Intergeneric and interspecific hybridizations among *Glebionis coronaria*, *G. segetum* and *Leucanthemum vulgare*

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ABSTRACT: Cross-hybridizations between *Glebionis coronaria* (L.) Spach and *G. segetum* (L.) Fourr., *G. coronaria* and *Leucanthemum vulgare* Lam. and *G. segetum* and *L. vulgare* by using ovule culture were successfully made the hybrid seedlings. RAPD primer OPA20 was found to isolate respective bands specific to *G. coronaria*, *G. segetum*, and *L. vulgare*. The F₁ hybrids between *G. coronaria* and *L. vulgare* and *G. segetum* and *L. vulgare* showed morphologically rather the maternal-side leaf characters.

KEYWORDS: *Glebionis coronaria*, *Glebionis segetum*, Intergeneric hybrids, Interspecific hybrids, *Leucanthemum vulgare*

The Asteraceae is evolutionally the most advanced family in the plant kingdom and has the largest tribe Anthemideae, so-called *Chrysanthemum sensu lato* (Kondo *et al.* 2010) that were considered to be evolved during the glacial epoch (Bremer and Humphries 1993; Kondo *et al.* 2003, 2009; Pellicer *et al.* 2013, 2014). Most of the generic members of the Anthemideae have the basic chromosome numbers of X=9 and moreover, they have diploid chromosome number of 2n=18 up to didiploid chromosome number of 2n=198 (Tahara 1915; Dowrick 1952) that seem to be very rare case in the higher plants. The reason why they have such extra-ordinary higher polyploid series could be the reason why they have large synteny (Kondo *et al.* 2003). Such large synteny centered in *Artemisia* and other close relatives were detected and explained by Pellicer *et al.* (2013, 2014).

The present study reports intergeneric hybridization between *Glebionis* and *Leucanthemum* and intrageneric hybridization between *Glebionis segetum* (perennial) X *G. coronaria* (annual) are readily hybridized.

MATERIALS AND METHODS

Plant materials Seeds of the annual *Glebionis coronaria* were purchased from Takii and Co., Ltd. and those of the perennial *G. segetum* were sent from the Royal Horticultural Society, United Kingdom and they were planted in pots. A few plants of *Leucanthemum vulgare* Lam. were purchased from a local nurseries for the present research. Four cross combinations among the three species were cross-pollinated to make hybridization and were, then, isolated with anti-pollination bags (Table 1). Within eight to 15 days after the last hand pollination, the inflorescences pollinated were harvested. Their ovaries were isolated under a stereomicroscope and were sterilized in 10% sodium hypochlorite solution for ten minutes, and then, put into the distilled steriled water for five min. for three times in the clean bench. Then, the surface-sterilized

ovaries were placed and planted on 1/2 MS medium supplemented with 3.0 (w/v)% sucrose, 0.2 (w/v)% gelrite, 0.2mg/l IAA and adjusted at pH 5.8 (Murashige and Skoog 1962).

Subculture Growing plantlets were subcultured in 1/2 MS medium (Murashige and Skoog, 1962) supplemented with 1.5 (w/v)%, sucrose, 0.3 (w/v)% gelrite and no growth-substance.

DNA-extraction, amplification and sequencing Total genomic DNA's of the cultured-materials were extracted and isolated from some pieces of leaflets or plantlets by the CTAB method (Doyle and Doyle 1987). They were, then, incubated in the waterbath at 65°C for 30 min. During this incubation, they were shaken well at an interval of every 10 min. Then, they were added 500μl of CIA (chloroform:isoamylalcohol=24:1) and messed up for 10 min. Then, they were centrifuged at 15,000 rpm at room temperature for 10 min. Then, the supernatant fluid of ca 450μl was transferred to another test-tube. Then, 1μl of Rnase (10μg/ml) were added and incubated at 37°C for 1h. They were, then, added again 450μl of CIA and gently mixed for 5 min before they were centrifuged at 15,000 rpm at room temperature for 10 min, and the supernatant ca 400μl was taken and transferred to new test-tube. Four hundreds μl isopropanol was added and mixed well and gently placed for five minutes. It was centrifuged at 10,000 rpm for 10 min, and its supernatant ca 400 μl isopropanol and left gently for five minutes and the supernatant was removed. Leftover pellet was added 1 ml of 70% ethanol stirred up well and centrifuged at 15,000 rpm for 5 min. Then, the supernatant was thrown away and the test-tube was turned upside-down for 15 min to get naturally drying. Then, 50μl 1X TE liquid was added for 4 °C storage, or if long-term storage at -20°C. Moreover, concentrations of extracted DNA was measured by

Table 1. Artificial cross hybridizations and ovule culture between *Glebionis coronaria* and *Leucanthemum vulgare*

Combinations of cross hybridization			Number of Ovules sown	Number of ovules germinated	Number of plants grown from ovules	Germination frequency
<i>Glebionis coronaria</i> ♀	X	<i>Glebionis segetum</i> ♂	32	8	0	25.0
<i>Glebionis coronaria</i> ♀	X	<i>Leucanthemum vulgare</i> ♂	107	38	16	35.5
<i>Leucanthemum vulgare</i> ♀	X	<i>Glebionis coronaria</i> ♂	89	27	9	30.0
<i>Glebionis segetum</i> ♀	X	<i>Leucanthemum vulgare</i> ♂	41	9	0	21.9

spectrophotometer, and adjusted and diluted down to 50 ng/μl.

RAPD analysis DNA extracted by CTAB method was used for RAPD analysis. The composition of PCR reagents were 2.8μl SDW, 5.0μl 2X buffer (TOYOBO), 1.0μl dNTPs (TOYOBO), 10μM primer 0.6μl (TOYOBO), 0.1μl KODFX (TOYOBO, DNA0.5μl, total 10μl. Total 20 primers used were among the random Primer Series KIT A (Operon). The condition of the earliest degeneration of PCR at 94°C for 1 min, annealing was at 40°C for 2 min, extension at 68°C for 2 min, made total for 45 cycles and stored at 4°C. PCR amplification was used by a thermalcycler (Gene Amp PCR System 9700, PE Applied Biosystems).

Electrophoresis PCR amplified products were determined by electrophoresis. Gel used for the electrophoresis was used and 200 bp DNA Ladder as the marker and 1.5 or 2.0% agarose gel (Takara: L03 TAKARA 50039). PCR product added loading buffer (Sigma; G-7654) and DNA sized marker 200 bp DNA ladder 2μl. Mupid-2 plus (Advance) as an electrophoresis equipment was used with electrophoresis tub TAE buffer filled out before 100 V electrified for 30 min., and then, the gel was put and shaken in ethidium bromide for 35 min in dark and stained and made electrophoresis again for 5 min.. Photography was made under UV exposure by Printgraph AE-6933FXCF-U (Atto Co.).

Hybridization approval by RAPD and morphological comparisons The hybrids obtained between *Glebionis coronaria* and *Leucanthemum vulgare* were approved by the paternal band restricted by RAPD primer OPA20. Thus, the RAPD primer OPA20 could identify the hybrid strain of *Glebionis coronaria* X *Leucanthemum vulgare* separated from the parentages.

RESULTS AND DISCUSSION

Since the members of the tribe Anthemideae, the family Asteraceae commonly have the protandrous flower, all of the pollen grains were easily removed before any pollination onto the stigmatoid tissues by the air spray or hitting shocks. Then, hybridizing pollinations could be made easily in the morning in the sunny day.

Flowering season of *Glebionis coronaria* in Japan is in the spring time and that in *G. segetum* in the summer to

autumn seasons. Flowering season of *Leucanthemum vulgare* is in the spring time. Four cross combinations among the three species were cross-pollinated to make hybridization and were, then, isolated with the anti-pollination bags. Within eight to 15 days after the last hand pollination, the inflorescences pollinated were harvested. Their ovaries were isolated under a stereoscopic microscope and were sterilized in 10% sodium hypochlorite solution for ten minutes, and then, put into the distilled sterilled water for five min. for three times in the clean bench. Then, the surface-sterilized ovaries were placed and planted on 1/2 MS medium supplemented with 3.0 (w/v)% sucrose, 0.2 (w/v)% gelrite, 0.2mg/l IAA and adjusted at pH 5.8 (Murashige and Skoog 1962).

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Fig. 1. Comparisons of band variability in RAPD primer OPA20. Easily Ladder, blank, *Glebionis coronaria*, *Leucanthemum vulgare*. Thus, the RAPD primer OPA20 could identify the hybrid strain of *Glebionis coronaria*, *Leucanthemum vulgare*, a hybrid between *Leucanthemum vulgare* and *Glebionis coronaria*.

RAPD analysis DNA extracted by CTAB method was used for RAPD analysis. The composition of PCR reagents were 2.8µl SDW, 5.0µl 2X buffer (TOYOBO), 1.0µl dNTPs (TOYOBO), 10µM primer 0.6µl (TOYOBO), 0.1µl KODFX (TOYOBO, DNA0.5µl, total 10µl). Total 20 primers used were among the random Primer Series KIT A (Operon). The condition of the earliest degeneration of PCR at 94°C for 1 min, annealing was at 40°C for 2 min, extension at 68°C for 2 min, made total for 45 cycles and stored at 4°C. PCR amplification was used by a thermalcycler (Gene Amp PCR System 9700, PE Applied Biosystems).

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