Natural interploidy hybridization among the key taxa involved in the origin of horticultural chrysanthemums

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Received 26 May 2021; Accepted 30 July 2021; Article first published online 18 August 2021

1 Introduction

Hybridization has played an important role in plant domestication and diversification through human history (Heslop-Harrison & Schwarzacher, 2007; Arnold, 2014; Cornille et al., 2014). Multiple important crops have been generated through hybridization either between wild species or through introgression from crop wild relatives into cultivated lineages. Major examples include modern strawberries (Fragaria ananassa Duch.) (Brinthurst & Voth, 1984) and triploid bananas (Musa ssp. L.) (Simmonds & Shepherd, 1955; Heslop-Harrison & Schwarzacher, 2007), but also ornamental species such as tree peonies (Paeonia suffruticosa Andrews) (Zhou et al., 2014), cherry (Prunus yedoensis Matsumura) (Baek et al., 2018), and dahlia (Dahlia variabilis L.) (Saar et al., 2003). Understanding the frequency, phylogenetic distribution, and propensity for hybridization in wild populations could not only inform breeders as to the possible range of interspecific hybrids, but could also reduce the laborious, time-consuming, and frequently unsuccessful process of artificial crossing and de novo hybrid generation (Lim et al., 2008; Kuligowska et al., 2016).

Hybridization occurs more easily between species of the same ploidy level than differing ploidy levels. For example, hybridization between diploid and tetraploid...
species is often limited as triploid hybrids are usually inviable and less fit, preventing backcross formation (Husband & Sabara, 2003; Wang et al., 2014; Zohren et al., 2016). However, species of contrasting higher ploidy levels appear to have weaker reproductive barriers and hybridize more easily than diploids and tetraploids (Sonnleitner et al., 2013; Sutherland & Galloway, 2017). For example, within the Campanula rotundifolia L. polyploid complex, postzygotic isolation was lower in tetraploid–hexaploid species than in diploid–tetraploid crosses (Sutherland & Galloway, 2017). To date, however, only a small number of studies have investigated hybridization between higher ploidy levels, and the prevalence of higher level cross-ploidy hybridization across plant families remains unclear.

Chrysanthemum L. (Asteraceae) provides an excellent model for studying hybridization between high ploidy levels, with ploidy ranging from diploid \((2n = 2x = 18)\) to decaploid \((2n = 10x = 90)\) (Tahara, 1915; Zhou & Wang, 1997; Li et al., 2015; Ma et al., 2015; Luo et al., 2017) and with different cytotypes within species (Dowrick, 1952, 1953; Chen, 2012; Liu et al., 2012; Yan et al., 2019). Chrysanthemum includes approximately 37 wild species, of which 17 occur in China (Shih & Fu, 1983) where they have captured great public interest. Multiple wild species have been crossed by humans to generate numerous cultivars, and they are among the most famous Chinese flowers, with significant commercial and medicinal value (Kim & Lee, 2005; Shahrajabian et al., 2019). The evolutionary history of polyploidy within Chrysanthemum is currently unknown, although some polyploids are thought to be allopolyploid in origin (Chen et al., 1996; Liu et al., 2012) and subject to multiple historical polyploidization events (Yang et al., 2006).

Chrysanthemums were first cultivated in China approxi-mately 1600 years ago, and were later introduced to Japan and Europe (Chen, 1985, 2012; Shih et al., 2011). Modern cultivated chrysanthemums are mainly hexaploids and hybridization and subsequent artificial selection are thought to give rise to numerous cultivars (Chen, 1985; Dai et al., 2002). The ancestry of modern chrysanthemums remains elusive, but several wild species are thought to be involved, including Chrysanthemum indicum \((4x)\), Chrysanthemum vestitum \((6x)\), Chrysanthemum lavandulifolium (Fischer ex Trautvetter) Kitamura \((2x)\), Chrysanthemum nankingense (Handel-Mazzetti) Cui \((2x)\), and Chrysanthemum zwedskii (Herbert) Tzvelev \((2x)\) (Chen, 1985; Dai & Chen, 1997; Fukai, 2003; Ma et al., 2016, 2020). Chrysanthemum indicum and C. vestitum are key species in the origin and evolution of cultivated chrysanthemums (Dai et al., 1998, 2002) based on two lines of evidence. First, ancient literature documents multiple uses for C. indicum in central China, which is consistent with the geographic origin of modern cultivars (see Chen, 2012). Second, artificial hybridization between C. indicum and C. vestitum can generate hybrids resembling the prototype of modern chrysanthemums (Chen, 2012).

In this study, we investigate whether natural hybrid-ization occurs between Chrysanthemum species with different ploidy levels, focusing on C. indicum and C. vestitum as well as varieties of those species. We ask: (i) Does interploidy hybridization naturally occur between tetraploid and hexaploid Chrysanthemum species, which are likely involved in origin of modern cultivated chrysanthemums? (ii) If hybridization occurs, is there evidence for a greater propensity at high ploidy levels? (iii) Do some modern chrysanthemum cultivars share chloroplast haplotypes with the three wild Chrysanthemum taxa, consistent with their involvement in their origins? To this end, we genotyped 317 samples at 13 microsatellite markers and sequenced chloroplast trnL-trnF for a subset of 103 samples. In addition, we extracted trnL-trnF from the chloroplast genomes of 28 taxa, representing 15 wild species of Chrysanthemum, 12 cultivars, and one sample of Ajania varifolia (Chang) Tzvelev. We analyze these data in a population genetic and phylogeography context, and use them to investigate a case where, traditionally, reproductive isolation caused by differences in ploidy levels would be expected to be strong.

2 Material and Methods

2.1 Study species

Chrysanthemum indicum is tetraploid with a wide distribution across China, although narrowly distributed diploid and hexaploid cytotypes have also been reported (Liu et al., 2012). Chrysanthemum vestitum is a hexaploid distributed across Hubei and Henan provinces in central China (Zhao & Chen, 1999); the variety C. vestitum var. latifolium is hexaploid with a restricted distribution in the Dabie Mountains in Anhui province.

The three taxa are outcrossing perennials (Chen, 2012) and are likely to be pollinated by bees (Wang N, pers. obs., 2020). Morphologically, C. indicum has yellow florets and smooth leaves with deep serrations. Both C. vestitum and C. vestitum var. latifolium have white florets and pubescent leaves and stems (Fig. 1). Putative hybrids either with an intermediate morphology or ploidy level between C. indicum and C. vestitum have been found in localities where the species co-occur (Nakata et al., 1992; Zhao & Chen, 1999). These hybrids exhibit continuous phenotypes between C. indicum and C. vestitum (Zhao & Chen, 1999), indicating the existence of putative hybrid swarms.

2.2 Sampling across hybridizing populations

To understand the extent and distribution of hybridization, we surveyed sympatric populations of the three focal taxa: C. indicum, C. vestitum, and C. vestitum var. latifolium. We identified and sampled five populations where C. indicum and C. vestitum co-occur and two where C. indicum and C. vestitum var. latifolium co-occur (Table S1). In addition, we collected C. indicum from two allopatric populations, TA and ZP (Table S1). Samples were collected at random within populations, ensuring spacing of at least 10 m between individuals. Healthy and pest-free leaf tissue was collected and stored in silica gel. A total of 317 samples were collected including between 14 and 74 from each of the seven hybridizing populations, five from TA, and three from ZP (Table S1). A global position system (UniStrong) was used to record the coordinates of each population. Sampling locations are illustrated in Fig. 2.
assessed on 1.0% agarose gels, and then diluted to a concentration of 10–20 ng/μl for genotyping and sequencing. Thirteen microsatellite loci were used for genotyping (Zhang et al., 2014; Jo et al., 2015). The 5′ terminus of the forward primer was labeled with FAM, HEX, or TAM fluorescent probes. Each microsatellite locus was amplified individually prior to being combined into four multiplexes (Table S2). The polymerase chain reaction (PCR) protocol follows Hu et al. (2019).

2.4 Population genetic analysis
It is often difficult to assign microsatellite genotypes for mixed ploidy species, as the frequency of different alleles can be difficult to quantify. In hexaploids, each microsatellite locus would be expected to have up to six alleles per individual. We chose to score each allele separately using the software GeneMarker 2.4.0 (SoftGenetics), and checked each genotype manually. We then calculated allele richness for each population using FSTAT 2.9.4 (Goudet, 1995) and undertook principal coordinate (PCO) analysis in POLYSAT 1.7.4 (Clark & Jasieniuk, 2011), based on Bruvo’s pairwise genetic distances (Bruvo et al., 2004).

We undertook STRUCTURE analysis for each hybridizing population separately using STRUCTURE 2.3.4 (Pritchard et al., 2000) with ploidy specified as 6n. We combined XG, NX, and PH into one hybridizing population as the three localities are separated by only a few kilometers. We set the number of genetic clusters (K) to 2 when analyzing the genetic structure of each hybridizing population as only two parental species are present. The allopatric C. indicum populations (TA and ZP) were used as a reference population.

In addition, to identify the most likely K value across populations we included all populations in a combined STRUCTURE analysis, testing K values from 1 to 10. The number of genetic clusters was estimated using the Evanno test (Evanno et al., 2005) in the program STRUCTURE HARVESTER 0.6.94 (Earl & vonHoldt, 2012). Ten replicates of the STRUCTURE analysis were carried out with 100,000 iterations and a burn-in of 100,000 for each run. The admixture model, with an assumption of correlated allele frequencies, was used. Individuals were assigned to clusters based on the highest membership coefficient averaged over the 10 independent runs. Replicate runs were grouped based on a symmetrical similarity coefficient of >0.9 using the Greedy algorithm in CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) and visualized in DISTRUCT 1.1 (Rosenberg, 2004). In populations where C. indicum and C. vestitum or C. indicum and C. vestitum var. latifolium co-occur, we estimated Q scores in STRUCTURE with 95% confidence intervals to define pure C. indicum, pure C. vestitum, pure C. vestitum var. latifolium, or putative hybrids. We distinguish individuals with confidence intervals overlapping 1 as pure C. indicum, with 0 as pure C. vestitum, and those remaining as putative hybrids, in populations where the two species co-occur. Similarly, in populations where C. indicum and C. vestitum var. latifolium co-occur, we distinguish individuals with confidence intervals overlapping with 1 as pure C. indicum, with 0 as pure C. vestitum var. latifolium, and those remaining as hybrids.

We compared the average allele number per individual between C. indicum, C. vestitum, C. vestitum var. latifolium,
and their hybrids at each microsatellite locus, using the Kruskal-Wallis test in the R package agricolae version 1.3-3 (de Mendiburu, 2020). We would expect that the average allele number is higher for *C. vestitum* and *C. vestitum* var. *latifolium* than for *C. indicum*. In addition, we tested whether introgression was symmetric between *C. indicum* and *C. vestitum* or between *C. indicum* and *C. vestitum* var. *latifolium*. Barplots on the left or the right of the STRUCTURE plot represent the number of individuals having different levels of genetic admixture. Populations XG, NX, and PH were analyzed together due to their close proximity. Allopatric populations TA and ZP served as controls. Blue, green, and orange represent *C. indicum*, *C. vestitum*, and *C. vestitum* var. *latifolium*, respectively. *C*. Admixture value for each population among *C. indicum*, *C. vestitum*, and *C. vestitum* var. *latifolium*. Hybrids were excluded for such comparisons. The map in the top left corner of (B) was supplied from the State Bureau of Surveying and Mapping (http://bzdt.ch.mnr.gov.cn/).

### 2.5 trnL-trnF sequencing and analysis

In order to detect the potential maternal parents of the hybrids, and to estimate the haplotype diversity of these taxa, we amplified trnL-trnF for a subset of 103 samples, including between five and 34 individuals from each population. Reactions were carried out in 20 μl volumes containing 13 μl ddH₂O, 5 μl 2× Taq PCR mix (Tiangen), 0.5 μl each primer (trnL and trnF; Taberlet et al., 1991), and 1 μl DNA template. The PCR products were outsourced for purification and sequencing to Qingdao. Sequences were manually edited and aligned using BioEdit version 7.2.5 (Hall, 1999). The R package pegas version 0.14 (Paradis, 2010) was used to construct haplotype networks, using default settings, with gaps treated as a fifth state. The total number of sites, polymorphic sites, parsimony informative sites, and nucleotide diversity were computed using DnaSP version 6.12.03 (Rozas et al., 2017). All sequences obtained in this study were submitted to GenBank with accession numbers MZ032043–MZ032145.

To aid in a broader phylogenetic analysis, we extracted the trnL-trnF region from the available whole chloroplast genomes for 15 wild taxa of *Chrysanthemum* occurring in China, 12 cultivars, and *Ajania variifolia*. A phylogenetic tree was estimated using the maximum likelihood method in
3 Results

3.1 Hybridization across ploidy levels inferred from microsatellites

Genetic diversity estimates were similar among the three taxa and hybrids, with allelic richness ranging from 3.74 to 4.26 and gene diversity from 0.75 to 0.84 (Table S3). On average, the number of alleles scored in Chrysanthemum vestitum and C. vestitum var. latifolium was significantly higher than Chrysanthemum indicum at eight and seven loci, respectively (P < 0.05); this was expected for a hexaploid possessing more chromosome copies than a tetraploid species (Fig. S1).

The PCO analysis based on Bruvo’s genetic distances among all samples revealed three clusters, with coordinates 1 and 2 explaining 13.8% and 7.8% of the total variation, respectively (Fig. 2). Coordinate 1 separated C. indicum from C. vestitum and C. vestitum var. latifolium and coordinate 2 separated C. vestitum from C. vestitum var. latifolium (Fig. 2). Most hybrids identified by the STRUCTURE analysis fell between C. indicum and C. vestitum in the PCO plot (Fig. 2).

The combined STRUCTURE analysis across all samples identified K = 3 as the optimal K value (Fig. S2A), with the three clusters corresponding to C. indicum, C. vestitum, and C. vestitum var. latifolium (Fig. 3A). At K = 2, C. indicum formed one cluster and C. vestitum and C. vestitum var. latifolium formed another cluster (Fig. S2B), supporting the close relationship between these two taxa and the inference that C. vestitum var. latifolium is a subspecies of C. vestitum. Interestingly, there are three C. vestitum individuals possessing a considerable level of introgression from C. vestitum var. latifolium and one C. indicum individual in population TZ with substantial introgression from C. vestitum (Fig. 3A).

A total of 20 hybrids were detected when STRUCTURE was used for each hybridizing population separately, of which 19 were hybrids between C. indicum and C. vestitum and one was a hybrid between C. indicum and C. vestitum var. latifolium. Fourteen hybrids between C. indicum and C. vestitum were from population XG, two from each of NX and PH and one from WFS. The only hybrid between C. indicum and C. vestitum var. latifolium was from population TZ. No hybrids were detected from population LJS where C. indicum and C. vestitum co-occur and from BMJ where C. indicum and C. vestitum var. latifolium co-occur (Fig. 3B).

Introgression occurred symmetrically between C. indicum and C. vestitum and between C. indicum and C. vestitum var. latifolium in all hybridizing populations except WFS, where only three C. indicum were collected (Fig. 3C). Introgression was limited in populations TZ, BMJ, and LJS but was extensive in population XG (Fig. 3C).

3.2 Plastid diversity and directionality of hybrid formation

A total of 17 chloroplast haplotypes were detected across samples, with 12, nine, and four in C. indicum, C. vestitum, and C. vestitum var. latifolium, respectively (Fig. 4; Table S4). Both C. indicum and C. vestitum harbored three private haplotypes and C. vestitum var. latifolium harbored one (haplotype H3). Chrysanthemum indicum shared six haplotypes with C. vestitum and three haplotypes with C. vestitum var. latifolium. By contrast, C. vestitum only shared the most common haplotype, H6, with C. vestitum var. latifolium. Frequencies of particular haplotypes varied considerably between species as some haplotypes found in C. indicum or C. vestitum were absent in C. vestitum var. latifolium (Fig. 4). Three haplotypes (H6, H6, and H17) were found in hybrids, with haplotype H6 shared among the three taxa, and haplotype H16 found to be private to the hybrid (Fig. 4). One hybrid shared haplotype H17 with C. indicum, whereas this haplotype was absent from all C. vestitum and C. vestitum var. latifolium samples (Fig. 4).

All modern Chrysanthemum morifolium Ramat. cultivars formed a clade with full support, and this clade was nested in a large monophyletic clade including Chrysanthemum dichrum Shih, Chrysanthemum zawadskii, and Chrysanthemum chanetii (Léveillé) Shih (Fig. 5). Five haplotypes (1, 3, 6, 16, and 17) from the three taxa and hybrid were also nested in this monophyletic clade (Fig. 5).

4 Discussion

In this study, we provide genetic evidence of natural interploidy hybridization between two pairs of taxa involved in the formation of modern Chrysanthemum horticultural hybrids. We detected more hybrids between Chrysanthemum indicum and Chrysanthemum vestitum than between C. indicum and C. vestitum var. latifolium, possibly due to different levels of reproductive isolation. In addition, we show that C. vestitum var. latifolium formed a genetic cluster distinct from C. vestitum, and as such deserves its varietal status. Here, we discuss the importance of hybridization between different ploidy levels in natural Chrysanthemum populations, before considering the dynamics of different hybrid swarms. We finish with the wider implications of our findings for understanding the origin of horticultural chrysanthemum hybrids.

4.1 Tetraploid–hexaploid hybridization and symmetrical introgression

Interploidy hybridization is more common in genera containing many polyploids, and where species readily co-occur and hybridize, such as Betula (Zohren et al., 2016; Hu et al., 2019) and Spartina (Ainouche et al., 2003). Diploid–tetraploid hybridization produces mostly sterile triploids; however, where hybrids are fertile, introgression usually occurs preferentially from diploids to tetraploids (Pinheiro et al., 2010; Moraes et al., 2013). In contrast, hybridization between tetraploids and hexaploids has been proposed to be easier, as pentaploid hybrids are formed frequently (Hülber et al., 2015) and are more fertile than triploids (Sutherland & Galloway, 2017). Consistent with this, we detected 20 hybrids among C. indicum, C. vestitum, and...
C. vestitum var. latifolium, indicating incomplete reproductive isolation. The number of hybrids is likely to be underestimated in our study, because of the stringent confidence threshold applied in the STRUCTURE analysis. Three individuals with admixture between 26.2% and 31.4% from population XG and two individuals with admixture between 34.5% and 48.7% were not supported by Q scores with 95% confidence intervals, but could prove to be hybrids such as later generation backcrosses.

Nineteen of the 20 hybrids were between C. indicum and C. vestitum and one was between C. indicum and C. vestitum var. latifolium. This difference might reflect different levels of reproductive isolation. Higher fruit set in artificial C. indicum–C. vestitum crosses than in artificial C. indicum–C. vestitum var. latifolium crosses partially supports this hypothesis (Zhou, 2009). However, environmental selection against hybrids between C. indicum and C. vestitum var. latifolium could also account for its rarity. Chrysanthemum indicum and C. vestitum occupy similar habitats and are usually intermixed in sympatric populations (Qi S, pers. obs., 2019). This could enhance their opportunity for hybridization, while their similar habitat preferences might reduce the chance of ecological selection on the hybrids. In contrast, C. indicum and C. vestitum var. latifolium are adapted to different conditions, and hybrids might fail to survive due to the breakdown of suites of coadapted genes.

Within some diploid–tetraploid systems, introgression is more common from diploids to tetraploids (Zohren et al., 2016; Wang et al., 2020). However, we observe symmetrical introgression between C. indicum and C. vestitum and between C. indicum and C. vestitum var. latifolium, indicating that hybrids can backcross with both parents. This is in line with recent studies showing that pentaploids can mediate gene flow between species with different ploidy levels (Peskoller et al., 2021). We could not distinguish clearly between male and female parental taxa because C. indicum and C. vestitum share some plastid haplotypes. However, C. indicum can serve as maternal parent so hybrid shares a haplotype with C. indicum, whereas this haplotype is absent from all C. vestitum samples. In addition, one hybrid had a unique haplotype (H16), which is possibly from unsampled C. indicum and C. vestitum or introgressed from other Chrysanthemum species.

### 4.2 Variable hybridizing among populations

The number of hybrids between C. indicum and C. vestitum varied substantially among the hybridizing populations (Fig. 3B). Fourteen of 19 hybrids were in population XG,
and no hybrids were found in population LJS. These populations are approximately 50 km apart, and differential reproductive isolation seems unlikely to account for such differences. However, we note that population LJS is closer to human habitation and human activities could have an impact on the persistence of hybrids.

Unexpectedly, one hybrid between *C. indicum* and *C. vestitum* var. *latifolium* is from population TZ and none are from BMJ. Moreover, the extent of genetic admixture in TZ seems to be higher than in BMJ (Fig. S1). In TZ, *C. indicum* and *C. vestitum* var. *latifolium* grow closely together, meaning there are enhanced opportunities for hybridization, and likely relaxed selection against hybrids. However, in BMJ, *C. indicum* and *C. vestitum* var. *latifolium* are segregated by altitude; this could limit the survival of hybrids due to environmental selection.

However, under future climate change, *C. indicum* might move to higher altitudes and come into closer contact with *C. vestitum* var. *latifolium*, producing more hybrids, as seen in population TZ. This has implications for conserving *C. vestitum* var. *latifolium*. *Chrysanthemum indicum* is widespread and abundant, whereas *C. vestitum* var. *latifolium* is restricted to the Dabie Mountains. Hybridization between abundant *C. indicum* and rare *C. vestitum* var. *latifolium* could be predicted to drive the rare species to extinction through genetic or demographic swamping (Todesco et al., 2016).

### 4.3 Presence of orphan hybrids

Hybrids usually occur in sympatry with their parental species, but sometimes they occur separately, due to (often human-mediated) long-distance dispersal of hybrids, or natural colonization of sterile hybrid taxa (James & Abbott, 2005). Alternatively, parental species might die out due to genetic swamping or competitive exclusion (Levin et al., 1996; Huxel, 1999). Regardless of the mechanism, these result in orphan hybrids (Marques et al., 2010; Groh et al., 2019).

We detected a few individuals showing considerable admixture between *C. vestitum* var. *latifolium* and *C. vestitum* in populations WFS and XG, where *C. vestitum* var. *latifolium* is not known to occur. We also detected one *C. indicum* individual showing considerable admixture from *C. vestitum* in population TA in Shandong province.
This indicates the presence of hybrids in the absence of one or both parental species, which has been reported in some plant species, such as oaks (Dodd & Afzal-Rafii, 2004) and pines (Lanner & Phillips, 1992). A plausible explanation is the existence of undetected C. vestitum var. latifolium near populations WFS and XG, or within travelling distance of pollinators, or seed dispersal.

4.4 Implications for the origins of cultivated chrysanthemums

Our results based on an analysis of plastid trnL-trnF showed a monophyletic clade composed of C. lavandulifolium, C. cheneti, C. zawadskii, and five haplotypes of C. indicum, C. vestitum, C. vestitum var. latifolium, and the hybrid between C. indicum and C. vestitum (Fig. 5). This indicates that either of these species potentially acted as the maternal parent of cultivated chrysanthemums. This is consistent with previous research implicating their involvement (Dai et al., 1998, 2005; Fukai, 2003). However, the specific maternal parental progenitor of modern cultivated chrysanthemums remains elusive. The modern cultivated chrysanthemums were placed in a monophyletic clade that was sister to C. lavandulifolium. However, the chloroplast genome of C. lavandulifolium has some unique mutations compared with cultivated chrysanthemums. This leads to the hypothesis that the maternal progenitor of modern cultivated chrysanthemums has gone extinct (Ma et al., 2020). However, this requires further evaluation as only one or two whole chloroplast genomes were included for each wild Chrysanthemum species and cultivar. This means the diversity of chloroplast genomes was not sufficiently represented. Given the high haplotype diversity of the three taxa and the fact that these haplotypes did not form a monophyletic clade (Fig. 5), we predict that the ultimate maternal progenitor of modern cultivated chrysanthemums could potentially be any of the wild Chrysanthemum species, and there could be multiple maternal progenitors for chrysanthemum cultivars.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (31770230, 31600295, and 31872710), Natural Science Foundation of Shandong Province, China (ZR2018PC022), and Funds of Shandong “Double Tops” Program (SYL2017XTTD13).

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