INTERNATIONAL JOURNAL OF BIOSCIENCES AND BIOTECHNOLOGY • Vol. 8 No. 2 • April 2021
eISSN: 2655-9994
pISSN: 2303-3371
https://doi.org/10.24843/IJBB.2021.v08.i02.p06

CYTOLOGICAL VARIATION OF $(AAG)_7$ REPEAT ON LETTUCE CHROMOSOMES BY FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

Winny Dewi Widarmi [1,2]*, Shinji Kikuchi [1], Hidenori Sassa [1], and Takato Koba [1]
[1]Laboratory of Genetics and Plant Breeding, Graduate School of Horticulture, Chiba University, Matsudo 648, Matsudo, Chiba 271-8510, Japan
[2]Corteva Agriscience (PT Dupont Agricultural Product Indonesia), Cibis Nine 10th floor, Jl. TB Simatupang No. 2, East Cilandak, South Jakarta 12560, Indonesia
*Corresponding author: winnydw@yahoo.com

ABSTRACT

Cytological studies using fluorescence *in situ* hybridization (FISH) technique provides phylogenetical information in closely related taxa and have been widely applied for karyotyping and studying chromosomal organization and evolution in plant species. In the present study, FISH using a microsatellite sequence of $(AAG)_7$ as the probe was performed in order to discriminate the chromosomes in four *Lactuca* species, *i.e.*, *L. sativa*, *L. serriola*, *L. salign*a, and *L. virosa*. The experiment was carried out in April to September 2018 at Laboratory of Genetic and Plant Breeding of Breeding of Graduate School of Horticulture, Chiba University, Japan. Different distribution patterns of $(AAG)_n$ signals were shown on the chromosomes in the four *Lactuca* species studied, In *L. sativa* and *L. serriola*, FISH with $(AAG)_7$ sequences revealed dispersed distribution patterns with one pair of bright signals, respectively. While in *L. saligna* and *L. virosa*, distinct signals with different intensities were observed in two pairs of chromosomes of *L. saligna* and five pairs of chromosomes of *L. virosa*. In conclusion, the AAG repeat signals could be used as cytogenetic landmarks for chromosome identification in *Lactuca* species.

Keywords: $(AAG)_7$ repeat, FISH, microsatellite, lettuce

INTRODUCTION

Cultivated lettuce (*Lactuca sativa* L.) is an economically important member of the genus *Lactuca*, which includes closely related wild species, *L. serriola*, *L. salign*a and *L. virosa.* All of them have $2n = 2x = 18$ chromosomes, and the estimated genome size of *L. sativa* is 2.7 Gb, with repetitive sequences accounting for 74.2% of the genome (Koopman *et al.,* 1998; Michelmore *et al.,* 1994; Reyes-Chin-Wo *et al.,* 2017). So far, karyotype analyses were carried out to demonstrate the relationships among the cultivated lettuce and its closely related species (Haque and Godward 1985; Koopman and De Jong 1996; Lindqvist 1960; Matoba *et al*., 2007). Koopman *et al*. (1993) applied chromosome banding techniques to discriminate the chromosomes of *Lactuca* species. Widarmi *et al. (2019*) and Matoba *et al.* (2007) applied FISH technique using 45S and 5SrDNAs to identify the chromosomes of *Lactuca* species. From these studies, it was clarified that chromosomal characteristics of

L. sativa and *L. serriola* were almost similar to each other, while cytological features of *L. saligna* are slightly different from *L. sativa/L. serriola*, and *L. virosa* is significantly different from the other species.

Fluorescence *in situ* hybridization (FISH) using repetitive DNA sequences as the probes results in chromosome-specific hybridization patterns that allows identification of all chromosomes within a species, making this technique a powerful tool for the karyotyping and studying chromosomal organization and evolution in many plant species (Mukai *et al.* 1993; Pedersen and Langridge 1997; Jiang and Gill 2006).

The distribution of microsatellite $(AAG)_n$ on the chromosomes of wheat, barley, and related species in Triticeae were well studied. The sequence $(AAG)_5$ was used as the probe for *in situ* hybridization to identify different chromatin classes in wheat (Cuadrado *et al.,* 2000; 2008). The oligonucleotide sequence $(AAG)_7$ was used for genome and chromosome identification via *in situ* hybridization in cultivated barley and related species of the Triticeae (Poaceae) (Pedersen *et al.*, 1996) The sequence $(AAG)_9$ was used for the complementary identification of all A-genome chromosomes in diploid and polyploid wheat (Badaeva *et al.*, 2015, 2016). Also the sequence $(GAA)_6$ was used to perform a more accurate karyotype analysis of *Zanthoxylum armatum* (Luo *et al.*, 2018). Thus, probing AAG repeats for *in situ* hybridization seems to be a useful diagnostic tool in cytogenetics in many plant species.

In the present investigation, we aim to perform FISH using $(AAG)_7$ as the probe in order to clarify the possibility of using the microsatellite repetitive sequences for discrimination and/or identification of the chromosomes in *Lactuca* species.

MATERIALS AND METHODS

Chromosomal study of lettuce chromosomes by FISH using $(AAG)_7$ as the probe was conducted in April to September 2018 at Laboratory of Genetic and Plant Breeding of Graduate School of Horticulture, Chiba University, Japan.

Plant materials and chromosome sample preparation

Seeds of *L. sativa* 'Shinano Hope', *L. saligna*, *L. serriola*, and *L. virosa* were kindly provided by Nagano Vegetable and Ornamental Crops Experimental Station, Japan. They were germinated and planted at the Graduate School of Horticulture, Chiba University, Matsudo, Japan. Root tips of 1-2 cm long were collected from the seedlings and young plants after a few weeks growth in 500-mL pots. The root tips were fixed in 3 ethanol: 1 acetic acid (v/v) at 4°C for least 1 day before preparation. Fixed root tips were washed three times with distilled water, and the samples

eISSN: 2655-9994
pISSN: 2303-3371
https://doi.org/10.24843/IJBB.2021.v08.i02.p06

were stored in 70% ethanol at 4°C. Chromosomes preparations were made through enzymatic maceration, *i.e.*, root tips were digested with 10 µl enzymatic mixture (4% Cellulase Onozuka RS, Yakult; 2% Pectolyase Y-23, Kyowa; and 1% Pectinase from *Aspergillus*, Sigma) at 37°C for 45-90 minutes. Squashing was made in 45% acetic acid and the slides were stored at -80°C for more than12 h. The glass covers were removed by forceps for the following FISH experiment.

DNA probe preparation

The $(AAG)_7$ probe was labelled with digoxigenin-11-dUTP (Roche) by terminal deoxynucleotidyl transferase (TdT) (Thermo Fisher Scientific) following the manufacturer's instructions.

FISH analysis

Chromosome slides were re-fixed in 1% paraformaldehyde/1× PBS for 10 min at room temperature. Procedures for hybridization and detection were followed as those described by Kikuchi *et al.*(2008). The slides were counter-stained with 4,6-diamidino-2-phenylindole (DAPI) in a VectaShield antifade solution (Vector Laboratories). All FISH images were captured with an Olympus BX53 fluorescence microscope with a CCD camera (CoolSNAP MYO; Photometrics). The FISH images were processed by Metamorph, Metavue imaging series version 7.8 and edited with Adobe Photoshop CC 2017. CHIAS IV (http://www2.kobeu.ac.jp/~ohmido/index03.htm) was used for the construction of the ideogram.

RESULTS AND DISCUSSION

RESULT

Images of mitotic metaphase of four *Lactuca* species visualized after FISH using $(AAG)_7$ sequence as the probe are shown in Figure. 1.

Different distribution patterns of $(AAG)_n$ signals were shown in all four *Lactuca* species studied. In *L. sativ*a and *L. serriola*, FISH with $(AAG)_7$ sequences revealed dispersed distribution patterns with one pair of bright signals, respectively. While in *L. saligna* and *L. virosa*, distinct signals with different intensities were observed in two pairs of chromosomes of *L. saligna* and five pairs of chromosomes of *L. virosa*.

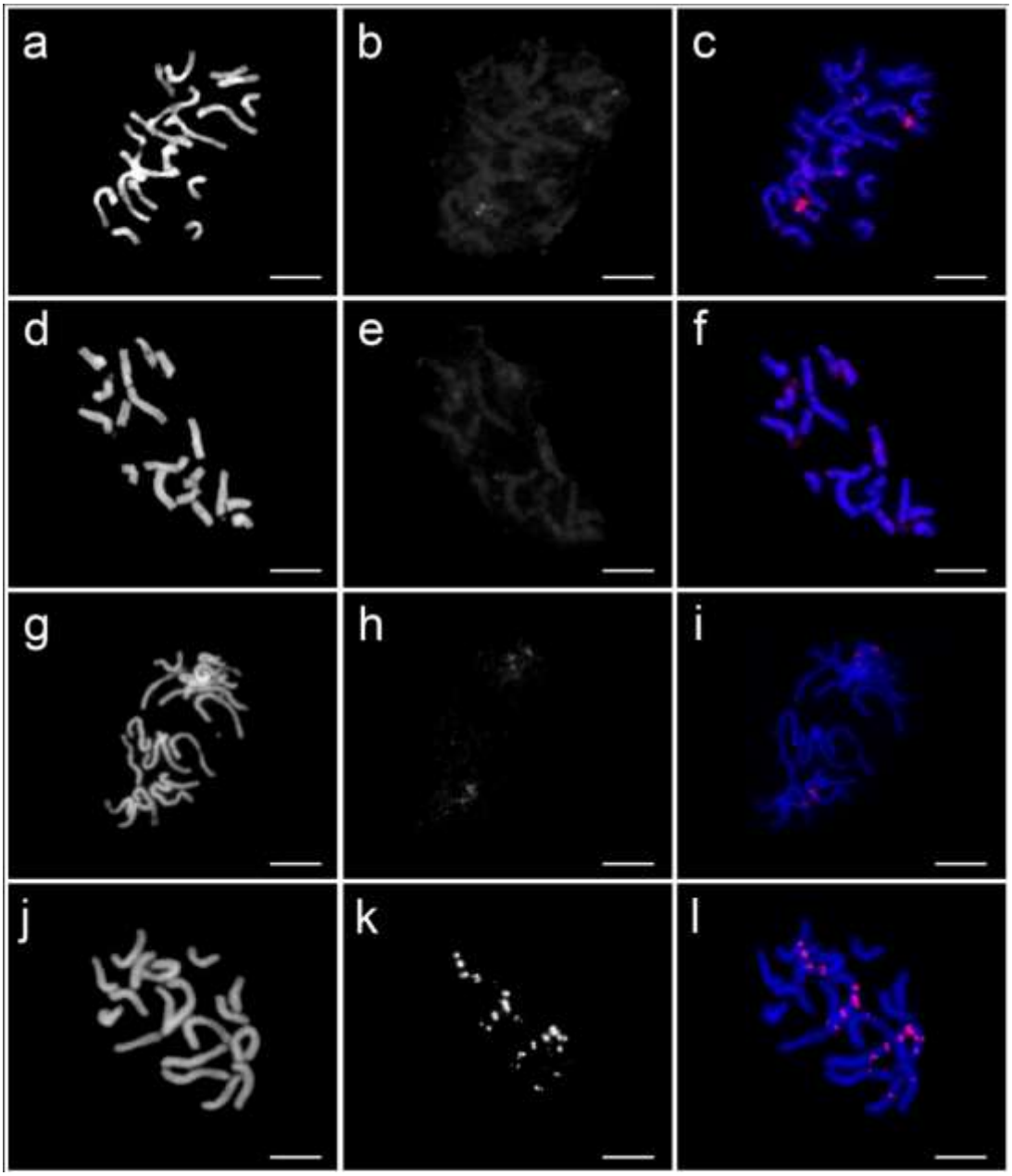

Fig. 1. FISH of $(AAG)_7$ (red) probe in four *Lactuca* species. (a-c) *L. sativa* cv. 'Shinano Hope' showing dispersed signals distribution in all chromosomes and two bright signal sites of $(AAG)_7$, (d-f) *L. serriola* showing dispersed signals distribution in all chromosomes with one pair of weak signals, (g-i) *L. saligna* showing bright signals in two pairs of chromosomes, (j-l) *L. virosa* showing strong signals. Scale bar=10 µm

Figure 2 shows FISH karyotypes of four *Lactuca* species, which were arranged according to the order of the lengths of the chromosomes based on the FISH images shown in Figure 1. Quantitative ideograms of the prometaphase chromosomes of the four lettuce species generated by CHIAS IV based on the characteristics of condensation patterns, chromosome lengths, and FISH signals are shown in Fig. 3.

eISSN: 2655-9994
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https://doi.org/10.24843/IJBB.2021.v08.i02.p06

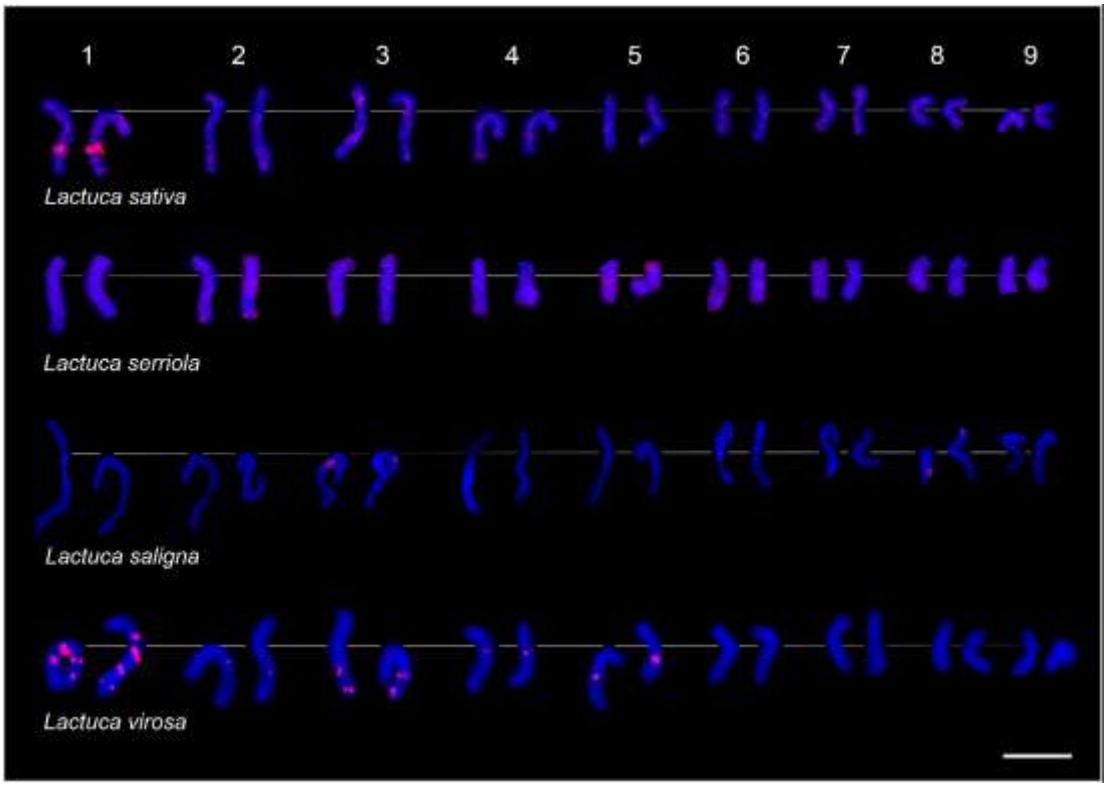

Fig. 2. FISH karyotypes of four *Lactuca* species with $(AAG)_n$ signals. Homologous pairs of chromosomes were arranged and numbered according to their lengths. Horizontal lines show the positions of the centromeres. Scale Bar=10 µm

Dispersed signal distribution was observed in prometaphase chromosomes of *L. sativa*, with one pair of strong pericentromeric signals on Chromosome 1. *L. serriola* also showed similar dispersed distribution as those of *L. sativa* chromosomes, with one pair of small telomeric signals on Chromosome 2. By contrast, distinct signals were observed on *L. saligna* and *L. virosa* chromosomes. In *L. saligna*, Chromosomes 3 and 8 showed clear signals at the interstitial regions of the long arm and subtelomeric region of the short arm, respectively. While, in *L. virosa*, Chromosome 1 had four major signals, two on the proximal region of the short and long arms, and two on the interstitial region of the long arm. Chromosome 3 showed two pairs of signals, one on the interstitial region and one on the subtelomeric region of the long arm. Chromosomes 2 and 5 showed one pair of clear signals on the long arms of the chromosomes and Chromosome 4 had one pair of signals on the proximal region of the chromosome. With their distribution patterns, Chromosomes 1 to 5 could be clearly discriminated by using $(AAG)_7$ probe.

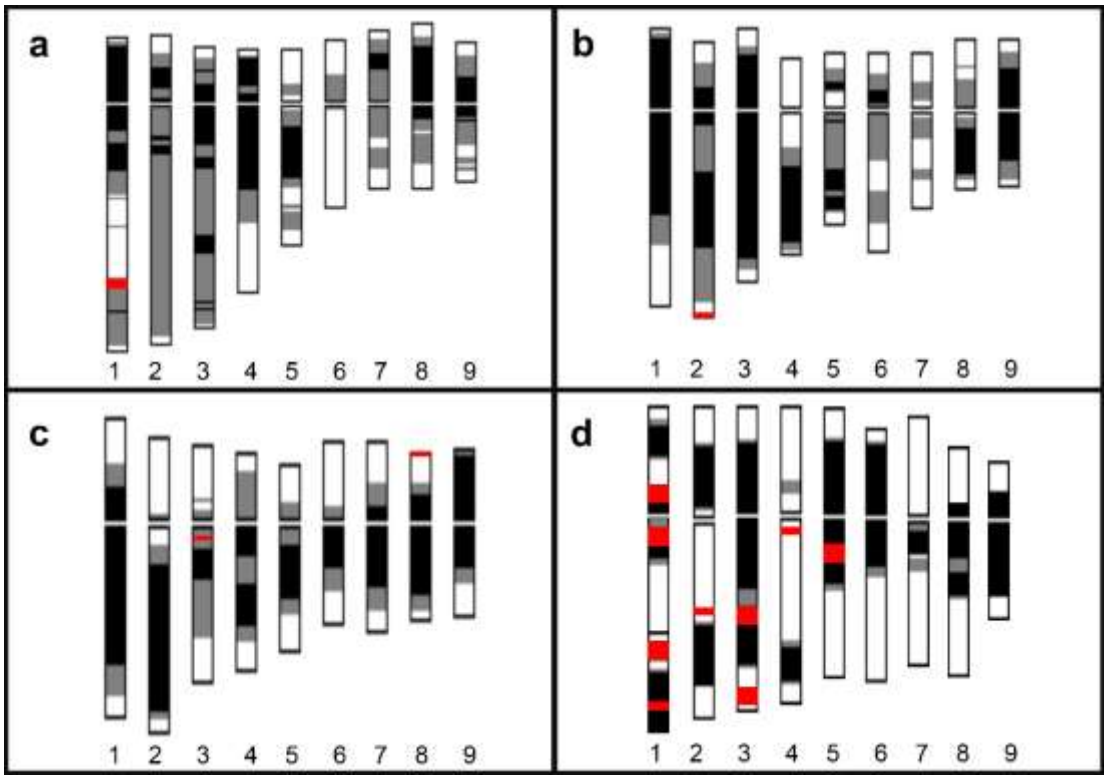

Fig. 3. FISH ideogram of prometaphase chromosomes of *L. sativa* (**a**), *L. serriola* (**b**), *L. saligna* (**c**) and *L. virosa* (**d**) with $(AAG)_n$ signals (red) constructed by CHIAS IV. Numerals denote the chromosome numbers according to their chromosome lengths. Black, gray, and white areas represent chromosome regions with heavy, intermediate and slight condensation, respectively. The gaps in the middle of the chromosomes indicate the centromere regions. Homologous pairs of chromosomes were arranged and numbered according to their lengths. Horizontal lines show the positions of the centromeres

DISCUSSION

Schmidt and Heslop-harrison (1996) demonstrated that microsatellites, representing a substantial fraction of the genome, showed chromosome-specific amplification in plants. The repeat AAG presented different *in situ* hybridization patterns that provided cytogenetic landmarks for chromosome identification in barley, *Hordeum vulgare* ssp. vulgare (Carmona *et al.*, 2013). Also, Sonah *et al.*, (2011) described that AAG repeat units are major contributors to the genomes of dicots.

As shown in the previous reports (Lindqvist 1960; Koopman and De Jong 1996), the karyotype analyses indicated the closest relationship between *L. sativa* and *L. serriola*. Our results showed that karyotype of *L. sativa* was similar to that of *L. serriola*, since the dispersed signal distribution patterns of $(AAG)_7$ repeats were similarly observed in both species studied. However, *L. sativa and L. serriola* could be distinguished easily by

eISSN: 2655-9994
pISSN: 2303-3371
https://doi.org/10.24843/IJBB.2021.v08.i02.p06

the presence of the AAG pericentromeric and telomeric signals on the Chromosomes 1 and 2, respectively. On the other hand, *L. saligna* and *L. virosa* showed their specific karyotype features with AAG signals compare to the other two species (Figure 2). Lindqvist (1960), Haque and Godward (1985), Koopman *et al.*, (1993), and Koopman and De Jong (1996) described that cytological features of *L. saligna* and *L. virosa* are different from those of *L. sativa*/*L. serriola*. Based on the sequence comparisons of chloroplast DNAs in *Lactuca* species, Wei *et al.* (2017) revealed that *L. sativa*/*L. serriola* group was different from that of *L. saligna*/*L. virosa*. This fact indicates that the distribution of microsatellite sequence on the chromosomes reflects the phylogenetic differentiation of the species in the genus *Lactuca*. Cytological analyses on the distribution of microsatellite sequences would provide insight into the phylogenetic differentiation of the species in the genus *Lactuca*.

CONCLUSIONS

In conclusion, the AAG repeat presented different *in situ* hybridization patterns that provided cytogenetic landmarks for chromosome identification in *Lactuca* species. These such variation results provide insight into the distribution pattern and evolution of SSRs in the genome of *Lactuca*.

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