Characterization of Lettuce Chromosomes Based on Condensation Patterns and Physical Mapping of 45S and 5S rDNAs Using Fluorescence *in situ* Hybridization

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Summary *Lactuca sativa* L. is one of the most important reference species for genome research in Compositae. In the present study, chromosome image analysis system IV (CHIAS IV) and fluorescence *in situ* hybridization (FISH) were applied to obtain detailed information of the characteristics of lettuce chromosomes. One lettuce cultivar of *L. sativa* and three wild species of *L. serriola*, *L. saligna* and *L. virosa* were used to analyze chromosome lengths, arm ratios and degrees of chromosomal condensation at prometaphase. Locations of 45S and 5S rRNA genes were determined by FISH technique. Our results showed that nine pairs of homologous chromosomes could be discriminated for all four species by their morphological characteristics, condensation patterns (CPs) and localization of rRNA loci. Statistical analysis showed that the total length of *L. saligna* chromosomes was significantly the shortest among the species tested. The present study is the first for the physical mapping of condensed regions and rDNAs using CPs and FISH of prometaphase chromosomes in lettuce. The information provided here would be valuable for practical breeding and genetics of lettuce.

Keywords CHIAS IV, Condensation pattern, FISH, Lettuce, rDNA.

Cultivated lettuce, *L. sativa* ($2n=18$), is one of the most important vegetables in the group of leafy plants produced commercially in many countries worldwide. World production of lettuce in 2016 was more than 26 million tons, and those were mainly produced in Asia, North and Central America and Europe (FAO 2017, <http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor>). *L. sativa* is an annual plant in the subtribe Lactucinae, tribe Lactuceae (Cichorieae), subfamily Cichorioideae of the family Compositae (Asteraceae) (Křístková *et al.* 2008). However, there are uncertainties about the phylogenetic relationships within the genus *Lactuca*, mainly due to the complex and variable morphological characters of the species (Zhen *et al.* 2017). Lebeda *et al.* (2004, 2009) divided the genus into seven sections and two geographical groups based on taxonomic and biogeographical criteria, *i.e.*, *Lactuca* (subsection *Lactuca* and *Cyanicae*), *Phaenioxpus*, *Mulgedium*, *Lactucopsis*, *Tuberosae*, *Micranthae* and *Sororiae*, and African and North American groups.

There are three wild species in the subsection *Lactuca*, namely, *L. serriola*, *L. saligna* and *L. virosa*; all being $2n=18$, self-pollinated plants and important genetic resources for lettuce breeding (Michelmore *et al.* 1994, Koopman *et al.* 1998). It is estimated that *L. serriola*, was at first domesticated in Egypt more than 4000 years

ago, and was the ancestral species of the presently cultivated lettuce (Harlan 1986).

So far, many cytological studies, karyotype analyses, chromosome banding and FISH techniques were carried out to demonstrate the relationships among the cultivated lettuce and its closely related species. Lindqvist (1960) was the first who studied the most comprehensive chromosome morphology of *Lactuca* species. He observed distinct differences in metaphase chromosome morphology between *L. virosa* and the other three species, *L. sativa*, *L. serriola* and *L. saligna* and found that the karyotypes of *L. sativa* and *L. serriola* were identical, and the measurements of the chromosomes revealed certain dissimilarities between these two species and *L. saligna*. Haque and Godward (1985) described the somatic metaphase chromosome pairs on the basis of their lengths, centromere positions, and presence or absence of satellites of three cultivars of *L. sativa*, *L. serriola* and a cultivar of *Cichorium intybus*. He found slight differences in chromosome lengths, the *L. sativa* cultivars and *L. serriola* being distinguishable by their mean lengths. Koopman *et al.* (1993) obtained chromosome banding patterns with C- and N-bandings, and AgNO₃ staining in somatic metaphase of four *Lactuca* species and found out that *L. sativa* and *L. serriola* had almost identical chromosome morphology, and *L. saligna* differed only slightly from them, but *L. virosa* was quite distinct from the other species. Koopman and de Jong (1996) applied a numerical analysis to study the evo-

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lutionary relationships among *L. sativa*, *L. serriola*, *L. saligna* and *L. virosa* using karyotypes and relative DNA content. They found that *L. sativa* and *L. serriola* were very closely related, and *L. saligna* differed significantly from *L. sativa/serriola* only in its relative DNA amount. *L. virosa* differed from *L. saligna* and *L. sativa/serriola* for all parameters. They also found the largest difference between *L. saligna* and *L. virosa*, and considered that *L. saligna* and *L. virosa* are advanced species that evolved in different directions.

Matoba *et al.* (2007) examined karyotypes and chromosomal distribution of 5S and 18S rDNAs in *L. sativa*, *L. serriola*, *L. saligna* and *L. virosa* by FISH. The karyotype analysis revealed dissimilarity between *L. virosa* and the other three species, and it was difficult to distinguish *L. sativa* and *L. serriola* based solely on karyotype analysis. FISH result showed one 5S rDNA and two 18S rDNA loci in all four species. They revealed that both 5S and 18S rDNA loci of *L. sativa*, *L. serriola* and *L. saligna* were identical in their numbers and locations. However, the locations of rDNAs of *L. virosa* were different from those of the other species.

Since metaphase chromosomes of horticultural plants are relatively small compared to those of other plants, *e.g.*, wheat and barley, it has been rather difficult to discriminate their individual chromosomes. In lettuce, all cytological studies, karyotype analyses, and chromosome banding have been conducted mainly using metaphase chromosomes, which are in highly condensed condition (Matoba *et al.* 2007). Fukui (1986) and Fukui and Mukai (1988) developed the CHIAS through measuring chromosome condensation patterns (CPs) in small plant chromosomes at prometaphase. So far, chromosomes of many species have been characterized with CHIAS, *e.g.*, in saltbush (Fukui and Mukai 1988), rice (Fukui and Iijima 1991), soybean (Yanagisawa *et al.* 1991), tea plant (Furukawa *et al.* 2017), and others. CP is considered to be a useful and reliable parameter to characterize morphological traits and identify the chromosomes in many plant species, having small chromosomes. On the other hand, FISH technique provides phylogenetical information in closely related taxa, and FISH technique using rRNA genes and telomere sequences as the probes have been widely applied for karyotyping and studying chromosomal organization and evolution in many plant species.

A combination of CP and FISH analyses have provided novel aspects of chromosome images *via* physical localization of repetitive sequences on the elongated prometaphase chromosomes. The aim of the present study is to identify and discriminate lettuce chromosomes in prometaphase cells through karyotype analysis based on the CPs using CHIAS IV and determining the locations of 4S and 5S rDNAs by FISH.

Materials and methods

Plant materials and chromosome sample preparation

The seeds of *L. sativa* cv. 'Patriot', *L. serriola*, *L. saligna* and *L. virosa* investigated were supplied from Nagano Vegetable and Ornamental Crops Experimental Station, Japan. Root tips of 1 cm long were collected from the seedlings or young plants grown for a few weeks after the seedling stage in 500-mL pots. They were fixed in the solution of 3 ethanol:1 acetic acid (v/v) at 4°C for at least one day before chromosome preparation. Fixed root tips were washed three times with distilled water, and were stored in 70% ethanol at 4°C until use. Chromosome preparations were made through enzymatic maceration, where root tips were digested with an enzymatic mixture [4% cellulase Onozuka RS (Yakult), 2% pectolyase Y-23 (Kyowa), and 1% pectinase (Sigma)] at 37°C for 45–90 min. Squashing was made in 45% acetic acid on the glass slides and the slides were stored at –80°C more than 12 h, and the coverslips were removed with a razor blade. Chromosomes were stained with 1% (v/v) Giemsa (Merck) in 1/15M sodium phosphate buffer (pH 6.8) for 2 min and air-dried.

DNA probe preparation

The 4S rDNA (pTa71, Gerlach and Bedbrook 1979) and 5S rDNA (Fukui *et al.* 1994) were used as the probes for FISH analyses. The 5S rDNA was amplified from the genomic DNA of *L. sativa* by polymerase chain reaction (PCR) using coding regions of 5S rDNA as the primers (5'-GATCCCATCAGAACTCCG AAG-3' and 5'-CGGTGA TTTAGT GCTGGTAT-3') (Fukui *et al.* 1994). PCR products with about 300 bp were inserted into the *EcoRV* site of pZER0-2 Vector (Invitrogen) according to the manufacturer's instruction. The plasmid inserted with 5S rDNA unit was used as the probe. The 4S and 5S rDNAs were labeled with DIG-Nick Translation Mix (Roche), and Biotin-Nick Translation Mix (Roche) according to manufacturer's instruction, respectively.

FISH analysis

Chromosome slides were re-fixed in 1% paraformaldehyde in PBS for 10 min at room temperature. Procedures for FISH were followed as described by Kikuchi *et al.* (2008). The 10 µL of hybridization cocktail containing 50% formamide, 10% dextran sulfate, 2×SSC, and 100–500 ng labeling probe was applied to the slide, covered with a coverslip, and then denatured for 3 min at 80°C on a heat block. After incubation at 37°C for one day, the slide was washed and briefly dried. Detection of the biotin- and DIG-labeled probes was performed with biotinylated streptavidin-FITC (Vector Laboratories) and anti-digoxigenin-rhodamine (Roche), respectively. The slides were counter-stained

with 5 $\mu\text{g/mL}$ of 4,6-diamidino-2-phenylindole (DAPI) in a VectaShield antifade mounting medium (Vector Laboratories). All FISH images were captured with an Olympus BX53 fluorescence microscope equipped with a CCD camera (CoolSNAP MYO, Photometrics). The FISH images were processed by MetaMorph, Meta imaging series version 7.8 (Molecular Devices, San Jose) and edited with Photoshop CC 2017 (Adobe).

Image analysis

CHIAS IV (<http://www2.kobe-u.ac.jp/~ohmido/index03.htm>) was used to analyze the images of the lettuce prometaphase chromosomes. Giemsa-stained chromosome images were converted to an 8-bit greyscale and the fluorescence signal images were captured as red-green-blue (RGB) images with 8-bit grey levels for the construction of the ideogram. Homologous chromosomes were detected by observing morphological characteristics and CPs, and designated as Chromosomes 1 to 9 according to their lengths, excluding satellite. Data for arm lengths and degrees of condensation of nine pairs of chromosomes were collected in six chromosome spreads. The arm ratio was calculated as the length of the long arm divided by the length of the short arm (Levan *et al.* 1964). The comparison of the mean lettuce chromosome lengths among the species was done by Student's *t*-test. For statistical analysis, StatPlus ver. 6 (AnalystSoft Inc., Walnut) was used.

Results

Morphology of lettuce prometaphase chromosomes

Squashed prometaphase chromosomes stained with Giemsa of the four species are shown in Fig. 1. In all species, chromosome pairs could be discriminated with their morphologies and condensation patterns; heavily condensed chromosome regions were recognizable as densely stained regions and were distinguished from weakly as loosely condensed regions.

Table 1 shows numerical data of the prometaphase chromosomes. The lengths of the chromosomes varied from 5.34 to 13.24 μm in four species. The four longest chromosomes were submetacentric, and the others were metacentric and submetacentric. The total chromosome lengths of *L. sativa* and *L. serriola* were almost the same (82.99 μm and 82.94 μm , respectively). *L. saligna* had the shortest total lengths (70.04 μm), and *L. virosa* had the longest total lengths (85.54 μm).

Physical mapping of 45S and 5S rDNAs

Physical locations of the 45S and 5S rDNAs on the chromosomes are shown in Fig. 2. Two pairs of 45S rDNA signals were detected at the secondary constrictions and satellites on the short arms of the chromosomes of all species used. A pair of 5S rDNA signals was detected at the interstitial regions of the short arms of chromosomes of all species. A pair of 5S signals was

Fig. 1. Giemsa-stained prometaphase chromosomes of *L. sativa* (A), *L. saligna* (B), *L. serriola* (C) and *L. virosa* (D). Numerals indicate the chromosome numbers according to their length (see text for explanation). Scale bars=10 μm .

Table 1. Morphology of lettuce at prometaphase chromosomes.

Species	Chromosome No	Length (mean $\pm$ SE μ m)	Arm Ratio ($r\pm$ SE)	Type	rDNA
<i>Lactuca sativa</i> L.	1	12.65 $\pm$ 0.19	2.76 $\pm$ 0.22	sm	45S
	2	11.64 $\pm$ 0.32	2.07 $\pm$ 0.28	sm	
	3	10.41 $\pm$ 0.35	1.92 $\pm$ 0.26	sm	
	4	9.80 $\pm$ 0.40	1.74 $\pm$ 0.17	sm	
	5	9.27 $\pm$ 0.25	2.44 $\pm$ 0.31	sm	45S, 5S
	6	8.46 $\pm$ 0.33	2.13 $\pm$ 0.24	sm	
	7	7.53 $\pm$ 0.23	1.59 $\pm$ 0.11	m	
	8	7.03 $\pm$ 0.17	1.69 $\pm$ 0.19	m	
	9	6.20 $\pm$ 0.38	1.26 $\pm$ 0.25	m	
	Total	82.99 $\pm$ 2.61 ^a			
<i>Lactuca saligna</i> L.	1	11.09 $\pm$ 0.45	2.65 $\pm$ 0.36	sm	45S
	2	10.13 $\pm$ 0.40	2.64 $\pm$ 0.14	sm	
	3	9.15 $\pm$ 0.39	2.84 $\pm$ 0.32	sm	
	4	8.02 $\pm$ 0.39	2.23 $\pm$ 0.38	sm	
	5	7.27 $\pm$ 0.32	2.03 $\pm$ 0.24	sm	45S, 5S
	6	6.75 $\pm$ 0.21	1.68 $\pm$ 0.22	m	
	7	6.37 $\pm$ 0.16	1.62 $\pm$ 0.15	m	
	8	5.92 $\pm$ 0.24	1.62 $\pm$ 0.12	m	
	9	5.34 $\pm$ 0.29	1.25 $\pm$ 0.11	m	
	Total	70.04 $\pm$ 2.84 ^b			
<i>Lactuca serriola</i> L.	1	12.61 $\pm$ 0.18	2.81 $\pm$ 0.30	sm	45S
	2	11.84 $\pm$ 0.26	2.92 $\pm$ 0.42	sm	
	3	10.78 $\pm$ 0.31	2.71 $\pm$ 0.46	sm	
	4	10.27 $\pm$ 0.42	2.65 $\pm$ 0.23	sm	
	5	9.11 $\pm$ 0.34	2.16 $\pm$ 0.44	sm	45S, 5S
	6	8.09 $\pm$ 0.19	1.79 $\pm$ 0.33	sm	
	7	7.37 $\pm$ 0.22	1.95 $\pm$ 0.24	sm	
	8	6.87 $\pm$ 0.22	1.67 $\pm$ 0.19	m	
	9	6.01 $\pm$ 0.14	1.53 $\pm$ 0.17	m	
	Total	82.94 $\pm$ 2.26 ^a			
<i>Lactuca virosa</i> L.	1	13.24 $\pm$ 0.17	2.16 $\pm$ 0.39	sm	45S
	2	12.28 $\pm$ 0.24	2.42 $\pm$ 0.30	sm	
	3	11.53 $\pm$ 0.13	2.51 $\pm$ 0.36	sm	
	4	10.36 $\pm$ 0.26	2.64 $\pm$ 0.36	sm	
	5	8.64 $\pm$ 0.38	1.69 $\pm$ 0.16	m	45S, 5S
	6	7.93 $\pm$ 0.26	1.76 $\pm$ 0.21	sm	
	7	7.40 $\pm$ 0.16	1.70 $\pm$ 0.36	m	
	8	6.95 $\pm$ 0.11	1.96 $\pm$ 0.46	sm	
	9	6.21 $\pm$ 0.12	1.80 $\pm$ 0.33	sm	
	Total	84.54 $\pm$ 1.83 ^a			

SE=Standard Error; Arm ratio, r =Long arm length/short arm length; Type: m, metacentric; sm, submetacentric; st, subtelocentric (Levan *et al.* 1964). Means with the same letter are not significantly different at the 5% level.

localized carrying one of the two pairs on 45S rDNA signal.

Characterization of lettuce chromosomes

Quantitative ideograms of the prometaphase chromosomes of the four lettuce species generated by CHIAS IV based on the characteristics of CPs, chromosome lengths, and FISH signals are shown in Fig. 3. It was clarified that the distal regions of the short and long arms of all *Lactuca* chromosomes studied were relatively heavily condensed than the proximal and interstitial regions.

Characteristics of the chromosomes of each species were as follows; i) *L. sativa*: Chromosomes 1 and 5 showed heavy condensation in the long arms. Chromosome lengths of this species ranged from 6.20 to

12.65 μ m (Table 1). Chromosome 1 to 6 were submetacentric and Chromosomes 7 to 9 were metacentric. Two pairs of 45S rDNA signals were detected at the end of the short arms of Chromosomes 3 and 5. One pair of 5S rDNA signals was detected in the interstitial regions of the short arms of Chromosome 5. ii) *L. saligna*: the chromosome lengths of this species were the shortest among the species used, ranging from 5.34 to 11.09 μ m (Table 1). Chromosomes 1 to 5 were submetacentric and Chromosomes 6 to 9 were metacentric. Four large 45S rDNA signals were observed at the end of the short arms of Chromosomes 3 and 5. Two 5S rDNA signals were detected in the interstitial regions of the short arms of Chromosome 5. Chromosome 4 and 5 had condensed regions on the short arm, while Chromosome 9 had a condensed region on the long arm. All chromosomes

Fig. 2. FISH prometaphase chromosome spreads (A, C, E, G) and karyotypes (B, D, F, H) of the lettuce showing 45S (red) and 5S (green) rDNAs signals in *L. sativa* (A, B), *L. saligna* (C, D), *L. serriola* (E, F) and *L. virosa* (G, H). Chromosomes are arranged according to their lengths. Scale bars=10 μ m.

Fig. 3. Ideogram of prometaphase chromosomes of *L. sativa* (A), *L. saligna* (B), *L. serriola* (C) and *L. virosa* (D), constructed by CHIAS IV. Numerals denote the chromosome numbers according to their chromosome lengths (see text for details). 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. Red and green circles demonstrate 45S and 5S rDNA loci, respectively.

had condensed regions in the centromeric regions except Chromosome 6. iii) *L. serriola*: the interstitial regions were slightly condensed, except Chromosome 6, which were highly condensed. The chromosome lengths ranged from 6.01 to 12.61 μ m (Table 1). Chromosomes 1 to 7 were submetacentric and Chromosomes 8 and 9 were metacentric. The positions of the rDNA signals in the karyotype were similar to those of *L. sativa* and *L. saligna*, in which four 45S rDNA signals were observed at the end of the short arms of Chromosomes 3 and 5. A pair of 5S rDNA signals was detected in the interstitial regions of the short arms of Chromosome 5. iv) *L. virosa*: chromosome length of this species was the longest among the species used, ranging from 6.21 to 13.24 μ m (Table 1). Chromosomes 1 to 4, and 6, 8 and 9 were submeta-

centric, and Chromosomes 5 and 7 were metacentric. Chromosomes 2 and 7 had highly condensed regions along the whole chromosomes. Locations of the 45S and 5S rDNAs were different from the other species, namely, two pairs of 45S and one pair of 5S rDNAs located at the end of the short arm of Chromosome 5 and interstitial region of Chromosome 7, respectively.

Discussion

In the present study, homologous chromosome pairs in four *Lactuca* species were identified by observing prometaphase chromosomes stained with the Giemsa solution. Condensed chromosome regions at prometaphase were recognized as densely stained regions distinguish

from other regions. Our results of karyotype were coincident with the previous reports (Lindqvist 1960, Haque and Godward 1985, Koopman *et al.* 1993, Koopman and de Jong 1996, Matoba *et al.* 2007); the karyotypes of *L. sativa* and *L. serriola* were almost similar to each other and they seemed to be closely related. Although it was reported that the karyotypes of them were difficult to distinguish based solely on the karyotype analysis of metaphase chromosomes (Matoba *et al.* 2007), the present results suggest that the use of prometaphase chromosomes are helpful to discriminate the chromosomes based on the CPs in *L. sativa* and *L. serriola*.

It is considered that the total chromosome length of a plant is reflected with the genome size of it. The genome sizes of *L. sativa*, *L. saligna*, *L. serriola* and *L. virosa* were reported to be 2.7Gb, 2.2Gb, 2.7Gb, and 3.3Gb, respectively (Koopman 2000, Reyes-Chin-Wo *et al.* 2017). The *L. virosa* genome is larger than those of the other lettuce species. Here, we can find a clear correlation between the genome sizes and the total chromosome lengths. The difference of the genome sizes might be due to the difference in the amounts of the condensed region on the interstitial and distal regions of short and long arms of the chromosomes. The majority of *L. virosa* chromosomes exhibited highly condensed (Figs. 1, 3) and the longest chromosome length (Table 1) in comparison with the other three species. It is suggested that genome sizes of the plants are strongly correlated with chromosome length mainly due to the amount of repetitive DNA sequences in *Lilium* (Du *et al.* 2017) and *Nigella damascena* (Kuznetsova *et al.* 2017). Therefore, the present results suggest that the abundance of repetitive DNA in the genomes might have contributed to the genome sizes in *Lactuca* species, and it is reflected as the CPs of the chromosomes.

Karyotyping using CHIAS IV and FISH mapping facilitated the construction of quantitative and physical ideograms of plant chromosomes. Through combining these two techniques, comprehensive lettuce chromosome maps would be constructed using DNA sequences.

The loci of the 45S and 5S rDNAs of *L. sativa*, *L. serriola* and *L. saligna* were identical in their numbers and locations, except that the locations of rDNAs of *L. virosa* were different from those of the other species. This result was similar to the previous study by Matoba *et al.* (2007) who applied 18S and 5S rDNAs as the probes on metaphase chromosomes.

In conclusion, the present study is the first for physical mapping of condensed regions and rDNAs using CP and FISH of prometaphase chromosomes in lettuce. The development of a quantitative chromosome map as shown in the present report is fundamental for physical mapping of the genes and quite useful not only for basic genome research and chromosome analysis but also practical breeding and genetics of lettuce. In future studies, other probes prepared from different kinds of re-

petitive sequences, single-copy genes and DNA markers would be localized for the construction of precise physical chromosome maps of lettuce.

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