RAPD Profiling of Three Japanese *Drosera* Species

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Summary 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 allopoloidal genome formation during *Drosera* speciation might obtain new beneficial genetic characters to survive and adapt to certain environments.

Key words Cytogenetics, *Drosera rotundifolia*, *Drosera spatulata*, *Drosera tokaiensis*, Genome, RAPD.

Carnivorous plants are a group with highly specialized morphology for insect trapping, and thus appeal to botanists (Juniper *et al.* 1989, Rivadavia *et al.* 2003, Bhau *et al.* 2009, Hoshi *et al.* 2010). The Droseraceae, which is a representative family of carnivorous plants, contains three genera of *Aldrovanda*, *Dionaea* and *Drosera* (Takahashi and Sohma 1982, Juniper *et al.* 1989, Conran *et al.* 1997, Williams *et al.* 1994, Rivadavia *et al.* 2003). In contrast to the two monotypic genera *Aldrovanda* and *Dionaea*, *Drosera* is one of the largest genus, consisting of approximately 150 species and distributed worldwide (Rivadavia *et al.* 2003). Many *Drosera* species, especially the Northern Hemisphere species, show the basic chromosome number of $x=10$ with differences in ploidy level and chromosome size (Hoshi *et al.* 2010), and most of the species 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. spreads widely in the Northern Hemisphere (Rivadavia *et al.* 2003). In Japan, this species occurs in the main Islands except the Ryukyu 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 the diploid with middle size ($2\text{--}3\mu\text{m}$) of metaphase chromosomes ($2n=2x=20M$) (Fig. 1A). *Drosera spatulata* Labill. is often treated as a complex, first

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Fig. 1. Fluorescence staining of mitotic-metaphase chromosomes with DAPI. A. *D. rotundifolia* ($2n=2x=20M$). B. *D. spatulata* ($2n=4x=40S$). C. *D. tokaiensis* ($2n=6x=20M+40S$). Bar= $5\mu m$.

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

reported in Tasmania, 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). Natural habitats of *D. spatulata* in Japan are located in the southern areas: 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 size (approximately $1\mu m$) of metaphase chromosome ($2n=4x=40S$) (Fig. 1B). *Drosera tokaiensis* (Komiya and Shibata) Nakamura and Ueda is a recently recorded hexaploid-species with hybrid origin ($2n=6x=20M+40S$) (Fig. 1C). The parental ancestors of *D. tokaiensis* are *D. rotundifolia* as the paternal origin and *D. spatulata* as the maternal origin (Shirakawa *et al.* 2012) (Fig. 2). *Drosera tokaiensis* is found only in Japan, particularly in the Kansai district, Shikoku, the Pacific side of the central parts of Honshu, Tokai district and spread out inland (Nakano *et al.* 2004). The leaves of this species show an intermediate leaf shape between obovate and spatulate (Nakano *et al.* 2004, Hoshi *et al.* 2008, Hoshi *et al.* 2010).

In contrast to many previous phylogenetic works of RAPD (Sharma *et al.* 1995), our recent preliminary investigation showed various RAPD DNA band patterns with no phylogenetic relation to 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

on *D. rotundifolia* and *D. tokaiensis*, but not *D. spatulata* (Hoshi *et al.* 2010).

The purpose of this research is to clarify the overall picture of appearance patterns of RAPD DNA fragments among the three Japanese *Drosera* species in molecular profiling using whole 1200 RAPD primers.

Materials and methods

Plant materials

The plant materials were collected from three *Drosera* species: *D. rotundifolia* L., *D. spatulata* Labill. and *D. tokaiensis* (Komiya and C. Shibata) T. Nakamura and Ueda (Table 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 in continuous light condition.

DNA extraction

Total genomic DNA extraction followed Hoshi *et al.* (2010). The DNAs were extracted from fresh leaves from *in vitro* cultured plants. The individuals were ground into powder with liquid nitrogen and homogenized in the buffer containing 1 M 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 pellet was washed with 70% ethanol, dried, and dissolved in 200 µL TE overnight. On the second day, 1 µL of 1 mg mL^{–1} RNase was added, and the solution was incubated at 37°C for 1 h in a water bath. The DNA was treated as in the procedures of the first day and finally dissolved in 50 µL TE.

RAPD amplification

We followed the instruction of 10 mer kit (Operon technologies, Alameda, CA, U.S.A.) to amplify the DNA samples with a number of 1200 primers. PCR was performed in a volume of 20 µL. The reaction mixture contained 0.1 µL *Taq* DNA Polymerase (TOYOBO CodeNo.TAP-211), 2 µL dNTPs (2 mM each), 2 µL 10×*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 PC-708, Astec). The standard amplification condition was 94°C for 5 min followed by 45 cycles of 94°C for 0.5 min, 42°C for 0.5 min, 72°C for 1 min, and the reaction was completed by a final extension step of 72°C for 10 min. Amplified PCR products were performed onto 1.0% agarose gel electrophoresis in 1×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^{–1} ethidium bromide for 15 min.

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

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

^aPlant sources of three Japanese species are described in Hoshi and Kondo (1998). ^bM: middle size chromosome, S: small size chromosome (see Hoshi and Kondo 1998).

Results and discussion

A total of 1200 decamer random sequences were initially screened to select the analysis primers to be used. For the purpose of clearing up the ambiguous bands in three *Drosera* species and its closed species bands, all species were used and primers that did not generate bands in the fingerprints were not retained.

A total of 534 primers selected from OPA-01 through OPBH-20 primers were tested in each of the three *Drosera* species accessions for examining the RAPD pattern. The typical results of RAPD fingerprinting in the three species are shown in Fig. 3. Using all primers from OPA to OPBH series, the RAPD generated many bands in the range of 100–3000 bp in *D. rotundifolia*,

Fig. 3. Electrophoresis patterns of DNA amplification products obtained by RAPD primers of OPA-10 (A), OPB-18 (B), OPC-09 (C) and OPBE-11 (D) 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).

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

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.

Primer series of OPD, OPI, OPJ, OPS, OPY, OPZ and OPAR revealed the presence of more than three bands shared by all *Drosera* species. In whole RAPD bands, 341 bands were common among all three species. Moreover, many common bands between two species were obtained. *Drosera rotundifolia* and *D. spatulata* showed 504 common bands, while *D. spatulata* and *D. tokaiensis* showed 1084 bands. *D. rotundifolia* and *D. tokaiensis* showed 1478 common bands. As for the electrophoresis result of specific DNA amplification, 756 and 505 bands were specific in *D. rotundifolia* and *D. tokaiensis*, respectively. In contrast, the number of specific bands observed in *D. spatulata*. *Drosera spatulata* was 1306, screened and selected from whole 1200 random primers (Fig. 4).

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

The relationship of genome compositions among the three species suggested that RAPD fragments were preferentially amplified from 20 middle size chromosomes in *D. rotundifolia* and *D. tokaiensis*. In consequence, most DNA fragments by RAPD had a priority to amplify from the genome set with middle size chromosome than small size chromosome, which resulted in the present study showing common bands between *D. rotundifolia* and *D. tokaiensis* (Fig. 4).

In a recent study on the anti-allergic effect of a fraction of *Drosera* plants to HMC-cells, *D. tokaiensis* had a stronger anti-allergic effect than *D. rotundifolia* and *D. spatulata* (Fukushima *et al.* 2009). The RAPD study showed that *D. tokaiensis* had not only a huge number of bands common to the other two species, but also many specific bands, even though *D. tokaiensis* is of amphiploid origin between *D. rotundifolia* and *D. spatulata* (Fig. 4). The DNA sequences amplified as RAPD bands specific to *D. tokaiensis* (Fig. 3D) might occur after speciation or chromosome differentiation.

Therefore, the chromosome information obtained from molecular studies, including the RAPD profiling, suggested that allopoloidal genome formation during *Drosera* speciation might produce new advantageous characteristics for environmental adaptation.

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