila*, *P. x hakkodensis*, *P. koraiensis* and *P. bungeana*, and a American species of *P. edulis* were observed by a fluorescent banding method using chromomycin A₃ (CMA) and 4',6-diamidino-2-phenylindole (DAPI). The chromosome number of all taxa was $2n=24$ and their karyotypes were composed of 22 long metacentric chromosomes and two short heterobrachial chromosomes as previous reports. CMA-bands appeared at the interstitial region and not at the proximal region of metacentric chromosomes. Interstitial CMA-bands were observed on 16-18 long metacentric chromosomes in chromosome complement of most species excepting for *P. bungeana* having six interstitial CMA-bands. The short chromosomes of most species have an interstitial CMA-band on their long arms and that of *P. edulis* had a proximal CMA-band. Clear proximal CMA- or DAPI-bands appeared in most species of subgenus *Pinus* were not observed in species of subgenus *Strobos* examined. DAPI-dots appeared at centromeric regions of all chromosomes and many thin DAPI-bands did at interstitial regions as appeared in the species of subgenus *Pinus*. The karyotype analysis combined with CMA- and DAPI-bandings was useful for identification of each homologous chromosome and for chromosomal relationship among species in haploxyline pines.

KEYWORDS: Chromosome, Fluorescent banding, Haploxyline pine, Karyotype, *Pinus*, *Strobos*

The genus *Pinus* is the largest one composed of 120-126 species in Pinaceae (Shaw 1914; Critchfield and Little 1966; Mirov 1967; Little and Critchfield 1969; Farjon 1984; Farjon and Styles 1997). They distribute widely in temperate to tropical climatic parts in north Hemisphere and one species, *P. merksii* expands over the equator at Sumatra Island, Indonesia (Critchfield and Little 1966; Farjon 1984; Farjon and Styles 1997; Price *et al.* 1998). The *Pinus* shows a great diversity in structure, phenology, morphology, ecology, genecology, geography, genetics and molecular genetics. On the basis of pioneer works of Shaw (1914), Mirov (1967) and, Little and Critchfield (1969) their taxonomic treatments are used widely and, subgenera *Pinus* and *Strobos* are accepted. Several revisions have made adjustments to the species status and modified the taxonomic treatment at the ranks of subgenera, sections and/or subsections (Farjon 1984; Farjon and Styles 1997; Gernandt *et al.* 2003, 2005; Price *et al.* 1998; Liston *et al.* 1999; Syring *et al.* 2007; Hernández-León *et al.* 2013). In recent years molecular phylogenetic analyses using various DNA sequences of cytoplasmic and nuclear genomes were applied frequently to phylogenetic and taxonomic studies on *Pinus* species, and revealed reliable phylogenetic relationships among species. Then molecular phylogenetic trees constructed were compared with taxonomic treatments (Chaw *et al.* 1997; Liston *et al.* 1999; López *et al.* 2002; Zhang and Li 2004; Gernandt *et al.* 2005, 2009; Eckert and Hall 2006;

Tsutsui *et al.* 2009) and coincident with subgenera and sections. The subgenus *Strobos* has about 50 species and separated into two sections *Strobos* and *Parrya* by taxonomy and also molecular phylogeny (Zhang and Li 2004; Eckert and Hall 2006).

In a point of cytogenetic view the *Pinus* species is very conservative in a common chromosome number of $2n=24$, has many long metacentric chromosomes forming a similar symmetric karyotype (Saylor 1972, 1983; Hizume 1988). Conserved karyotype is suitable for precise comparative karyotype analysis after an identification of each homoeologous chromosome and expected to supply valuable phylogenetic information. For chromosome identification of each homologous pair in chromosome complement, several techniques such as C-banding (Borzan and Papeš 1978; Tanaka and Hizume 1980; MacPherson and Filion 1981), G-banding (Drewry 1982), fluorescent banding (Hizume *et al.* 1983, 1989b, 1990; Jacobs *et al.* 2000; Islam-Faridi *et al.* 2007) and *in situ* hybridization (ISH) including fluorescent *in situ* hybridization (FISH) of rDNA probes (Hizume *et al.* 1992; Doudrick *et al.* 1995; Lubaretz *et al.* 1996; Jacobs *et al.* 2000; Liu *et al.* 2003; Cai *et al.* 2006; Islam-Faridi *et al.* 2007; Shibata *et al.* 2016) and, other probes such as *PCSR* and telomere sequences (Hizume *et al.* 2002b; Islam-Faridi *et al.* 2007; Shibata *et al.* 2016) were used for chromosome analysis in *Pinus* species. The fluorescent banding using CMA and DAPI displayed many

Table 1. Locality or source of eight taxa of *Pinus* subgenus *Strobus*.

Taxon	Locality (source)
<i>Pinus armandii</i> Franch. var. <i>armandii</i>	Lijian, Yunnan, China Kunming, Yunnan, China
<i>P. armandii</i> var. <i>amamiana</i> (koiz.) Hatsushima	Kumage, Kagoshima, Japan (FFPRI)
<i>P. parviflora</i> Sieb & Zucc.	Fujiyoshida, Yamanashi, Japan (FFPRI) Higashiakaisi, Ehime, Japan
<i>P. pumila</i> (Pallas) Regel	Yatsugatake, Nagano, Japan (FFPRI)
<i>P. hakkodensis</i> Makino	Ioudake, Aomori, Japan (FFPRI)
<i>P. koraiensis</i> Sieb. & Zucc.	Tohoku Brabch of FFPRI
<i>P. bungeana</i> Zucc. Ex Endl.	Beijing, China
<i>P. edulis</i> Engelm.	Coconia, Arizona, USA (U.S. For. Tree Seed Center, Georgia)

FFPRI: Seed Bank of Forestry and Forest Products Research Institute, Tsukuba.

fluorescent bands at interstitial and/or centromeric regions of most chromosomes in *Pinus*. Their band patterns were useful for chromosome identification and comparative karyotype analysis which revealed relationships among three Japanese species in subgenus *Pinus* (Hizume *et al.* 1983, 1989b, 1990). Recently the FISH with two or more probes was adapted to species of subgenera *Pinus* (Hizume *et al.* 2002b; Liu *et al.* 2003; Islam-Faridi *et al.* 2007; Shibata *et al.* 2016) and *Strobus* (Cai *et al.* 2006; Shibata *et al.* 2016). Shibata *et al.* (2016) showed a usefulness of multi FISH for chromosome identification and comparative karyotype analysis in many *Pinus* species. The multi FISH analysis seems the most excellent technique for comparative karyotype analysis but contains somewhat complex and difficult procedures, and high cost chemicals. The fluorescent banding using CMA and DAPI is very easy and highly reproducible procedure including just staining.

In this report the fluorescent banding patterns of chromosomes in eight taxa of *Pinus* subgenus *Strobus* were described to supply basic chromosomal information in the diversity of *Pinus* genome.

MATERIALS AND METHODS

Seeds of seven taxa of *Pinus* subgenus *Strobus* were collected in natural forests and supplied from the resource centers showed in Table 1. Seeds were sowed on a filter paper wetted or sterilized sand in a pot and incubated. After two or more weeks of sowing primary roots of germinates were collected for chromosome observation and were prepared chromosome slides by a procedure of Hizume *et al.* (1983). The chromosome preparations were stained sequentially with CMA and DAPI following a protocol of Kondo and Hizume (1982) and Hizume *et al.* (1983). The fluorescent banded chromosome images under an epi-fluorescence microscope were recorded on a high sensitive film.

RESULTS

Eight *Pinus* taxa belonging to subgenus *Strobus* had $2n=24$ chromosomes in somatic cells and the chromosome number confirmed a chromosome number of previous reports (Saylor 1983; Hizume 1988). Their karyotypes were composed of 11 pairs of long metacentric chromosomes of about ranging 15 to 12 μm and one pair of short somewhat heterobrachial chromosomes (about 10 μm), which were also similar to previous reports of species of subgenus *Strobus* (Saylor 1983; Hizume 1988). Fluorescent band patterns with CMA and/or DAPI were described briefly below in each species of subgenus *Strobus*.

1. *Pinus armandii* var. *armandii* and var. *amamiana*
CMA-bands appeared at the interstitial regions of one arm of 16-18 long metacentric chromosomes and not on six long metacentric chromosomes in a chromosome complement in both varieties (Figs. 1A, 2). A pair of chromosomes had interstitial CMA-bands on their both chromosome arms. In var. *amamiana* had more two weak CMA-band at interstitial of two chromosomes. Two short submetacentric chromosomes had a CMA-band at the interstitial region of their long arm. Number of interstitial CMA-bands varied somewhat among seedlings (Table 2). In typical pattern four CMA-bands located at terminally interstitial regions, eight at the medium interstitial regions and six at the intermediate interstitial regions. One pair of long metacentric chromosomes has a CMA-band associating with thin CMA-band or CMA-dots. The location and size of CMA-bands was different among chromosome pairs and allowed identifying homologous chromosomes having CMA-band. In var. *armandii*, DAPI-banded chromosomes were observed (Fig. 1B). Many thin DAPI-bands appeared at the interstitial region of most chromosomes and DAPI-dots appeared at centromeric regions. Negative DAPI-bands were observed at interstitial CMA-band regions (Fig. 1). Combination of

Fig. 1. Fluorescent banded chromosomes of *P. armandii* var. *armandii*. A: CMA banding, B: DAPI-banding. Bar=10 μ m.

Fig. 2. Fluorescent banded chromosomes banded with CMA in *P. armandii* var. *amamiana*. Bar=10 μ m.

Fig. 3. Fluorescent banded chromosomes of *P. parviflora*. A: CMA banding, B: DAPI-banding. Bar=10 μ m

CMA- and DAPI-band patterns supplied useful information for exact chromosome identification.

2. *Pinus parviflora*

CMA-bands appeared on 16-18 long metacentric chromosomes and two short submetacentric chromosomes (Fig. 3A). Sixteen interstitial CMA-bands of the long chromosomes were observed frequently among plants (Table 2). All CMA-bands appeared at the interstitial region of a single arm of each chromosome. The location of CMA-bands was at terminally or not proximally interstitial region. Short submetacentric chromosomes also had a CMA-band at the interstitial region of their long arm. All chromosomes had a pair of DAPI-dots at the centromeric regions (Fig. 3B). Several thin DAPI-bands also appeared at the interstitial regions of most long metacentric chromosomes. Chromosome regions of CMA-band were dark on bright chromosome arms with DAPI. Information combined of CMA- and DAPI-band patterns could identify each homologous chromosome in a chromosome complement.

3. *Pinus pumila*

CMA-bands appeared on 16-18 long metacentric chromosomes and two short submetacentric chromosomes

(Fig. 4). Four long metacentric chromosomes had interstitial CMA-bands at near chromosome end. One pair of long metacentric chromosomes carrying an interstitial CMA-band had thin CMA-band or pair CMA-dots appeared at closely distal side of thick CMA-band. Other interstitial CMA-bands also appeared at distal or near medium region and not at proximal region. Two small submetacentric chromosomes were located at the interstitial region of long arm as observed in the other species. On the basis of location of CMA-band and chromosome length most homologous chromosome pairs were identified.

4. *Pinus x hakkodensis*

Interstitial CMA-bands appeared on 16-18 long metacentric chromosomes and two short submetacentric chromosomes (Fig. 5). An intensity of fluorescence of CMA-band varied among chromosome pairs. The CMA-band of the small submetacentric chromosomes was located at the interstitial region of long arm as observed in *P. parviflora* and *P. pumila*. The CMA-band patterns of these three taxa are similar and seems support that this taxon is a hybrid species between *P. parviflora* and *P. pumila*.

Fig. 4. Fluorescent banded chromosomes of *P. pumila*. A: CMA banding, B: DAPI-banding. Bar=10 μ m.

Fig. 5. Fluorescent banded chromosomes of *P. x hakkodensis* stained with CMA. Bar=10 μ m.

5. *Pinus koraiensis*

CMA-bands were observed at the interstitial regions of certain arms of 15-16 long metacentric chromosomes and at the interstitial regions of the long arms of the short submetacentric chromosomes (Fig. 6A). Four CMA-bands appeared at near terminal interstitial region than other CMA-bands. Other six chromosomes have no CMA-band. After DAPI-banding a pair of DAPI-dots appeared at centromeric regions of all chromosomes and thin DAPI-bands also appeared at the interstitial regions of most chromosomes (Fig. 6B). The fluorescent band patterns with thin DAPI-band and thick CMA-bands permits to identify nearly all homologous pairs in the chromosome complement.

6. *Pinus bungeana*

Four pairs of chromosomes had CMA-bands at the interstitial region. Four thick CMA-bands were located at interstitial region of one arm in long metacentric chromosomes and two did at interstitial region of short arm in the shortest chromosomes (Fig. 7A). Thin CMA-bands appeared at interstitial region of both arms in a pair of long metacentric chromosomes. Number of thick interstitial CMA-bands of this species is six that is less than other taxa of subgenera *Strobus* and *Pinus* examined. After DAPI-staining DAPI-dots appeared at the centromeric region of four chromosome pairs and did not clear in other chromosomes. Several clear interstitial DAPI-bands appeared on most chromosome arms (Fig. 7B). The regions

Fig. 6. Fluorescent banded chromosomes of *P. koraiensis*. A: CMA-banding, B: DAPI-banding. Bar=10 μ m.

Fig. 7. Fluorescent banded chromosomes of *P. bungeana*. A: CMA-banding, B: DAPI-banding. Bar=10 μ m.

Fig. 8. Fluorescent banded chromosomes of *P. edulis*. A: CMA-banding, B: DAPI-banding. Bar=10 μ m.

of CMA-bands were observed negative one on the chromosome arms fluoresced with DAPI. The sites of clear DAPI-bands were also CMA-negative. These fluorescent banding patterns of CMA and DAPI, and morphology of chromosomes allowed identifying each homologous chromosome in a chromosome complement.

7. *Pinus edulis*

Above seven taxa are Asian species and *P. edulis* is one of American species. The CMA-bands appeared at the interstitial regions of 16-18 long metacentric chromosomes (Fig. 8A). In certain plants one or two long metacentric chromosomes had weak CMA-bands at the interstitial regions of both arms (Table 2). The number of chromosomes with no CMA-band was four to six. The short submetacentric chromosomes had a weak CMA-band at the proximal region. Pairs of DAPI-dots appeared at the centromeric regions of all chromosomes and many thin DAPI-bands appeared at the interstitial regions of most chromosomes (Fig. 8B). CMA- and DAPI-band patterns and chromosome morphology allowed identifying most

homologous chromosome pairs in a chromosome complement.

DISCUSSION

The CMA- and DAPI-band patterns of karyotype have been reported in many conifers of Pinaceae (*Abies*: Puizina *et al.* 2008, Hizume *et al.* 2016; *Cedrus*: Dagher-Kharrat *et al.* 2001; *Keteleeria*: Hizume *et al.* 1993b; *Larix*: Hizume *et al.* 1988c, 1993a; *Picea*: Hizume *et al.* 1989a, Hizume and Kuzukawa 1995, Lubaretz *et al.* 1996, Siljak-Yakovlev *et al.* 2002; *Pinus*: Hizume *et al.* 1983, 1989b, 1990, Doudrick *et al.* 1995; *Pseudolarix*: Hizume 2015b; *Pseudotsuga*: Hizume and Kondo 1992, Hizume and Akiyama 1992; *Tsuga*: Hizume 2015a), Cupressaceae (Kondo and Hizume 1982; Hizume *et al.* 1988a; Hizume and Fujiwara 2016), Podocarpaceae (Hizume *et al.* 1988b; Davis *et al.* 1997), and Araucariaceae (Hizume and Kan 2016). In common with angiosperm species some thick CMA-bands appear at the secondary constrictions in all conifer species investigated. Feature of DAPI-band pattern varied depending on genera.

Table 2. Number of plants and number of chromosome with various CMA-band type in eight taxa of *Pinus* subgenus *Strobos*.

Taxon of subgenus <i>Strobos</i>	No. of plants	Number of chromosomes after CMA-banding							
		mI	mII	mCI	mC	m-	smI	smC	sm-
<i>P. armandii</i> var. <i>armandii</i>	7	14	2	0	0	6	2	0	0
	1	14	2	0	0	5	2	0	0
	3	14	2	0	0	4	2	0	0
<i>P. armandii</i> var. <i>amamiana</i>	2	15	1	0	0	6	2	0	0
	1	16	0	0	0	6	2	0	0
	3	18	0	0	0	4	2	0	0
<i>P. parviflora</i>	12	16	0	0	0	6	2	0	0
	4	17	0	0	0	5	2	0	0
	4	18	0	0	0	4	2	0	0
<i>P. pumila</i>	3	16	0	0	0	6	2	0	0
	1	17	0	0	0	5	2	0	0
	1	18	0	0	0	4	2	0	0
<i>P. hakkodensis</i>	5	18	0	0	0	4	2	0	0
<i>P. koraiensis</i>	1	15	0	0	0	7	2	0	0
	14	16	0	0	0	6	2	0	0
<i>P. bungeana</i>	8	4	2	0	0	16	2	0	0
<i>P. edulis</i>	1	15	2	0	0	5	0	2	0
	2	16	0	0	0	6	0	2	0
	6	16	1	0	0	5	0	2	0
	1	16	2	0	0	4	0	2	0
	3	17	0	0	0	5	0	2	0
	1	17	1	0	0	4	0	2	0
	3	18	0	0	0	4	0	2	0

Symbols show m: long metacentric chromosomes, sm: shortest chromosomes.

CMA-band types are expressed in C: centromeric CMA-band, I: interstitial CMA-band,

II: interstitial CMA-bands on both arm, -: no CMA-band.

In *Pinus* and *Larix* the fluorescent banding pattern supplied valuable information for chromosomal relationships among species.

In four diploxylon species of *Pinus* (Hizume *et al.* 1983, 1989b, 1990) the fluorescent band pattern is useful for an identification of homologous chromosomes in each chromosome complement and for reveal of interspecific relationships of chromosomes among species. Many CMA-bands appeared at the interstitial and/or centromeric regions of most chromosomes. DAPI-bands appear at the proximal region of some chromosomes and many thin DAPI-bands at the interstitial region of most chromosomes. A pair of DAPI-dots appeared at the centromeric region of each chromosome. Comparison of fluorescent band patterns among these *Pinus* species showed an obvious difference in fluorescent bands of proximal region. The four species investigated belonging to subgenus *Pinus* have thick CMA- or DAPI-band at the proximal region of most chromosomes, but the species of subgenus *Strobos* examined this time have no proximal thick band. This point seems an exact difference between subgenus *Pinus* and *Strobos*. There is similarity in

presence of interstitial thick CMA-bands, interstitial thin DAPI-bands, and centromeric DAPI-dots between subgenera.

In subgenus *Strobos* seven taxa had 16-18 interstitial CMA-bands in each chromosome complement and *P. bungeana* showed less number of six interstitial CMA-bands (Table 2). This difference might coincident with position of phylogenetic trees of subgenus *Strobos* (Eckert and Hall 2006; Wang and Wang 2014) and it needs to analyze the fluorescent banding in more species of this subgenus to confirm a phylogenetic importance of number of interstitial CMA-bands. The American species, *P. edulis* showed a proximal CMA-band on the short submetacentric chromosomes. This character should be also confirmed by a survey in other American species of this subgenus.

Molecular cytogenetic studies have performed in seven species of subgenus *Strobos* (Cai *et al.* 2006; Shibata *et al.* 2016). All interstitial FISH signals of 45S rDNA probe seems correspond to thick CMA-bands as reported in subgenus *Pinus* (Hizume *et al.* 1992, 2001, 2002b) and *Strobos* (Cai *et al.* 2006; Shibata *et al.* 2016).

In seven species of subgenus *Strobos* FISH signals of 5S rDNA appeared in the close distal side of 45S rDNA signal at the terminally interstitial region of chromosomes. The closely associated 45S and 5S rDNA signals seems that close double CMA-bands containing each 45S and 5S rDNA were observed sometimes on not-well condensed chromosome preparation (Fig. 4). 5S rDNA loci appear on CMA-band in other conifers (Hizume and Kuzukawa 1995; Hizume *et al.* 1996; Vischi *et al.* 2003; Shibata *et al.* 2004; Shibata and Hizume 2008). In all the species of subgenus *Pinus* examined, several chromosomes had proximal FISH signals of *PCSR* but the species of subgenus *Strobos* had not at all (Shibata *et al.* 2016). This phenomenon corresponds to an absence of a thick fluorescent band at proximal region of any chromosome in the subgenus *Strobos*.

In the species of subgenera *Pinus* (Hizume *et al.* 1983, 1989b, 1990) and *Strobos* (Figs. 2, 5-7) examined many interstitial DAPI-bands appeared commonly on their chromosome arms. As DAPI binds preferentially AT-base pairs of DNA sequence bright DAPI-bands indicate that certain AT-rich sequences are localized in the DAPI-band region. In species of subgenus *Pinus* the FISH using a probe of telomere sequence showed many interstitial signals on thin DAPI-bands in most chromosomes (Hizume *et al.* 2002b; Islam-Faridi *et al.* 2007; Shibata *et al.* 2016) but did not appear at any chromosomes in two species of subgenus *Strobos* (Shibata *et al.* 2016). The phenomenon indicates that AT-rich DNA sequences localizing in the interstitial DAPI-bands in genus *Pinus* are different between subgenera. It is desired an isolation of AT-rich sequence locating at an interstitial DAPI-band of subgenus *Strobos* species and application as a probe for FISH. The centromeric DAPI-dots in all chromosomes of both subgenera are expected to contain unknown common DNA sequence in *Pinus*. When the DNA sequence locating at DAPI-dots is identified, the centromeric sequence will be important for understanding of *Pinus* or conifer chromosome structure.

In taxonomic system of *Pinus* the subgenus *Strobos* is divided into two sections *Strobos* and *Parrya* and, each section dose into two and five subsections, respectively (Farjon and Styles 1997; Gernandt *et al.* 2003, 2005; Price *et al.* 1998). Molecular phylogenetic studies were performed using ITS sequence (Liston *et al.* 1999) and chloroplast DNA sequences (Eckert and Hall 2006), and supported the sections and some subsections. Below is a correspondence between species investigated and subdivisions of subgenus *Strobos*.

Section *Strobos*

- subsection *Cembrea* ----- *P. koraiensis*, *P. pumila*
- subsection *Strobi* ----- *P. armandii*, *P. parviflora*

Section *Parrya*

- subsection *Cembroides* ----- *P. edulis*
- subsection *Gerardianea* ----- *P. bungeana*

The band pattern in number and location of CMA-bands (Table 2) is similar among species of section

Strobos examined excepting for *P. bungeana* showing small number of interstitial CMA-bands. The short chromosomes of most species had interstitial CMA-band on long arm but only *P. edulis* have a proximal CMA-band. The two species were put in section *Parrya* and belonged to different subsections. The fluorescent banded karyotypes suggest that the taxa in section *Parrya* might show higher diversity than other section *Strobos*. Our observation on fluorescent chromosome banding was achieved on only eight taxa out of about 50 species in subgenus *Strobos*. When fluorescent banded karyotypes are carefully observed in more species and compared with reliable molecular phylogenetic relationship, the chromosomal relationships among the species or a feature of chromosomes in phylogenetic diversity of subgenus *Strobos* will be revealed.

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