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## Natural hybridization between *Iris minutoaurea* Makino and *Iris odaesanensis* Y. N. Lee in Korea: evidence from cytological traits

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**Abstract.** Interspecific hybridization resulting in diploid-homoploid hybrids is relatively rare in natural populations. The *Iris* genus, which comprises numerous economically important species, is a taxonomically complex group in which polyploidization and hybridization frequently contribute to taxon diversification and speciation in natural populations. In Korea, populations of the diploid *Iris odaesanensis* ( $2n = 28$ ) and *Iris minutoaurea* ( $2n = 22$ ) come into contact with each other, leading to homoploid hybrids, as recently demonstrated by molecular phylogenetic and morphological surveys. In this study, we aimed to confirm whether the chromosome number, genome size, and pollen viability of the putative hybrids corresponded to the hybrid nature of their diploid parental species. As previously demonstrated, the hybrids exhibited intermediate tepal color traits between the parental taxa. *Iris minutoaurea*, *I. odaesanensis*, and the putative hybrids were consistently diploid ( $2n = 22, 28,$  and  $25,$  respectively), confirming that homoploid hybridization mainly occurred in natural populations. The genome size of the putative hybrids (mean: 3.84 pg, range: 3.80–3.86 pg) was additive when compared with those of the parental diploid species (i.e., 3.72 pg in *I. odaesanensis* and 3.95 pg in *I. minutoaurea*). No fertile pollen grains were found in the putative hybrids, which may have prevented the establishment of hybrid lineages and backcrosses with the parental species *I. odaesanensis* or *I. minutoaurea*. Together, these data confirm the existence of natural homoploid *Iris* hybrid populations in Korea and shed light on the dynamics of interspecific hybridization in the *Iris* genus.

**Keywords:** chromosome number, genome size, homoploid hybrid, *Iris*, pollen abortion.

### INTRODUCTION

Hybridization between closely related angiosperm taxa contributes to the diversification and speciation of natural populations (Soltis and Soltis 2009; Whitney et al. 2010; Yakimowski and Rieseberg 2014; Goulet et al. 2017; Kim et al. 2023). Natural hybridizations are collectively regarded as a crucial evolutionary mechanism, resulting in the most complex diversification of the Iridaceae family (Arnold et al. 1990; Young 1996; Makarevitch et al. 2003; Son et al. 2015; Samad et al. 2016; Niketić et al. 2018). A new hybrid lineage may form through allopolyploidy accompanied by the duplication

of chromosome sets or through homoploid hybrid speciation between species of the same ploidy level (Soltis and Soltis 2009; Kadereit 2015; Schumer et al. 2014). To date, numerous homoploid hybrid species from natural populations have been recognized in many economically important plant families (Asteraceae–Lipman et al. 2013; Poaceae–Jiang et al. 2013; Amaryllidaceae–Smirnov et al. 2017; Lamiaceae–Arabaci et al. 2021; Iridaceae–Xiao et al. 2021; Isoëtaceae–Suissa et al. 2022). An increasing number of interspecific hybrids of the genus *Iris* have occasionally been reported, e.g., *I. nelsonii*, a natural hybrid between *I. hexagona* and *I. fulva* (Arnold 1993), *I. verticolor*, a hybrid between *I. virginica*, and *I. setosa* (Lim et al. 2007), and *I. ×ampliflora*, a hybrid between *I. japonica* and *I. wattii* (Xiao et al. 2021). However, little is known regarding the morphological, ecological, genetic, and cytological context between natural homoploid hybrid species (Yakimowski and Rieseberg 2014; Feliner et al. 2017).

*Iris* L. ser. *Chinense* (Diels) Lawrence includes eight species that are mainly distributed in Korea, China, and Japan according to a recent taxonomic study (Wilson 2020). Four *Iris* species (*I. odaesanensis* Y. N. Lee, *I. minutoaurea* Makino, *I. koreana* Nakai, and *I. rossii* Baker) are recognized in Korea (Lee 2003; Sim 2007; Choi et al. 2020a, 2022a, b, c). *Iris odaesanensis* is known to be an endemic species in Korea (Sim 2007). However, some researchers have considered that the species are sub-endemic in Korea, with populations also being found in Jilin, China (Zhao et al. 2000; Son et al. 2015; Choi et al. 2020; Wilson 2020). However, whether the *Iris* population in China represents *I. odaesanensis* requires further investigation. Among the Korean *Iris* species within the *Chinenses* series, *I. koreana* was hypothesized to be of hybrid origin based on cytological data (i.e., chromosome number and genome size variation; Choi et al. 2020a). Subsequent molecular cytogenetic data substantiated *I. koreana* as a putative allopolyploid species, with genomic contributions from two closely related species, *I. minutoaurea* and *I. odaesanensis* (Park et al. 2022). Interestingly, putative hybrid plants between *I. minutoaurea* and *I. odaesanensis* have been documented in Gasan, Korea, based on both molecular analysis and morphological data, revealing intermediate characteristics in tepal color and position of the ovary when compared with the parental species (Son et al. 2015; Yang et al. 2020). Despite increasing interest in these putative hybrid plants from natural populations, debate continues regarding whether these plants show conserved chromosome numbers, genome sizes, and karyotype features compared with their diploid parents, *I. odaesanensis* and *I. minutoaurea*.

We recently investigated sympatric populations of *I. odaesanensis* ( $2n = 28$ ) and *I. minutoaurea* ( $2n = 22$ ) using molecular cytogenetic and complete plastome data (Choi et al. 2020a; Park et al. 2022). In those investigations, we identified sympatric populations of putative hybrid individuals that appeared to be derived from two distinct genetic clusters corresponding to diploid *I. odaesanensis* and *I. minutoaurea*. To verify the plausible hybrid origin and relationship with *I. odaesanensis* and *I. minutoaurea*, we compared the chromosome numbers, genome size values, and pollen characters with those of the putative parental species using both light microscopy and scanning electron microscopy (SEM). In addition, we tested the pollen viability of the putative hybrid.

## MATERIALS AND METHODS

### *Plant materials*

We selected two mixed-growing natural populations of *I. minutoaurea* and *I. odaesanensis* and putative hybrids between them for this study (Table 1; Figure 1A–D). Living plants were transplanted at Chungnam National University for cytological observations. We examined one to six different individuals of each species and the putative hybrids to check their chromosome numbers and pollen viabilities and estimate their genome sizes (Table 1). All voucher specimens were deposited in the Herbarium of Chungnam National University (CNUK, Daejeon, Korea).

### *Chromosome numbers, karyotypes, and pollen viabilities of putative hybrid plants*

We determined chromosome numbers in root tip metaphasic plates using the standard Feulgen staining technique as described by Choi et al. (2020a). We placed pollen grains from two anthers per hybrid individual into aniline blue dye solution to distinguish between fertile and sterile pollen grains (Choi et al. 2020b; Kim et al. 2021). To make detailed palynological observations, we dehydrated mature anthers from putative hybrid individuals in an ethanol series (70%, 90%, 95%, and 100%) at room temperature over 30 min. The dehydrated anthers were immersed in liquid carbon dioxide for critical point drying (Leica Microsystems, EM CPD300, Germany). All dried samples were mounted on aluminum stubs and coated using an ion-sputtering device (Hitachi, E-1010, Japan). The pollen grains in the dried anthers were gently removed using a needle and analyzed using SEM (Hitachi, S3000N, Japan) at 20 kV

**Table 1.** Plant material used for cytological analysis of *I. odaesanensis*, *I. minutoaurea*, and their putative hybrids in natural populations in Korea.

| Taxon; collection number                         | Locality: global positioning satellite (GPS) coordinate | Chromosome number | Genome size 1C ± S.D. (pg) | Reference         |
|--|---|-------------------|----------------------------|-------------------|
| <i>I. odaesanensis</i> *                         |   |                   |                            |                   |
| JCKC1932271                                      | Gasán, Kyungsang  | 2n = 28           | 3.76 ± 0.021               | Choi et al. 2020a |
| JC041904   | Gasán, Kyungsang  | 2n = 28           | 3.74 ± 0.042               | Choi et al. 2020a |
| JC041903   | Gasán, Kyungsang  | 2n = 28           | 3.67 ± 0.015               | Choi et al. 2020a |
| JC041940   | Gasán, Kyungsang  | 2n = 28           | 3.73 ± 0.018               | Choi et al. 2020a |
| Mean   |   |                   | 3.72                       |                   |
| <i>I. minutoaurea</i> Makino                     |   |                   |                            |                   |
| JC041913*  | Gasán, Kyungsang  | 2n = 22           | 4.09 ± 0.042               | Choi et al. 2020a |
| Gasán_8*   | Gasán, Kyungsang  | 2n = 22           | 3.92 ± 0.008               | Choi et al. 2020a |
| Che-01   | Mt. Cheonma, Gyeonggi; N37°41'24", E127°24'36", 157 m   | 2n = 22           | 3.85 ± 0.009               | This study        |
| Che-02   | Mt. Cheonma, Gyeonggi; N37°41'24", E127°24'36", 157 m   | 2n = 22           | 3.94 ± 0.008               | This study        |
| Go-1   | Go-Nam, Gyeonggi; N37°40'22", E127°15'17", 157 m        | 2n = 22           | 3.93 ± 0.009               | This study        |
| Hwa-3  | Mt. Hwa-Ya, Gyeonggi; N36°56'25", E127°16'47", 162 m    | 2n = 22           | 4.02 ± 0.004               | This study        |
| Mean   |   |                   | 3.95                       |                   |
| <i>I. minutoaurea</i> × <i>I. odaesanensis</i> * |   |                   |                            |                   |
| JC041927   | Gasán, Kyungsang  | 2n = 25           | 3.86 ± 0.017               | This study        |
| JC041906   | Gasán, Kyungsang  | 2n = 25           | 3.85 ± 0.009               | This study        |
| JC041924   | Gasán, Kyungsang  | 2n = 25           | 3.80 ± 0.051               | This study        |
| Mean   |   |                   | 3.84                       |                   |

\* The GPS coordinates, latitude, and longitude of the collection sites are not indicated for protection purposes, although the latter species has recently been excluded from the list of endangered Korean taxa.

with a working distance of 10 mm, as described by Kim et al. (2021).

#### Genome-size measurements

The genome sizes of four *I. minutoaurea* individuals and three putative hybrids were measured via flow cytometry, with *Solanum pseudocapsicum* L. (1C = 1.2946 pg; Temsch et al. 2010) serving as an internal standard. Genome-size estimations for *Iris* species were carried out as described by Choi et al. (2020a). Due to the variable genome-size data in our previous research, ranging from 3.70 pg/1C to 4.09 pg/1C with *I. minutoaurea* (Choi et al. 2020a), we included additional 1C values for the same species from another different natural population to clarify the range of genome-size data (Table 1).

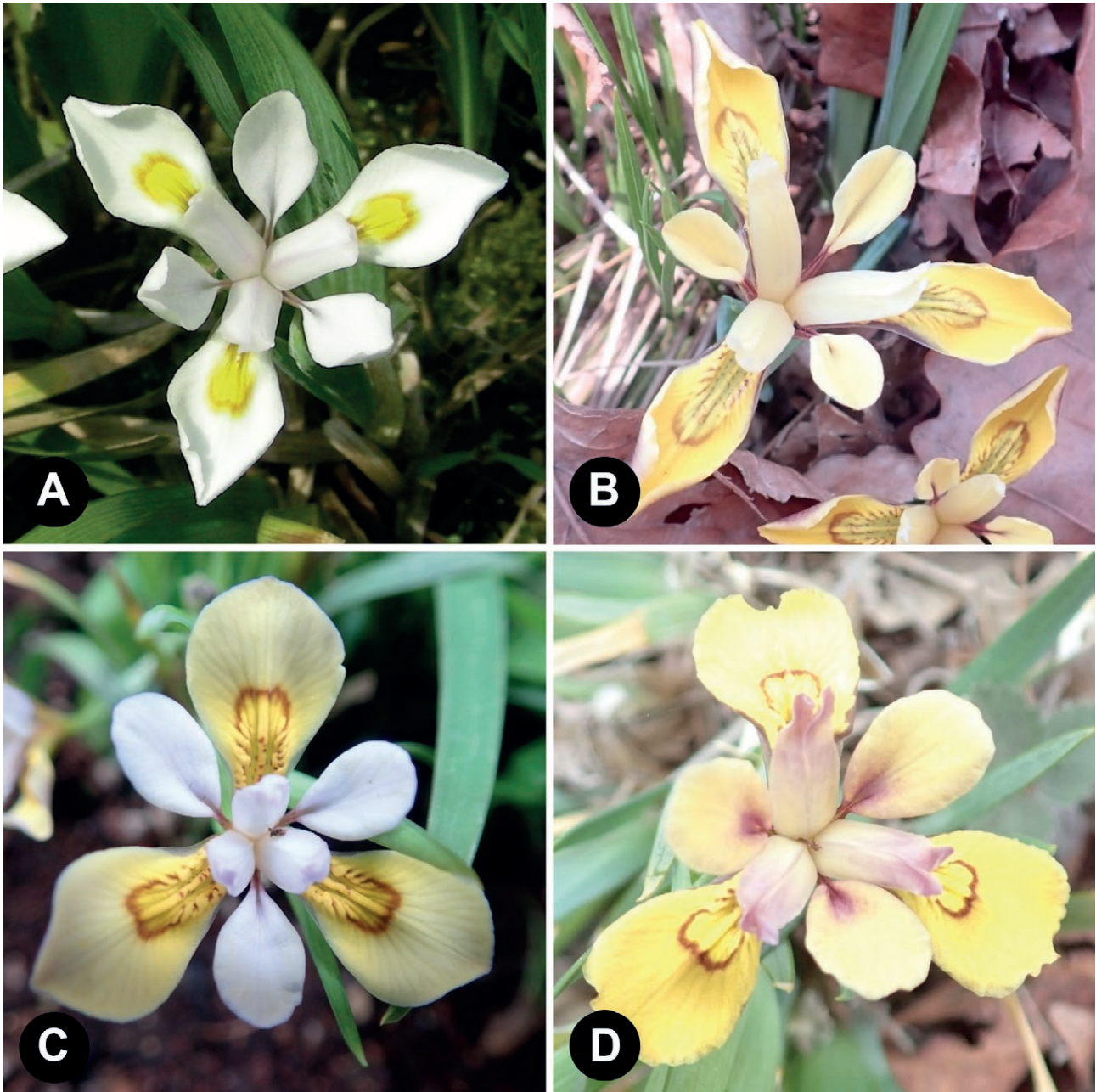
## RESULTS

### Confirmation of homoploid hybridization based on the chromosomal number and genome size

Despite floral color variations among the investigated taxa (e.g., *I. odaesanensis* in Figure 1A, *I. minuto-*

*aurea* in Figure 1B, and their putative hybrids in Figure 1C–D), cytological analysis showed that *I. minutoaurea*, *I. odaesanensis*, and their putative hybrids were consistently diploid, with 2n = 22, 28, and 25, respectively (Figure 2), confirming that homoploid hybridization occurred in natural populations (Figures 1–2). The karyotypes in the putative hybrid plants could not be directly compared with their homologous pairs because of their odd chromosome numbers (2n = 25); thus, they were analyzed with reference to their parental karyotypes. The karyotypes of the hybrid plants were mostly composed of metacentric, sub-metacentric, and acrocentric chromosomes, regardless of their parental chromosomes (Figure 2B). Although satellites and nucleolar-organizer regions did not normally show clear localization in mitotic cells due to high chromosome condensation (Figure 2A–B), they were occasionally visible in hybrid plants (Figure 2C).

Flow cytometric analyses yielded high-resolution histograms with distinct sample peaks and internal standards for all seven individuals investigated (Table 1). The results of our genome-size (1C) analysis are shown in Table 1, together with our previous data. The genome size of *I. minutoaurea* was higher (range: 3.85–4.09 pg) than that of *I. odaesanensis* (range: 3.67–3.76 pg). The

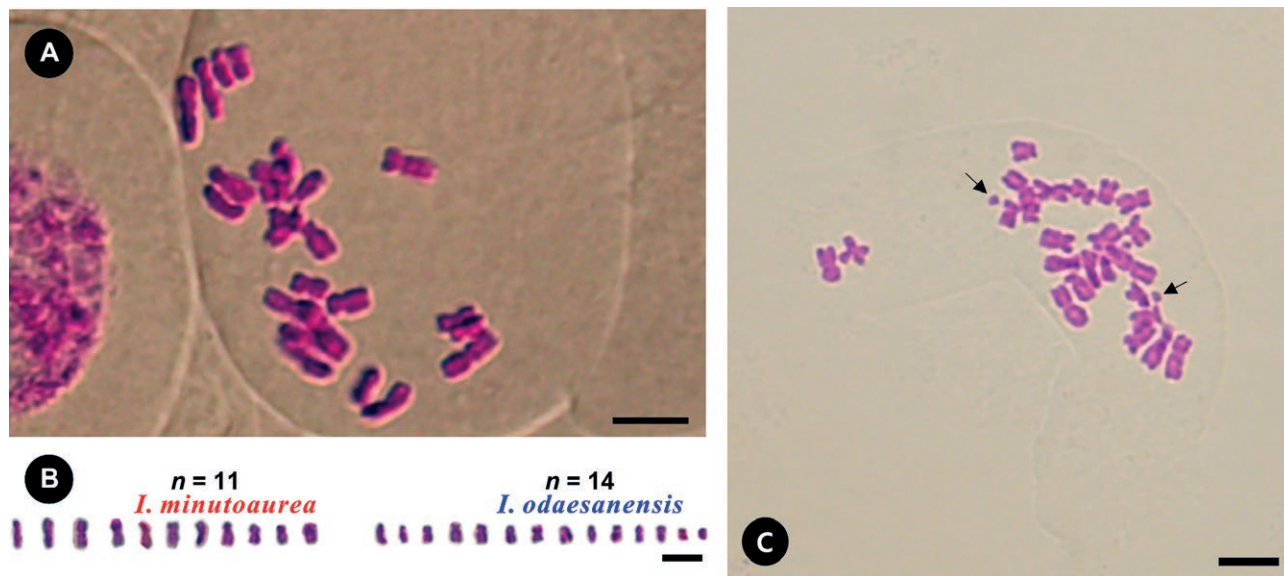


**Figure 1.** Floral morphological variations in the studied *Iris* taxa. **A.** *Iris odaesanensis*: standards and styles were white, and falls were white with lower central half highlighted in yellow. **B.** *Iris minutoaurea*: standards and styles were yellow, and falls were yellow with its central lower half highlighted in dark purple. **C–D.** Putative hybrids between *I. odaesanensis* and *I. minutoaurea*. The putative hybrids showed tepal colour variations: with white standards and styles and yellowish falls with its lower central half highlighted in dark purple (**C**), yellow standards and falls with their lower basal part highlighted in dark purple and yellow styles with its apical margin coloured in purple (**D**). Photographs were taken by T.-S. Jang.

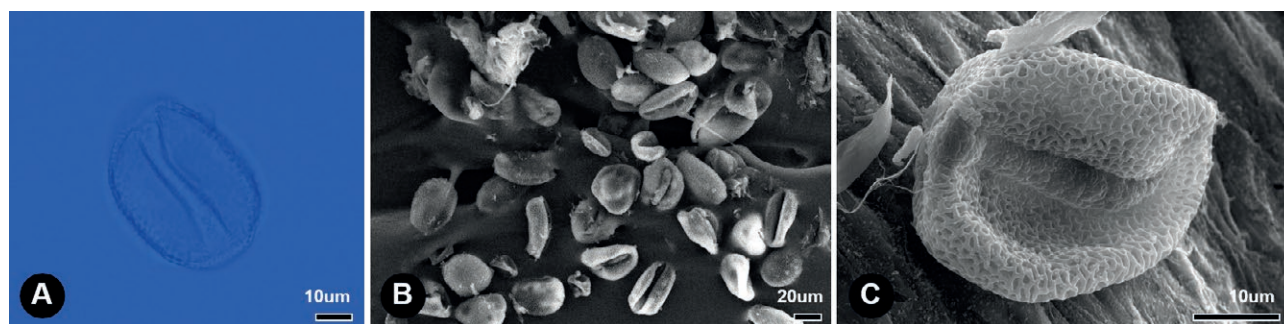
IC mean value of the putative hybrids (mean: 3.84 pg, range: 3.80–3.86 pg) was additive with respect to the diploid parental species (i.e., 3.72 pg in *I. odaesanensis* and 3.95 pg in *I. minutoaurea*).

#### *Pollen fertility in the putative hybrids*

Mature anthers from all hybrid plants were removed, and all pollen grains were stained with



**Figure 2.** Chromosome numbers and karyotypes of putative hybrids ( $2n = 2x = 25$ ) of *I. minutoaurea* ( $2n = 2x = 22$ ) and *I. odaesanensis* ( $2n = 2x = 28$ ). **A, C.** Mitotic metaphase chromosomes with  $2n = 25$  (arrows indicate satellites of putative hybrids). **B.** Karyotype of a putative hybrid including a haploid chromosome of  $n = 11$  (*I. minutoaurea*) and  $n = 14$  (*I. odaesanensis*). Scale bars =  $5 \mu\text{m}$  ( $n$  indicates a haploid chromosome number; karyotypes were analyzed in comparison to previous chromosome-counting data [Choi et al. 2020a]).



**Figure 3.** Light and scanning electron microscopic photographs (LM and SEM) of sterile pollen grains in putative hybrids between *Iris odaesanensis* and *I. minutoaurea*. **A.** An unstained pollen grain (sterile pollen grains) in aniline blue dye solution. **B, C.** Sterile pollen grains observed by SEM. **B.** Stamens of putative hybrids produced sterile pollen grains. **C.** Enlargement of a sterile pollen grain by SEM.

aniline blue dye solution to assess the fertility of the putative hybrid plants (Figure 3A). However, despite staining, no fertile pollen grains were found, indicating that most or all pollen grains were infertile (Figure 3). SEM analysis performed on pollen samples from the putative hybrid individuals revealed that the pollen grains were monosulcate and mostly curled up or shriveled with folded aperture membranes (Figure 3B–C). The exine ornamentation of the putative hybrids was reticulate with continuous muri (very rarely discontinuous).

## DISCUSSION

Here, we provide cytological and palynological evidence for the presence of homoploid hybrids between *I. minutoaurea* and *I. odaesanensis* (*I. ser. Chinense*) in natural Korean populations, in agreement with previous results based exclusively on molecular and morphological evidence (Son et al. 2015; Yang et al. 2020). Both diploid parental species differ in terms of morphology, chromosome number, genome size, and complete chloroplast genome composition (Sim 2007; Choi et al. 2020a; Park et al. 2022). Based on molecular phylogenetic analyses of their *matK* and nrITS sequences, the

putative hybrids did not form a clearly separated monophyletic group when analyzed together with their parental species (*I. minutoaurea* and *I. odaesanensis*; Son et al. 2015). The present chromosome number and genome-size data for the homoploid hybrids appeared to be more conclusive (Table 1). Thus, our results are consistent with the hypothesis that hybridization in the *Iris* genus of the Iridaceae family may have been important for taxon diversification and speciation (Arnold et al. 1990; Young 1996; Lim et al. 2007; Gao et al. 2021; Park et al. 2022).

Natural homoploid hybrid speciation accompanied by reproductive isolation from putative parents has often been reported for the Iridaceae family (Arnold et al. 1990). The intermediate morphological variations of the *Iris* putative hybrid were not influenced by environmental factors. This lack of influence was attributable to a sympatric distribution in their parental diploid species (Table 1). Instead, these variations seemed to correlate with cytological differences, as frequently observed with other homoploid hybrid species (Peruzzi et al. 2012; Musiał et al. 2020; Xiao et al. 2021; Pellicer et al. 2022). Hybridization in plants can be affected by overlapping flowering periods in sympatric areas (Paun et al. 2009; Abbott et al. 2010; Jiang et al. 2013; Kim et al. 2023) and is mainly documented for apomictic groups (Paun et al. 2006; Hörandl 2010; Hojsgaard and Hörandl 2019). Our results confirmed that homoploid hybrid species of Korean irises may have evolved partial intrinsic reproductive isolation from both diploid parents considering their different base chromosome numbers ( $x = 11$  in *I. minutoaurea* and  $x = 14$  in *I. odaesanensis*; Table 1). Such different base chromosome numbers and odd chromosome number could lead to infertility in individuals and prevent the establishment of hybrid lineages and back-crosses with the parental species (Carnicero et al. 2023). However, only limited data are available regarding the emergence of *Iris* hybrids from natural populations, their potential for sexual and asexual reproduction (e.g., by rhizome disruptions), or morphological divergence from the parental taxa (Arnold et al. 1990; Niketić et al. 2018; Yang et al. 2020; Gao et al. 2021).

Efforts to maintain homoploid hybrids from natural populations are likely to fail because of their infertile offspring (Stebbins 1958; Yakimowski and Rieseberg 2014). Pollen abortion in putative hybrid *Iris* plants could be reflected by impaired chromosomal pairing during meiosis between the two unequal parental genomes ( $x = 11$  and 14). These hybrid plants are not amenable to sexual reproduction, suggesting that the hybrids probably represent a natural first-generation hybrid species, as has been reported for other species (Denduangboripant et al. 2007; Saito et al. 2007; Tseng and Hu 2014). Although such hybrid taxa may not even-

tually produce well-differentiated hybrid species, they can facilitate the testing of key predictions from models of hybrid speciation, as observed with other plant groups (Barton 2001; Chen et al. 2022). Further investigation using molecular cytogenetic techniques and genomic *in situ* hybridization of the putative hybrid and its parental species will shed light on the speciation and diversification of *Iris* ser. *Chinense*.

## CONCLUSION

In summary, our comprehensive data demonstrate the existence of populations of natural homoploid *Iris* hybrids. Natural hybrids of *I. odaesanensis* ( $2n = 28$ ; 3.72 pg/1C)  $\times$  *I. minutoaurea* ( $2n = 22$ ; 3.95 pg/1C) were additive in terms of the chromosome number ( $2n = 25$ ) and genome size (i.e., 3.84 pg/1C). The odd chromosome number in the homoploid hybrid could affect the formation of infertile pollen grains, thereby preventing the establishment of hybrid lineages and backcrosses with the parental species *I. odaesanensis* or *I. minutoaurea*.

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