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Intergeneric hybridization and relationship of genera within the tribe Anthemideae Cass. (I. *Dendranthema crassum* (kitam.) kitam. × *Crossostephium chinense* (L.) Makino)

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Abstract An intergeneric cross has been made between *Dendranthema crassum* (kitam.) kitam. ($2n = 90$; ♀) and *Crossostephium chinense* (L.) Makino ($2n = 18$; ♂). Most of the hybrid embryos aborted at an early developmental stage. Using ovule rescue, it was possible to establish a single intergeneric hybrid plant showing $2n = 54$ chromosomes. The leaf length, leaf width and epidermal hair density of the hybrid were all intermediate between those of the parents. However the flower diameter, number of tubular florets, epidermal hair height and epidermal hair length exceeded those of both parents. A genomic in situ hybridization approach was able to distinguish between the parental genomes in the hybrid plant.

Keywords *Dendranthema crassum* (kitam.) kitam. · Fluorescent genomic in situ hybridization · *Crossostephium chinense* (L.) Makino · Intergeneric cross · Anthemideae · *Chrysanthemum sensu lato*

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Introduction

The Asteraceae tribe Anthemideae Cass., more commonly referred to as *Chrysanthemum sensu lato*, comprises 12 subtribes, 108 genera and 1,741 species (Bremer and Humphries 1993). There is a general consensus that the uniformly diploid group *Crossostephium* is primitive, and Bremer and Humphries (1993) clustered this genus along with *Dendranthema* within one Artemisiinae subtribe, but separated by *Ajania* and some other genera. However, the variation in ploidy level (from $2x$ to $10x$) of *Dendranthema* species suggests that it is probably of relatively recent origin (Nakata et al. 1987; Iwatsuki et al. 1995). Because of either extensive character divergence or convergence and plesiomorph deficiency, taxonomic relationships and circumscription among the Anthemideae genera *sensu stricto* are rather uncertain (Hoffmann 1894; Bremer and Humphries 1993).

Intergeneric hybrids is a powerful means of assessing the relationships between genomes. A number of such crosses have been successfully made between genera within the Anthemideae (Ohishi et al. 1996; Kondo et al. 1999; Abd El-Twab et al. 1999; Abd El-Twab and Kondo 1999, 2001a, b, 2004, 2006; Fukai et al. 2000; Zhao et al. 2008a). In many cases, however, these wide crosses are not feasible, for a variety of reasons (Davis and Heywood 1963). Non-viable hybrids can arise due to disharmony between the parental genomes (Wolff and Rijin 1993), with hybrid embryos failing to develop beyond an early

stage (Tanaka and Watanabe 1972). Some success in overcoming this incompatibility has been achieved by the introduction of ovary or embryo culture (Watanabe 1977), leading to the production of a number of viable hybrid combinations between Asteraceae species (Kondo et al. 1999; Abd El-Twab et al. 1999; Abd El-Twab and Kondo 1999, 2001a, 2006). However, as yet, no hybrids between species belonging to *Dendranthema* and *Crossostephium* have been reported.

Genomic in situ hybridization (GISH) determines levels and incorporated positions of alien chromatin and is applied to intergeneric hybrids in *Chrysanthemum sensu lato* (Ogura and Kondo 1998; Abd El-Twab et al. 1999; Kondo et al. 1999; Abd El-Twab and Kondo 1999, 2001a, b, 2004, 2006). Many F1 hybrids were studied by using GISH between *Dendranthema*, *Chrysanthemum*, *Ajania*, *Tanacetum*, *Brachanthemum*, *Elachanthemum*, *Leucanthemella* and *Nipponanthemum* (Ohishi et al. 1996; Abd El-Twab et al. 1999; Abd El-Twab and Kondo 2001b; Fukai et al. 2000; Zhao et al. 2008a). It may provide more information to clarify and justify intergeneric relationship among the members of *Chrysanthemum sensu lato*.

Crossostephium chinense (L.) Makino ($2n = 2x = 18$) has an ornamental leaf with white dense flosses. It is of particular interest for chrysanthemum improvement as it is characterized by enhanced levels of both salt tolerance and insect resistance (Chen et al. 1995; Li et al. 2008). *Dendranthema crassum* (kitam.) kitam. is a typical decaploid species, with $2n = 10x = 90$ (Hotta et al. 1996). In this paper, we report the successful hybridization between these two species, the taxonomic relationship between these two genera was also discussed.

Materials and methods

Plant materials and artificial crossing

The accessions of *D. crassum* and *C. chinense* are both conserved within the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. *Dendranthema crassum* has an outer ring of ligulate flower surrounding a central mass of tubular florets while *C. chinense* does not. Tubular florets of the former were removed for emasculation, and the ligulate flower petals were docked to expose the stigma, before covering with a paper bag. Two days after emasculation, pollen taken from freshly opened flowers of *C. chinense* was transferred to the stigma of the emasculated flower of *D. crassum* with a brush, and the pollinated flower was re-enclosed in a paper bag.

Pollen germinability

Pollen was collected on a soft brush during the morning on sunny days, and was germinated in vitro on a cavity slide in ME_3 medium containing 30% PEG1500 for 12 h at 20°C using a hanging drop method (Zhao et al. 2005). Pollen grains which had extended a tube longer than the radius of the pollen grain, as observed by light microscopy, were assumed to be viable (Zhang and Croes 1982). Ten samples of at least 50 pollen grains each were assessed to derive an estimate of the percentage of viable pollen. A sample of ten ligulate flowers (2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, 4 d, 6 d and 8 d after pollination) was fixed in FAA (1:1:18 formalin: glacial acetic acid: 70% ethanol). After removal of the ovary, the pistils were softened by treating with 1 M NaOH for 12 h, stained with 0.1% (w/v) decolourized aniline blue in 0.1 M K_3PO_4 and squashed under a cover slip. The presence of pollen tubes was detected by fluorescence microscopy (BH-2; Olympus Co. Ltd.) under UV light.

Megagametophyte and embryo development

Ten flower buds per developmental stage and 50 fertilized ligulate flowers (sampled each day for one month after pollination) were fixed in FAA and embedded in paraffin wax (Li 2001). Serial sections of thickness 6–14 μm were stained in Heidenhain's haemaoxylin and mounted in Canada balsam. Then, observations are made with light microscope (BX41; Olympus Co. Ltd.) using permanent slides.

Embryo rescue

Ovaries were surface sterilized by immersion in 70% ethanol for 30 s, followed by 10% H_2O_2 for 10 min, and four rinses in sterile water. The ovary coats were aseptically removed to extract the ovules, which were

transferred to a Murashige and Skoog (1962) medium containing 2 mg/l 6-BA and 0.5 mg/l NAA. The cultures were held at 25°C with a 16 h day provided by cool white fluorescent lamps (36 mmol m^{-2} s^{-1}). The single rescued plantlet was removed to fresh medium every 4 weeks, until it developed roots 1 cm in length, at which point it was potted into a 1:1:1 mixture of vermiculite, perlite and soil. The rooted seedling was held under a 16 h photoperiod (4 mmol m^{-2} s^{-1}) at 19 ± 1°C, 90% relative humidity and fed weekly with half-strength Murashige and Skoog medium for a week. After this period, the humidity was gradually lowered over a period of 1 month, before transplanting into the field.

Morphological identification

Flower shape, leaf shape and epidermal hair characteristics were compared for the putative hybrid and the two parental plants. Flower shape was defined by a flower diameter index, a ligulate flower quantity index and a tubular florets quantity index (Li 1993), which were derived from measurements taken from ten flowers. Leaf shape comprised a combination of length, width and a petiole length index (Li 1993), measured from the fifth leaf below the apex, sampled from ten leaves. Epidermal hair was characterized by its density and height, along with a length index (Li 1993). Measurements of these characters represented the mean of ten observations. Data were analyzed by one-way ANOVA using SYSTAT 7.0 (SYSTAT 1997).

Chromosome counting and GISH analysis of putative intergeneric hybrid

Young root tips (ca. 1 cm in length) were collected from cuttings of two parents and putative intergeneric hybrid, held in ice water for 20–24 h, fixed in Carnoy's solution (3:1 ethanol: glacial acetic acid) and stored at 4°C for 24 h. The fixed root tips were squashed under a glass slide in a drop of 45% glacial acetic acid, and chromosome spreads observed and photographed under phase contrast microscopy (BX41; Olympus Co. Ltd.).

For GISH analysis, genomic DNA of *C. chinense* was extracted from fresh young leaves using the CTAB method (Doyle and Doyle 1987) and labelled with biotin 16-dUTP (Roche Ltd.) by nick translation. The methods for prehybridization, probe denaturation and in situ hybridization followed Heslop-Harrison et al. (1991), with some modifications. Cover slips were removed by the liquid nitrogen freezing method and the preparations were dried at room temperature. The slides were then denatured in 75% (v/v) deionized formamide at 78°C for 70 s, dehydrated by passing through an ethanol series (5 min in 75%, 95% and 100%) and air-dried at room temperature. The hybridization mixture was denatured by boiling for 7 min, and then quenched for 15 min. A 15 µl aliquot of the hybridization solution was applied to each slide, which was then covered with a 20 mm × 20 mm cover slip and placed in a humid chamber at 37°C for 12 h. The slides were thereafter washed four times in 2× SSC at 42°C and in 1× PBS for 5 min at room temperature. After air-drying at room temperature, the preparations were stained with 100 µg/ml propidium iodide (Sigma) for 5 min and washed with 1× PBS. Finally they were mounted in Vectashield medium. Biotinylated DNA was detected with FITC, which fluoresces yellow when excited with blue light. Fluorescence signals were observed by epifluorescence microscopy (BH-2; Olympus Co. Ltd.).

Results

Intergeneric hybridization, pollen germinability, megagametophyte and embryo development, and embryo rescue

A total of 1,300 ligulate *D. crassum* flowers were artificially pollinated with *C. chinense* pollen, but no hybrid seeds were obtained. The in vitro germination percentage of *C. chinense* pollen was 51%, a level sufficient for effective cross breeding (Fig. 1a). On the stigma, the initiation of germination began 2 h after pollination and continued for a further 2 d (Fig. 1b, c). In all, 260 *D. crassum* megaspore mother cells were scored, and 149 of these (~57%) developed into a normal female gametophyte from a hypodermal archesporical cell. In *D. crassum*, the embryo sac develops by a series of divisions from the megaspore mother cell (Fig. 2a–g), but in 111 (~43%) of the megagametophytes, development was arrested at the megaspore mother cell stage. Embryo abortion was the norm among the sample of 180 pollinated ovaries, with only a single globular embryo (at 15 d post pollination) being observed (Fig. 2h, i). Embryos at other developmental stages

were not seen. Most of the ovaries harvested 15 d post pollination were empty and discoloured. Finally, 160 plump ovules (at 15 d post pollination) were selected for in vitro culture, but only one of these generated a plantlet after 8 weeks of culture.

Morphology of the putative hybrid

The mature plant derived from the putative hybrid and the two parental plants differed significantly from one another for all the eight morphological characters, except in the number of ligulate flowers (Fig. 3h; Table 1), for which the putative hybrid was as productive as the maternal plant. The mean leaf length of the putative hybrid was 6.3 cm while that of *D. crassum* and *C. chinense* were, respectively, 9.7 cm and 4.1 cm (Fig. 3g; Table 1). Similarly, the mean leaf width of the putative hybrid was 4.1 cm, while that of *D. crassum* was 6.2 cm and that of *C. chinense* 1.2 cm (Fig. 3g; Table 1). The mean epidermal hair density of the putative hybrid was 32.0 mm^{-2}, while that of *D. crassum* and *C. chinense* were, respectively 23.2 mm^{-2} and 173.6 mm^{-2} (Fig. 3d, e, f; Table 1). The mean flower diameter, number of tubular florets, epidermal hair height and epidermal hair length of the putative hybrid were, respectively 4.6 cm, 219.8, 140.9 μm and 664.6 μm, while the equivalent values for the *D. crassum* and *C. chinense* parents were 3.7 cm and 0.6 cm, 160.0 and 82.4, 95.2 μm and 94.5 μm, and 522.9 μm and 334.6 μm (Fig. 3h, i, d, e, f; Table 1). The mean petiole length of the putative hybrid was 1.8 cm, while those of *D. crassum* and *C. chinense* were respectively 4.1 cm and 2.4 cm (Fig. 3g; Table 1).

Chromosome number and GISH analysis of the putative intergeneric hybrid

As expected, *D. crassum* showed $2n = 10x = 90$ and *C. chinense* $2n = 2x = 18$, and the putative hybrid $2n = 6x = 54$ (Fig. 4). GISH was able to distinguish between the *D. crassum* and *C. chinense* genomes in the nuclei of the putative hybrid. Thus, among the 54 chromosomes in the putative hybrid, nine were labelled by the avidin-FITC assay when the probe comprised genomic DNA of *C. chinense*, while the remaining 45 did not hybridize with the probe (Fig. 5).

Discussion

Intergeneric hybridization between *D. crassum* and *C. chinense*

Many wild genera/species possess elite attributes such as resistance to biotic or abiotic stresses (Liu et al. 2005). Utilization of this germplasm has great potential for genetic improvement. Intergeneric hybridization is a promising approach for intergration of excellent parental traits into their hybrid which is of considerable practical interests to the breeding (Eeckhaut et al. 2007). The F_1 embryos commonly collapsed during early developmental stage, thus distant hybridization of *Chrysanthemum sensu lato* always failed to yield the F_1 hybrids (Tanaka and Watanabe 1972; Wolff and Rijin 1993). While the barrier of distant hybridization of *Chrysanthemum sensu lato* can be overcome by ovary culture (Watanabe 1977).

Dendranthema is a genus of high horticultural interests such as use in cut flowers, pot plants and gardens. But most of *Dendranthema* cultivars are susceptible to diseases, pests and other environmental stress, which seriously hampered its output and quality (Li 1993; Jiang et al. 2002; Zhang et al. 2005). Our previous studies showed that intergeneric hybridization is a prominent and practical way to improve the traits of *Dendranthema* (Zhao et al. 2008b). In present study, in order to introduce excellent traits of *C. chinense* to *Dedranthema*,

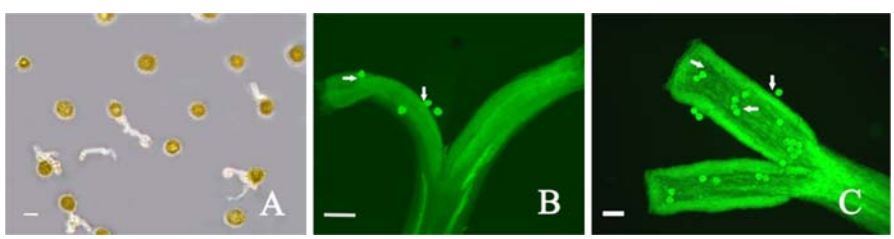

Fig. 1 Pollen germinability of *C. chinense*. **a** Pollen grains germinating in vitro, *bar* = 10 μm. Pollen germination on the stigma (**b**) 2 h and (**c**) 2 d post pollination, *bar* = 100 μm

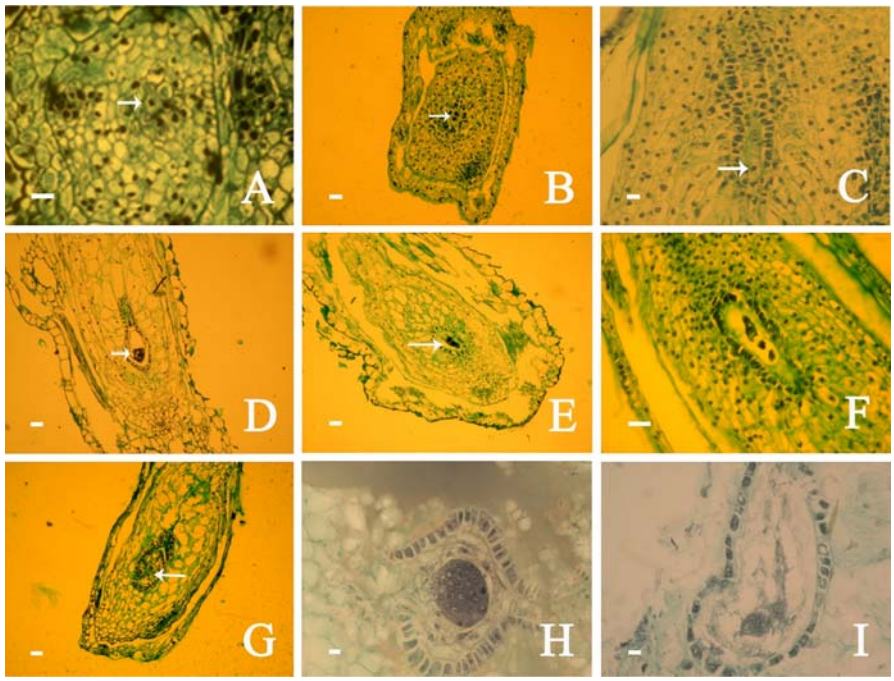

Fig. 2 Megagametophyte development of *D. crassum*, and embryo development of the hybrid. **a** The megaspore mother cell (*arrow*). **b** Two haploid nuclei (*arrow*). **c** Four haploid nuclei, in which the megaspore at the chalazal end is functional (*arrow*) while the other three have degenerated. **d** The mononuclear embryo sac, the functional megaspore shown by an *arrow*. **e** The binucleate embryo sac (*arrow*). **f** The tetranucleate embryo sac. **g** The mature embryo sac, with the egg cell shown by an *arrow*. **h** Globular embryo (*arrow*). **i** Embryo is collapsed during early developmental stage (*arrow*). **a–g** *bar* = 10 μm, **h–i** *bar* = 20 μm

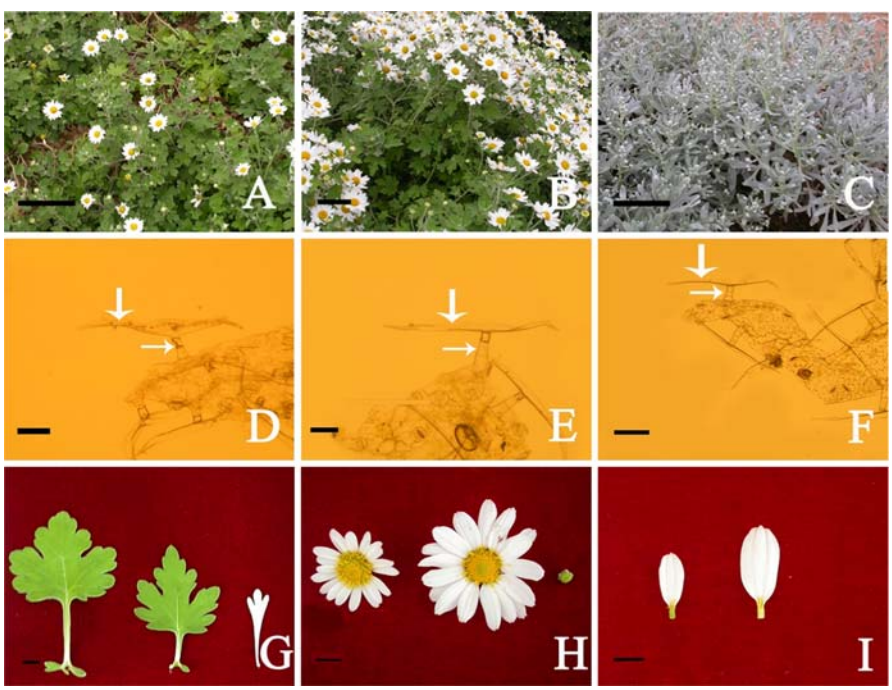

Fig. 3 Morphological characteristics of *D. crassum*, *C. chinense* and the putative hybrid. **a–c** Plant morphology at the flowering stage. (**a**) *D. crassum*, (**b**) The putative hybrid, (**c**) *C. chinense*, *bar* = 10 cm. **d–f** Epidermal hair morphology, the *large arrow* indicating hair length, and the *small arrow* hair height. (**d**) *D. crassum*, (**e**) The putative hybrid, (**f**) *C. chinense*, *bar* = 100 μm. **g** Leaf morphology of *D. crassum* (*left*), the putative hybrid (*middle*) and *C. chinense* (*right*). **h** Flower morphology of *D. crassum* (*left*), the putative hybrid (*middle*) and *C. chinense* (*right*). **i** Petal morphology of *D. crassum* (*left*) and the putative hybrid (*right*). **g–i** *bar* = 1 cm

Table 1 Morphology difference analysis of *D. crassum*, the hybrid and *C. chinense*

Characters	Species		
	D. crassum	The hybrid	*C. chinense*
Leaf length (cm)	9.75 ± 0.76^{a}	6.28 ± 0.59^{b}	4.07 ± 0.25^{c}
Leaf width (cm)	6.18 ± 0.42^{a}	4.07 ± 0.24^{b}	1.20 ± 0.12^{c}
Petiole length (cm)	4.14 ± 0.44^{a}	1.78 ± 0.26^{c}	2.40 ± 0.35^{b}
Ligulate flower quantity	20.60 ± 1.08^{a}	21.40 ± 1.78^{a}	
Tubular florets quantity	160.00 ± 17.04^{b}	219.80 ± 14.35^{a}	82.40 ± 3.95^{c}
Flower diameter (cm)	3.75 ± 0.28^{b}	4.58 ± 0.10^{a}	0.57 ± 0.08^{c}
Epidermal hair height (μm)	95.20 ± 3.16^{b}	140.90 ± 2.60^{a}	94.50 ± 2.80^{b}
Epidermal hair length (μm)	522.90 ± 6.59^{b}	664.60 ± 8.29^{a}	334.60 ± 9.61^{c}
Epidermal hair density (mm^{-2})	23.20 ± 2.53^{c}	32.00 ± 4.64^{b}	173.60 ± 6.20^{a}

Note: Values with different superscript indicate significant differences at $P < 0.01$ according to Tukey's test. Values represent mean ± SE

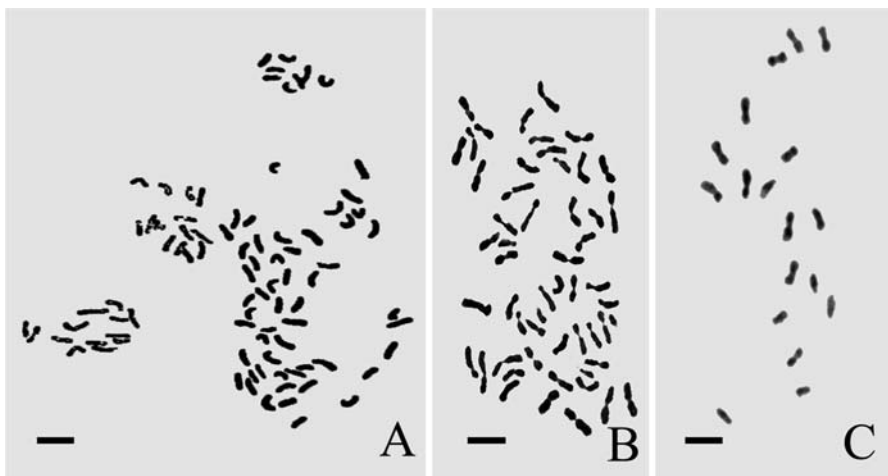

Fig. 4 Chromosome counting at metaphase of mitosis of *D. crassum*, *C. chinense* and their putative hybrid. **a** *D. crassum*. **b** The putative hybrid. **c** *C. chinense*. *Bar* = 2 μm

D. crassum was pollinated by *C. chinense*. When the intergeneric hybridization was conducted, no hybrid seeds were produced, however, both in vitro and in planta germination assays showed that the *C. chinense* pollen was viable, and a few *D. crassum* megaspores did develop into a mature embryo sac. Since the development of the hybrid embryo was apparently arrested at the globular stage, it seems that hybrid embryo abortion is the major means of preventing the formation of this intergeneric hybrid combination. A single putative hybrid plant was obtained by the application of ovule rescue, and its hybridity was confirmed by both chromosome counts and GISH analysis. In particular, the GISH assay showed that the hybrid was fully symmetric, with nine *C. chinense* and 45 *D. crassum* chromosomes. We believe that this represents the first report of an intergeneric hybrid between *D. crassum* and *C. chinense*.

The intergeneric hybrid between *D. crassum and C. chinense* grew vigorously in the field, and its morphology differed markedly from that of either of its parents. Specifically, its leaf length, leaf width and epidermal hair density were intermediate between those of its parents, but its flower diameter, number of tubular florets, epidermal hair height and epidermal hair length were greater than those of either parent. These variable traits suggesting that intergeneric hybridization is an effective way for creating novel genetic variations and improving the ornamental value of the *Dendranthema*. The hybrid acts as an

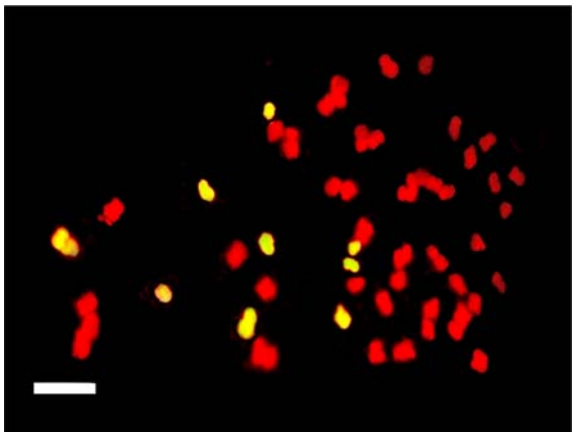

Fig. 5 GISH in mitotic-metaphase chromosomes of the hybrid between *D. crassum* and *C. chinense*. *C. chinense* chromosomes *fluoresce yellow*, while those of *D. crassum* stained *red*. *Bar* = 5 μm

intermadiate material for the introduction of novel genetic variation into *Dendranthema* cultivars. Moreover, this hybrid can also serve as a means for clarifying the genome relationship (such as GISH analysis) between two parental genera.

The genetic relationship between *Dendranthema* and *Crossostephium*

The intensity of fluorescence in GISH preparations reflects sequence homology between the probe DNA and the target, and thus can be used as a measure of homology between distinct genomes (Kondo et al. 1999; Abd El-Twab and Kondo 2001a; Kondo and Abd El-Twab 2002). *Ajania remotipinna* is readily crossable with *Dendranthema lavandulifolia* and *D. chanetii*, and hybrids are obtainable without recourse to embryo rescue (Abd El-Twab et al. 1999; Kondo and Abd El-Twab 2002). For these combinations, a large excess of blocking DNA is necessary to achieve genome discrimination in the hybrid (Abd El-Twab et al. 1999; Kondo and Abd El-Twab 2002). In contrast, in *Dendranthema* × *Tanacetum*, *Leucanthemella* × *Nipponanthemum*, *Chrysanthemum* × *Nipponanthemum*, and *Dendranthema* × *Nipponanthemum* hybrids (which require embryo rescue for their success), GISH analysis suggests that sequence homology is less close, as no blocking DNA is needed to fully discriminate between the parental genomes (Kondo et al. 1999; Abd El-Twab and Kondo 2001b, 2004, 2006). The ease with which GISH could discriminate between *D. crassum* and *C. chinense* thus indicates that their genetic relationship to one another is relatively distant. The difficulty of creating intergeneric hybrids increases along with the phylogenetic distance between the parents (Sharma 1995; Liu et al. 2005). Therefore difficulty in obtaining an intergeneric hybrid between *D. crassum and C. chinense* suggested distant phylogenetic relationship between the two genera. The conclusion was supported by a phylogenetic analysis based on sequence variation in the internal transcribed spacer of nuclear ribosomal DNA (unpublished data) as well.

Taken together, intergeneric crosses and GISH analysis suggested that *Dendranthema* are more distant to *Crossostephium* than to *Ajania*. It may be one of interpretations that *Dendranthema* and *Crossostephium* were separated by *Ajania* in phylogenetic trees by Bremer and Humphries (1993).

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