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Full Length Article

A Genetic Linkage Map of Wild *Chrysanthemum* Species Indigenous to Korea and its Challenges

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Abstract

Chrysanthemum is widely used as an ornamental plant because it has tremendously diverse in morphologies including flower shapes, sizes, colors and plant architecture. However, genetic study on this crop is not intensively performed and many of genetic features are not known yet. Although several linkage maps of *Chrysanthemum* based on a variety of molecular markers were developed, all of them used *Chrysanthemum* cultivars which are mostly hexaploids. In the current study, we present a linkage map of an F1 population crossed between two parents, *C. boreale* (2n = 2x = 18) and *C. indicum* (2n = 2x = 18), which are wild *Chrysanthemum* species collected in South Korea. Forty-eight linkage groups were formed but a few linkage groups had clustered markers which are very tightly linked. Thus, cytogenetic analysis was performed to explain this phenomenon. As a result, chromosomal rearrangements including reciprocal translocation seems to be involved in the two parents used in the current study. We discuss what these chromosomal rearrangements cause to construct genetic linkage maps. We also suggest possible solutions to improve the quality of this linkage map in the near future. The results in the current study suggest that it needs to be very cautious to choose species for breeding in *Chrysanthemum*. © 2018 Friends Science Publishers

Keywords: Chromosome abnormality; Cytogenetics; Genotype-by-sequencing (GBS); Reciprocal translocation; Single nucleotide polymorphism (SNP)

Introduction

Chrysanthemum (*Chrysanthemum* × *morifolium*) is one of the most important ornamentals worldwide because it has tremendously diverse in morphologies including flower shapes, sizes, colors, and plant architecture. The genus *Chrysanthemum* contains about 41 species that are well-distributed in East Asia of which diversity is thought to be originated in China (Bremer, 1993). The floriculture industry in north-east Asian countries including China, Japan, and Korea as well as some European countries bears large volume of *Chrysanthemum* production in the forms of cut flowers, pot plants, gardening and landscaping, and even medicinal usage (Silva, 2003; Zhang *et al*., 2011). With vigorous breeding activity, over 6,000 cultivars were developed to be quite variable among species in this genus in terms of morphology and ploidy level (Dorwick, 1953; Li *et al*., 2013) and most of them are allohexaploids and aneuploids although the most of them are as in 2n = 6x = 54 (Zhang *et al*., 2010, 2013). However, genetic improvement of *Chrysanthemum* is very difficult especially because of its genome complexity with its mega genome size, high level of heterozygosity caused by its outcrossing nature, self-incompatibility, and inbreeding depression (Xu *et al*., 2009).

Linkage maps of *Chrysanthemum* utilizing amplified fragment length polymorphism (AFLP) (Zhang *et al*., 2010), inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), single nucleotide polymorphisms (SNPs) markers (Van Geest *et al*., 2017a, b), and sequence related amplified polymorphism (SRAP)

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markers (Zhang *et al*., 2011) have been developed so far. However, all of them used *Chrysanthemum* cultivars which are hexaploids. Two parents in the current study, *C. boreale* (2n = 2x = 18) and *C. indicum* (2n = 2x = 18), are wild *Chrysanthemum* species collected in South Korea (Hwang *et al*., 2013; Kim *et al*., 2014). They are utilized widely as an ornamental crop and a pharmaceutic medical crop falling into the same group in the cluster analysis of compositions of volatile compounds, which makes them very valuable natural resources (Lee *et al*., 2009; Kim *et al*., 2014). They are believed to be a wild progenitor of the hexaploid cultivars, and has long been a source to breed new forms of garden *Chrysanthemum* (Yang *et al*., 2006). They share genetically close relationship based on molecular markers (Lee and Kim, 2000; Khaing *et al*., 2013; Kim *et al*., 2015). Consequently, their morphological phenotypes are similar to each other according to a phylogenetic analysis using Korean native *Chrysanthemum* species (Kim *et al*., 2014). However, there are morphological variations in phenotypes such as leaf shapes even within same species, which makes it hard to classify them accurately (Lee *et al*., 2007). Likewise, there are large variations within each species in karyotypes (Kim *et al*., 2003; Won *et al*., 2013). Their results may imply the evidence of some changes in chromosomal structure like reciprocal translocations. This would interrupt a fine genetic mapping although the ploidy level of two parents (diploids unlike commercial cultivars) would make it less complicated to construct a genetic map.

Various molecular markers were utilized for genetic studies of *Chrysanthemum*. Among them, RAPD profiles were first used to know where the cultivated *Chrysanthemum* (Dai *et al*., 1998) followed by AFLP to determine relationships between various *Chrysanthemum* species is originated (Zhou, 2002) and used to detect polymorphisms and to confirm hybridity (Lema-Rumiñska *et al*., 2004). ISSR markers were developed to evaluated polymorphisms of cultivated *Chrysanthemum* (Miao *et al*., 2007) and used to test the genetic stability of *in vitro* cultured *Chrysanthemum* (Wang *et al*., 2007). However, these molecular markers are difficult to be directly compared with other close species for various comparative genomics approaches due to limited DNA sequence information. On the other hand, genetic linkage maps provide transferable genetic information as well as many other applications in breeding. The first genetic map of *Chrysanthemum* was constructed using RAPD, ISSR, and AFLP (Zhang *et al*., 2010), and SRAP markers added to make a denser map (Zhang *et al*., 2011) although these maps are not sufficient to provide high resolutions. The high level of heterozygosity in *Chrysanthemum* as an outcrossing species should apply the double pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994). This method has been applied in constructing genetic map in outcrossing tree species (Bratteler *et al*., 2006), lawn grasses (Warnke *et al*., 2004), grasses such as sugarcane (*Saccharum* spp.) (Ming *et al*., 1998) and switchgrass (*Panicum virgatum*) (Missaoui *et al*., 2005), and ornamental crops (Dunemann *et al*., 1999).

The whole-genome sequence analyses for various crops in a time efficient manner became possible due to next generation sequencing (NGS) technologies in a short time (Metzker, 2010). With NGS, a new genotype-by-sequencing (GBS) technology has been developed and applied to the sequencing of multiplexed samples (Poland and Rife, 2012). GBS, one of the latest applications in NGS, is very useful tool to search and genotype SNPs in the targeted genomes via reduced representation libraries by simultaneously performing molecular marker discovery and genotyping. The SNPs detected by GBS are ready to use for constructing genetic maps with another co-dominant marker, simple sequence repeats (SSR). These molecular markers can be used for high-density genetic map of *Chrysanthemum*.

Karyotyping is a valuable tool to observe chromosomal variations such as the presence/absence of chromosomes with different morphological traits. It can detect karyotype changes such as chromosomal rearrangements (Kim *et al*., 2008). These rearrangements and duplications/deletions of chromosome segments due to unequal crossing-over between homologous chromosomes, segment inversions, and segment translocations between non-homologous chromosomes (Rieseberg, 2001). Thus, the diverse karyotypes in chrysanthemum species result from chromosome rearrangements. This could be the case of those species in the current study. Thus, the karyotypes were also investigated in the current study. This could help to interpret the genetic map constructed in this study.

The objective of this study is to construct genetic maps using F_1s from the cross between *C. boreale* and *C. indicum* which are collected in South Korea using SNPs markers detected by GBS. This map will be interpreted and discussed by integrating with the results of cytogenetic experiment. The findings from the current study would elucidate the challenges on *Chrysanthemum* breeding indigenous to Korea and provide clues to improve the quality of the genetic map.

Materials and Methods

Plant Materials

Diploids of *C. boreale* and *C. indicum* (both 2n=2x=18) were chosen and *C. boreale* as a female and *C. indicum* as a male were crossed to generate 79 progenies of F_1s in National Institute of Horticultural & Herbal Science, Rural Development Administration, Wanju, Korea between 2016 and 2017. The gDNA of each plant sample was extracted using a CTAB method (Xin and Chen, 2006) and diluted at 40 ng/*u*L. Each samples were frozen with liquid nitrogen and homogenized with mortar and pestles or a mechanical homogenizer to obtain intact DNA (Guillemaut and Maréchal-Drouard, 1992) followed by phenol-chloroform-isoamyl alcohol (PCI) extraction (Zhu *et al*., 1993) to obtain

clean DNA samples so that restriction enzymes are not blocked by junk proteins during digestions.

GBS Library Construction

A little modification of protocol for constructing GBS library was applied from Poland and Rife (2012). The adapters for each enzyme combination are listed in Supp. Table 1. Genomic DNA was digested using two sets of enzyme combinations for ligation between barcoded adapters and individual samples. The first set comprises of two restriction enzymes, *NsiI-HF* (New England Biolabs, Ipswich, MA, United States) and *MseI* (Enzynomics, Daejeon, Korea) and the second set consist of *XhoI* (Enzynomics, Daejeon, Korea) and *MseI*. After pooling samples from plate into a 15 ml falcon tube, it was cleaned by a PCI extraction method to be re-precipitated with 2-propanol. Then, it was size-selected with AMPure XP beads (Beckman Coulter, High Wycombe, UK) to exclude small fragments for amplified by polymerase chain reaction (PCR). The product from PCR went through size selection again to be the final product for further process.

To construct genetic maps, three raw FASTQ files generated from three NGS reactions with two different Illumina sequencing platforms were combined. Among three, two FASTQ files were obtained by the Illumina's HiSeq2500 and one was by Illumina's NextSeq500. Since reference genome was not available, a "Mock reference" was generated using GBS SNP-Calling Reference Optional Pipeline (GBS-SNP-CROP) (Melo *et al*., 2016). The GBS-SNP-CROP builds a population tailored "Mock reference" from the same GBS data using clustering strategy. Illumina adapters and low quality sequences were removed using Trimmomatic v.0.33 (Bolger *et al*., 2014). The Trimmed reads were aligned to "Mock-reference" using BWA-mem algorithm (Li and Durbin, 2009_ENREF_57). After alignment, read groups were added using Picard (version 2.10.10), AddOrReplaceReadGroups (http://broadinstitute.github.io/ picard/) and duplicates were removed using the MarkDuplicates embedded in the Picard. The Genome Analysis Toolkit, Haplotype Caller (GATK, version 3.8-0) was used for calling SNPs. To reduce the false discovery rate, SNPs with a fisher strand (FS) less than 30 and quality by depth (QD) less than 2 were excluded.

Genotyping and Analysis of Segregation

Genotyping of polymorphisms of markers were scored as following:

When *C. boreale* is homozygous and *C. indicum* is heterozygous, *C. boreale* is scored as "nn" and *C. indicum* is scored as "np". When *C. boreale* is heterozygous and *C. indicum* is homozygous, *C. boreale* is scored as "lm" and *C. indicum* is scored as "ll". When both of them are heterozygous, each of them is scored as "hk".

The testcross markers (segregation of markers heterozygous in one parent) were tested against a Mendelian segregation ratio of 1:1 using a chi-square test ($P < 0.05$), while those intercross markers (heterozygous in both parents) were tested against a 3:1 ratio ($P < 0.05$). Those markers which did not fit the ratio describe above were considered as distorted markers to be excluded from genetic mapping.

Genetic Map Construction

Two parental segregation patterns were formed based on markers. Each pattern contained the testcross markers segregating from the respective parent and the intercross markers presenting in both parents, *C. boreale* for female and *C. indicum* for male, according to the double pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994). Homologous linkage maps were generated using JoinMap version 4.10 with LOD threshold of ≥ 3.0 (Van Ooijen, 2006). The calculation of the linkage maps utilized all the pair-wise recombination estimation of < 0.30, a LOD score > 0.01 with Kosambi mapping function. The resulting linkage maps were visualized by using MapChart 2.1 software (Voorrips, 2002).

Karyotyping

C. boreale was obtained from the National Institute of Horticultural & Herbal Science, Rural Development Administration in South Korea. The young root tips were treated with 2 m*M* 8-hydroxyquinoline for 5 h in 20°C water bath. Aceto-ethanol (3:1) was used to fix the roots overnight, and 70% ethanol was used to preserve the roots at 4°C. The root tips were washed with distilled water to be digested in the condition of [0.3% of Cellulase R-10 (Sigma, USA), cytohelicase (Sigma, USA), and pectolyase γ-23 (Duchefa, Germany)] for 90 min in a 37°C chamber and squashed in 60% acetic acid.

PCR using the genomic DNA of *C. boreale* generated the 5S rDNA probe; the forward primer 5′-GATCCCATCAGAACTCC-3' and the reverse primer 5′-GGTGCTTTAGTGCTGGTAT-3′ (Koo *et al*., 2002). PCR was performed using the following reaction: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min, and final elongation at 72°C for 10 min. The 5S rDNA was labeled with biotin 16-dUTP.

The method used by Lim *et al*. (2001) was followed with some modification. The hybridization mixture contained formamide, 10% sodium dodecyl sulfate, 50% dextransulfate, 20X SSC, salmon sperm, and 50–100 ng of DNA probe. The mixture was placed in a water bath for DNA denaturation at 90°C for 10 min and then on ice for 5 min. Each slide received 40 μL of the mixture and hybridization at 37°C for 18 h.

The washing conditions w 2X SCC for 10 min at room temperature (RT), 0.1X SCC for 40 min at 42°C, and 2X SSC for 10 min at RT. This was followed by incubation in

an ethanol series. For the hapten-labeled probe, washing conditions were 2X SSC for 15 min at RT, 0.1X SSC for 40 min at 42°C, and 2X SSC for 15 min. The slides were quickly submerged in 1X detection buffer for 5 min at RT. Streptavidin CY3 (Roche, USA) was used to detect the labeled DNA. The slides were counterstained with DAPI/Vectashield (1:100).

The chromosomes were observed at ×1000 magnification with an Olympus BX53 fluorescence microscope (Olympus, Japan) with a charge coupled device camera (CoolSNAP™ cf, Photometrics, USA). Genus™ version 3.1 (Applied Imaging, USA) was used to analyze FISH images and Adobe Photoshop CC was did to do final image adjustments.

Results

Genotyping

A total of 2,224,623,618 high quality paired-end reads were generated from 81 samples including parental species. 1,481,179,182 reads were generated by Illumina HiSeq2500 and 743,444,436 reads were generated from Illumina's NextSeq500. Average percentage of paired reads which could be used was 87.3%. As a genotyping results, total 1,361,156 SNPs were retained with the average depth of 3.07. Only 11,306 SNPs genotyped more than 80% in all the samples were used for downstream analysis.

Genetic Linkage Map

Of the 11,306 SNPs detected, 1,521 SNPs were informative were in genotyping the F_1 population for mapping. Of the 1,521 SNPs, 682 SNPs (44.8%) were testcross markers and the rest 18 SNPs (1.2%) were intercross markers. Distorted segregation (*P* values at 0.05) was seen in 821 SNPs (54.0%) (Table 1). The linkage maps followed the double pseudo-testcross mapping strategy with the markers polymorphic in each of the parents to be developed separately and combined into integrated maps using JoinMap v4.1. A total of 700 SNPs (including testcross and inter-cross markers) were used for linkage analysis and 549 SNPs (78.4%) were grouped on the two parental maps with 151 (21.6%) markers unlinked. 48 linkage groups were formed with two major groups and 46 minor groups (Fig. 1 and Table 2). The average map distance between adjacent markers was 1.4 cM. The linkage group ranged in size from 0 cM to 59.9 cM (Fig. 1). Further, the total number of marker per linkage group ranged from 2 to 34.

Karyotyping

C. boreale was observed as a diphas nine pairs of chromosome (Fig. 2). Two loci of 5S rDNAs (green fluorescence) are positioned in the pericentromeric regions of chromosome 4 in *C. boreale* (there is no data for *C. indicum* due to no sample ready currently) (Fig. 3).

Table 1: Segregation analyses for the markers scored in the *Chrysanthemum* progeny using

Characteristics	Number of SNP markers
Informative SNP markers	11,306
Number of polymorphic SNP markers used for mapping	1,521
Number of testcross markers (1:1) present in both parents	682
Number of intercross markers (3:1)	18
Number of distorted markers	821

SNP analysis

Table 2: Analysis of linkage group of F_1 population between *Chrysamthemum boreale* and *C. indicum*

Characteristics	Counts
Linkage groups	48
Number of linkage groups having 2 markers or less	33
Unlinked markers	682
Total SNP markers in the linkage groups	700
Number of polymorphic SNP markers used for mapping	1,521

The homologous chromosomes that bear 5S rDNA loci are to some extent have differed in length, especially in chromosome 5 (Fig. 2). Further, the intensity of signal between the 5S rDNA-bearing chromosomes is also noticeable in which one homologue carries a more intense signal than the other one in chromosome 4 (Fig. 3).

Discussion

Clustered SNPs in Two Linkage Groups

The linkage map in the current study has three interesting features. One, makers are clustered in a few linkage groups. Two, the distance between markers in the clustered linkage groups are extremely close. Three, linkage groups with small numbers of markers are prevailing. There are three major possibilities to explain these three interesting features.

First, there are not enough SNP markers and genotyping error detected form GBS method to cover the whole genome. Based on simulation study on applying GBS, the highly qualified SNPs with large numbers are important (Beissinger *et al*., 2013). Although the not enough number of markers could generate the similar number of linkage groups with the targeted plant, it would be hard to achieve proper linkage groups without one-to-one correspondence because of missing information on a subset of the chromosome (Wang *et al*., 2004). Those segmented linkage groups could be linked one another with more markers added. Also, the low number of intercross markers linked to the testcross markers in *C. boreale* as well as the failure to find homologues between the two parents might be due to the limited intercross markers. The additional markers are should be added in the follow up study. In fact, although GBS, which can analyze genotype fast and accurately, can detect numerous SNPs,

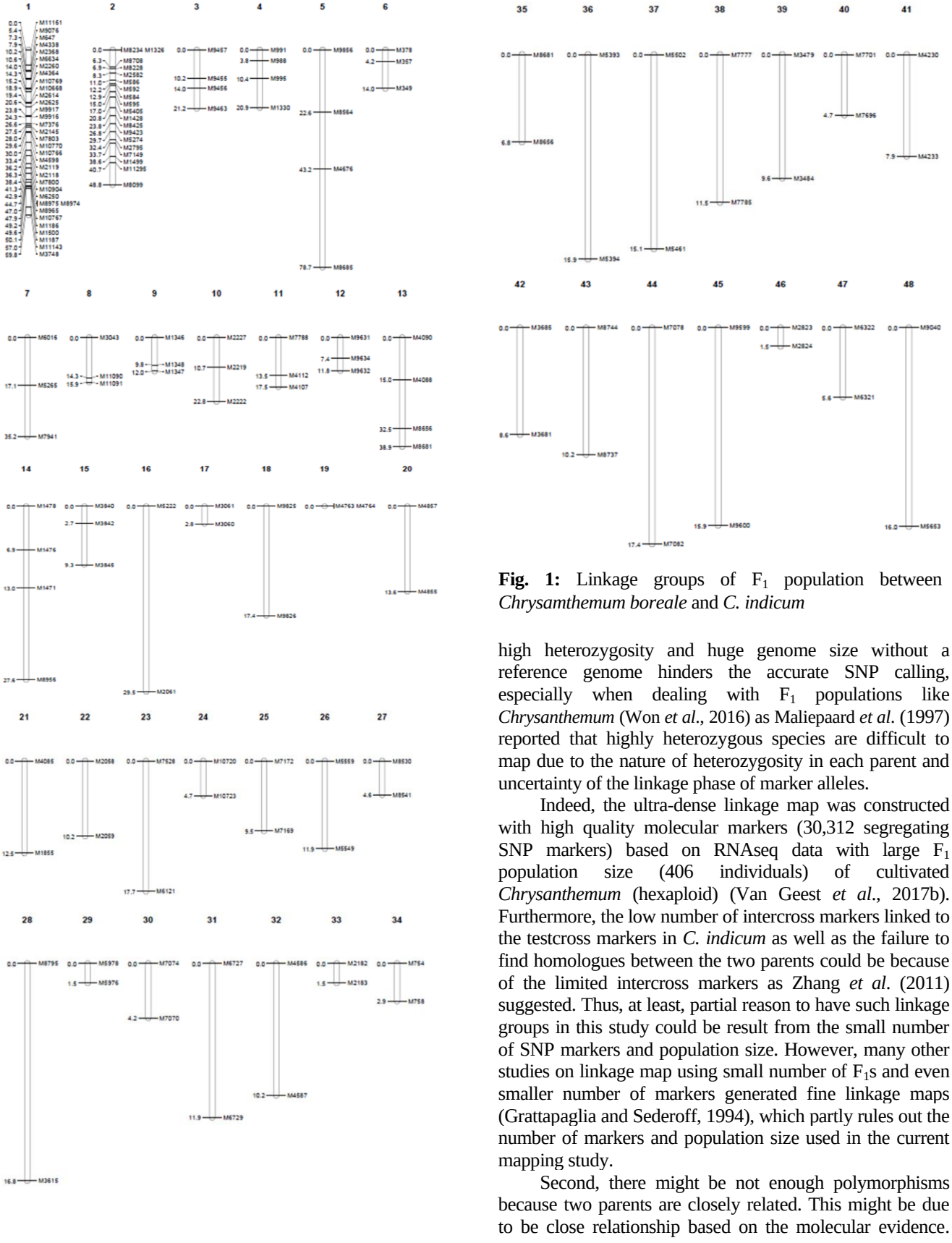

Fig. 1: Linkage groups of F_1 population between *Chrysamthemum boreale* and *C. indicum*

high heterozygosity and huge genome size without a reference genome hinders the accurate SNP calling, especially when dealing with F_1 populations like *Chrysanthemum* (Won *et al*., 2016) as Maliepaard *et al*. (1997) reported that highly heterozygous species are difficult to map due to the nature of heterozygosity in each parent and uncertainty of the linkage phase of marker alleles.

Indeed, the ultra-dense linkage map was constructed with high quality molecular markers (30,312 segregating SNP markers) based on RNAseq data with large F_1 population size (406 individuals) of cultivated *Chrysanthemum* (hexaploid) (Van Geest *et al*., 2017b). Furthermore, the low number of intercross markers linked to the testcross markers in *C. indicum* as well as the failure to find homologues between the two parents could be because of the limited intercross markers as Zhang *et al*. (2011) suggested. Thus, at least, partial reason to have such linkage groups in this study could be result from the small number of SNP markers and population size. However, many other studies on linkage map using small number of F_1s and even smaller number of markers generated fine linkage maps (Grattapaglia and Sederoff, 1994), which partly rules out the number of markers and population size used in the current mapping study.

Second, there might be not enough polymorphisms because two parents are closely related. This might be due to be close relationship based on the molecular evidence.

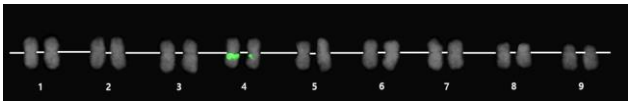

Fig. 2: Chromosome paring of *Chrysamthemum boreale*

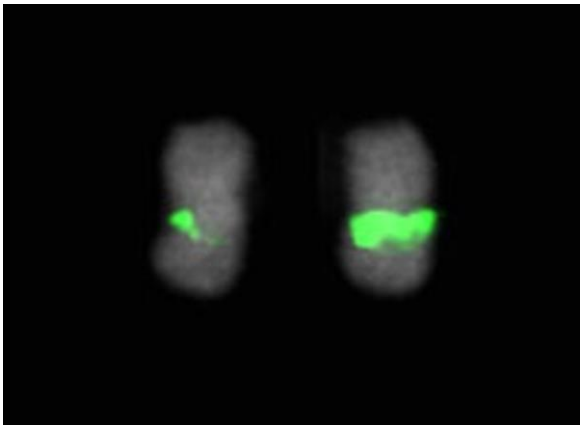

Fig. 3: The intensity of 5S rDNA signals differ in one pair of signals of *Chrysamthemum boreale*

However, this cannot explain the whole phenomenon to have such a clustered linkage map, because the karyotype of *C. boreale* and *C. indicum* are clearly different from each other (Kim *et al*., 2003) although different karyotyping does not necessarily mean the different genetic background. Two linkages maps using *Chrysanthemum* cultivars by Zhang *et al*. (2010, 2011) may provide a clue. They used the same F_1 population in both studies. The marker numbers are not significantly different, 557 and 869, respectively. However, the percent of the marker number following the expected segregating ratio of each map are 69.1% and 77.1%, and the unlinked marker ratio is 40% and 15–17%, respectively. The marker numbers to construct linkage map are higher in the second study. The distinctive difference between two linkage maps are the kind of molecular markers. The latter study switched the molecular makers from AFLP, ISSR, RAPD, and SRAP. Among those markers, SRAP is more efficient to discriminate genetic diversity among closely related cultivars compared with other molecular markers (Budak *et al*., 2004). Thus, one might conclude that the cross between closely related species resulted in less polymorphisms using SNP. Nonetheless, three points are not explained yet. One is the smaller genome coverage than expected (65–66%) in the linkage map with SRAP markers although it was improved from the previous map (51–55%). Second is the number of linkage groups increased with SRAP markers (55–57 linkage groups) compared to the previous linkage map (33-44 linkage groups). Just like variations in the loci in the map, a various number of recombination events in each parent could result in the different map length (Lerceteau-Köhler *et al*., 2003). The last one is the severely clustering markers into a few linkage groups that were not much improved in the linkage map with SRAP markers. Consequently, we may need to think that there should be additional issues involved in the linkage map of the current study.

The last feature is chromosomal rearrangements such as reciprocal translocation, which could have a huge impact on the linkage map (Liu *et al*., 2016). This could be the reason why recombination hot spots exist when the population size is too small (He *et al*., 2014), causing the clustering of molecular markers in some specific linkage groups. Zhang *et al*. (2010) also pointed this out in their study. A coefficient of coincidence greater than one, which leads the negative coefficient, occurs in a linkage experiment with the offspring by heterogeneous meiotic events, which could be caused by reciprocal translocation (Säll and Bengtsson, 1989). The negative interference causes high recombination frequency in the interstitial segment resulting in more double crossing-over events; consequently, those markers near each side of break-point do not segregate (Sybenga and Mastenbroek, 1980: Larsson, 1985; Tadmor *et al*., 1987). Thus, the tightly linked markers in the current study could be due to high negative interference, which are frequently observed in crosses involving tightly linked markers (Beck, 1980). Another problem with reciprocal translocations is a 'pseudo-linkage'. Reciprocal translocations involve the exchange of two terminal segments between two non-homologous chromosomes suppressing recombination between loci around the translocation breakpoints (Farré *et al*., 2011). Consequently, pseudo-linkage between markers, which are from different chromosomes, is created in those area, which could partially explain many fragmented linkage groups with a few markers in the current study. Last problem could be caused by reciprocal translocation resulting in the segregation distortion although there are many other mechanisms including self-incompatibility and inbreeding depression to induce distortion (Zhang *et al*., 2010). The severe deviation of distorted markers from the expected Mendelian ratios also support the high possibility of translocation because distortions are due to pairing abnormalities which lead to an abortion of heterozygous embryos (Quillet *et al*., 1995). Severe segregation distortion is problematic because it significantly affects the estimation of genetic distances (Hackett and Broadfoot, 2003_ENREF_80). This may explain there are many unlinked markers (21.6%) in the current study.

Unusual Chromosomal Structures of *Chrysanthemum* spp.

Klie *et al*. (2014) concluded that *Chrysanthemum* is a segmental allopolyploid based on the cytological studies and their analysis of molecular markers indicates a partial polysomic inheritance. However, Van Geest *et al*. (2017a) refuted that a predominance of bivalents does not necessarily mean the disomic inheritance. They

argued that bivalents forming should be due to the genetic control in *Chrysanthemum*. This suggests that cultivated *Chrysanthemum* should be classified as a hexaploid with polysomic inheritance, which enables progress in the development of genetic linkage mapping. Indeed, in the follow-up study successfully generated an ultra-dense linkage map for hexaploid *Chrysanthemum* cultivars (Van Geest *et al*., 2017b_ENREF_11). However, low rate of chiasma, uneven chromosome paring, reciprocal translocation found in wild *Chrysanthemum* diploids may need another strategy to construct a genetic linkage map owing to those reasons discussed above unlike cultivars although the addition of qualified markers could improve the map.

In fact, the evidence of reciprocal translocations was found in many *Chrysanthemum* species; in the bivalents, at least one or two distinct bivalents highly likely due to reciprocal translocations were observed, resulting in gametes with different chromosome complement (Gupta *et al*., 2013) including the same species used in the current study (Kim *et al*., 2003; Hwang *et al*., 2013). Indeed, chromosome length difference in chromosome 5 and signal intensity in chromosome 4 were found in *C. boreale* in the current study, which could be the evidence of chromosome abnormality, possibly due to reciprocal translocation although there may need further investigation.

The basal chromosome number of *Chrysanthemum* spp, is 9 and it has a variety of ploidy levels (2x–25x) having large-size chromosomes, the occurrence of reciprocal translocations, and localization of chiasmata in which they are formed with different frequencies in other chromosomal area (Gupta *et al*., 2013). This is consistent with the many other chromosome studies of *Chrysanthemum* showing that new forms originate as a result of the loss and gain of chromosomal materials during mitosis with various abnormalities given rise to cells where these has been changed in the chromosome number or to cells where chromosome breakage has resulted in the production of fragment chromosomes (Dowrick and El-Bayoumi, 1966). Likewise, *C. indicum* and *C. boreale* collected from wild in Korea appear to have prevalent chromosome rearrangements, such as reciprocal translocations and unequal crossing-over, resulting in gametes with different chromosome complements (Rieseberg, 2001; Kim *et al*., 2008). Gametes of fertile pollen, resulting from such chromosomal rearrangements with high genetic diversity in wild *Chrysanthemum* populations, would result in diverse somatic karyotypes, as found in our previous study (Kim *et al*., 2003). As a result, the seed setting rates of F_1 hybrids between tetraploid *C. boreale* (2n=4x=36) and *C. japonense*, which are collected from wild in Japan, were very low ranging from 0 to 34.9% (Watanabe, 1981). They found many trivalent in pollen mother cell and even reported that one of the small bivalents which paired loosely looked to have only matrix connection and divided precociously at the first meiotic anaphase, indicating that there may be reciprocal translocations in each parent deduced by the fact that the measurements of each somatic chromosome of *C. japonense* in their study showed large variations.

Rana (1965) reported that the segments exchanged during chromosomal interchanges caused by reciprocal translocation are either almost same length or very different in sizes. The original symmetry of the karyotype will not be disturbed by the former kind of interchange. However, the latter should result in huge changes in the original chromosomal patterns. According to the author, the interchanged chromosome segments in *Chrysanthemum* are to be equal size in general, which is symmetric, which is very similar with the high degree of multiple formation in the interchange heterozygotes. More importantly, the equal length of interchanged segments and the fact that there was no formation of chiasma in the interstitial segments are the factors of determining co-orientation favor a balanced segregation of the chromosomes (Rana, 1965). However, Hwang *et al*. (2013) reported that *C. boreale* collected in Korea has asymmetric chromosomes, which leads to the alteration chromosomal patterns. Further, clear difference exists in karyotype between *C. boreale* and *C. indicum* as stated above (Kim *et al*., 2003). These implies that there are variations in chromosomal patterns depending on cultivars and wild *Chrysanthemum* species. Depending on the species of *Chrysanthemum* to be crossed, the fertility varies due to embryo abortion caused by many functional genes and proteins, especially those associated with cell senescence and death as well as self-incompatibility and inbreeding depression (Schubert and Lysak, 2011). Thus, it may need careful caution to choose parents for crossing. In other words, morphology of chromosomes of each parent also needs to be considered in addition to karyotyping for counting chromosome numbers.

In this sense, non-homologous chromosomes bearing species like *C. boreale* and *C. indicum* would generate very low chiasma as the low chiasmata rates per bivalent of *C. indicum* was observed (Gupta *et al*., 2013); consequently, many missing segments would be induced in the linkage map of the following generation as observed in the current study. Further, the non-homologues pairing of chromosomes from each parent in F_1 would hinder to find informative polymorphisms in unpaired regions of chromosomes.

Conclusion

The results in the current study show that it would be very difficult to construct a linkage map in wild *Chrysanthemum* species indigenous to Korea. The linkage groups presented in this study could be improved with more qualified makers. However, we highly suspect reciprocal translocation involved in both parents so that there would be a limit for the improvement based on the karyotyping results. Under this assumption, we emphasize that the chromosomal

morphologies of each parent should be carefully investigated before crossing the wild *Chrysanthemum* species, which would be as important as high quality molecular markers with large numbers. Further, with karyotyping alone, it is difficult to know whether it is from single event or secondary chromosomal rearrangements including inversion and deletion (Schubert and Lysak, 2011). Hence, it would be worth using comparative genomics approaches by investigating the chromosomal rearrangement in *C. boreale* and *C. indicum* for better understanding the evolutionary history of these genomes not only for biology itself but also for planning future breeding strategy in *Chrysanthemum* indigenous to Korea.

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