Chromosome number and phenotypic variations in hybrid populations of diploid and triploid in Hemerocallis

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Research Article

Keywords: Hemerocallis, polyploidy, chromosome number, phenotypic variations

Posted Date: November 23rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2093042/v1

Abstract

The main objective of the present study was to identify chromosome number variation in the progenies from triploid × diploid (3x × 2x) crosses in daylilies to analyze the survival of aneuploid embryos and the trait variation of progeny (e.g., plant height, flower diameter, and flower opening and closing time), and fully tap into the potential of triploid daylilies for daylily breeding. The results showed that the fruits of crosses fully developed, and seedlings were obtained from the two hybrid groups, indicating that male sterile triploid *Hemerocallis fulva* can be used as a female. Chromosome preparation showed that the progenies were predominantly aneuploid. Based on megasporogenesis, we deduce that the endosperm of 3x × 2x is 7x in *H. fulva* and conclude that the aneuploid embryo survival of 3x × 2x in *Hemerocallis* is the result of the euploid endosperm. The analysis of trait variation showed that most of the morphological characters were separated in the F1 generation. The F1 plants were nocturnal-flowering. The results suggest that flower opening time in the evening is partially dominant to that in the morning. The main objectives of our study were to evaluate daylily tolerance to karyotypic imbalance and enhance our understanding of flowers' ability to predict and adapt to daily environmental changes.

Introduction

Hemerocallis plants are widely used in landscape construction and landscaping due to their strong stress resistance, wide adaptability, easy cultivation, and high ornamental value. Previous studies have shown that the basic chromosome number of *Hemerocallis* is 11; most wild species and cultivars are diploid (2n = 2x = 22), and some cultivar is tetraploid (2n = 4x = 44) (Li et al. 2009, Vendrame et al. 2009, Zhang et al. 2014). Fifty-two taxa of daylily have been studied, out of which forty-five have 22 chromosomes in the somatic complement, six have 33 chromosomes including the species *Hemerocallis. fulva* var. *Europa*, *H. fulva* var. *cypriana*, and one cultivar 'Mrs. David Hall' has 44 chromosomes (Roy 1976)

Polyploidy, divided into aneuploidy and euploidy, is widely distributed and has played an important role in plant evolution. Aneuploidy refers to deviations of one or more chromosomes from the basic genome in monoploids or from multiples of the basic genome in diploids and polyploids. Different ploidy plant hybridization is important for gene exchanges and generating variants. Aneuploid plants have mainly been obtained from crosses between triploid and diploid plants in many species. Reciprocal crosses between diploid and triploid plants were performed to generate aneuploid plants. Crosses with diploid female parents yielded much higher diploid progenies than those with triploid female parents, while crosses with triploid female parents yielded more aneuploid progeny (Li et al. 2017). Crosses between the diploid *Hylocereus polyrhizus*, as the female parent, and the tetraploid *Selenicereus megalanthus*, as the male parent, yielded triploid and aneuploid hybrids (Tel-Zur et al. 2005)

Flowering plants can be classified as nocturnal and diurnal. These are stable traits that are regulated by an endogenous oscillator and the external environment. The genetic mechanisms of circadian flowering are poorly understood, perhaps because of the weak circadian flowering rhythms in the model plant. The triploid daylily (*H. fulva*) is not only a good potential source for breeding aneuploid cultivars, but also a

significant source for the study of flowering rhythms. Flowers of *H. fulva* open in the morning and closes in the evening, while *Hemerocallis citrina* opens at dusk and closes in the early morning. The diurnal (*H. fulva*) and nocturnal flowers (*H. citrina*) provide the potential for the genetic study of flowering rhythms (Matsumoto et al. 2015). In flowering plants, pollination success is strongly dependent on the timing of when flowers start to bloom and when they start to close. A series of genetic studies has revealed that a few major genes or even a single gene can cause differences in flowering time (Nitta, et al. 2010). The hybridization between *H. citrina* and *H. fulva*, their natural hybrid population blooming and withering time showed a discontinuous bimodal distribution. Most F1 hybrids showed diurnal flowering. These results indicate that only a few genes have a strong phenotypic effect on the determination of flowering time in *Hemerocallis* (Hasegawa et al. 2006). In addition, in an analysis of the artificial hybrid population of the two species, individuals of the F1 generation showed high variability in flower opening time even within an individual, while among the F2 hybrids, the ratio of morning flowering and evening flowering did not deviate from 1:1, and the ratio of evening closing and morning closing did not deviate from 3:1. These results suggest that the time of flower opening and that of closing are regulated by different major genes (Nitta et al. 2010). In contrast, studies on the hybridization breeding between *Iris. dichotoma* and *Iris. domestica*. Flowers of *I. dichotoma* open in the afternoon and closes in the evening, while *I. domestica* opens in the early morning and closes at dusk. In an analysis of the flower opening time of hybrid progeny, flower opening time in the early morning was found to be partially dominant to that in the afternoon, while flower closure at dusk was found to be dominant to closure in the evening. These results indicated that flower opening and closing times are controlled by different polygenes (Liu et al. 2018).

In this study, crosses between diploid and triploid plants were conducted, and the number of chromosomes and flowering rhythms of the progeny were analyzed. The main objectives of our study were to evaluate the tolerance of daylilies to karyotypic imbalances and enhance our understanding of flowers' ability to predict and adapt to daily environmental changes.

Materials And Methods

Three species were used as parents in the experiments. They were planted in the Xiaotangshan Nursery of National Engineering Research Center for Floriculture (40°09' N, 116°26' E, Beijing). *H. fulva*, and *Hemerocallis lilioasphodelus* L. are wild species and 'siyuehua' is a cultivar. *H. lilioasphodelus* and 'siyuehua' are diploids with 22 chromosomes (2n = 22). *H. fulva* is triploid with 33 chromosomes (2n = 33) (TAKENAKA 2009). While *H. fulva* has diurnal flowers, *H. lilioasphodelus* and 'siyuehua' are nocturnal flowers.

H. fulva was crossed with the pollen of *H. lilioasphodelus* and 'siyuehua'. The population was established by artificial hybridization during the full blooming period of daylilies from June to August. The populations used for phenotypic measurement and analysis were all greater than 30.

Based on previous studies on the processes of flowering (Nitta et al. 2010), Experiments were conducted during the full-blossom period of *Hemerocallis* spp. The species and cultivars could be divided into two

modes according to times of opening: diurnal-flowering plant (open between 3:00–15:00) and nocturnal-flowering plant (open between 15:00–3:00 the next day) (Ren et al. 2019). The plant height, scape height, leaf width, petal length, petal width, calyx width, calyx length, number of leaves, and number of flowers per stem were measured at the same time for each genotype. Observation results were recorded, and the statistical techniques employed for analysis were descriptive statistics, one-way ANOVA, and one sample t-tests. Data analysis was performed using Microsoft Excel 2020 and SPSS 24.0

The hybrid progeny root tips taken from plants maintained in growth at the Xiaotangshan Nursery of National Engineering Research Center for Floriculture. Root tips (1–2 cm) were pre-treated in a saturated solution of 1-bromonaphthalene for 4–5 h and fixed in 3:1 (ethanol: glacial acetic acid) for 24 h. They were then hydrolyzed in 1 M HCl at 60℃ for 12 min and transferred to a 2.5% pectinase solution for 30 min at 37℃. Root tips were then stained with Carbol fuchsin for 15 min. Root tips were covered with a plastic cover slip and tapped several times with a dull needle to break up the tip and disperse the cells. The slide was placed between two pieces of folded filter paper and moderate thumb pressure was applied to flatten the cells. The chromosome number was counted under a light microscope.

Results

Seed setting and germination rate

Because the flowering time of the three daylilies did not overlap and the ploidy was different, the cross compatibility between *H. fulva* and daylily was very low. In this study, fruits of two crosses developed. The seed setting rates of *H. fulva* × *H. lilioasphodelus* and *H. fulva* × 'siyuehua' were 64% and 32% respectively (Table 1). The seed setting rate of *H. fulva* × 'siyuehua' was higher than *H. fulva* × *H. lilioasphodelus.* In contrast, the seed germination of *H. fulva* × *H. lilioasphodelus* was higher than *H. fulva* × 'siyuehua'. The results showed that the fruits of crosses fully developed, and seedlings were obtained from the two hybrid groups, indicating that male sterile triploid *Hemerocallis fulva* can be used as a female.

Table 1
seed setting rate and germination rate of progeny from crosses between triploid and diploid in daylily

Cross combinations	No. of flowers crossed	Ovary swelling	No. of seeds	No. of seeds germination	Seed - setting rate	Germination rate
H. fulva × 'siyuehua'	267	25	173	36	64%	21%
H. fulva × *H. lilioasphodelus*	412	69	453	208	32%	45%

Flower opening and closure in hybrids

H. fulva flower opening time is 4:00–8:00, and the florescence of a single flower lasts 13 h; the flower opening time of *H. lilioasphodelus* is 17:00–19:00, and the florescence of a single flower lasts 13 h; and 'siyuehua' flower opening time is 17:00–18:30, and the florescence of single flower lasts 11 hours. For flower opening time, the F1 populations of the two hybrids were nocturnal-flowering. The flower opening time of the F1 generation of *H. fulva* × *H. lilioasphodelus* was between 17:00–23:15 (Table 2), with two peaks at 17:00–18:45 and 21:00–23:15. Twenty-one plants opened at 17:00–18:45, and 9 plants flowered at 21:00–23:15. The flower opening time of the F1 population hybrids of *H. fulva* × 'siyuehua' was 17:45–23:30 (Fig. 1). For flower closing time, the progenies of hybrids were extremely variable. The flower closing time of *H. fulva* × *H. lilioasphodelus* was 3:00–10:15. Most of the flowers started closing at 3:00–5:30. In the cross of *H. fulva* × 'siyuehua', flower closing time was 10:00–12:30 (Fig. 2). The florescence of a single flower of the F1 progeny of *H. fulva* × *H. lilioasphodelus* lasted 6.5–13.5 h. The *H. fulva* × 'siyuehua' progenies' single-flower durations were 11.5–19.5 h. The single flower duration of the hybrids between diurnal and nocturnal flowers extended with a delay of closure time.

Table 2
Opening and closure times in 24 combinations.

Cross combinations	Opening time	Closing time	florescence of single- flower /h
H. lilioasphodelus	17:00–19:00	7:30 – 8:30	13
'siyuehua'	17:00–18:30	2:30 – 4:00	11
H. fulva	4:00–8:00	19:00–21:00	13
H. fulva ×*H. lilioasphodelus*	17:00–23:15	10:00–16:30	9.4
H. fulva × 'siyuehua'	17:00–23:30	10:00–12:30	14.9

Variation of morphological characters

The flower color of *H. fulva* is orange, and those of *H. lilioasphodelus* and 'siyuehua' are yellow. The progeny flower color of the two groups was orange, similar to that of *H. fulva* (Fig. 3), and the flower color was greatly affected by the female parent. In the *H. fulva* × 'siyuehua' group, the F1 generation showed variability in scape height. For example, the highest scape was 129.50 cm in height, while the shortest scape was 46 cm in height (Table 4). The traits with large variation coefficients concentrated in the number of flower buds per scape was 50.06 and 65 respectively. The average flower buds per plant in the cross of *H. fulva* and *H. lilioasphodelus* was 19.9 (Table 3); the number of flower buds significantly increased. There were two plant types in the F1 population. One had overlapping basal leaves, was flattened upwards, and had a leaf width of approximately 1.5 cm (Fig. 4. d). The plant type was similar to the daylily (Fig. 4. b). The other had flat basal leaves, the leaf was flattened on both sides, and had a leaf width larger than 2 cm (Fig. 4. c). The type was similar to the female parents *H. fulva* (Fig. 4. a). The ratio of the type numbers did not deviate from 1:1. These findings indicate that only a few genes have strong phenotypic effects on the determination of plant type in *Hemerocallis*.

Table 3

Phenotypic statistic of *H. fulva*, *H. lilioasphodelu* and their F1 generation

Characters	*H. fulva*	*H. lilioasphodelus*	F1	CV (F1, %)
PH (cm)	56.2	53.61	43.57 ± 1.50	36.00
SH (cm)	82.7	86.47	83.48 ± 3.49	19.9
LL (cm)	73.00	80.97	71.88 ± 1.76	17.07
LW (cm)	1.93	1.30	1.74 ± 0.08	19.71
LN	12.00	8.00	10.90 ± 0.27	15.98
FN	13.00	15.00	19.19 ± 2.31	50.06
FD (cm)	9.30	8.74	10.00 ± 0.42	13.36
PL (cm)	6.93	7.45	8.00 ± 0.36	18.90
PW (cm)	2.60	1.56	2.2 ± 0.39	20.59
CL (cm)	7.10	7.35	7.94 ± 0.10	18.90
CW (cm)	1.60	1.26	1.27 ± 0.38	18.50

Statistics were collected from 30 random individuals in each population. *PH* = plant height; *SH* = scape height; *LL* = leaf length; *LW* = leaf width; *LN* = number of leaf; *FN* = number of flower per; *FD* = flowers diameter; *PL* = petal length; *PW* = petal width; *CL* = calyx length; *CW* = calyx width

Table 4
Phenotypic statistic of *H. fulva* and ‘siyuehua’ and their F1 generation

Characters	*H. fulva*	‘siyuehua’	F1	CV (F1, %)
PH (cm)	56.20	52.91	43.81 ± 3.17	27.77
SH (cm)	82.70	81.34	78.57 ± 6.00	27.96
LL (cm)	73.00	77.90	69.03 ± 2.90	19.76
LW (cm)	1.93	1.97	2.12 ± 0.17	25.20
LN	12.00	10.00	9.71 ± 0.83	37.74
FN	13.00	25.24	16.48 ± 0.17	65.00
FD (cm)	9.30	12.20	11.17 ± 1.37	18.58
PL (cm)	6.93	10.17	9.29 ± 0.64	19.82
PW (cm)	2.60	1.70	2.49 ± 0.61	18.79
CL (cm)	7.10	9.67	9.08 ± 0.12	20.90
CW (cm)	1.60	1.20	1.62 ± 0.62	21.53

Chromosome counting in hybrids

Chromosome preparation showed that *H. fulva* is triploid with 33 chromosomes. *H. lilioasphodelus* and ‘siyuehua’ are diploids with 22 chromosomes. The chromosome preparation of F1population showed variability. 30 progeny plants from two hybrids groups were tested. Chromosome preparation showed that the progenies of these 3x × 2x crosses were predominantly aneuploid with chromosome numbers from 17 to 33(Fig. 5; Table 5). Based on the results, it was derived that the functional eggs contributed by triploids in 3x × 2x crosses were predominantly aneuploid.

Table 5
Chromosome numbers of the progenies obtained from triploid × diploid crosses showing the contribution by the male and the female.

Chromosome numbers	Contributed by male	Contributed by female	Number of plants
17	11	6	1
22	11	11	2
24	11	13	3
25	11	14	2
26	11	15	3
27	11	16	2
28	11	17	9
29	11	18	2
30	11	19	2
31	11	20	1
32	11	21	2
33	11	22	1

Discussion

Progeny seed germination and ploidy state analysis

The triploid *H. fulva* was the female parent, and the fruits of both combinations were swelling and seed-bearing after pollination. This shows that the female gametes of triploid *H. fulva* can be successfully fertilized by diploid daylilies, and that the hybridization obstacle may occur after fertilization. As can be seen from the differences in fruit swelling rate and seed setting, both combinations had high abortion rates. The difference in the number of seeds obtained and the germination rate of seeds indicated that the cause of fruit abortion may be related to the abnormal meiosis of triploid *H. fulva* megaspores. The unequal segregation of chromosomes during meiosis in triploid *H. fulva* results in a mismatch of chromosome numbers between embryo and endosperm, and the dysplasia of endosperm. The Endosperm Balance Number (EBN) hypothesis states that in the endosperm of hybrids, the endosperm can develop normally only when the genetic composition ratio of the female parent and the male parent is 2:1 (Johnston et al. 1980). In theory, when the parental ploidy is different, the ratio of the genome of the female to the male in the endosperm is not 2:1, fruit abortion occurs. The ploidy of endosperm is the primary factor in seed development or abortion (Haig et al. 1991, Birchler et al. 1993, Johnston et al. 1980). Triploid *H. fulva* is generally highly sterile because of the unequal segregation of chromosomes during meiosis. An analysis of the microsporogenesis chromosome pairing of *H. fulva* from different

populations showed that the chromosome pairing in metaphase I of meiosis is very high. Although monovalents and bivalents are formed, trivalents are the majority. Thus, the pollen of triploid *H. fulva* is sterile (Mao 1988). In this study, two hybrid groups of seeds and plants were obtained. The results show that unlike the sterile pollen, megaspores are fertile.

The megasporogenesis of triploid *H. fulva* may *Fritillaria*-type plants, such that a secondary nucleus of *Fritillaria*-type embryo sac is equal to the sum of the nuclei of four daughter cells (Maheshwari 1948). Because the sum of nuclear DNA of the four meiotic aneuploid daughter cells also originates from the duplication of the nuclear DNA of a megasporocyte, the secondary nucleus of triploid *H. fulva* may be hexaploid (6x), based on the theory that the endosperm of 3x × 2x crosses is 7x (Fig. 6). Based on megasporogenesis of *Fritillaria*-type plants, the endosperm of 3x × 2x is euploid, and it is considered that the euploid endosperm of 3x × 2x crosses is responsible for the survival of the aneuploid embryos resulting from these crosses. According to megasporogenesis, a triploid *Fritillaria*-type plant produces an aneuploid egg cell (1.5x) and hexaploid secondary nucleus (6x) (Maheshwari 1948). So in our study the number of plants with 28 chromosomes in the F1 progenies was the largest.

When the triploid *Lilium* was crossed as the female with diploid and tetraploid lilies, the majority of progenies were aneuploid (Natenapit et al. 2010, Barba-Gonzalez et al. 2006). The progenies of triploid × diploid/tetraploid (3x × 2x/4x) crosses in *Lilium* were aneuploid. Based on megasporogenesis, it has been deduced that the endosperm of 3x × 2x is 7x and that of 3x × 4x is 8x in *Lilium* and it was concluded that the aneuploid embryo survival of 3x × 2x/4x in *Lilium* is the result of the euploid endosperm (Zhou et al. 2011). In *Tulipa* crosses, most progenies in 2x × 3x crosses were triploid with the exception of a small number of aneuploids (3x + 1 and 3x − 1) or 4x and 5x progenies. In this case, the triploid female had contributed predominately diploid, triploid, or near-triploid gametes (3x) (Marasek-Ciolakowska et al. 2014). The difference in results could be explained by the different type of embryo sacs. In the genus *Tulipa*, some species follow the *Fritillaria* type, a few the *Adoxa* type, and one the *Drusa* type (Maheshwari 1948). In contrast, according to a recent study, 3x × 2x hybrids usually produce euploids such as diploid, tetraploid, and pentaploid progenies. In a study on the cross between diploid (2n = 18) sexual *Erigeron strigosus* and triploid (2n = 27) agamospermous *Erigeron annuus*, the distribution of F1 chromosome numbers is bimodal, centering on diploid and triploid but with an underrepresentation of diploids (Noyes 2000). Based on our results, we suggest that *H. fulva* could be used as a female to be crossed with an appropriate diploid male. The progeny of triploid and diploid hybrids are mainly aneuploid, while a few were diploid and triploid. It is well-known that a change in chromosome number can bring about large variation in ecological characteristics.

Progeny flower opening and closing time

The flower opening time of triploid *H. fulva* is 4:00–7:00, and it is fully opened at 8:00. The flowering time of the two daylilies is 17:00–18:00, and they are fully opened at 20:00. The results show that *H. fulva* flower opening time is during the dark-to-light transition, while daylily plants bloom before the light-to-dark transition. In different latitudes, the two species maintain the same opening method. This shows that regardless of when dusk begins, diurnal-flowering plants can accurately predict the beginning of

dawn and perform flower opening activities suitably. Likewise, regardless of when dawn begins, nocturnal-flowering (daylilies) plants will accurately predict the time of dusk, and suitably perform flower opening activities. In an analysis of the flower opening time of *H. fulva* and 'siyuehua', two species were transferred to 12 h dark-12 h light conditions after 12 h light-12 h dark treatment, and after 24 h light-to-dark cycle reversal treatment (12D-12L), the flower opening time and closing time also showed an inverted rhythm, and still maintains periodicity, which is consistent with that of the natural environment (Ren 2020). The flower opening time of the two species were affected by the external light and synchronized with the external light-dark transition, indicating that the internal physiological rhythms of the two species were reset by the light-to-dark or dark-to-light transitions. In a study on *Pharbitis nil*, there were two distinct clock systems in *Pharbitis*. The circadian system strongly reset by the transition of light-dusk, enables generation of dusk-set circadian rhythms throughout the year without being affected by changes in the time of dawn (Hayama et al. 2018, Hayama et al. 2007).

In this study, the flower opening time of the F1 populations of *H. fulva* and *H. lilioasphodelus* was 17:00–23:15. The flowering time of the combination of *H. fulva* and 'siyuehua' was 17:45–23:30. The plants of the two hybrids' offspring were nocturnal-flowering (Fig. 1). This suggests that flower opening time in the evening is partially dominant to that in the morning. The timing of flower opening and closing are at specific times. The fact that these rhythms are maintained under constant light or dark conditions suggests a circadian clock involvement. An analysis of the temporal floral movements in *Arabidopsis thaliana* shows that the circadian clock induces flower opening redundantly with unknown light-sensing pathways, and flower closing is completely dependent on clock control (Muroya et al. 2021). Transcriptome sequencing analysis of the F1 populations of 'naomi ruth' and 'siyuehua' identified 23 key circadian clock genes that were differentially expressed in different daylilies. The results showed that the circadian clock input, core and output genes formed a coordinated and antagonistic regulatory network, which was highly correlated with the regulation of different flower opening time traits of daylilies (Ren et al.2021). These studies proved that the circadian clock controls flower opening time.

Aneuploidy, especially trisomic plants derived from 3x × 2x crosses, is useful in genetic studies locating genes and linkage groups on particular chromosomes. The change of chromosome number can bring about large variation in morphological, physiological, and ecological characteristics. The triploid daylily may be a good potential source for breeding of aneuploid daylily cultivars. In the progeny chromosome of the two hybrid combinations, the male daylily provides 11 chromosomes, and the female *H. fulva* provides 6–22 chromosomes. Even if the female *H. fulva* provides a higher number of chromosomes than the father in some plants of the offspring, the flower opening time was always at night. This showed that flowering at night was dominant. This result was different from a Japanese study. Triploidy contributes significantly to the rate of autopolyploid formation regardless of mating system, and to allopolyploid formation in outcrossing taxa.

Based on our results, we suggest that triploid *H. fulva*, regardless of their male sterility, could be used as a female to cross with appropriate diploid male. We also conclude that the progenies of 3x × 2x are aneuploid in *Hemerocallis*. It is well known that a change in chromosome number can bring about large

variation in morphological, physiological, and ecological characteristics. This research provides genetic mechanisms of circadian flowering and has shown that flower opening time in the evening is partially dominant to that in the morning. In addition, flowering is also influenced by genes relating to environmental conditions. The triploid *H. fulva* may be a good potential source for breeding aneuploid daylily cultivars.

Declarations

Funding

This research was supported by the National Natural Science Foundation of China (No. 31971706),which named 'The Mechanism of Circadian Clock Regulation on Flower Opening Time in Hemerocallis'.

Competing Interests

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

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Chunjing Guan. The first draft of the manuscript was written by Chunjing Guan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

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

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Figures

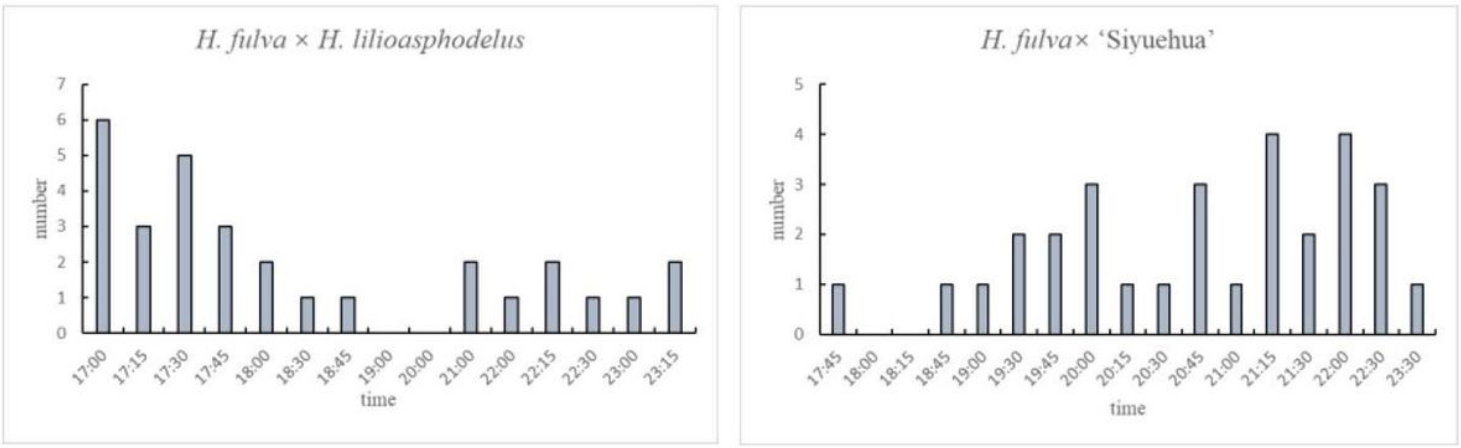

Figure 1

Distribution patterns of flower opening time in F1 hybrids

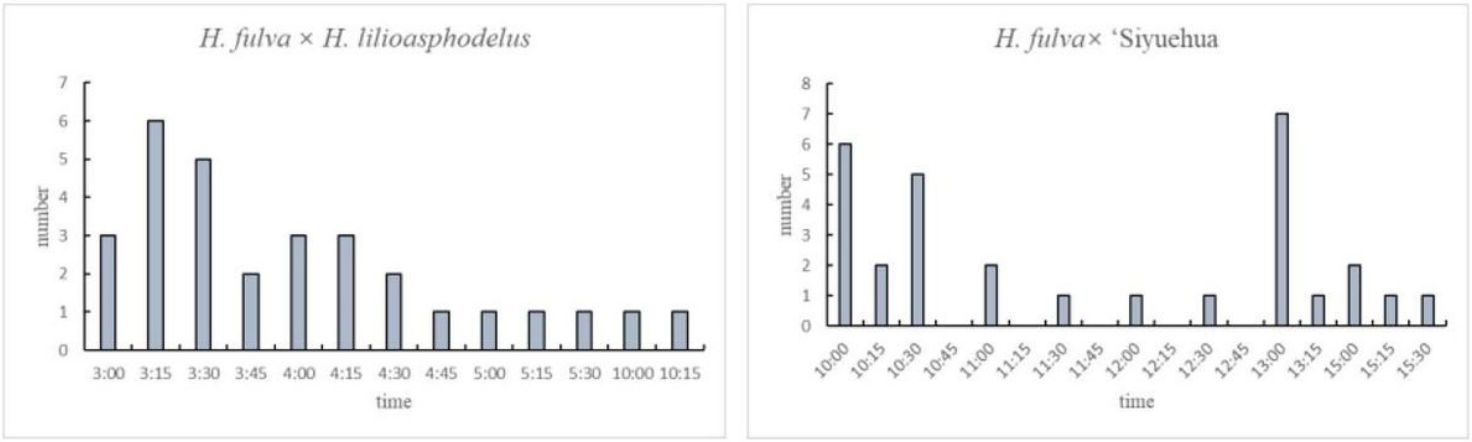

Figure 2

Distribution patterns of flower closing time in F1 hybrids

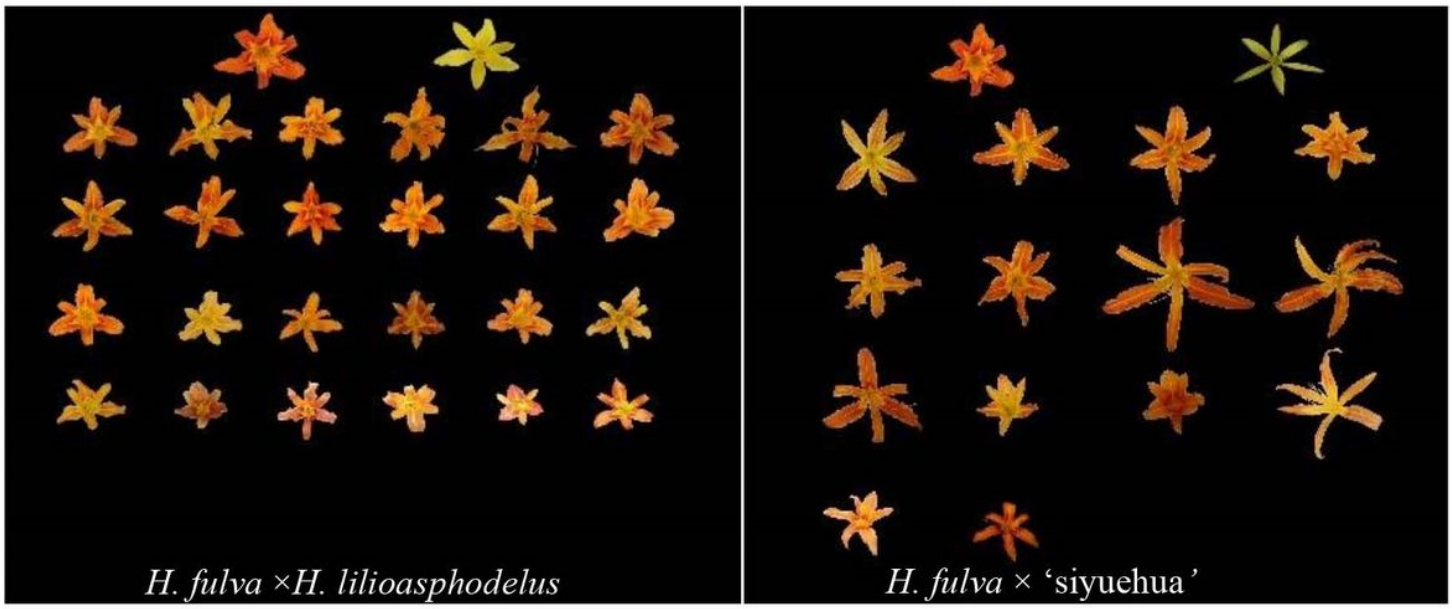

Figure 3

Individuals with novel flower colour in F1 (bar 1 cm)

Figure 4

The plant type of the parents and F1 populations

a: *H. fulva* **b:** *H. lilioasphodelus* **c, d** Individuals of F1

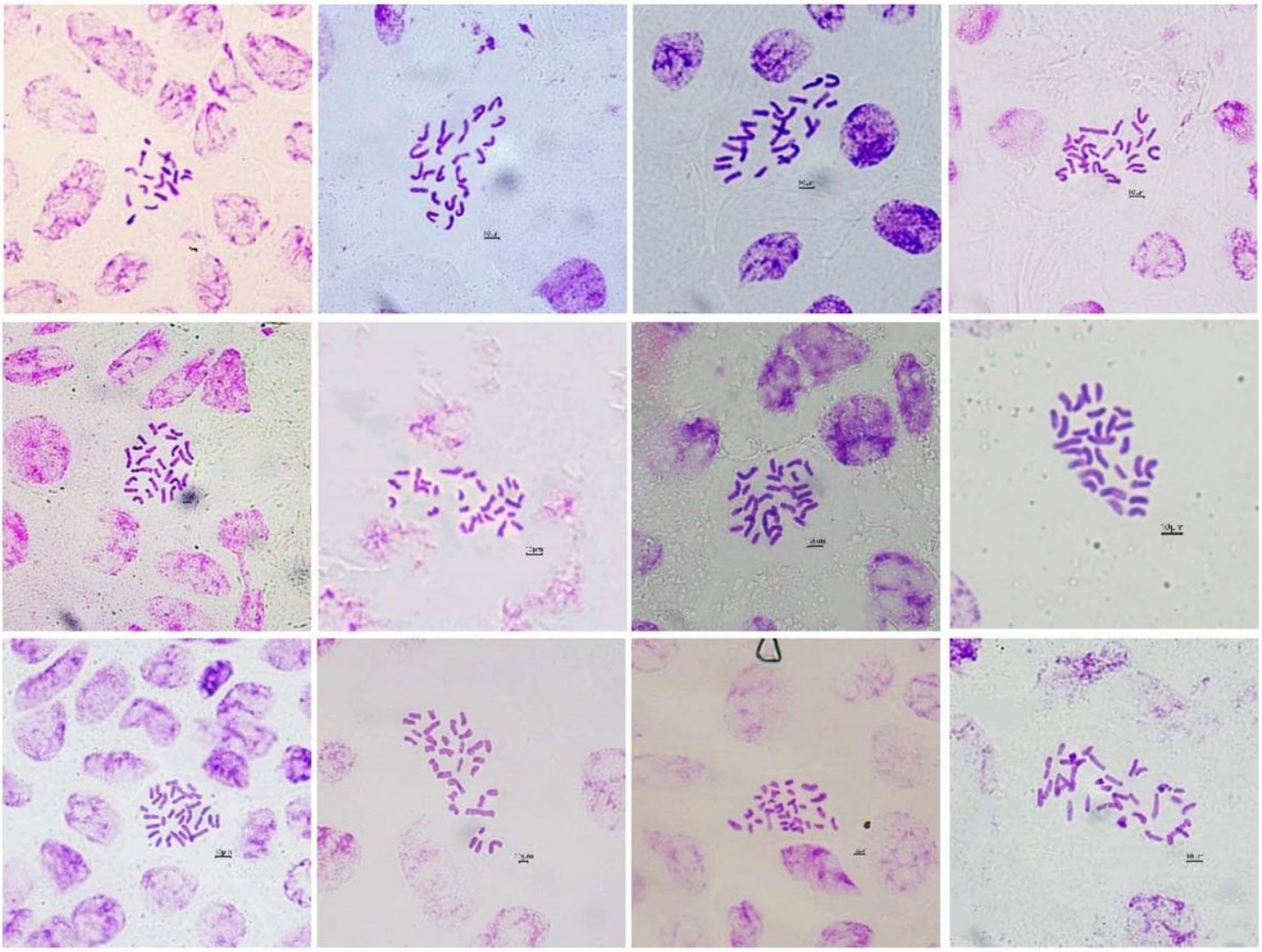

Figure 5

The chromosomes at mitotic metaphase from root tips of the representative 3x · 2x progenies.

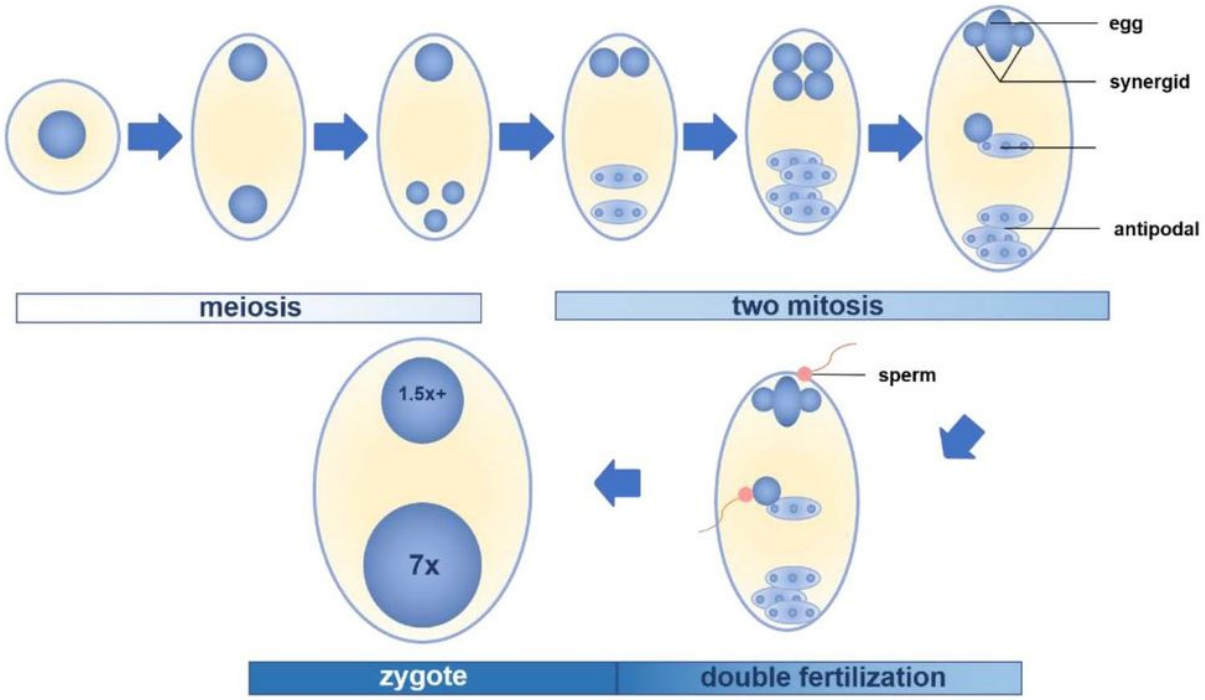

Figure 6

Ideogram of megasporogenesis of Fritillaria-type embryo sac and double fertilization in *H. fulva*.