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RESEARCH ARTICLE

FISH Karyotype Comparison between Wild and Cultivated *Perilla* Species Using 5S and 45S rDNA Probes

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ABSTRACT *Perilla* species (Lamiaceae) have been used as a resource for oilseeds and vegetables, and medicinal purposes. Cytogenetic studies based on chromosomal composition are essential to understand the basic genome structure of a species and to provide vital information for crop improvements. However, only a few studies have assessed the cytogenetic aspects of *Perilla* species. Fluorescence *in situ* hybridization (FISH) karyotypes using 5S and 45S rDNA probes were analyzed for the wild and cultivated species of *Perilla*: *P. citriodora* and *P. frutescens*. Chromosome complements were diploid in *P. citriodora* and allotetraploid in *P. frutescens*. The chromosome length ranged from 3.07 to 4.92 μm and 2.41 to 5.73 μm in the diploid and allotetraploid variants, respectively. The karyotypic formula was 2n = 12m + 8sm (2 satellites) for *P. citriodora* and 2n = 20m + 20sm (2 satellites) for *P. frutescens*. A pair of 5S signals was detected in the telomeric region of chromosome pair 7, while a pair of 45S rDNA signals was detected in the telomeric region extending through the satellite region of chromosome 2 of *P. citriodora*. However, two pairs of 5S signals were detected from the interstitial to the telomeric regions of chromosome 7 and 17, and a pair of 45S rDNA signals was located on the satellite region of chromosome 20 of *P. frutescens*. This result will provide useful information to develop a breeding program and to construct the chromosomal backbone for the ongoing genome sequence assembly project.

Keywords *Perilla*, Fluorescence *in situ* hybridization (FISH), 5S rDNA, 45S rDNA

INTRODUCTION

Perilla is an upright, bushy annual plant, belonging to the mint family Lamiaceae (Nitta *et al*. 2005; Vaughan and Geissler 2009). The genus consists of one tetraploid species, *P. frutescens* (L.) Britt. and three diploid species, *P. citriodora* (Makino) Nakai, *P. hirtella* Nakai, and *P. setoyensis* G. Honda (Nitta *et al*. 2005; Ito and Honda 2007). *P. frutescens* is an allotetraploid species derived from a diploid donor, *P. citriodora* (Honda *et al*. 1994). However, the three diploid species might also be the parents of the allotetraploid species (Nitta *et al*. 2005).

The members of the genus *Perilla* are generally known for having square stems, four stamens (Brenner 1993) and wrinkled, serrated, and broad ovate leaves with medium green and purple colorations (Lee and Ohnishi 2001). The plants naturally grow near roadsides, wastelands, and farm fields (Sa *et al*. 2015). The limited distribution of *Perilla* species is a result of biotic and abiotic external conditions and physiological reactions to various stress factors in the environment such as temperature fluctuation (Larcher 2003).

These annual weeds that are common in the Eastern United States (Brenner 1993) have been traditionally cultivated for commercial reasons, particularly in Korea, Japan, China, and other Asian countries (Nitta *et al*. 2005; Vaughan and Geissler 2009) as well as in Europe, Russia, and USA (Nitta *et al*. 2005). It is one of the economically valuable plants producing essential oils along with *Sesame*

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indicum (Ito *et al*. 2000). *Perilla* has been used for a long time in Korean cuisine (Mo *et al*. 2017). Its chemical derivate perillartine is commonly used as an artificial sweetener (Vaughan and Geissler 2009), in fresh vegetable dishes, and as the main ingredient in pickles (Nitta *et al*. 2005) because of the aromatic leaves (Seo and Baek 2009). It has also been used for years as traditional medicine, particularly in Southeast Asian countries such as China and Japan (Yamamoto and Ogawa 2002).

Cytogenetic investigations based on chromosome number, structure, and ploidy are needed for breeding and development purposes of the species, due to the growing demand for *Perilla* species in agriculture as a vegetable and for seed oil for medicinal purposes (Kato *et al*. 2005). A molecular cytogenetic study using fluorescence *in situ* hybridization (FISH) is a promising technique in the research on genome functions, ploidy levels, alien gene introgressions and genome evolution (Hwang *et al*. 2009). Clarifying genome structures, identifying chromosome counts, physically locating repeats and genes, and knowing genome rearrangements like chromosomal segment inversions, duplications, and translocations (Pellerin *et al*. 2018; Zhou *et al*. 2018) offer an overview of the chromosome constitution, organization, and pairing (Han *et al*. 2008).

Cytogenetic markers such as tandem repeats of 5S and 45S rDNAs provide excellent information on homologous chromosome pairing, DNA sequence location and genome organization (Heslop-Harrison 2000; Waminal *et al*. 2018). The highly conserved coding regions of these rDNAs are essential to ribosome functioning (Lapitanz 1992). The 5S and 45S rDNAs typically appear in closely related groups (Bergeron and Drouin 2008) in independent loci in the eukaryotic phylogenetic lineage (Heslop-Harrison 2000; Wicke *et al*. 2011). These commonly called eukaryotic 'universal gene families' showed intra- and inter-populational, numerical, and positional variations in most species (Mantovani *et al*. 2005). Changes in the chromosomal distribution correlate to the rate of speciation (Heslop-Harrison 2000).

The 120 bp coding gene of the 5S rDNA repeat unit with its relatively short intergenic spacer (IGS) of around 100 to 1,000 bp (Galián *et al*. 2012) has gene copies varying from 2,000 to 75,000. Copies of these genes can be found in either the inter- or intra-chromosomal region and at multiple loci (Wicke *et al*. 2011). The nucleolar organizing region (NOR) of the 45S rDNA repeat unit consists of three coding regions (18S, 5.8S, and 25S/26S/28S), internal transcribed spacers between genes (ITS1 and ITS2), and a long intergenic spacer (IGS) separating adjacent repeats. The NOR-forming 45S rDNA is a highly conserved region consisting of up to 10% plant genome (Heslop-Harrison 2000; Galián *et al*. 2012).

In this study, we carried out a FISH karyotype analysis on the two species of *Perilla*: the cultivated *P. frutescens* (L) Britt. and the wild *P. citriodora* (Makino) Nakai, using 5S rDNA and 45S rDNA probes. The results could provide elemental information of the genome which can be used in a breeding program and clarify interspecific relationships in the genus *Perilla*.

MATERIALS AND METHODS

Plant samples

The seeds of *Perilla citriodora* and *P. frutescens* 'Deulkkae' were provided by Professor Lee Ju Kyong (Kangwon National University) and Dr. Lee Myoung Hee (National Institute of Crop Science, RDA), respectively. In a petri dish containing a paper moistened with distilled water, seeds were cautiously germinated at room temperature until roots of 2 cm in length were harvested. The root samples were pretreated with 2 mM 8-hydroxyquinoline for 5 hours at 18℃, fixed in Carnoy's fixative for 2 hours, and stored in 70% ethanol (Pellerin *et al*. 2018).

Chromosome spread preparation

Somatic chromosome spreads were prepared following Waminal *et al*. (2018) and Zhou *et al*. (2018). The pretreated meristematic root tips were dissected and enzymatically digested in a 1% pectolyase and 2% cellulase for 1.5 hours at 37℃. Root tips were then washed with distilled water and resuspended in an aceto-ethanol solution (9:1 v/v). The suspension was pipetted onto pre-warmed slides in a humid chamber, air-dried, and fixed in 2% formaldehyde for 5 minutes (Vrána *et al*. 2012). A series of ethanol treatments were used for dehydration (70%, 90%,

and 100%) (Pellerin *et al*. 2018).

Probe preparation

Complying to Waminal *et al*. (2018), a 9 kb 45S (18S-5.8S-25S) rDNA (Gerlach and Bedbrook 1979) fragment was labeled with biotin-16-dUTP by nick translation (Roche, Germany). The 5S rDNA was obtained according to the procedure described by Hwang *et al*. (2009) and labeled with digoxigenin-11-dUTP using a PCR DIG Probe Synthesis Kit (Roche, Germany) and using genomic DNA as a template following the protocol described by (Koo *et al*. 2002).

Fluorescence *in situ* hybridization

Flourescence *in situ* hybridizaztion (FISH) karyotypes using 5S and 45S rDNA probes were analyzed for the wild and cultivated species of *Perilla*: *P. citriodora* and *P. frutescens*. The procedures were carefully adapted from Pellerin *et al*. (2018). The hybridization mixture comprised of 50% formamide, 10% dextran sulfate, 2× saline-sodium citrate buffer (SSC), 5 ng/μL salmon sperm DNA, and 500 ng/μL per DNA probe and was repleted with nuclease-free water to a total volume of 40 μL per slide. The mixture was denatured at 90℃ for 10 minutes, and 40 μL was added to each slide. Chromosomes were denatured at 80℃ for 5 minutes and incubated overnight in a humidity chamber at 37℃. Later, the slides were stringently washed using 2 × SSC at 20℃ to 25℃ for 10 minutes, 0.1 × SSC at 42℃ for 25 minutes, and 2 × SSC at room temperature for 5 minutes; followed by dehydration in an ethanol series (70%, 90%, and 95%) at room temperature. The slides were air-dried and counterstained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Labs, H-1000, USA), and observed under an Olympus BX53 fluorescence microscope (Olympus America Corp., USA) equipped with a Leica DFC365 FS CCD camera (Leica microsystem Inc., Germany) using an oil lens (×100 magnification). The captured images were sorted out using Cytovision ver. 7.2 (Leica Microsystems, Germany). Adobe Photoshop CS6 was used for image enhancement and ideogram preparation. Mitotic metaphase chromosome spreads were used to analyze the FISH karyotype. The chromosomes were paired according to chromosomal size, centromeric position, and ribosomal signals, and arranged in a decreasing order based on total chromosome length (Levan *et al*. 1964; Waminal and Kim 2018).

RESULTS

The seeds from *Perilla* species yielded healthy root tips after 3-5 days of germination, which is required for metaphase spread preparation. Clear metaphase spreads without overlapping chromosome arms could easily be observed under a fluorescence microscope by counter-staining with DAPI. The chromosome spreads were successfully investigated through the FISH technique.

FISH karyotype

The FISH metaphase spreads and the karyograms of the diploid *P. citriodora* and the allotetraploid *P. frutescens* are shown in Fig. 1 and Fig. 2, respectively. Chromosome complements of the two species consisted of 2n = 20 in *P. citriodora* and 2n = 40 in *P. frutescens*, and a pair of satellite chromosome was observed in both species (Figs. 1 and 2A, B). Karyotypic data analysis according to chromosome length, type, and FISH signals is shown in Tables 1 and 2. The total chromosome length of *P. citriodora* ranged from 3.07 to 4.92 μm, in which the short arm ranged from 1.03 to 2.43 μm and the long arm from 1.69 to 3.46 μm (Table 1). For *P. frutescens*, the total chromosomal length ranged from 2.41 to 5.73 μm, in which the short arm ranged from 0.73 to 2.16 μm, and the long arm from 1.68 to 4.24

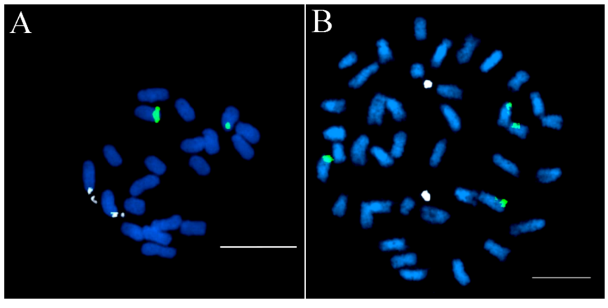

Fig. 1. Dual-color FISH metaphases of *Perilla citriodora* (A) and *P. frutescens* (B) showing the 5S (green) and 45S (white) rDNA signals. Bar = 5 μm.

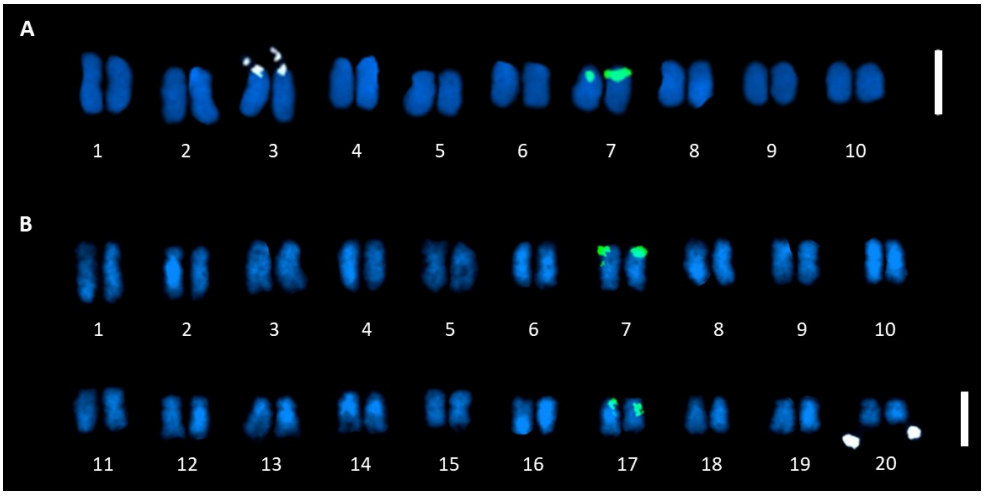

Fig. 2. FISH karyotypic idiogram of diploid *Perilla citriodora* (A) and allotetraploid *P. frutescens* (B) showing 20 homologous pairs arranged in decreasing lengths. Bar = 5 μm.

Table 1. FISH karyotype data of *Perilla citriodora* showing its per chromosome length, arm ratio, type, and rDNAs signals.

Chr. pair no.	Chromosome length (μm)			Arm ratio (L/S)	Chromosome type	FISH signal	
	Short arm (S)	Long arm (L)	Total (S + L)			5S	45S
1	2.42-2.43	2.48-2.49	4.91-4.92	1.02-1.03	m††	-	-
2	1.31-1.41	3.24-3.46	4.55-4.87	2.45-2.47	sm†	-	-
3	1.31-1.37	3.05-3.06	4.36-4.43	2.24-2.33	sm*	-	two chromosomes
4	1.73-1.84	2.36-2.42	4.15-4.20	1.29-1.40	m	-	-
5	1.03-1.06	3.02-3.03	4.06-4.08	2.85-2.95	sm	-	-
6	1.62-1.64	1.94-1.97	3.58-3.59	1.19-1.21	m	-	-
7	1.03-1.04	2.43-2.47	3.46-3.49	1.45-2.40	sm	two chromosomes	-
8	1.35-1.38	2.01-2.06	3.40-3.41	1.45-1.53	m	-	-
9	1.38-1.46	1.87-1.91	3.29-3.32	2.28-1.38	m	-	-
10	1.38-1.42	1.69-1.71	3.07-3.13	1.21-1.22	m	-	-
				total	12m + 8sm	two chromosomes	two chromosomes

†Submetacentric, ††Metacentric, *Satellite. +: rDNA signal is present, -: absent.

μm (Table 2).

In terms of chromosomal type, *P. citriodora* exhibited 12 metacentric and eight submetacentric chromosomes with a pair of satellites in the 3rd pair of chromosomes (Table 1). The karyotype formula is 2n = 12m + 8sm (2 satellites). *P. frutescens* revealed 20 metacentrics and 20 submetacentric chromosomes with a pair of satellites in chromosome 20 (Table 2). The karyotype formula is 2n = 20m + 20sm (2 satellites). The chromosome complement of *P. frutescens* somehow resembles its possible parent *P. citriodora*.

Localization of rDNA probe

The dual-color FISH analysis showed that the *P. citriodora* genome contained single pair of 5S and 45S rDNA signals (Fig. 1A) while *P. frutescens* has two pairs of 5S rDNA and one pair of 45S rDNA signals (Fig. 1B). The 5S rDNA was localized at the telomeric region of chromo-

Table 2. FISH karyotype data of *Perilla frutescens* showing its per chromosome length, arm ratio, type, and rDNAs signals.

Chr. group no.	Chromosome Length (μm)			Arm ratio (L/S)	Chromosome type	FISH signal	
	Short arm (S)	Long arm (L)	Total (S + L)			5S	45S
1	1.39-1.48	4.03-4.24	5.42-5.73	2.86-2.28	sm†	-	-
2	1.28-1.48	3.36-3.81	4.96-5.09	2.11-2.97	sm	-	-
3	2.09-2.10	2.81-2.87	4.90-4.95	1.34-1.37	m††	-	-
4	2.09-2.12	2.82-2.87	4.94-4.95	1.33-1.34	m	-	-
5	1.92-2.11	2.73-2.85	4.77-4.83	1.29-1.48	m	-	-
6	2.13-2.16	2.33-2.36	4.49-4.50	1.08-1.11	m	-	-
7	1.37-1.55	2.94-2.98	4.36-4.48	1.90-2.18	sm	two chromosomes	-
8	1.99-2.11	2.26-2.42	4.37-4.41	1.07-1.22	m	-	-
9	2.01-2.02	2.35-2.38	4.35-4.39	1.17-1.19	m	-	-
10	1.86-1.96	2.31-2.32	4.17-4.27	1.18-1.25	m	-	-
11	1.80-1.98	2.16-2.35	4.14-4.15	1.09-1.31	m	-	-
12	1.08-1.33	2.75-3.02	3.99-4.10	2.06-2.78	sm	-	-
13	1.17-1.21	2.79-2.82	3.99-4.00	2.30-2.41	sm	-	-
14	1.42-1.64	2.23-2.40	3.82-3.87	1.36-1.68	m	-	-
15	1.70-1.81	2.00-2.09	3.79-3.81	1.10-1.23	m	-	-
16	1.00-1.18	2.49-2.58	3.58-3.68	2.11-2.57	sm	-	-
17	0.89-0.90	2.58-2.66	3.56-3.58	2.95-2.96	sm	two chromosomes	-
18	1.02-1.08	2.38-2.44	3.44-3.46	2.21-2.39	sm	-	-
19	0.98-1.03	2.40-2.46	3.43-3.44	2.32-2.50	sm	-	-
20	0.73-1.00	1.68-1.90	2.41-2.91	1.89-2.31	sm*	-	two chromosomes
				total	20m + 20sm	four chromosomes	two chromosomes

†Submetacentric, ††Metacentric, *Satellite. +: rDNA signal is present, -: absent.

some 7 in *P. citriodora* (Fig. 2A) whereas in *P. frutescens*, it was detected in the interstitial region extending through the telomeric region of chromosomes 7 and 17 (Fig. 2B). The 45S rDNA signals were located interstitially and extended to the satellite region of chromosome pair 3 for *P. citriodora* (Fig. 2A), unlike *P. frutescens* where the signals were located entirely at the satellite region of chromosome 20 (Fig. 2B). Probe signal size varied between the two species, however, they both showed a strong signal intensity for the 5S and 45S rDNAs probe (Fig. 1). The chromosome complement of the cultivated species resembles the complement analysis of the diploid species.

DISCUSSION

The advancement in molecular cytogenetic techniques and FISH in particular, provides excellent information on chromosomal structures and functions (Heslop-Harrison 2000). The technique uses repetitive DNAs as primary probes to clarify genomic structures at the chromosome level. Unique repeat probes can be used as cytogenetic markers for chromosome identification (Hwang *et al*. 2009; Waminal *et al*. 2018) and their distribution in the genome can be useful to trace phylogenetic relationships among species and in the plant breeding program (Sybenga 2012).

The ploidy results demonstrated that *P. citriodora* is diploid with 2n = 20, whereas *P. frutescens* is tetraploid with 2n = 40, which is in accordance to the previous reports

by Honda *et al*. (1994), Ito and Honda (2007), and Diao *et al*. (2009). Both species exhibited single pair of satellite chromosomes (Diao *et al*. 2009). Among *Perilla* species, the wild *P. citriodora* with one pair of 5S rDNA was considered the most closely related and possible parent to the cultivated *P. frutescens* with two pairs of 5S rDNA. This conclusion is based on the phylogenetic relationship (Nitta *et al*. 2005), the genetic diversity (Woo *et al*. 2016) and the nucleotide sequences of geraniol synthases (Ito and Honda 2007) of both species. The phylogenetic pattern and genetic diversity among *Perilla* species are highly diverse and based on their endemic widespread and continental distribution, particularly in China, Japan, Korea, Vietnam, Taiwan, and other nearby Asian countries (Nitta *et al*. 2005). Thus, the variation and distribution, especially in the 5S and 45S rDNA gene of both species, could be a result from unequal chromosomal crossover through genetic recombination during the gradual distribution and agricultural breeding across the country (Diao *et al*. 2009). The karyotypic formula for *P. citriodora* was 2n = 20 = 12m + 8sm (2 satellites) and 2n = 40 = 20m + 20sm (2 satellites) for *P. frutescens*. The chromosome numbers of both species is also consistent with the study of Zhang *et al*. (2008).

In this study, the NOR-forming 45S rDNA and non-NOR-forming 5S rDNA were used as FISH probes (Singh *et al*. 2009) as these are highly repetitive DNA sequences with a distinct species-specific chromosomal distribution. In addition, 45S rDNA and 5S rDNA can be found interspersed with other multicopy genes in lower eukaryotes. However, these genes have often been shown to be located in separate areas of the genome in higher eukaryotes (Martins and Galetti 1999), which is important to identify distinctly labeled probes in the chromosomes. The probes are widely used for plant taxonomic and evolutionary studies (Volkov *et al*. 2017), since 5S and 45S rDNAs are usually not linked to the same chromosome, and conversion and crossing-over are be more probable (Martins and Galetti 1999) The genomic rearrangements resulting from conversion or crossing-over are also common mechanisms in the evolution of multiple tandem arrays (Dover 1989). There is a varying number of 5S and 45S rDNA loci, sizes, and patterns of distribution within the related species. The higher number of signals of 5S rDNA in the tetraploid species compared to the 45S rDNA signals are in contrast to many other plant species. In some rice species (Ohmido *et al*. 2010), *Cucurbita* (Han *et al*. 2008), *Passiflora* (De Melo and Guerra 2003), and *Brassica rapa* (Lim *et al*. 2005), the transpositions and dispersions of 45S rDNA were higher compared to 5S rDNA in the genome. The specific features that may contribute to the intensive changes in multiple tandem repeats make these rDNA sequences cytologically essential in identifying species (Eickbush and Eickbush 2007).

Furthermore, simple sequence repeat (SSR) analysis concluded that the original cultivation of the tetraploid species started in China (Sa *et al*. 2015) and thrived in Korea, followed by Japan and other Asian countries (Nitta *et al*. 2005). Thus, the phylogenetic relationship possibly resulted from a transfer of desirable traits from both parents during hybridization and introgression of the species (Soltis and Soltis 2009).

In conclusion, FISH karyotypes of the diploid wild species *P. citriodora* and the tetraploid cultivated species *P. frutescens* were established, and the results will be useful for identifying the species and elucidating interspecific relationship in *Perilla*. In addition, the study provides essential information for breeding (Sybenga 2012) and a chromosome backbone for the ongoing genomic sequencing research. FISH karyotype analysis using other major repeat probes is needed to refine the genomic organization in *Perilla*.

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