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A study of genome size changing in higher plants (高等植物におけるゲノムサイズ変化に関する研究)

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A STUDY OF GENOME SIZE CHANGING IN HIGHER PLANTS

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ABSTRACT

The genus *Drosera* (Droseraceae) comprises more than 150 species distributed mainly in the Southern Hemisphere, with some in the Northern Hemisphere. Most of the northern hemisphere species, which belong to section Drosera, are diploids with basic chromosome number of x=10. To infer genome structures and chromosome differentiations with genome size changing among these three *Drosera* species, we applied cygogenetic works with two parental genomic probes of *D. rotundifolia* (2n=20) and *D. spatulata* (2n=40) to somatic metaphase chromosomes of *D. tokaiensis* (2n=60).

In the first approach, RAPD analysis was carried out using 1200 decamer random sequences to clarify the whole fingerprint of DNA fragment appearance patterns among three Japanese *Drosera* species: *D. tokaiensis*, *D. rotundifolia* and *D. spatulata*. The RAPD analysis results showed that *D. tokaiensis* had not only magnificent bands common to other two species, but also many specific bands, although *D. tokaiensis* is of amphiploidal origin between *D. rotundifolia* and *D. spatulata*. The specific bands of *D. tokaiensis* might generate after the speciation or amphiduplication. Therefore, the relationship of genome compositions among the three species suggested that RAPD fragments were preferentially amplified from 20 middle-sized chromosomes in *D. rotundifolia* and *D. tokaiensis*. Thus, the chromosome information and the RAPD profiling suggested that alloploidal genome formation during Drosera speciation might obtain new beneficial genetic characters to survive and adapt to certain environments.

An *in vitro* technique was connected with application to produce colchicine-induced hexaploid from the artificial-crossing triploid hybrid of *Drosera rotundifolia* and *D. spatulata*. The colchicine treated plants of artificial-crossing triploid hybrid showed morphologically mutated characteristics. Three hexaploid strains by chromosome doubling were produced after screening of clones treated with 0.05% and 0.1% colchicine solutions for 1 or 3 days. Each of the strains had 2n=60 with 20 middle size and 40 small size chromosomes (2n=6x=20M+40S). The stomata guard cell sizes of colchicine-induced hexaploid and the wild hexaploid species *D. tokaiensis* were larger than that of the artificial-crossing triploid hybrid. Flow cytometry analysis showed that 2C DNA-values of *D. rotundifolia* (2n=2x=20M), *D. spatulata* (2n=4x=40S) and *D. tokaiensis* (2n=6x=20M+40S) were 2.73, 1.41 and 3.74 pg, respectively. In contrast, the artificial-crossing triploid hybrid (2n=3x=10M+20S) and colchicine-induced hexaploid (2n=6x=20M+40S) were 2.31 and 4.41 pg, respectively. The genome size of artificial-crossing triploid hybrid (2.31 pg) was nearly half size of colchicine-induced hexaploid (4.41 pg). Compared to colchicine-induced hexaploid, the genome size of *D. tokaiensis* was unexpectedly small, even though they contained same genome constitutions.

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CHAPTER I

General introduction

Among eukaryotes, the amount of DNA in picograms within a haploid nucleus is known as the 1C value (the genome size). Leitch *et al*. (2009) reported the genome size of eukaryotes varied 40,000-fold. The smallest genome recorded is found in *Encephalitozoon intestinalis*, a parasitic microsporidian with a genome size of only 0.003 pg or 2.9 Mbp DNA (1 pg = 978 Mbp) (Doležel *et al.* 2003). The largest genome recorded is in the marbled lungfish, *Protopterus aethiopicus*, at more than 130 pg or 130,000 Mbp (Pedersen 1971). With regard to eukaryotic genome size, most groups exhibit a narrower range of variation, from 3- to 4-fold in mammals and 14-fold in gymnosperms (Bennett and Leitch 1995; Gregory 2005; Leitch *et al*. 2009). DNA C-values in angiosperms show an extraordinarily wide range of approximately 2000-fold. C-values also vary extensively among closely related organisms of apparently comparable complexity (Fig. 1-1) (Li 1997; Fedoroff 2012).

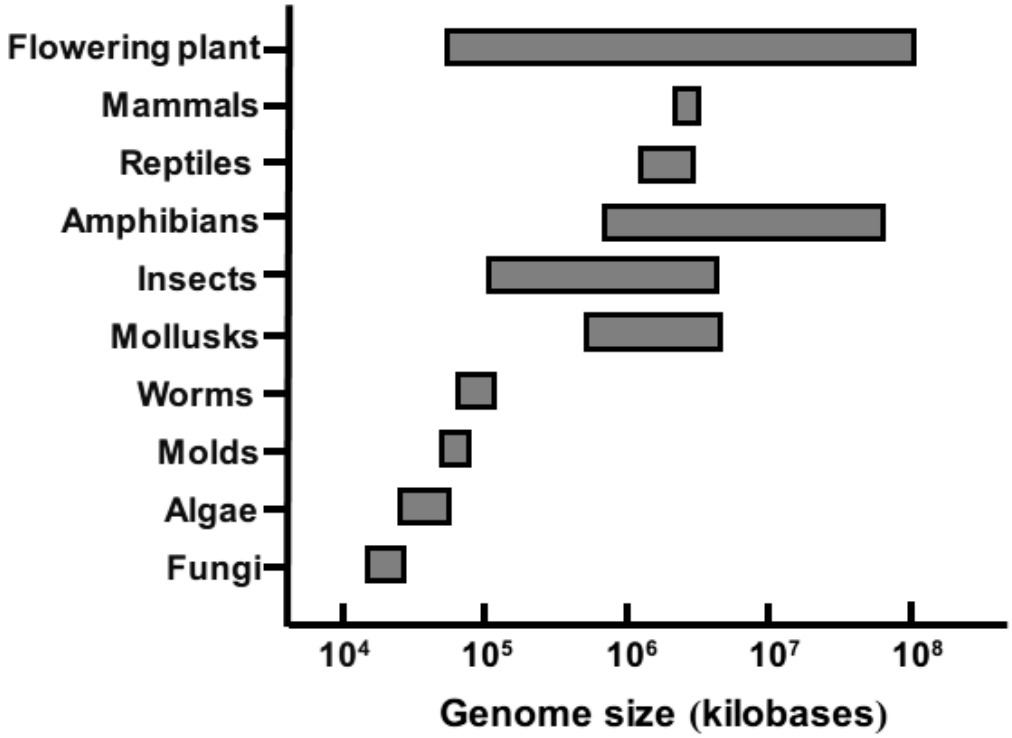

Fig. 1-1. Haploid genome-size range among different organisms. The illustrated image includes current information and basic data from Li (1997).

A giant genome is found in *Fritillaria* species (Liliaceae), which is reported to have DNA amounts of approximately 130 pg (130,000 Mbp) (Leitch *et al*. 2009; Zonneveld 2010) and numerous chromosomes as large as 10–20 μm (Kamari and Phitos 2006). On the other hand, the smallest angiosperm genome was recently found among the carnivorous plants of Lentibulariaceae: *Genlisea margaretae* with a size of only 0.06 pg or 63 Mbp, and *G. aurea* with 64 Mbp. Chromosome number and chromosome structure are not well known in Lentibulariaceae. The smallest chromosome size in *Genlisea* is 2.1 Mbp, which is the same size as a bacterial genome (Greilhuber *et al.* 2006). However, most angiosperms have a small or medium genome size (*ca.* 1 pg or 2–3 pg, respectively) and chromosome size (*ca.* 1 μm or 2–3 μm, respectively).

The plant groups widely used as phylogenetic markers include the three large families Asteraceae, Fabaceae, and Poaceae (Jansen and Palmer 1987; Doyle *et al.* 1996; Kim *et al*. 2005), as well as Liliaceae and Orchidaceae, with wide genome size ranges. The carnivorous sundew group, especially Droseraceae and Drosophyllaceae, exhibit unusually high genome size variation and small to large chromosomes.

Droseraceae consists of three genera: *Aldrovanda, Dionaea,* and *Drosera* (Takahashi and Sohma 1982; Juniper *et al*. 1989; Albert *et al*. 1992; Williams *et al*. 1994; Conran *et al.* 1997; Rivadavia *et al*. 2003; Shirakawa *et al*. 2011). The genus *Drosera*, in

particular, has essential compounds that are natural sources of medication (Banasiuk *et al.* 2012). It has been reported that some species have anti-inflammatory and antibacterial effects, including *D. intermedia* Hayne*, D. anglica* Huds, *D. madagascariensis* DC., and *D. peltata* Smith. "Droserae Herba," which is comprised mostly of *D. rotundifolia*, has been used to treat pertussis or whooping cough (Paper *et al.* 2005; Fukushima *et al.* 2009; Kačániová *et al.* 2014). In addition, a strong anti-allergic effect was recently found in *D. tokaiensis* (Fukushima *et al*. 2009).

Plant DNA C-values and genome sizes are significant biodiversity variables (Bennett and Leitch 1995; Bennett *et al.* 2000). However, few genome studies have focused on carnivorous plant families, which have a wide range of genome sizes. There have been very few calculated C-values, molecular genetic studies, and karyomorphological analyses of these species in the years since the original plant DNA C-values were reported by Rothfels and Heimburger in 1968.

The three Japanese *Drosera* species with complex trapping systems to capture insects are a good model to study genome organization (Dixon *et al*. 1980; Zhang *et al.* 2010). *Drosera rotundifolia* is a diploid species ($2n=2x=20$) with mid-sized chromosomes. A native plant of *D. spatulata* in Japan is a tetraploid species ($2n=4x=40$) with small-sized metaphase chromosomes. *Drosera tokaiensis* is a hexaploid species ($2n=6x=60$),

originating as a natural hybrid between *D. spatulata* and *D. rotundifolia* (Hoshi *et al.* 2010), and it has 20 mid-sized chromosomes and 40 small-sized metaphase chromosomes (Hoshi *et al.* 2008; Hoshi *et al.* 2010). *Drosera rotundifolia* is distributed across the Northern Hemisphere and has generally been used as a traditional medicine (Fukushima *et al*. 2009). *Drosera spatulata* is distributed mostly in Southeast Asia, including parts of Japan and Australia, and has no anti-allergy effect (Fukushima *et al.* 2009). *Drosera tokaiensis* is found only in Japan and has recently been reported to have a high anti-allergy effect (Fukushima *et al*. 2009).

Many previous phylogenetic studies have used RAPD data (Sharma *et al.* 1995). Our preliminary investigation showed various RAPD DNA band patterns with no phylogenetic significance in the Japanese *Drosera* species (Hoshi *et al*. 2010). Interestingly, although *D. rotundifolia* and *D. spatulata* are the parental species of *D. tokaiensis*, the main RAPD bands preferentially occur in *D. rotundifolia* and *D. tokaiensis*, but not in *D. spatulata* (Hoshi *et al*. 2010). A molecular study, including RAPD profiling, might identify new beneficial characteritics related to environmental adaptation.

Induction of polyploidy is a useful tool for breeding many plants and for generating mutations in ornamental crops (Hancock, 1997; Väinölä 2000; Ascough *et al.* 2008; Thompson *et al.* 2010). Polyploid plants exhibit considerable evolutionary genetic

diversity (Xing *et al.* 2011). Colchicine is an alkaloid that effectively induces polyploid plants by disrupting microtubule formation in all stages of the cell cycle and causing chromosomal aberrations (Ascough *et al.* 2008; Campos *et al*. 2009). It can be useful for plant breeders treating sterile triploid hybrids from different plant species.

Drosera tokaiensis, which is a natural hexaploid, has strong anti-inflammatory effects (Fukushima *et al.* 2009) as well as complex genetic and chromosomal structures (Shirakawa *et al.* 2011) that make it interesting for the study of genome evolution.

However, the triploid hybrids between *Drosera spatulata* and *D. rotundifolia* are sterile. A synthetic hexaploid generated from diploids and tetraploids created by colchicine-induced doubling might possess the strong anti-inflammatory activity same as *D. tokaiensis*.

CHAPTER II

RAPD profiling of three Japanese *Drosera* species

Introduction

Carnivorous plants are a group with highly specialized morphologies for insect trapping and thus they appeal to botanists (Junipers *et al*. 1989; Rivadavia *et al.* 2003; Bhau *et al.* 2009; Hoshi *et al.* 2010). Droseraceae, which is a typical family of carnivorous plants, contains three genera: *Aldrovanda, Dionaea,* and *Drosera* (Takahashi and Sohma 1982; Juniper *et al*. 1989; Williams *et al*. 1994; Conran *et al*. 1997; Rivadavia *et al*. 2003). In contrast to the two monotypic genera *Aldrovanda* and *Dionaea*, *Drosera* is a large genus, which consists of approximately 150 species distributed worldwide (Rivadavia *et al*. 2003). Many *Drosera* species, especially in the Northern Hemisphere, have a basic chromosome number of x=10, with different ploidy levels and chromosome sizes (Hoshi *et al.* 2010), and most of them belong to section Drosera of subgenus Drosera (Seine and Barthlott 1994).

Three related species in this section with x=10 are found in Japan. *Drosera rotundifolia* L. is distributed widely in the Northern Hemisphere (Rivadavia *et al*. 2003), and in Japan it occurs on the main islands (Horikawa 1976; Nakano *et al*. 2004). The leaves of the species are spoon shaped (Nakano *et al*. 2004; Shirakawa *et al.* 2012; Hoyo

and Tsuyuzaki 2013), and all plants are diploid with mid-sized (2–3 μm) metaphase chromosomes (2*n*=2*x*=20M) (Fig. 2-1A). *Drosera spatulata* Labill. is often treated as a complex, first reported in Tasmania, but mainly distributed in Australia, New Zealand, some parts of Southeast Asia, Taiwan, southern China, and Japan (Merrill 1923; Van Steenis 1953; Allan 1961; Marchant and George 1982; Nakano *et al.* 2004). The natural habitats of *D. spatulata* in Japan are in the south: Ryukyu, Kyushu, and Shikoku Islands, and the west coastal regions of Honshu (Nakano *et al.* 2004). The leaves of the species are spatulate in shape (Nakano *et al.* 2004; Hoshi *et al.* 2008; Hoshi *et al.* 2010). The plants native to Japan are tetraploid and have small-sized (approximately 1 μm) metaphase chromosomes (2*n*=4*x*=40S) (Fig. 2-1B). *Drosera tokaiensis* (Komiya and C. Shibata) T. Nakamura and Ueda is a recently recorded hexaploid species of hybrid origin (2*n*=6*x*=20M+40S) (Fig. 2-1C).

The ancestors of *D. tokaiensis* are *D. rotundifolia* (paternal) and *D. spatulata* (maternal) (Shirakawa *et al.* 2012) (Fig. 2-2). *Drosera tokaiensis* is found only in Japan, especially in the Kansai district, Shikoku, the Pacific side of the central parts of Honshu, Tokai district, and adjacent inland areas (Nakano *et al.* 2004). The leaves of this species show an intermediate shape between obovate and spatulate (Nakano *et al.* 2004; Hoshi *et al.* 2008; Hoshi *et al.* 2010).

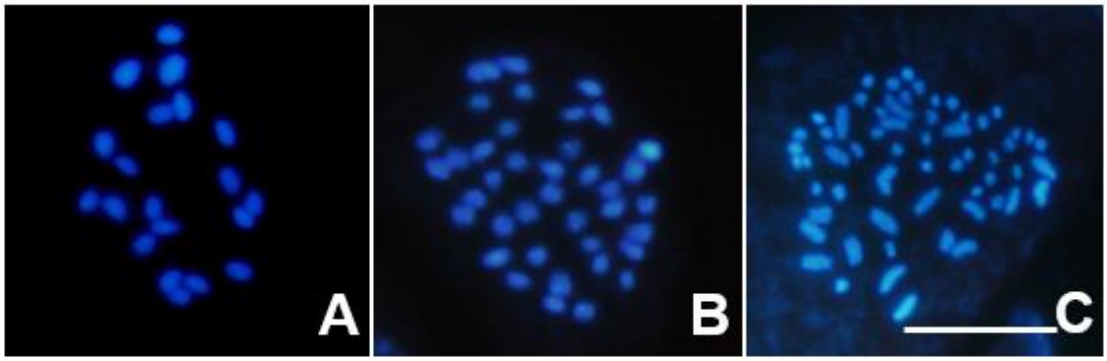

Fig. 2-1. Fluorescence staining of mitotic-metaphase chromosomes with DAPI.

A. *D. rotundifolia* (2*n*=2*x*=20M). B. *D. spatulata* (2*n*=4*x*=40S).

C. *D. tokaiensis* (2*n*=6*x*=20M+40S). Bar = 5 μm.

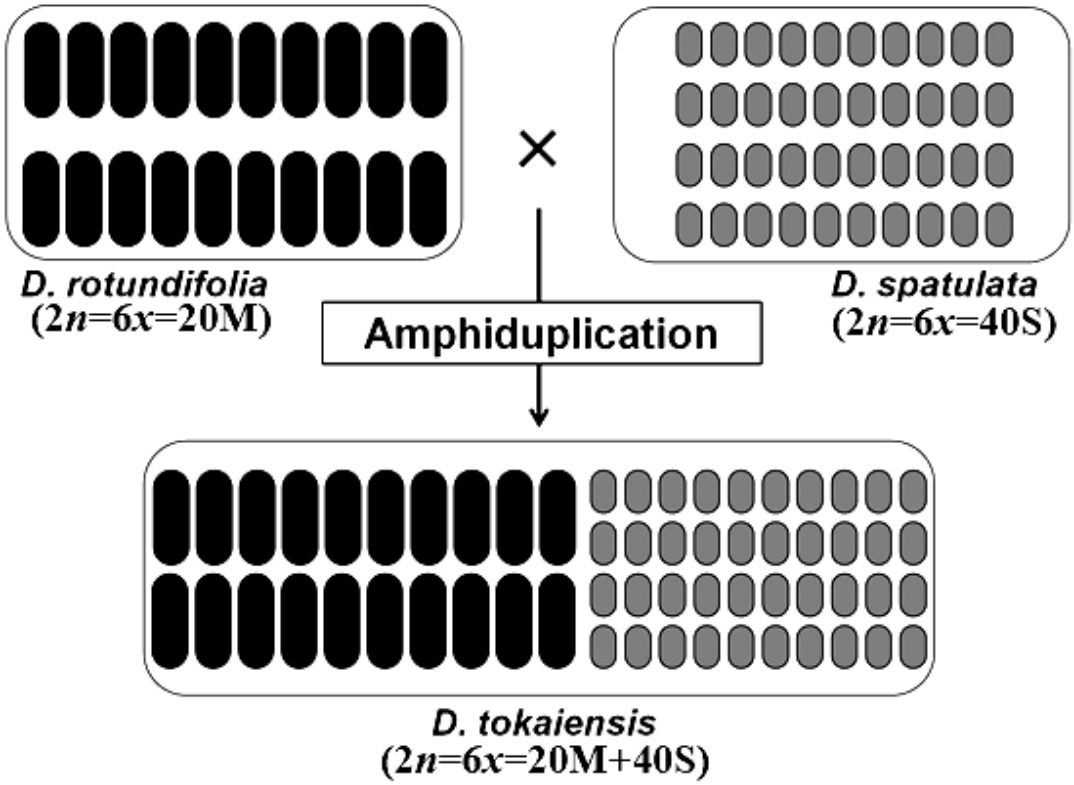

Fig. 2-2. Schematic illustration of chromosomal relationship among three Japanese species, *D. rotundifolia*, *D. spatulata* and *D. tokaiensis*.

In contrast to many previous phylogenetic studies using RAPD (Sharma *et al.* 1995), our preliminary investigation showed that the various RAPD DNA band patterns had no phylogenetic significance in the Japanese *Drosera* species (Hoshi *et al*. 2010). Interestingly, even though *D. rotundifolia* and *D. spatulata* are both parental species of *D. tokaiensis*, the main RAPD bands preferentially occur in *D. rotundifolia* and *D. tokaiensis*, but not in *D. spatulata* (Hoshi *et al*. 2010).

The purpose of the present study was to clarify the patterns of RAPD DNA fragments among the three Japanese *Drosera* species using 1200 RAPD primers.

Materials and methods

Plant materials

The plant materials used were three *Drosera* species: *D. rotundifolia* L.*, D. spatulata* Labill., and *D. tokaiensis* (Komiya and C. Shibata) T. Nakamura and Ueda (Table 2-1). Leaf materials of the plants were obtained from tissue culture in the Laboratory of Plant Environment Science, Department of Plant Science, School of Agriculture, Tokai University. The plants were cultivated on half-strength Murashige-

Skoog basal medium (Murashige and Skoog 1962), supplemented with 3.0% sucrose and 0.2% gellan gum at 25 °C under continuous light conditions.

Table 2-1. Source of the materials of three species of *Drosera spatulata* complex and its related species

Species	Accession number[a]	Chromosome number (2*n*)	Karyotype formula[b]	Ploidy level	Used plants for DNA extraction
D. rotundifolia	010816 Sera-1	20	20M	Diploid	*in vitro* culture
D. spatulata	Jpn Ha4x-6	40	40S	Tetraploid	*in vitro* culture
D. tokaiensis	Jpn Ha6x-9	60	20M + 40S	Hexaploid	*in vitro* culture

[a] Plant sources of three Japanese species are described in Hoshi and Kondo (1998)

[b] M: middle size chromosome, S: small size chromosome (see Hoshi and Kondo 1998)

DNA extraction

Total genomic DNA extraction was performed following Hoshi *et al.* (2010). The DNA was extracted from fresh leaves of plants cultures *in vitro*. The leaves were ground into powder with liquid nitrogen and homogenized in a buffer containing 1M Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% cetyltrimethylammonium bromide, and 0.5% mercaptoethanol. The homogenate was extracted three times with an equal volume of chloroform-isoamyl alcohol (24:1) for 10 min. The DNA was precipitated with isopropyl alcohol at -20 °C for 30 min. The DNA pellets were washed with 70% ethanol, dried, and dissolved in 200 µl Tris-EDTA (TE) overnight. On the second day, 1 µl of 1 mg/ml RNase was added, and the mixture was incubated at 37 °C for 1 h in a water bath. The DNA was

again treated following the procedures from the first day and then finally dissolved in 50 µl TE.

RAPD amplification

Following the manufacturer's instructions, we used a 10-mer kit (Operon technologies, Alameda, CA, USA) to amplify the DNA samples with 1200 primers. PCR was performed in a volume of 20 µl. The reaction mixture contained 0.1 µl *Taq* DNA Polymerase (TOYOBO Code No. TAP-211), 2 µl dNTPs (2 mM each), 2 µl 10X *Taq*-buffer, 13.9 µl MillQ water, 1 µl primer (10 pmol of each primer), and 1 µl template DNA (1 ng). The DNA amplification was performed on a PCR thermal cycler (Program Temp Control System, Astec, PC-708). The standard amplification conditions used were: 94 °C for 5 min followed by 45 cycles of 94 °C for 0.5 min, 42 °C for 0.5 min, and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Amplified PCR products were subjected to 1.0% agarose gel electrophoresis in 1X TAE buffer at 100 V for 40 min. The DNA bands were visualized using a UV transilluminator (Funakoshi, NTM-20) after staining with 0.5 µg/ml ethidium bromide for 15 min.

Results and discussion

A total of 1200 decamer random sequences were initially screened for use as analysis primers. For the purpose of clearing up the ambiguous bands in the three *Drosera* species, all species were used and primers that generated no bands in the fingerprints were not retained.

A total of 534 primers selected from OPA-01 through OPBH-20 were tested in each of the three *Drosera* species and their RAPD patterns were examined. Typical results of RAPD fingerprinting in the three species are shown in Fig. 2-3. Using all the primers from the OPA to OPBH series, RAPD generated many bands in the range of 100–3000 bp in *D. rotundifloria*, 200–3000 bp in *D. spatulata,* and 100–3000 bp in *D. tokaiensis*. The maximum number of major amplicons generated by RAPD was 16, obtained with primer OPZ-11.

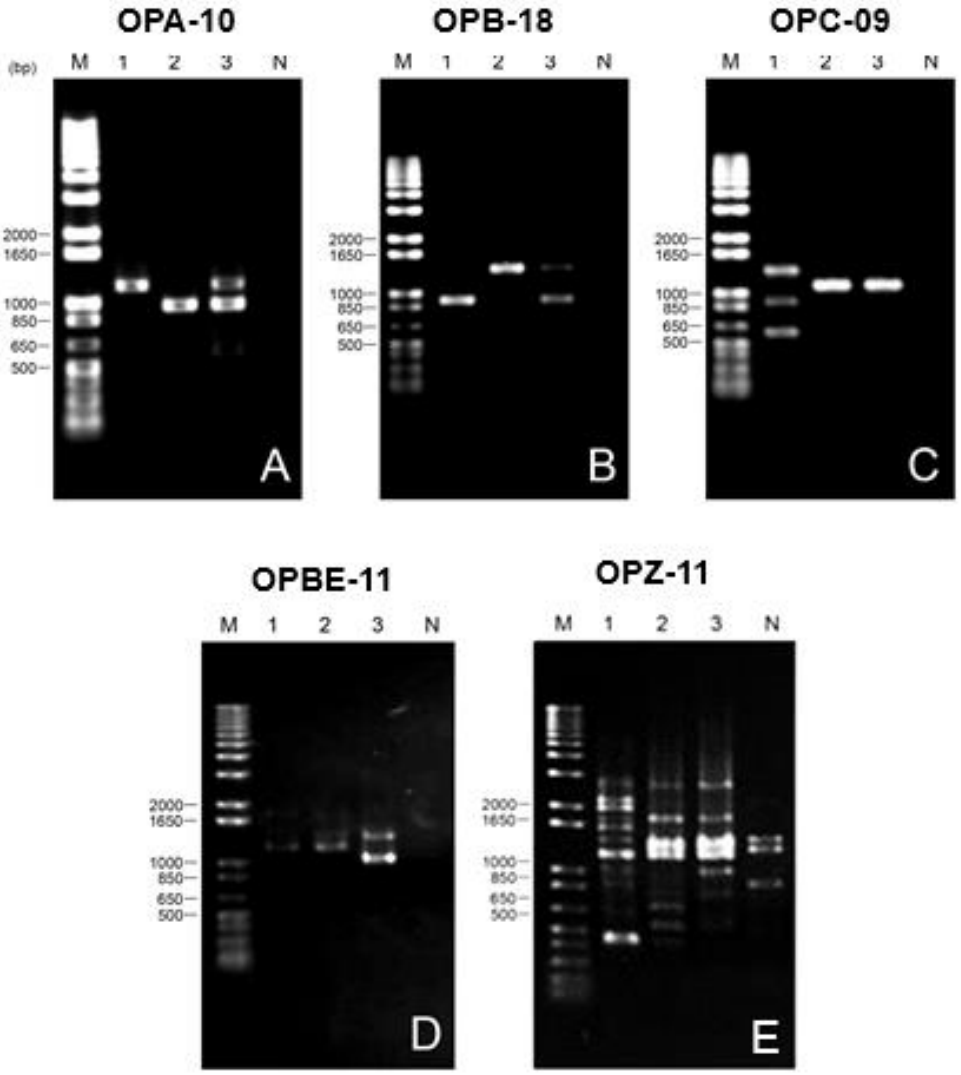

Fig. 2-3. Electrophoresis patterns of DNA amplification products obtained by RAPD primers of OPA-10 (A), OPB-18 (B), OPC-09 (C), OPBE-11 (D) and OPZ-11 (E) with template DNAs of *D. rotundifolia* (1), *D. spatulata* (2) and *D. tokaiensis* (3), without any template DNA (N). 1-kb Plus DNA Ladder was used for molecular marker (M).

The primer series OPD, OPI, OPJ, OPS, OPY, OPZ, and OPAR revealed the presence of more than three bands shared by all *Drosera* species. Overall, 341 bands were common among all three species. Moreover, many bands common to two species were obtained. *Drosera rotundifolia* and *D. spatulata* had 504 bands in common, while *D. spatulata* and *D. tokaiensis* had 1084 bands in common. The largest number of bands in common, 1478, was between *D. rotundifolia* and *D. tokaiensis*. A total of 756 and 505 bands were specific to *D. rotundifolia* and *D. tokaiensis*, respectively. In contrast, *D. spatulata* had 1306 bands screened and selected from 1200 random primers (Fig. 2-4).

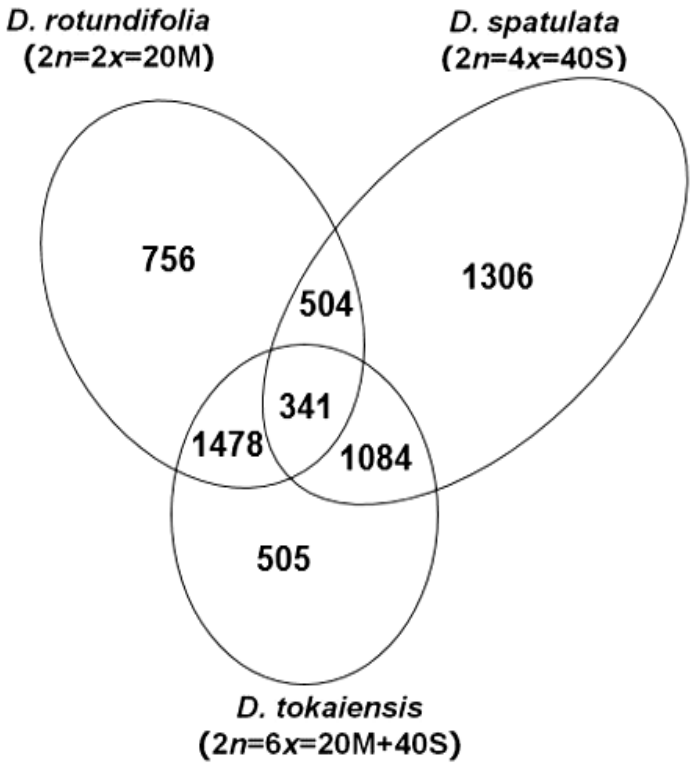

Fig. 2-4. Diagram of RAPD profiling between the three *Drosera* species**.**

The result of the RAPD analysis showed that *D. rotundifolia* and *D. tokaiensis* have the highest percentage of bands in common among the three species. This high degree of similarity between *D. rotundifolia* and *D. tokaiensis* seems to show a close relationship between the two species. However, previous molecular and cytogenetic studies clearly demonstrate that *D. tokaiensis* is an allopolyploid ($2n=6x=60$, hexaploid) with a hybrid origin from *D. rotundifolia* ($2n=2x=20$, diploid), the paternal ancestor, and *D. spatulata* ($2n=4x=40$, tetraploid), the maternal ancestor (Hoshi *et al*. 2008; Hoshi *et al*. 2010; Shirakawa *et al.* 2012).

The genome composition relationships among the three species suggest that RAPD fragments were preferentially amplified from the 20 mid-sized chromosomes in *D. rotundifolia* and *D. tokaiensis*. In consequence, the DNA fragments from these mid-sized chromosomes were preferentially amplified by RAPD, in contrast to fragments from the small-sized chromosomes, and this resulted in the observed pattern of bands in common between *D. rotundifolia* and *D. tokaiensis* (Fig. 2-4).

In a recent study on the effects of a *Drosera* plant fraction on HMC-cells, *D. tokaiensis* was shown to have a stronger anti-allergic effect than *D. rotundifolia* and *D. spatulata* (Fukushima *et al.* 2009). Our RAPD study showed that *D. tokaiensis* had not only a large number of bands in common with the other two species, but also many

specific bands, even though *D. tokaiensis* has an amphiploidal origin from *D. rotundifolia* and *D. spatulata.* The DNA sequences amplified as RAPD bands specific to *D. tokaiensis* might have arisen after speciation or chromosome differentiation (Fig. 2-4).

The chromosome information and RAPD profiling from this molecular study thus suggest that alloploidal genome formation during *Drosera* speciation may produce new advantageous characters for environmental adaptation.

CHAPTER III

Breeding and cytogenetic characterizations of new colchicine-induced hexaploid *Drosera* strains from triploid hybrids of *D. rotundifolia* and *D. spatulata*

Introduction

Carnivorous plants are unusual because of their highly specialized morphological adaptations for insect trapping (Juniper *et al.* 1989; Rivadavia *et al.* 2003; Hoshi *et al.* 2010). Droseraceae, a representative carnivorous plant family, consists of three genera: *Aldrovanda, Dionaea,* and *Drosera* (Takahashi and Sohma 1982; Juniper *et al*. 1989; Albert *et al*. 1992; Williams *et al*. 1994; Conran *et al.* 1997; Rivadavia *et al*. 2003; Shirakawa *et al*. 2011). In contrast to the two monotypic genera *Aldrovanda* and *Dionaea*, the genus *Drosera* consists of more than 150 species (Rivadavia *et al*. 2003) with extensive genetic diversity and with essential compounds that are natural sources of medication (Banasiuk *et al*. 2012). It has been reported that some species have anti-inflammatory and antibacterial effects, including *D. intermedia*, *D. anglica*, *D. madagascariensis,* and *D. peltata*. Moreover, a traditional medicine, "Droserae Herba" which is comprised mainly of *D. rotundifolia*, has been used for the treatment of pertussis or whooping cough (Paper *et al.* 2005; Fukushima *et al.* 2009; Kačániová *et al.* 2014). In

addition, a strong anti-allergy effect was recently found in the Japanese species *D. tokaiensis*, but no such effect was found in *D. spatulata* (Fukushima *et al*. 2009).

The three Japanese *Drosera* species, including *D. tokaiensis*, are useful models for studying genome organization in relation to the trapping system used to capture insects (Dixon *et al*. 1980; Zhang *et al.* 2010), the anti-inflammatory effects of the plant (Fukushima *et al.* 2009), and genetic diversity and chromosome evolution (Shirakawa *et al.* 2011). Geographically, *Drosera rotundifolia* is distributed across the Northern Hemisphere, and *Drosera spatulata* ranges across Southeast Asia including part of Japan and Australia. *Drosera tokaiensis* is found only in Japan. Cytologically, *D. rotundifolia* is a diploid species with mid-sized chromosomes ($2n=2x=20$M), while *D. spatulata* is a tetraploid species with small-sized chromosomes ($2n=4x=40$S). In contrast, *D. tokaiensis* is an amphidiploidal hexaploid species ($2n=6x=20$M+40S) with a hybrid origin from *D. spatulata* and *D. rotundifolia* (Hoshi *et al.* 2010).

Artificial triploid hybrids from *D. spatulata* and *D. rotundifolia* have been successfully produced. This artificial triploid hybrid is sterile and thus is unable to produce seed. In *Drosera*, spontaneous natural triploid hybrids occur occasionally and are usually sterile (Hoyo and Tsuyuzaki 2013). The fertility of these triploid hybrids can be restored by chromosome doubling (Campos *et al.* 2009). The induction of polyploids is

valuable tool for breeding in several plant species and for generating useful mutations in ornamental crops (Hancock 1997; Väinölä 2000; Ascough *et al.* 2008; Thompson *et al.* 2010). Colchicine, which is a representative alkaloid chemical that inhibits microtubule formation at all mitotic stages (Hancock 1997; Chen and Ni 2006; Ascough *et al.* 2008), is widely used to induce chromosome doubling in many plants (Campos *et al*. 2009).

Various techniques are available to determine the ploidy level of chemically treated plants, including chromosome counting, which is a direct approach to ploidy analysis. On the other hand, guard cell measurement is convenient tool, as are other plant morphology measurements, because stoma size is usually larger in the polyploid than in untreated plants. However, these methods are time consuming when a dealing with a large number of individuals (Campos *et al.* 2009). In contrast, flow cytometry analysis is an effective high-throughput method of analysis for nuclear DNA content (Shapiro 2003; Cousin *et al.* 2009). Although fast, stable, and simple estimates of ploidy levels have been made in many plants (Ajalin *et al.* 2002), accurate evaluations of new strains with chromosome doubling require use of the chromosome counting method because the detection of chromosomal aberrations or of small numbers of chimeric cells per plant are difficult using flow cytometry. Thus, three methods are necessary to screen and establish new polyploid strains. Polyploids show the importance of genetic diversity in plant evolution

(Xing *et al*. 2011; Zahedi *et al.* 2014), and often exhibit larger leaves, more vigorous stems, and larger flowers (Ascough *et al*. 2008).

The aim of the present study was to induce and characterize hexaploid strains made from an artificially crossed triploid hybrid of *Drosera rotundifolia* and *D. spatulata*. We initially examined the effect of colchicine on morphological characteristics, and then screened and identified ploidy levels by chromosome counting, stomatal guard cell measurement, and flow cytometry analysis. Finally, the newly established strains of colchicine-induced hexaploids were compared with the donor triploid hybrids, the wild species *D. tokaiensis*, and its parental species.

Materials and methods

Plant materials

The plant materials used were *Drosera rotundifolia* L. (accession No. 010816-sera1), *D. spatulata* Labill. (accession No. Jpn Ha4x-6), an artificially crossed triploid hybrid (accession No. 10 RS-01) of *D. rotundifolia* × *D. spatulata*, and *D. tokaiensis* (Komiya and C. Shibata) T. Nakamura and Ueda (accession No. Jpn Ha6x-9). The internal standards used in the flow cytometry measurements were *Oryza sativa* L. 'Hinohikari' and *Miscanthus sinensis* Andersson (the Sashiki-strain as a 2C standard, kindly given by

Kyushu Okinawa Agricultural Research Center, National Agriculture and Food Research Organization). All of the plants used were cultivated in the Laboratory of Plant Environment Science, Department of Plant Science, School of Agriculture, Tokai University.

Production of new plantlets from leaf explants

Leaf materials of the triploid hybrid (10 RS-01) were obtained from *in vitro* tissue culture clones. Induction of new plantlets from young leaves of the triploid hybrids was performed in ¼ B_5 liquid medium supplemented with 2.0% sucrose after transferring.

Hexaploid induction from triploid hybrids (10 RS-01)

The cultured leaf explants, which produced new buds, shoots, and plantlets, were soaked in 0%, 0.01%, 0.05%, 0.1%, and 0.5% colchicine solutions for 1, 2, and 3 days (20 plantlets per treatment). They were then cultured on half-strength Murashige-Skoog (MS) basal medium (Murashige and Skoog 1962), supplemented with 3.0% sucrose and 0.2% gellan gum at 25 °C under continuous light conditions.

Chromosome observation

Root tips of treated plants were pretreated with 0.05% colchicine for 2 h at 18 °C. They were then fixed in 45% acetic acid for 30 min on ice. The root tips were hydrolyzed in a mixture of 1 M HCl and 45% acetic acid (2:1) at 60 °C for 7 s. Root meristems were cut and squashed in 45% acetic acid. The preparations were air-dried at room temperature. After removing the coverslips, the samples were stained with 5 μg/ml DAPI solution. The chromosomes stained with DAPI were observed with a U filter. To check for chromosomal aberrations and chimeric cells in colchicine-induced hexaploid strains, more than 100 metaphase cells were observed in each strain.

Observation of stomatal guard cells

Guard cell lengths and widths in the colchicine-induced hexaploids were measured on 30 guard cells per species. The examined guard cells were from the lower epidermis in the middle parts of the leaves. Measurements were made using Image J software.

Flow cytometry analysis

In vitro leaves of the *Drosera* strains used this study were chopped in propidium iodide (PI) buffer containing with 1% (v/v) Triton X-100, 140 mM 2-mercaptoethanol,

0.5 M Na_2SO_3, 0.5 M Tris-HCl (pH 7.5), 0.2 mM $MgCl_2$, 20 mg/ml polyvinylpyrrolidone (PVP), and 1 mg/ml PI. The samples were then filtered through a 48-μm nylon mesh, incubated for 5 min, and stained with PI chopping buffer. The DNA content was measured using flow cytometry (Guava EasyCyte™ 12 HT Flow Cytometer). *Oryza sativa* (1C = 0.5 pg, calculated from Bennett and Smith 1976) and *Miscanthus sinensis* (1C = 2.65 pg, calculated from Nishiwaki *et al.* 2011) were used as reference standards to calculate genome size in absolute units.

Results and discussion

Survival rate after colchicine treatment

A few treatments caused phytotoxic effects. The survival rate was recorded after 60 days. The control group had the highest survival rate. The results showed that as the colchicine concentration increased, survival rate decreased (Table 3-1). The highest lethality was observed in the treatments with 0.5% colchicine solution for 3 days. Toxic colchicine effects were correlated with concentration and duration time. It has been previously reported that a long soaking period in a higher dose of colchicine solution negatively influences the vigor and rooting of plantlets (Trojak-Goluch and Skomra 2013).

Table 3-1. Survival rate and frequency of polyploidization in Triploid hybrid (10RS-01) after treated with colchicine 60 days.

Concentration of colchicines (%)	Treatment time (days)	Total number of explants	Survival rate after 60 days (%)	Number of mixoploid	Number of hexaploid	Frequency of mixoploid (%)	Frequency of hexaploid (%)
0	1	20	95	0	0	0	0
	2	20	90	0	0	0	0
	3	20	85	0	0	0	0
0.01	1	20	85	0	0	0	0
	2	20	55	0	0	0	0
	3	20	75	0	0	0	0
0.05	1	20	70	4	0	20	0
	2	20	85	1	0	5	0
	3	20	85	1	1	5	5
0.1	1	20	85	1	1	5	5
	2	20	85	1	0	5	0
	3	20	75	3	0	15	0
0.5	1	20	55	4	0	20	0
	2	20	70	1	0	5	0
	3	20	20	0	1	0	5

Changes in morphological characteristics

The morphological characteristics of the artificially crossed triploid hybrids are shown in Fig. 3-1. After 0.1% colchicine treatment for 3 days, some leaves changed from their normal shape to heart-shaped (Fig. 3-1A, arrow). Rarely, under the same treatment conditions, a separation at the basal part of leaf blade was observed (Fig. 3-1B, arrow). After 0.1% colchicine treatment for 1 day, some of leaves became bell-shaped (Fig. 3-1C, arrow).

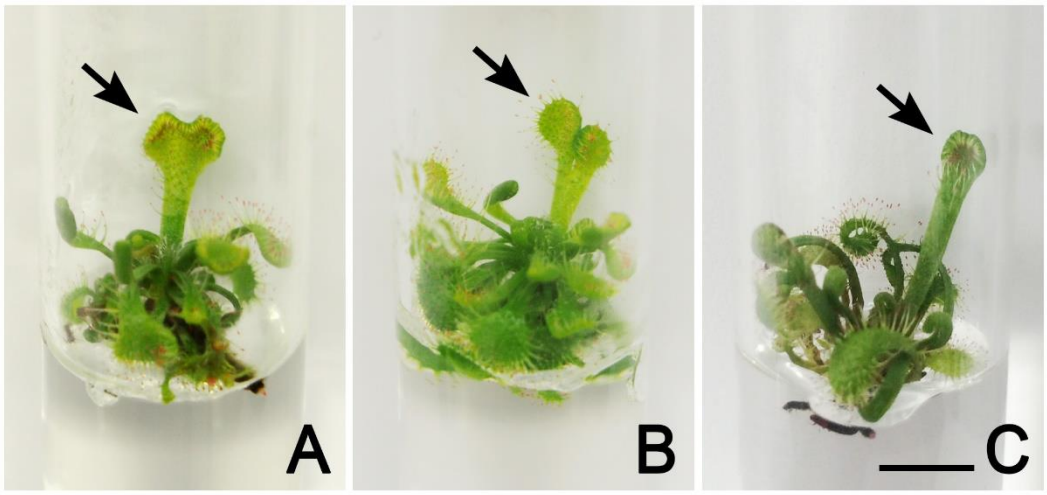

Fig. 3-1. Morphological characteristics changes 60 days after colchicine treatment. ‘heart shaped’ leaf (A), ‘double-headed (two leaf blade)’ leaf (B) and ‘bell shaped’ leaf (C) were observed in the 0.1% colchicine treatment for 1 day (C) and 3 days (A and B). Bar=1 cm.

Colchicine treatment resulted not only in chromosome doubling but also in morphological changes in leaf shapes. These leaf changes, including changes in size, margin, and doubled and forked apexes of leaves, have been previously described (Blakeslee and Avery 1937; Hewawasam *et al.* 2004; Obute *et al*. 2006; Obute *et al*. 2007). These leaf morphological changes may be due to chromosomal instability, reductions in

auxin levels and enzymatic activity, and variation in ascorbic acid levels (Franzke and Ross 1952; Obute *et al*. 2007).

Frequency of polyploids

Using flow cytometry (FCM), mixoploid plants could be detected after colchicine treatment. The highest mixoploid score was 20% on 1 day with either 0.05% or 0.5% colchicine treatment (Table 3-1). These mixoploids can be either mericlinal chimeras, having cells with different chromosome numbers in one meristem (Harbard *et al.* 2012), or periclinal chimeras, having cells of the same ploidy in parallel layers in one plant (Harbard *et al.* 2012). We isolated the pure synthetic hexaploid from several mixoploid clones and established from it three colchicine-induced hexaploid strains (Tables 3-1 and 3-2).

Table 3-2. Chromosome numbers and genome sizes of three Japanese and related species.

Species	Accession number	Chomosome number (2*n*)	Ploidy level	Karyotype formula[a]	Present data	
					2C (pg)	DNA amount of 2C (Mbp)
D. rotundifloria	010816 Sera-1	20	2*x*	20M	2.73	2745
D. spatulata	Jpn Ha4x-6	40	4*x*	40S	1.41	1549
D. tokaiensis	Jpn Ha6x-9	60	6*x*	20M + 40S	3.74	3784
Triploid hybrid	10 RS-01	30	3*x*	10M + 20S	2.31	2265
Induced hexaploid	No.63,243,283	60	6*x*	20M + 40S	4.41	4324

[a] M: middle size chromosome, S: small size chromosome (see Hoshi and Kondo 1998)

Chromosome counting

Cytological analysis revealed that the artificially crossed triploid hybrid possessed a chromosome number of 2*n*=30 (2*n*=3*x*=10M+20S) (Fig. 3-2A). From the 300 explants, only three colchicine-induced hexaploid strains, No. 63-Santhita-01-1-3, No. 243-Santhita-005-3-3, and No. 283-Santhita-05-3-3, were induced, from 0.1% treatment for 1 day, 0.05% treatment for 3 days, and 0.5% treatment for 3 days, respectively. These three strains possessed a chromosome number of 2*n*=60 (2*n*=6*x*=20M+40S) without any chimeric cells (Fig. 3-2B).

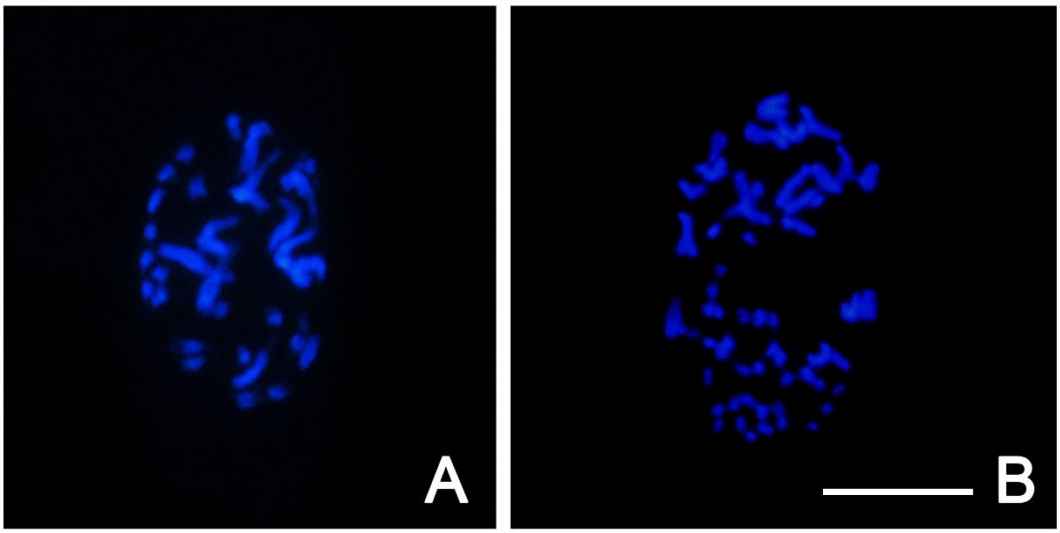

Fig. 3-2. Fluorescence staining of mitotic-metaphase chromosomes with DAPI. A. Artificial-crossing triploid hybrid (2*n*=3*x*=10M+20S). B. Colchicine-induced hexaploids (2*n*=6*x*=20M+40S). Bar=5 μm.

Comparison of leaf morphology

Ninety days later after colchicine treatment, the colchicine-induced hexaploids was morphologically compared with artificially crossed triploid hybrids and *D. tokaiensis*. The leaves of the triploid hybrids tended to be rounded, while the leaves of the colchicine-induced hexaploids and the wild hexaploid species *D. tokaiensis* were intermediate between round and spatulate and were similar to each other. The leaf blade length of the colchicine-induced hexaploids, however, was shorter than that of *D. tokaiensis*. Moreover,

the leaves of the colchicine-induced hexaploids were darker, thicker, and wider than those of the artificially crossed triploid hybrids. Additionally, the neighboring tentacles of the colchicine-induced hexaploids were thicker than those of the artificially crossed triploid hybrids (Fig. 3-3). Usually, polyploids have higher chloroplast numbers than diploids, and thus the total number of photosynthetic cells per unit of leaf area is consistently reduced with rising ploidy from diploid to hexaploid (Murti *et al.* 2012). Polyploid plants typically tend to have darker green leaves, thicker stems, and a higher leaf width/length ratio (Nonaka *et al*. 2011). Thus, the typical general trend of morphological changes caused by polyploidization was seen in the genus *Drosera*.

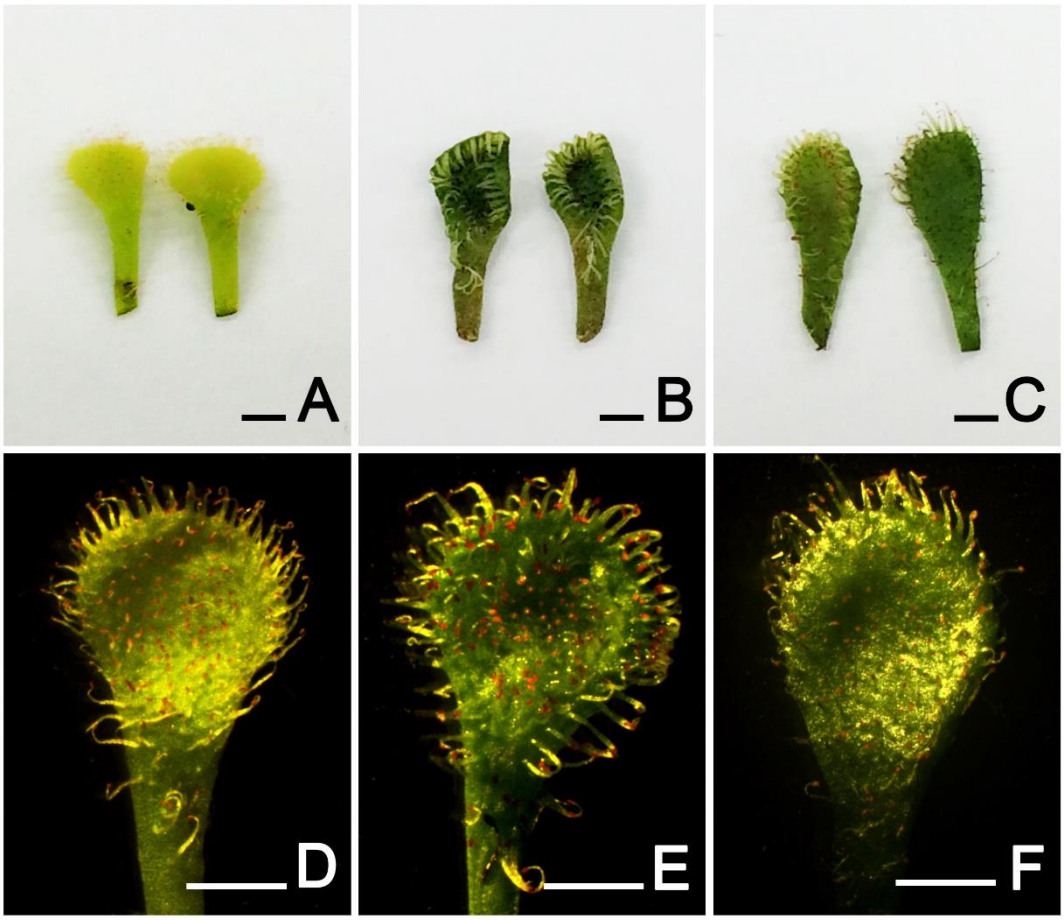

Fig. 3-3. 60-day (A-C) and 90-day (D-E) old leaves of artificial-crossing triploid hybrid (2*n*=3*x*=10M+20S) (A and D), colchicine-induced hexaploids (2*n*=6*x*=20M+40S) (B and E), and *D. tokaiensis* (2*n*=6*x*=20M+40S) (C and F). Bar=5 mm.

Evaluation of stomatal guard cell size

The effect of colchicine treatment on stomatal guard cell length and width was investigated in the artificially crossed triploid hybrids, the colchicine-induced hexaploids, and the wild hexaploid species *D. tokaiensis*. The results showed that the stomatal guard cells of the colchicine-induced hexaploids and the wild hexaploid species *D. tokaiensis*, both of which have the same constitutions ($2n$=$6x$=20M+40S) as the genomes of *D. spatulata* and *D. rotundifolia*, were larger than those of the artificially crossed triploid hybrids (Fig. 3-4, Table 3-3). Interestingly, the stomatal guard cell width of the colchicine-induced hexaploids was significantly greater than that of *D. tokaiensis*, while stomatal guard cell length of *D. tokaiensis* was significantly greater than that of the colchicine-induced hexaploids (Fig. 3-4, Table 3-3). Our previous paper demonstrated that the hexaploid *D. tokaiensis* had an amphidiploidal hybrid origin from *D. spatulata* and *D. rotundifolia* (Hoshi *et al.* 2010). Since the artificially crossed triploid hybrids were made from *D. spatulata* and *D. rotundifolia*, the genome constitution of the colchicine-induced hexaploids was the same as *D. tokaiensis*. Thus, the present results with respect to guard cell size and leaf shape showed unexpected morphological differences between colchicine-induced hexaploids and wild hexaploids, suggesting the existence of variation within their hexaploid genomes. Indeed, a wild habitat where hybrids occur

spontaneously will have environmental selection gradients that affect the genetic diversity and morphological composition of populations (Culumber *et al*. 2011; Hamilton and Aitken 2013).

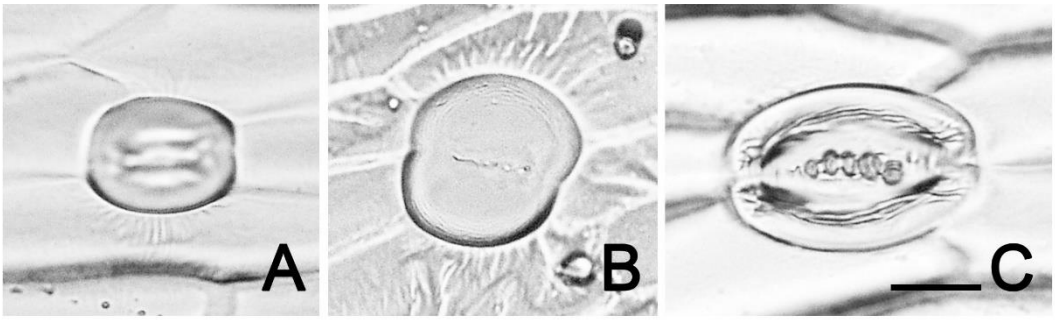

Fig. 3-4. Comparison of the stomata guard cell size of triploid hybrid (2*n*=3*x*=30) (A), artificial hexaploid (2*n*=6*x*=60) (B) and *D. tokaiensis* (2*n*=6*x*=60) (D). Bar = 20 μm.

Table 3-3. Effect of colchicine concentration and duration period on stomata guard cell size.

Plants	Mean stomata guard cell length ± SD (μm)	Mean stomata guard cell width ± SD (μm)
D. tokaiensis	44.42 ± 6.91[c]	22.47 ± 4.65[a]
Triploid hybrid	23.21 ± 4.75[a]	21.31 ± 3.29[a]
Induced hexaploid	30.46 ± 4.17[b]	30.87 ± 2.56[b]

[abc]Different letters within the same columns indicate statistically significance difference using the Duncan's test at 95% ($p < 0.05$)

2C DNA value estimation by flow cytometry

Recent FCM data has established new C-values for some *Drosera* genome sizes. The data from the different strains within the same species confirm the intraspecific stability of the 2C DNA value. The present study showed that the 2C-value of the Japanese strain of *D. rotundifolia* (2*n*=2*x*=20M) was 2.73 pg. This result strongly supports the previous results of Greilhuber (2008). In contrast, the 2C-value of Japanese *D. spatulata* (2*n*=4*x*=40S) was 1.41 pg. The value for the artificially crossed triploid hybrid (2*n*=3*x*=10M+20S) between *D. rotundifolia* and *D. spatulata* was 2.31 pg, while the value for the colchicine-induced hexaploids (2*n*=6*x*=20M+40S) made from the artificially crossed triploid hybrids by chromosome doubling was 4.41 pg. Thus, the observed 2C DNA value of the artificially crossed triploid (2.31 pg) was close to the expected hybrid 2C-value (2.07 pg), calculated as the sum of the haploid genomes of *D. rotundifolia* (2.73 pg) and *D. spatulata* (1.41 pg) (Fig. 3-5). The genome size of the artificially crossed triploid hybrid (2.31 pg) was nearly half the size of the colchicine-induced hexaploids (4.41 pg) (Fig. 3-6). Thus, these genome size estimations indicated an additive cytogenetic effect of the DNA content from the parental species and triploid donor plants. In allopolyploids, these doublings of homoeologous chromosome segments or genes are known in different donor taxa to be part of the polyploidy mechanism (Doyle

and Gaut 2000). When combining two different species with related genomes, we should expect genomic additivity related to both parental species (Liu and Wendel 2002).

There are some interesting reports that speciation accompanied by polyploid formation can both increase and decrease DNA amounts (*e.g.,* Johnston *et al.* 2005). The phenomenon of polyploidy is due to interspecific hybridization (allopolyploidy) (Renny-Byfield *et al.* 2012). It has been proposed that polyploid formation might cause reproducible, rapid, and directional changes in the parental sub-genomes within the polyploid (Comai *et al.* 2003; Ozkan *et al.* 2003; Chen and Ni 2006; Feldman and Levy 2009). Compared to the colchicine-induced hexaploids (4.41 pg), the genome size of *D. tokaiensis* (3.74 pg) is unexpectedly small, even though both contain the same genome constitutions. Non-additive change and DNA loss seem to be due to genomic stress after allopolyploidal hybridization, as has been discussed in the case of *Triticum*, *Gossypium*, and *Brassica* (Ozkan *et al.* 2003). In cases of genome upsizing, the associated partial downsizing may be associated with transposon elements (TEs) (Fedoroff 2012). TEs, including retrotransposons, contain many repeats in the genome. A TE-based mechanism could efficiently increase or perhaps decrease the genome size (Bennetzen *et al.* 2004). In the case of DNA loss, unequal intrastrand homologous recombination between these repeats might result in a net loss of native DNA and could eliminate a certain amount of

the repeated DNA (Devos *et al.* 2002). In *Arabidopsis*, the genome experienced a surge of retrotransposon amplification in a short time (Devos *et al.* 2002). It has been thought that genome size decreases through recombinant mechanisms. One of the possible mechanisms is the existence of solo LTRs, single "long terminal repeat sequences" that make large-scale deletion possible in the genome (Zhou and Cahan 2012). Solo LTRs were first found in yeast (Roeder and Fink 1980), and the mechanism of deletion by solo LTR has also been described (Devos *et al.* 2002). Recently, solo LTRs have been found in vascular plant species that contain many retrotransposons (SanMiguel and Vitte 2009). Unequal homologous recombination between two LTRs has happened in *Oryza sativa* (Shirasu *et al.* 2000; Vitte and Panaud 2003) and has also been reported in *Arabidopsis* (Devos *et al.* 2002; Vitte and Panaud 2003). However, solo LTRs have rarely been found in *Zea mays* (SanMiguel *et al*. 1996; Devos *et al.* 2002; Vitte and Panaud 2003). These new reports suggest that solo LTR recombination or a similar mechanism might occur in the *Drosera* genome.

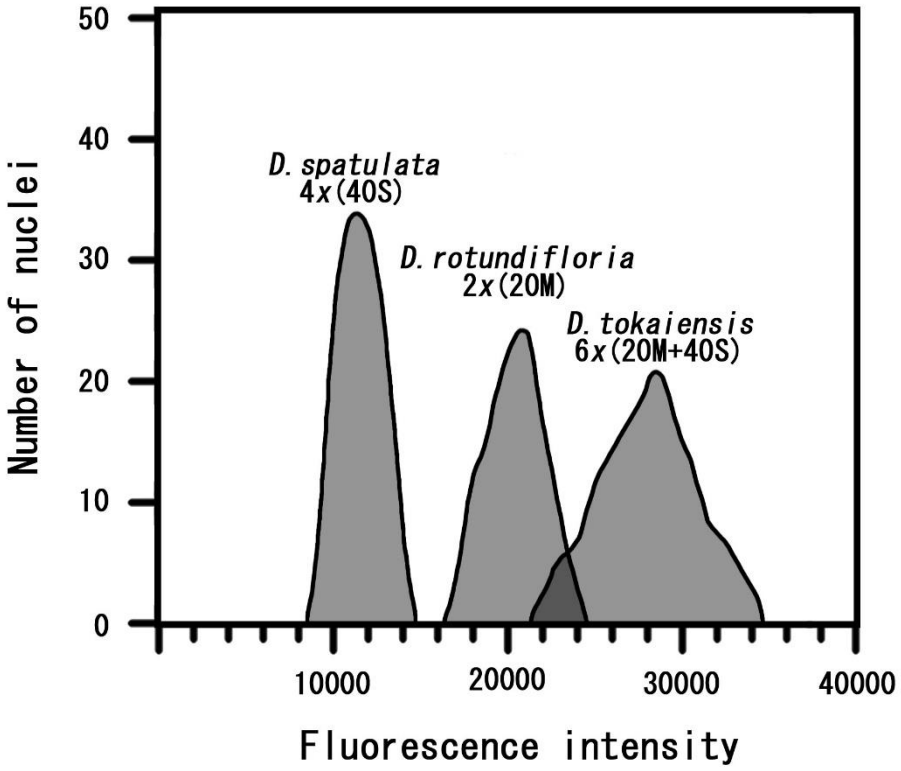

Fig. 3-5. The relative fluorescence intensity of nuclei isolated from *D. rotundifloria* (2n=2*x*=20M), *D. spatulata* (2n=4x=40) and *D. tokaiensis* (2n=6x=60).

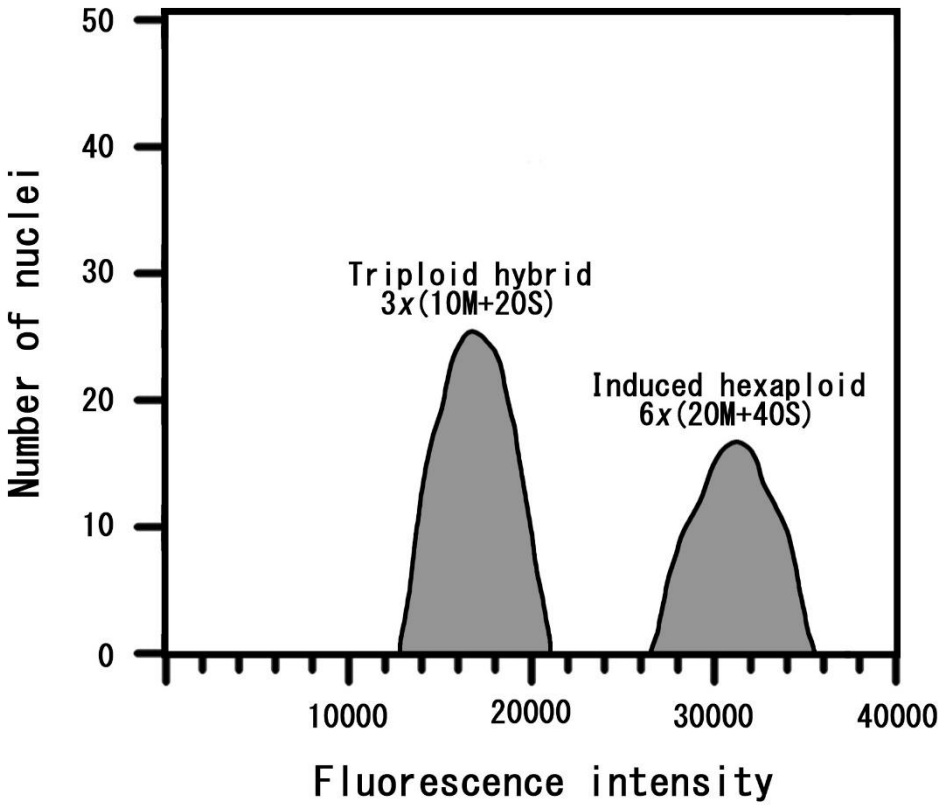

Fig. 3-6. The relative fluorescence intensity of nuclei isolated from artificial-crossing triploid hybrid (2*n*=3*x*=10M+20S) and colchicine-induced hexaploids (2*n*=6*x*=20M+40S).

CHAPTER IV

General discussion

Terminologically, it is important to discriminate between the nucleic DNA amount (*n* or 2*n*) and the DNA amount of the basic chromosome number (*x*) (Greilhuber *et al*. 2005; Greilhuber *et al*. 2006), and to recognize that 1C is the DNA content of the unreplicated holoploid complement with *n* chromosomes, while 1C*x* is the DNA content of the unreplicated monoploid set of *x* chromosomes (*x* being the basic chromosome number of a polyploid series). Because higher polyploidy is seen in *Drosera*, putative 1C*x* values were also estimated in this study. The genome size derived from the basic chromosome number (1C*x*), which is obtained by comparison with the basic chromosome number and putative C-value, is particularly useful for providing further insight into *Drosera* chromosome differentiation, especially in polyploid groups. In section *Drosera*, with a basic chromosome number of *x*=10, the present results show that the genome size data clearly distinguish one group from another. As expected, regardless of the level of polyploidy, the species with genome sets comprised of all small-sized chromosomes (S-type) show smaller DNA amounts in the basic set of chromosomes, while the species with genome sets consisting of all mid-sized chromosomes (M-type) show larger DNA amounts in the basic set of chromosomes. Moreover, *Drosera* appears to show a direction

in its chromosome evolution. Based on the molecular tree (Rivadavia *et al*. 2003) and many cytological studies, it is clear that *Drosera* chromosome size exhibits an evolutionary trend toward large increases in chromosome size and genome size in several Northern Hemisphere species with M-type chromosomes (Hoshi and Kondo 1998; Hoshi *et al*. 2008; Hoshi *et al*. 2010). Evolutionary chromosome-size increase is rather common in angiosperms, although an evolutionary size decrease in DNA amount is occasionally seen in some plants families such as Malvaceae (Wendel *et al*. 2002), Brassicaceae (Johnston *et al*. 2005), and Poaceae (Price *et al*. 2005). The mechanisms of DNA loss are not clearly understood to date.

Changes in genome size are considered to be greatly affected by the number of repetitive sequences and the ploidy levels in plant species (Bennetzen 2000; Grover and Wendel 2010). Although differences in ploidy levels result in large changes through the doubling of genome sets, the number of repetitive DNA sequences may increase or decrease within a haploid genome set. Thus, an increase-decrease event in the repetitive sequences that is related to transposable elements may directly contribute to an unreplicated monoploid chromosome set in a plant genome. Additionally, transposable elements, especially retrotransposons, cause repetitive DNA to disperse uniformly over all chromosomes in the genome (Kamm *et al*. 1996; Galasso *et al*. 1997; Harrison *et al*.

1997; Miller *et al*. 1998; Vicient *et al*. 1999; Hanson *et al*. 2003). Indeed, the retrotransposons responsible for size increases occupied more than 40% and 90% of the genome in *Vicia* (Pearce *et al*. 1996) and *Triticum* (Flavell 1986), respectively. Transposons are responsible for amplification and activation in the genome, and therefore can be correlated with biodiversity, genetic variation, and eventually speciation (Flavell *et al*. 1997; Galasso *et al*. 1997; Heslop-Harrison and Schwarzacher 2011).

With regard to a number of recent reviews of genome size change in a variety of species, Grover and Wendel (2010) presented interesting ideas concerning relevant environmental factors and ecological influences such as population size, population bottlenecks, and drift, as well as epigenetic factors such as DNA methylation and heterochromatinization of chromosome segments. With respect to environmental effects, Hanson *et al*. (2001) have already pointed out the mineral-poor environment of carnivorous plants as a possible factor in genome size reduction. It may impose pressure to reduce the redundant phosphorus-rich nucleic acids in non-essential repeated DNA sequences and hence tend to minimize genome sizes. Distinctive differences in genome sizes are seen between *Drosera* and *Drosophyllum*. Hanson *et al*. (2001) also found that *Drosophyllum* was more able than *Drosera* to acquire scarce water and phosphorus from the soil, supporting the hypothesis of genome size reduction in carnivorous plants.

In future genome research with the next generation technologies, the species of Droseraceae will be useful genetic resources that can contribute new evidence and insights on genome organization and chromosome evolution, not only in carnivorous plants but also in angiosperms generally. Further experimental studies on genome size estimation and molecular cytogenetic characterization are required.

PUBLICATIONS AND RELATED PAPERS

Publications

(1) Tungkajiwangkoon S.; Inagaki A.; Shirakawa, J. and Hoshi, Y. 2015. RAPD Profiling of Three Japanese Drosera Species. Cytologia 80(4): 393-398.

(2) Tungkajiwangkoon S.; Inagaki A.; Shirakawa, J. and Hoshi, Y. 2015. Breeding and Cytogenetic Characterizations of New Hexaploid *Drosera* Strains Colchicine-Induced from Triploid Hybrid of *D. rotundifolia* and *D. spatulata*. Cytologia (Accepted).

Conference presentations

(1) Santhita Tungkajiwangkoon, Ayato Inagaki, Junichi Shirakawa, and Yoshikazu Hoshi. RAPD Profiling and Genome Reading of *Drosera spatulata* Complex (Droseraceae). The eighth annual meeting of ISCB held at Hiroshima Jogakuin University: 7.

(2) Santhita Tungkajiwangkoon, Ayato Inagaki, Junichi Shirakawa, and Yoshikazu Hoshi. Assessment of Antiallergic Effect of Three Japanese Drosera Species of *Drosera* Complex. The 17th Asian Agricultural Symposium held on the Kumamoto Campus in Kumamoto, Kyushu. Tokai University

(3) Santhita Tungkajiwangkoon and Yoshikazu Hoshi. Cytology and Flow Cytometry of Three Japanese Drosera Species. Proceedings of ICCGE 2015. The 17th International Conference on Chromosomal Genetics and Evolution (World Academy of Science Engineering and Technology held at Bangkok, Thailand: 2203.

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