

Interploidy hybridization enhances floral phenotypic diversity of *Iris × norrisii* tetraploids

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Abstract

Interploidy hybridization has been widely utilized in plant breeding and cultivar improvement. In ornamental species, interploidy hybridization between diverse diploid lines and synthetic tetraploids enables the transfer of rich diploid variation to polyploid backgrounds. Although previously applied in the breeding of Irises and other ornamentals, this approach remains insufficiently explored in synthetic polyploid cultivars. To address the limited floral variation observed in artificial tetraploid lines of *Iris × norrisii*, we performed interploidy crosses between tetraploid plants and both diploid and mixoploid lines. Hybrid progenies were evaluated based on floral traits, chromosome counts, and the meiotic behavior of microspore mother cells in parental plants. Crosses using tetraploid plants as female parents yielded tetraploid or near-tetraploid aneuploid progeny, whereas those with diploid female parents produced only diploid offspring. Cytological analysis revealed meiotic abnormalities in both diploid and tetraploid, contributing to the formation of unreduced male gametes. Interploidy hybrids displayed a broader range of perianth colors than control tetraploid crosses, whereas floral morphological variation was similar between the two groups. These findings demonstrate that diploid *I. × norrisii* contributes to the diversification of floral traits in tetraploid lines via the transmission of genetic variation through unreduced male gametes. Interploidy hybridization is a promising approach for enhancing phenotypic diversity in polyploid ornamental breeding.

Introduction

Interploidy hybridization has been extensively applied in plant breeding programs. Crosses between diploid and tetraploid parents have been employed to develop asexual triploid fruits, such as seedless *Citrullus lanatus* (Thomas et al. 2003) and citrus varieties (Aleza et al. 2012). In ornamental breeding, varieties of desirable traits from diploid cultivars have been successfully introgressed into tetraploids, as demonstrated in Bearded Irises (Schreiner 1945; Pane-Joyce 1995) and Aril Irises (Warburton and Hamblen 1978). Additionally, the use of mixoploid Louisiana Iris cultivars has facilitated the improvement of floral diversity in tetraploid populations (Mertzweiler 1986).

Despite these successful applications, the potential of interploidy hybridization to enrich floral trait diversity in newly induced polyploid ornamental species remains underexplored. Recently, tetraploid plants of *Iris × norrisii* were successfully induced through colchicine treatment (Ding et al. 2023). Although these tetraploids exhibited larger flowers and seeds compared to their diploid counterparts, their floral phenotypes were strikingly uniform, predominantly exhibiting purple flowers. Given that effective breeding in polyploid ornamental plants often relies on generating and selecting diverse phenotypes, expanding the floral variation of tetraploid *I. × norrisii* is essential for developing novel cultivars with enhanced ornamental value.

To address this challenge, interploidy hybridizations were conducted between tetraploid *I. × norrisii* plants and both diploid and mixoploid lines, leveraging the rich floral diversity present in diploid populations. The resulting progenies were evaluated for floral traits, performed chromosome counts, and examined the meiotic behavior of parental microspore mother cells to elucidate the cytological mechanisms underlying hybrid formation. This study aims to assess the feasibility of using interploidy hybridization to diversify floral traits in tetraploid *I. × norrisii*, offering insights into accelerating polyploid breeding efforts in ornamental plants.

Materials and methods

Plant materials

Tetraploid plants of the C1 generation of *I. × norrisii* were obtained as previously reported by Ding et al. (2023). The C2 generation was obtained through self-pollination and intercrossing among C1 individuals. Both C1 and C2 generation were served as the tetraploid parents in the hybridization.

The diploid hybrid population was generated by crossing diploid *I. × norrisii* individuals with elite cultivars. Additionally, mixoploid plants were induced via colchicine treatment, following the method of Ding et al. (2023). All plant materials were cultivated in an open-field nursery at Beijing Forestry University (40°09' N, 116°26' E).

Hybridization experiment

Reciprocal crosses were performed between tetraploids and diploids (designated as 4X × 2X and 2X × 4X) and between tetraploids and mixoploids (designated as 4X × C0 and C0 × 4X), using mixed pollen for pollination. Self-pollination and intra-ploidy crosses within diploid and C2 populations served as controls (designated as 2X × 2X and 4X × 4X, respectively).

For each cross, the numbers of pollinations, fruits, plump ovules per fruit, and seeds were recorded. Additionally, fruit set rate, germination rate, and seedling rate were calculated. The number of plump ovules per fruit was determined by dividing the total number of plump ovules produced after 14 days of pollination by the number of fruits. Seedling rate was calculated as the number of progeny obtained divided by the number of pollinations

Chromosome counting in hybrid progeny

Chromosome numbers were determined following the protocol of Ding et al. (2023). Root tips and developing ovaries were collected, pretreated with saturated p-dichlorobenzene in the dark, rinsed with distilled water and fixed in Carnoy's solution (ethanol: acetic acid = 3:1, v/v). After fixation, tissues were hydrolyzed in hydrochloric acid, rinsed again, and stained with Carbol fuchsin (Solarbio, Beijing, China). Chromosomes were observed using a Soptop CX40 microscope.

Meiotic observation in diploid and tetraploid parents

Meiosis behavior in microspore mother cells (MMCs) of diploid and tetraploid parents was examined. Developing flower buds were fixed in a fixative solution (ethanol: acetic acid: chloroform = 3: 2: 5, v/v), rinsed with distilled water, and dissected to extract anthers.

The other contents were squashed on microscope slides, stained with Carbol fuchsin (Solarbio, Beijing, China), and examined under a Soptop CX40 microscope. The total number of MMCs and those showing chromosomal abnormalities were recorded at various meiotic stages, and the frequency of abnormalities was calculated for each stage.

Evaluation of hybrid progeny phenotypes

Floral traits of the hybrid progeny were evaluated in the outdoor nursery at Beijing Forestry University. Each individual was assessed for floral morphology, including the characteristics of perianth segments, pistils, and stamens, and classified into floral types.

Perianth color was recorded, analyzed statistically, and documented through digital photography.

Result

Hybridization experiment

The number of pollinations, fruits, plump ovules per fruit, seeds, fruit set rate, germination rate and seedling rate of each hybrid combination are summarized in Table 1.

Fruit set rates in interploidy crosses were lower than those observed in the control groups (2X × 2X and 4X × 4X). Among interploidy crosses, fruit set rates were higher when tetraploids served as the maternal parent compared to the backcrossing. Notably, the seedling rate of 4X × C0 (37.72%) was higher than that of the 4X × 4X control (19.57%). Conversely, the seedling rates of 4X × 2X, 2X × 4X, and C0 × 4X were significantly lower than that of the 4X × 4X control.

Table 1 Results of hybridization experiment							
Crossing Types	No. of Pollinations	No. of Fruits	Fruit Set Rate (%)	No. of Plump Ovules per Fruit	No. of Seeds	Germination Rate (%)	Seedling Rate (%)
4X × 2X	650	100	15.38	1.97	82	10.98	1.38
2X × 4X	865	108	12.49	3.59	23	34.78	0.92
4X × C0	167	26	15.57	6.54	164	38.41	37.72
C0 × 4X	290	27	9.31	1.15	28	3.57	0.34
4X × 4X	184	33	17.93	9.88	198	17.17	19.57
2X × 2X	95	38	40.00	1.89	-	-	-

Chromosome counting of hybrid progeny

Several individuals from the progenies of 4X × 2X, 2X × 4X, and 4X × C0 crosses were randomly selected for chromosome counting. Among the four offspring from the 4X × 2X crosses, three were tetraploid (2n = 4x = 64, Fig. 2a) and one was aneuploid (2n = 4x - 2 = 62, Fig. 2b). All five offspring from the 2X × 4X crosses were diploid (2n = 2x = 32, Fig. 2c). Among the five samples from the 4X × C0 crosses, three were tetraploid (2n = 4x = 64, Fig. 2d), while the remaining two were aneuploid (2n = 4x - 2 = 62, Fig. 2f).

Observation of the meiosis in diploid and tetraploid microspore mother cells

A total of 609 tetraploid and 547 diploid microspore mother cells (MMCs) were examined to assess meiotic behavior.

Both ploidy levels exhibited common meiotic abnormalities. During metaphase I, over 40% of MMCs at both ploidy levels exhibited chromosomes failing to congress at the equatorial plate (Table 2, Fig. 2c). Subsequently, unequal chromosome segregation was observed at early anaphase I (Fig. 2d), and chromosome bridges (Fig. 2e) were noted from early to late anaphase I, with the highest frequencies recorded during early anaphase I (29.2% in diploids; 33.3% in tetraploids). Chromosomes involved in bridges formation either separated rapidly or broke directly. Lagging chromosomes were primarily observed in anaphase I (Table 2, Fig. 2f), leading to micronuclei that persisted through meiosis II and into the tetrad stage. Ultimately, both diploid and tetraploid MMCs produced triads at the tetrad stage (Fig. 2j).

Despite these similarities, notable differences were evident. Tetraploid MMCs exhibited a higher frequency of meiotic abnormalities compared to diploids. At diakinesis, diploid MMCs displayed univalents (Fig. 2a), whereas tetraploid MMCs exhibited both univalents and multivalents (Fig. 2b). At early Anaphase I, unequal chromosome segregation was more frequent in tetraploids. Additionally, tetraploid MMCs displayed a low frequency of chromosome bridges during metaphase I and exhibited occasional perpendicular spindles during prophase II and polyads at the tetrad stage.

Table 2
Frequency of meiotic anomalies in microspore mother cells in tetraploids and diploid

Ploidy Level	Total Abnormal Rate (%)	Abnormal rate of different phases of meiosis of microspore mother cells							
		Metaphase II (%)		Early Anaphase I (%)		Late Anaphase II (%)		Prophase II (%)	
		chromosomes failing to congression	Chromosome Bridge	Unequal Segregation	Chromosome Bridge	Lagging Chromosomes	Chromosome Bridge	Micronucleus	Perpendicular Spindle
4X	42.1	46.3	4.5	14.3	33.3	51.7	10.3	28.0	2.0
2X	26.5	41.7	0.0	0.0	29.2	35.7	7.1	18.5	0.0

Phenotypic analysis of hybrid progeny

All hybrid progeny exhibited distinct floral phenotypes, which were categorized into two major types based on petal and stigma morphology: Iris dichotoma-type and Iris domestica-type. Iris domestica-type flowers displayed outer perianths without noticeable reflexing and revolute margins, inner perianths with triangular notches at the tips, and obtuse, rounded stigmatic appendages (Fig. 3g). Conversely, Iris dichotoma-type flowers were characterized by outer perianth segments that were transversely reflexed at the middle with revolute margins, inner perianths with trapezoidal notches at the tips, and pointed stigmatic appendages (Fig. 3h).

Further phenotypic variation was observed among different crossing combinations. In the 4X × 4X control group, 20 seedlings were evaluated for perianth color: 16 exhibited purple (Fig. 3a), two displayed light purple (Fig. 3b), one showed white (Fig. 3c), and one exhibited orange perianth color (Fig. 3e). Notably, the orange-flowered individual exhibited the Iris domestica-type morphology, whereas the purple, light purple, and white flowers corresponded to the Iris dichotoma-type.

Among the 4X × 2X crosses, six of nine offspring flowered, producing three purple, one white, one orange, and one pink (Fig. 3d) perianth colors. Similarly, in the 2X × 4X crosses, five of eight offspring flowered, yielding two pink, two orange, and one pink-yellow bicolor perianths.

The 4X × C0 crosses produced 59 offspring. Most individuals exhibited Iris domestica-type morphology, with 23 orange and 31 burgundy-colored flowers (Fig. 3f). Only two individuals displayed Iris dichotoma-type morphology, bearing purple and light purple perianth colors. Additionally, perianth spotting varied considerably, ranging from strip-like to punctate patterns, with distribution areas extending from full to partial petal coverage and varying in density from sparsely to densely merged patterns.

Discussion

Preferential production of unreduced male gametes diploid *I. × norrisii*

A key finding of this study is the preferential production of unreduced male gametes over unreduced female gametes in diploid *I. × norrisii*, which offers insights into reproductive barriers between different ploidy levels.

In 4X × 2X crosses, tetraploid and near-tetraploid aneuploids were obtained, suggesting that unreduced (2n) male gametes were produced by diploid individuals. Cytological evidence further supports this inference, as triads containing 2n pollen grains were observed in diploid MMC. When such unreduced pollen fertilizes normal female gametes from tetraploids, the resulting offspring can be tetraploid or aneuploid. Similar patterns of 2n pollen production through triads have been reported in other species such as *Populus × euramericana* (Zhang et al. 2009) and *Dimocarpus longan* (Li et al. 2022), indicating a conserved mechanism across diverse taxa.

Conversely, in 2X × 4X crosses where diploid individuals served as maternal parents, only diploid offspring were obtained. This result suggests two key inferences. First, *I. × norrisii* exhibits a limited capacity for producing unreduced female gametes, suggesting relatively greater stability during megaspore meiosis compared to microspore meiosis. Second, the tetraploid parent likely contributed haplotype male gametes containing 16 chromosomes, possibly due to meiotic abnormalities. This pattern differs from that observed in species such as *Hieracium echinoides* (Peckert and Chrtek 2006) and *Campanula rotundifolia* (Sutherland and Galloway 2017), where interploidy crosses produced a small number of diploid progeny but commonly yielded triploid and tetraploid progeny. These differences may reflect species-specific variation in the formation of unreduced female gametes.

Several meiotic irregularities observed in both diploid and tetraploid parental lines likely contribute to the production of unreduced male gametes and aneuploid offspring. These include univalents and multivalents during pachytene, lagging chromosomes, chromosome bridges, and micronuclei. Univalents and multivalents can lead to unequal chromosome segregation, while lagging chromosomes and bridges—hallmarks of chromosomal instability (Rodríguez-Muñoz et al. 2022)—may give rise to micronuclei and trigger breakage-fusion-bridge cycles (De Marco Zompit and Stucki 2021). These abnormalities likely underlie the production of unreduced pollen with abnormal chromosome numbers, ultimately resulting in the observed aneuploid progeny.

Diploids and mixoploids enrich floral phenotypic diversity in tetraploids via unreduced male gametes

The results of this study demonstrate that diploid and mixoploid individuals can enhance the floral phenotypic diversity of *I. × norrisii* tetraploid progeny through interploidy hybridization.

The tetraploid parental plants (C1 and C2 generations) exhibited limited perianth coloration, primarily purple and white. In contrast, novel perianth colors such as light purple and orange were observed in the $4X \times 4X$ control crosses, while interploidy crosses ($4X \times 2X$ and $4X \times C0$) produced even greater diversity, including light purple, orange, burgundy, and pink flowers. Additionally, distinct floral morphologies resembling *Iris dichotoma*-type and *Iris domestica*-type were observed in both control and interploidy progeny.

The diploid parental population was a genetically diverse hybrid group with highly variable perianth coloration, while the mixoploid individuals were obtained by colchicine treatment of seeds derived from this genetically diverse diploid population. As a result, both diploid and mixoploid parents carried abundant genetic variation. Combined with cytological observations, these findings suggest that diploids and mixoploids transmitted their genetic diversity to the tetraploid progeny via unreduced male gametes, thereby enriching floral phenotypic traits in the tetraploid background. Additionally, a higher seedling rate was observed in the $4X \times C0$ crosses than in the $4X \times 2X$ crosses, implying that mixoploids may exhibit greater reproductive compatibility with tetraploids than diploids do.

This phenomenon of generating phenotypic diversity via interploidy hybridization has significant evolutionary implications. Several recently formed polyploid species, such as *Senecio cambrensis* and *Mimulus peregrinus* (Edger et al. 2025), have formed multiple times in nature. The recurrent formation of allopolyploids, alongside interploidy hybridization processes as observed in *I. \times norrisii* and other plant groups, contributes significantly to the genetic diversity that underlies the evolution of allopolyploid species. Insights from these natural events can inform and refine strategies for polyploid breeding in ornamental plants.

Conclusion

Interploidy hybridization has proven to be an effective strategy for accelerating polyploid breeding in *I. \times norrisii*. Chromosome counting of hybrid progeny, along with cytological observations of meiotic behavior in parental MMCs, indicated that diploid individuals produce unreduced male gametes. These gametes transmit abundant genetic variation from diploid parents to tetraploid offspring, thereby enriching floral phenotypic diversity. However, this pathway is largely inactive diploids serve as the maternal parent. The use of mixoploid individuals increased the yield of tetraploid progeny, improving the overall efficiency of interploidy hybridization. While the ornamental traits of *I. \times norrisii* tetraploids has been broadened, future investigations will focus on further enhancing tetraploid floral phenotypic diversity and ornamental characteristics that surpass those found in diploids.

Declarations

Author contributions All authors contributed to the study conception and design. Material preparation was conducted by J. T., L. D., R. L. and J. X.. Data collection and analysis were performed by J. T. and L. D.. The first draft of the manuscript was written by J. T.. Y. G., Y. L. and Z. W. commented on previous versions of the manuscript. Y. G. acquired funding. All authors read and approved the final manuscript.

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Data availability The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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Figures

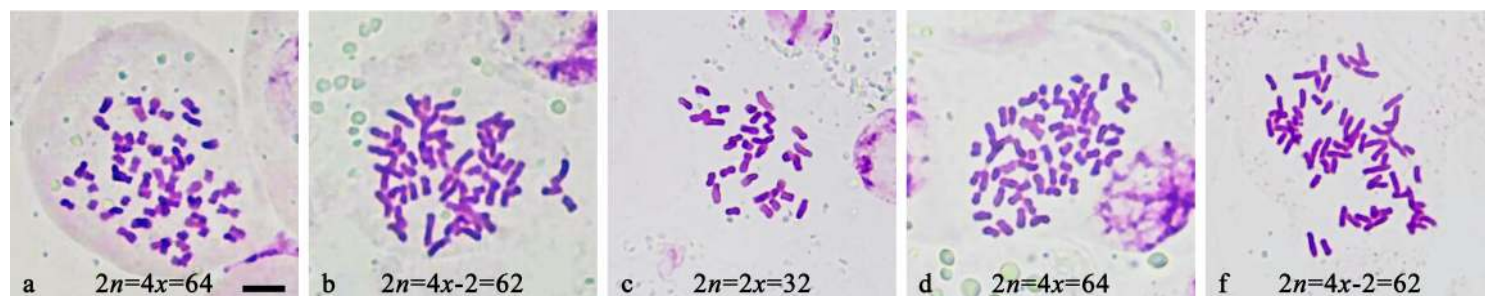


Figure 1

Chromosome counting results of hybrid progeny. Bar = 10 μm . a $4X \times 2X$ progeny, $2n = 4x = 64$; b $4X \times 2X$ progeny, $2n = 4x - 2 = 62$; c $2X \times 4X$ progeny, $2n = 4x = 64$; d $4X \times C0$ progeny, $2n = 4x = 64$; e $4X \times C0$ progeny, $2n = 4x - 2 = 62$

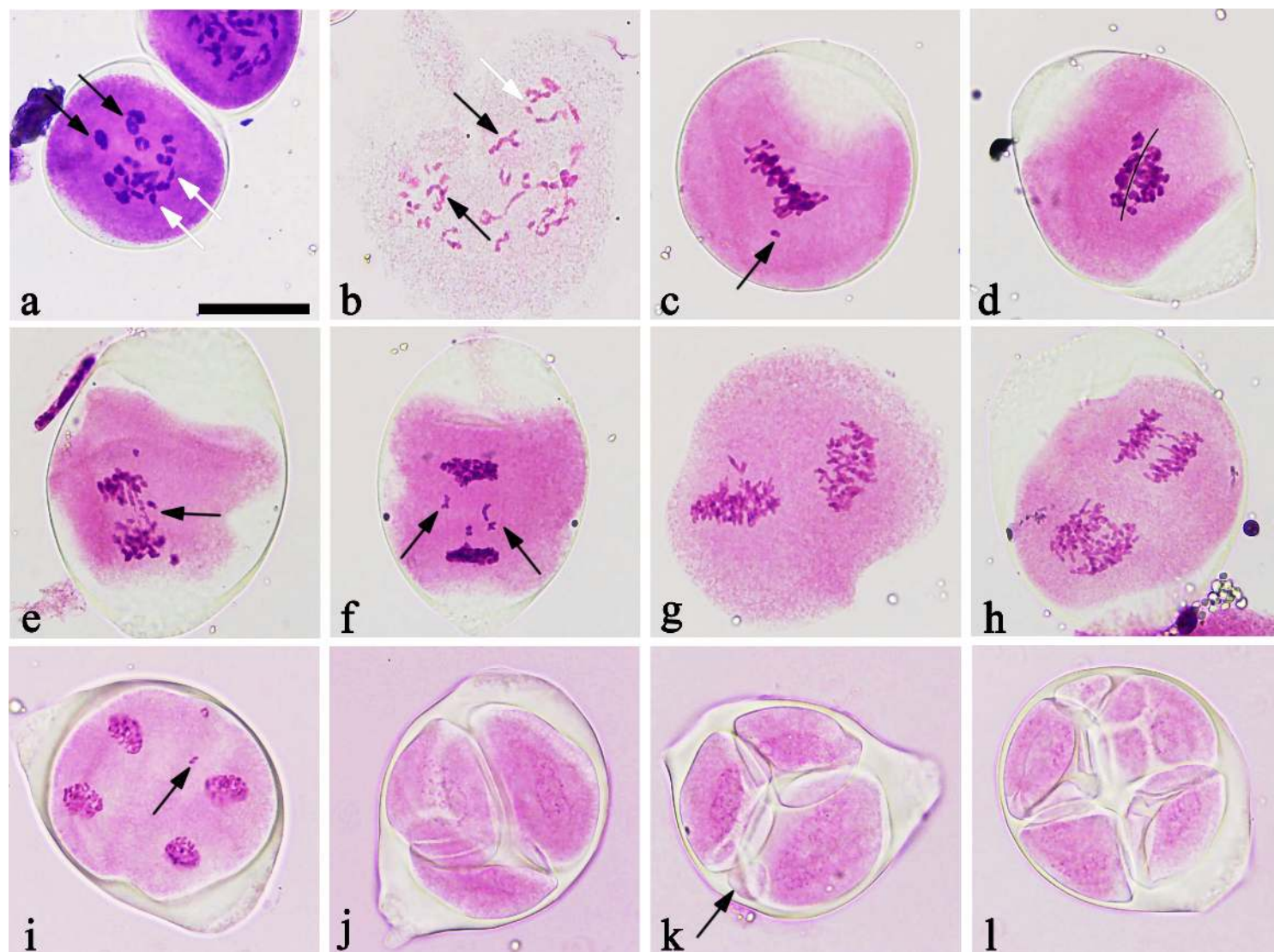


Figure 2

Chromosome abnormalities in meiotic divisions of diploid (a) and tetraploid (b-l) *I × norrisii* microspore mother cells. Bar = 50µm. a Diakinesis, bivalents (black arrow) and univalents (white arrow); b Diakinesis, multivalents (black arrow) and univalents (white arrow); c Metaphase I, chromosomes failing to congression (black arrow); d Early anaphase I, unequal segregation; e Late anaphase I, Chromosome bridge; f Late anaphase I, Lagging chromosomes; g Prophase I, perpendicular spindle; h Anaphase I, Asynchronous separation; i Telophase I, micronucleus; j Triad; k c (black arrow); l Polyad Phenotypic analysis of hybrid progeny

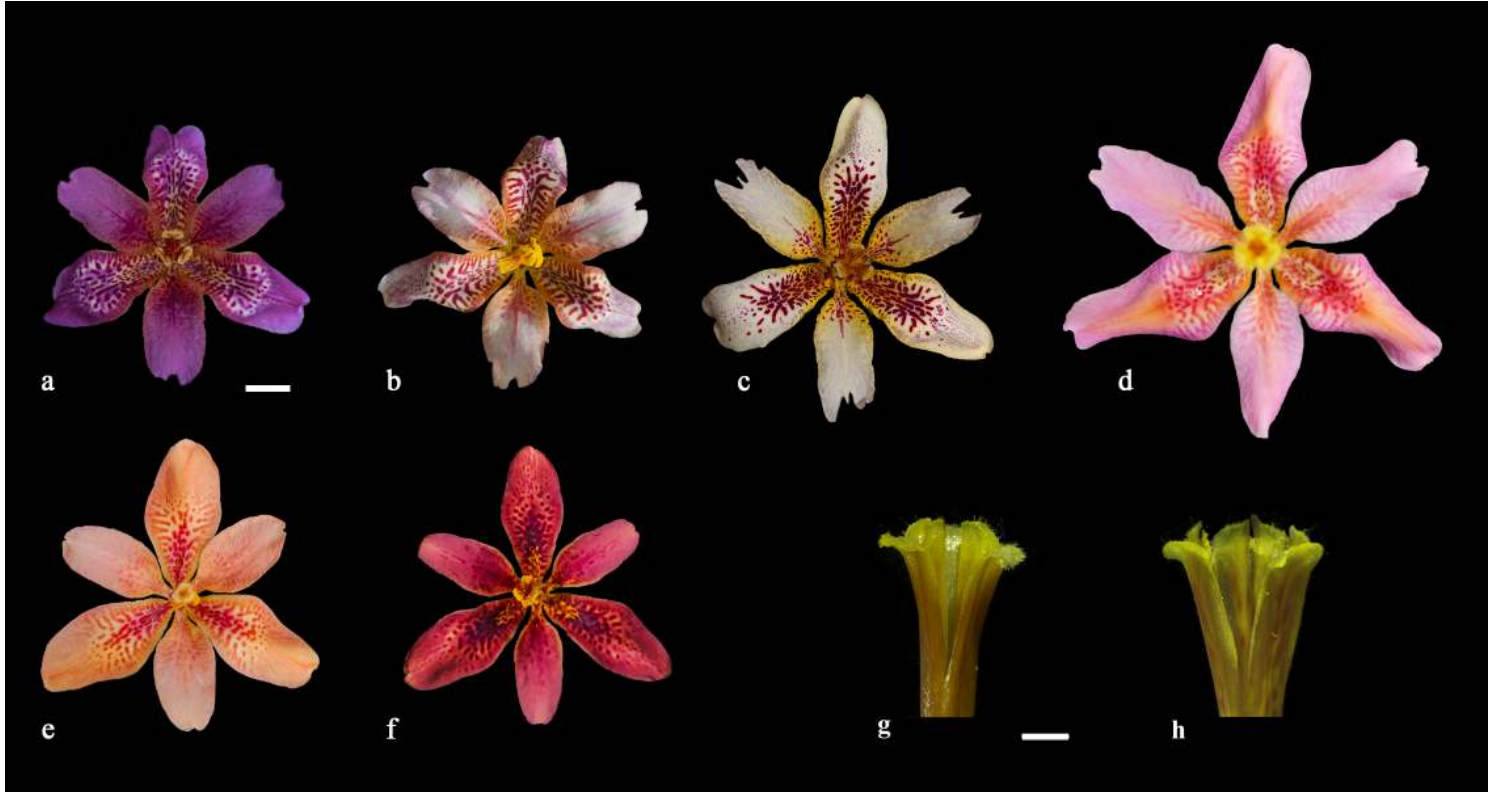


Figure 3

Flower colour and flower morphology of *I × norrisii* tetraploid hybrid progeny, and stigma morphologies of *Iris domestica*-type flower and *Iris dichotoma*-type flower. a-f Bar = 1 cm; g-h Bar = 2 mm. a C3 generation plants, purple, *Iris dichotoma*-type flower; b C3 generation plants, light purple, *Iris dichotoma*-type flower; c 4X × 2X progeny, white, *Iris dichotoma*-type flower; d 4X × 2X progeny, pink, *Iris dichotoma*-type flower; e 4X × C0 progeny, orange, *Iris domestica*-type flower; f 4X × C0 progeny, burgundy, *Iris domestica*-type flower; g *Iris domestica*-type stigma morphology; h *Iris dichotoma*-type stigma morphology