Revealing the abnormal meiosis and the variation of the functional female gametes of aneuploid lily (Lilium) using genomic in situ hybridization (GISH)

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Research Article

Abstract

Aneuploid lilies (*Lilium*) could be obtained from the LAA/LAAA × AA/AAAA hybridization; however, the characteristics of their meiosis and fertility has not been reported. In this study, an aneuploid lily, J1614, was extensively investigated for its microsporogenesis, fertility and functional eggs using conventional and modern cytogenetic methods. The results indicated that J1614 was an aneuploid Longiflorum-Asiatic (LA) lily (2n = 48 = 7L + 39A + 2L/A) while 'Pearl Jason' was an autotetraploid Asiatic lily (2n = 48A); L-chromosomes of J1614 usually formed univalent while A-chromosomes associated not only predominantly trivalents, but also tetravalents, bivalents, and even univalent at metaphase I as well; clearly, both univalents and other associated chromosomes were separated and moved to opposite poles at anaphase I; besides, lagging chromosomes and micronuclei were observed during microsporogenesis. Hybridization showed that J1614, regardless of its male sterility, had better partial female fertility when tetraploid a lily was used as male than when a diploid as male. The nine seedlings of J1614 × AAAA were all aneuploid with variable total chromosomes ranging from 46 to 53, meaning that the functional eggs produced by J1614 contained variable chromosomes ranging from 22 to 29. Based on the present results, we concluded that once good lines are selected from aneuploid lilies, they may not only become cultivars through vegetative propagation, but also become parents to breed new aneuploids and realize introgression breeding. In addition, the mechanism of abnormal meiosis of aneuploid lilies was hypothesized.

Introduction

Lily, one of the most important bulb flowers worldwide, refers to the genus *Lilium* of the family Liliaceae and its derived cultivars. The genus consists of 100 wild species, and they are classified into seven sections (De Jong 1974). Modern lily cultivars, such as Asiatic (A), Longiflorum (L), Oriental (O), and Trumpet lily (T) are breed from intra-section hybridization; meanwhile the cultivar groups, like LA, LO, OA, OT etc., are obtained from inter-section hybridization (Van Tuyl et al. 2000; Van Tuyl and Arens 2011). Most intra-sectional lilies are diploid (2n = 2x = 24), while most inter-sectional lilies are allotriploid (2n = 3x = 36), such as LAA, LOO, OTO, etc., and a few are odd-allotetraploids (2n = 4x = 48), like LAAA and LLLO (Zhang et al. 2012; Zhou et al. 2013). Although triploid and odd-allotetraploid lilies have abnormal meiosis during gametogenesis and they are male sterile, they could be used as female to cross with suitable males to produce aneuploid progenies (Lim et al. 2003; Barba-Gonzalez et al. 2006; Zhou 2007; Khan et al. 2009, Zhou et al. 2011, 2012; Xiao et al. 2019). Most aneuploid lilies grow weak or die early because of chromosomal or gene unbalanced, fortunately some are vigor and can be as cultivars since their genes are rebalanced in the aneuploid lilies (Zhou 2011-12; 2014). They are also used for lily introgression breeding (Zhong et al. 2022). However, their characteristics of meiosis and functional gametes have not been systematically studied.

In this study, an aneuploid lily was extensively investigated for its microsporogenesis, fertility, and genome composition of its female functional gametes, and the significance of aneuploid on lily introgression breeding was discussed.

Materials and methods

Plant materials

An aneuploid lily, coded J1614, was used as female parent. The aneuploid was obtained by 'Honesty' (LAAA) × 'Nello' (AAAA). Two Asiatic lilies were as males – one is diploid, J1707 (AA), the other is a tetraploid, 'Pearl Jason' (AAAA). A Longiflorum lily 'White Fox' (LL) was used to extract its genomic DNA as probe for genomic in situ hybridization (GISH). All lilies were grown in the greenhouse of Jiangxi Agricultural University.

Microsporogenesis

Microsporogenesis was analyzed referring to Xiao et al. (2019). When the flower buds were 28–32 mm, their anthers were treated with a fixative, containing three parts ethanol and one acetic acid by volume, at least 30 min, then, some pollen mother cells (PMCs) were mixed with a drop of 2% Carbol Fuchsin (Beijing Solarbio Science & Technology Co. Ltd.) on a slide, and then covered with a square cover glass for check under a light microscope (ZEISS Scope. A1).

Chromosome preparation at metaphase I and anaphase I

The meiotic chromosome slides were prepared almost the same as described above, except that a drop of 2% Carbol Fuchsin was replaced by a drop of 45% acetic acid. A gentle squash was done with thumb after the slides were covered; then, they were stored at -80 °C for about 1 h, following their covers were removed with a knife, and then immersed in ethanol for 1 min, and then dried on a slide rack. The slides were checked with a phase contrast microscope (ZEISS Scope. A1).

The types and germination of pollen grains

Fresh pollen grains were scattered on a medium, containing100 g/L sucrose, 5 g/L bacteriological agar, 20 mg/L H_3BO_3, and 200 mg/L $Ca(NO_3)_2$, and cultured at 25 °C for 4h, then observed under microscope (ZEISS Scope. A1).

Pollination and embryo rescue

The pollination and embryo rescue were referred to Zhou et al. (2012). When the flowers of J1614 were open, they were pollinated with fresh pollen of 'Pearl Jason' and J1707 using normal pollination. Having pollinated, the stigmas were wrapped with aluminum foil. When the fruits matured, they were harvested. In a fume hood, their embryo sacs or developed ovules were selected out and put on lily embryo rescue medium (pH = 5.8), containing 2.2 g·L^{-1} MS (Duchefa Biochemie), 60 g·L^{-1} sucrose and 4 g·L^{-1} gelrite (Duchefa Biochemie). They were stored in paper cases at 25 °C for 40–60 d and then transferred to lily propagation medium (pH = 5.8), containing 2.2 g·L^{-1} MS, 50 g·L^{-1} sucrose and 4 g·L^{-1} gelrite at 25 °C and 2000 lx light intensity for about 10 weeks.

Mitotic chromosome preparation

The mitotic chromosomes were prepared according to Wu et al. (2021). The root tips were cut off; and incubated in 0.7 mM cycloheximide (Duchefa Biochemie) at room temperature for 4 h, then stored in a fixative at least 30 min. The root tips were treated with 1% (w/v) cellulase RS (Duchefa Biochemie) and 1% (w/v) pectinase Y23 (Duchefa Biochemie) mix, at 37 °C for 1 h. Their meristem was mixed with 16 μL 45% acetic acid on a glass slide, covered with a cover glass and then gently squashed. The slides were checked with a phase contrast microscope (ZEISS Scope. A1).

In situ hybridization

5S rDNA and 45S rDNA were used as probe to analyze mitotic chromosomes of 'Pearl Jason' using fluorescence in situ hybridization (FISH) according to Lan et al. (2018).

The genomic DNA of 'White Fox' (LL) was extracted with CTAB method, and then labeled with biotin using nick translation kit (Roche 11745824910) as probe, meanwhile, HS (herring sperm) DNA, cooked for 30 min at 100 °C, as block for genomic in situ hybridization (GISH).

The hybridization mix (40μL) consisted of 50% deionized formamide, 10% dextran sulphate (Amresco 0198), 2x SSC (0.3M NaCl plus 30 mM sodium citrate, pH 7.0), 0.25% SDS, 25-50ng probe DNA, 25-50ng probe and 1−3μg block DNA. In situ hybridization was performed according to the method described by Barba-Gonzalez et al. (2004). Biotin signal was detected and amplified with Streptavidin-CY3 (Invitrogen, Camarillo, CA) and Biotinylated anti-Streptavidin (Vector Laboratories, Burlingame, CA). After mounted with VECTASHIELD (H-1200, Vector Laboratories, Burlingame, CA), the slides were observed under a fluorescence microscope (ZEISS Scope. A1).

Results

Genome composition of J1614 and 'Pearl Jason'

J1614 grew well and its flowers were deep red (Fig. 1a). It had seven L-chromosomes, two L/A recombinant chromosomes and 39 A-chromosomes, totally 48 chromosomes (Fig. 1b). The results showed that the lily was an aneuploid or pseudotetraploid as its total chromosome number was 48. Its formula was written as 2n = 48 = 7L + 39A + 2L/A.

As shown in Fig. 2, 'Pearl Jason' had orange-yellow flowers with strong reflex tepals and 48 A-chromosomes (2n = 4x = 48A), and FISH karyotype clearly indicated that it was autotetraploid Asiatic lily.

Microsporogenesis of J1614

As shown in Fig. 3, its meiosis was abnormal at metaphase I and anaphase I. (1) At metaphase I (Fig. 3a & b), the A-chromosomes were predominantly associated into trivalents (white "III"), and bivalents (white "II"), tetravalents (white "IV") and even univalents (white "I") were also rarely possible; however, most of

the L-chromosomes did not pair with any other L- or A- chromosomes and exist as univalents (red "I"), except that a few formed bivalent or multivalents with L- or A- chromosomes (white "I" + red "I" or "II"). (2) At anaphase I (Fig. 3c-e), clearly, most of the sister L-chromosomes were separated and move to two opposite poles; seemingly, most of the associated A-chromosomes also disjoined and moved to two poles; a few recombinant chromosomes were also observed, conforming that a few L- and A-chromosomes associated at metaphase I. (3) Microspores contained different free micronuclei (Fig. 4a and b) which be consequences of lagging chromosomes or acentric fragments. The types and sizes of their pollen grains were variable and near all of them did not germinate (Fig. 4c and d), showing that J1614 was highly male sterile.

Female fertility of J1614

J1614 was male highly sterile; however, its fruits developed well when it was used as female to cross with diploid or tetraploid Asiatic lily. The developed fruits between the two crosses seemingly similar, but their compatibilities were different. 12.5 embryo sacs were isolated from J1614 × AA per fruit and no seedling was obtained, while 60 embryo sacs were isolated from J1614 × AAAA and 9.5 seedlings were obtained per fruit. The results indicated that the aneuploid lily, J1614, regardless of its male sterility, had partial female fertile and its compatibility with tetraploid Asiatic lily was much better than that with diploid.

Genome composition of functional eggs of J1614

GISH showed that the nine progenies of J1614 × AAAA had variable total chromosomes ranging from 46 to 53, and they consisted of variable L-, A-, and L/A recombinant chromosomes (Fig. 5, Table 1). L-chromosomes or L-fragments in the progenies were less than those in J1614, suggesting that aneuploid lily would be a way to realize lily introgression breeding. Since tetraploid Asiatic lily 'Pearl Jason' contributed 24 A-chromosomes, the genome composition of functional egg cells produced by aneuploid J1614 were deduced and shown in Table 1. Obviously, the recombinant chromosome numbers in the functional eggs were agreement with the association between L- and A-chromosomes at metaphase I during microsporogenesis. The results indicated that aneuploid lily can produce functional aneuploid eggs regardless of its male sterility.

Table 1

The genome compositions of the progenies of J1614 × AAAA and those of functional eggs produced by J1614. The number of L-chromosomes, A-chromosomes, and L/A recombinant chromosomes are represented by L-, A-, and L/A-, respectively.

Progenies	Chromosomes of progenies				Chromosomes of functional eggs			
	Total	L-	A-	L/A-	Total	L-	A-	L/A-
20106-1	53	3	47	3	29	3	23	3
20106-4	49	3	43	3	25	3	19	3
20106-5	47	3	42	2	23	3	18	2
20106-7	50	2	48	0	26	2	24	0
20106-8	49	5	42	2	25	5	18	2
20106-10	51	5	45	1	27	5	21	1
20106-13	49	2	45	2	25	2	21	2
20106-15	46	1	44	1	22	1	20	1
20106-17	51	5	44	2	27	5	20	2
Average	49.44	3	44.66	1.78	25.44	3	20.66	1.78

Discussion

Kinetochore and chromosome separation during abnormal meiosis

The present study and previous reports show that all the abnormal meiosis have interesting features although each have some its own characteristics in distant F_1 hybrids (Zhou et al. 2008; Luo et al. 2013), allotriploid (Cui et al. 2022) and odd-allotetraploid lilies (Xiao et al. 2022). 1) Univalents are quite common at metaphase I and their sister-chromatids usually separated and moved to the two opposite poles at anaphase I. At metaphase I, in distant F_1 hybrids, such as LA and OA, most homoeologous chromosomes do not pair and thus form univalent (Barba-Gonzalez et al. 2004; Zhou et al. 2008); In allotriploid (LLO), all the homologous L-chromosomes prefer to pair each other and O-chromosomes tend to form univalent (Cui et al. 2022); Similarly, odd-allotetraploid (LAAA), all the homologous A-chromosomes prefer to associate multivalents and L-chromosomes tend to be univalent (Xiao et al. 2022). Intriguingly, the sister-chromatids of such univalents are clearly separated and moved to the opposite poles at anaphase I. The same phenomenon is also observed in allotriploid Alstroemeria (Kamstra et al. 2004). This is totally different from normal meiosis I, in which the chromosomes of bivalents separate rather than sister-chromatids. Univalents are commonly reported in plant F_1 hybrids, and they are the reason for 2n gametes resulting from an abnormal meiosis in many plants (Ramanna and Jacobsen 2003), such as Trifolium (Ansari et al. 2022), wheat/rye hybrids (Silkova et al. 2013), etc.

Interestingly, the sister-chromatid separations of univalents, which are formed by premature separation of bivalents at meiosis I, are observed in aging-related oocytes of mice and human, and the cohesion loss and splitting of sister kinetochores are regarded as the reasons for the abnormal phenomenon (Zielinska et al. 2015; Nakagawa and FitzHarris 2017). Akera and Lampson (2016) suggested that sister kinetochores should have been fused during normal meiosis I and sister kinetochores of univalents were split at abnormal meiosis I (Fig. 6(1)). However, according to GISH analysis on lily abnormal meiosis, it is more plausible to explain the abnormal meiosis I in a modified way shown as in Fig. 6(2). This is because a centromere on each chromosome has two kinetochores which are attached to spindle microtubules in normal mitosis; however, during normal meiosis, a centromere on each chromosome of a bivalent only has one kinetochore attached to spindle microtubules (www.wikipedia.org). Possibly, one chromosome has one kinetochore in its centromere, and kinetochore is possibly duplicated accompanying DNA replicating in mitosis (Fig. 6(2d)); however, during normal meiosis I, kinetochore duplication is hindered by synapses (Fig. 6(2e)) or the two duplicated kinetochores on sister chromatids are so close and function as one due to cohesion and chiasmata; and during abnormal meiosis I (Fig. 6(2f)), some bivalents premature and become univalent, or some chromosomes do not pair and remain univalents, and their kinetochores could replicate because no chiasmata or cohesion suppress kinetochore duplication. Since then, it is reasonable that both univalents and bivalents are disjoined and move to opposite poles during abnormal meiosis I in F_1 distant lily hybrids (Zhou et al. 2008). 2) Bivalents are also common in allotriploid lilies, and are also formed in F_1 distant LA or OA hybrids at metaphase I. They are usually disjoined at anaphase I as normal meiosis (Zhou et al. 2008; Xiao et al. 2022; Cui et al. 2022). The similar result is also reported in allotriploid *Alstroemeria* (Kamstra et al. 2004). 3) Trivalents or other multivalents are often found in odd-allotetraploid (Xiao et al. 2022), and occasionally occur in allotriploid (Cui et al., 2022) and distant hybrids (Zhou et al., 2008). They are disjoined evenly or unevenly at anaphase I (Zhou et al. 2015). Besides, lagging chromosomes and micronuclei are common in abnormal meiosis not only in lily (Zhou et al. 2008; Zhang et al., 2017; Cui et al. 2022; Xiao et al. 2022), but also in rice hybrids (Liu et al. 2021), *Saccharum* hybrids (Li et al. 2021), *Populus* hybrids (Wang et al. 2015), and *Musa* (Ahmad et al. 2021).

The partial female fertile of aneuploid lilies

It is confirmed that F_1 distant hybrids can be female parents to be backcrossed and produce allotriploid lilies regardless their male sterility (Zhou 2007; Liu et al. 2021); Similarly, triploid, allotriploid and odd-allotetraploid can be female parents to hybridize with appropriate males though they are highly male sterile (Lim et al. 2000; 2003; Khan et al. 2009; Natenapit et al. 2010; Xie et al. 2010; Zhou et al. 2011, 2012, 2014; Chung et al. 2013; Wang et al. 2015; Suzuki and Yamagishi 2015; Xi et al. 2015; Xiao et al. 2019; Cui et al. 2022). The phenomena seemingly look strange, not only because triploids are usually seedless, but also one plant's male fertility should be similar to its female fertility due to same genetic materials, i.e., same meiosis of gametogenesis. So, why are there such big difference between male and female fertility in these lilies? The question was well explained by comparative analysis between Fritillaria embryo sac and Polygonum embryo sac (Zhou 2007). According to megasporogenesis, in a

polygonum embryo sac, the nucleic DNA of its central cell is twice that of its egg cell, while in a fritillaria embryo sac, the nucleic DNA of its central cell is twice that its somatic cell (Fig. 7) (Zhou 2007; Zhou et al. 2011, 2012). So, for triploid watermelon as an example of polygonum-type plant, in its embryo sac, both egg and central cell are aneuploid, once double fertilized with a diploid, both embryo and endosperm are aneuploid; thus, triploid watermelon is seedless. By contrast, for a triploid lily, in its fritillaria-type embryo sac, its egg is aneuploid, but its central cell is hexaploidy; Once double fertilized with a diploid or tetraploid, endosperm is euploid and it develops well, and then make some aneuploid embryos survival (Zhou 2007; Zhou et al. 2011, 2012). This is the reason why all the male sterile lilies have some partial female fertility. In the present study, the fertility of the aneuploid lily is similar to that of triploid or odd-allotetraploid lilies. Once embryo sacs of an aneuploid lily are double fertilized by diploid or tetraploid male, both embryo and endosperm are aneuploid; surprisingly, some seeds develop well rather than seedless in triploid watermelon. How to explain its partial female fertility? Aneuploid lilies are usually less vigor and die early, however some grow well, like J1614 in the present research, indicating that it has balanced genes though it is an aneuploid. The nucleic DNA of each central cell in its embryo sac is invariably twice that of a somatic cell, meaning that all its central cells have balanced genes. Once fertilized with tetraploid male, its endosperm has the balanced genes and could develop and make aneuploid embryos survival (Fig. 7).

Chromosome numbers of progenies or functional gametes of aneuploid lilies

In the present study, J1614, containing 48 chromosomes, is aneuploid because of its unbalanced chromosomal composition. Its progenies have variable chromosomes, ranging from 46–53, when tetraploid as male. This is like aneuploid progenies obtained from 2x/4x × aneuploid (Zhong et al. 2022) and 3x × 2x/4x hybridizations (Lim et al. 2000, 2003; Khan et al. 2009; Xie et al. 2010; Natenapit et al. 2010; Zhou et al. 2011, 2012, 2014; Chung et al. 2013; Wang et al. 2015; Suzuki and Yamagishi 2015; Xi et al. 2015; Cui et al. 2022). All of them indicate that functional gametes produced by aneuploid or triploid lilies usually have much higher chromosome numbers when tetraploid used as male than when diploid used as male (Lim et al. 2003; Khan et al. 2009; Zhou et al. 2011, 2012; Wang et al. 2015; Xi et al. 2015). Besides, 3x × 4x is much more successful than 3x × 2x in *Lilium* (Zhou et al. 2011, 2012). The present study also shows that aneuploid × 4x is much more successful than aneuploid × 2x in *Lilium*.

Conclusions

Based on the present research and above discussion, it is concluded that lilies have huge chromosomes because of large genomes, which make them ideal to do cytogenetics research; and they produce Fritillaria embryo sacs, which makes F_1 distant hybrids, triploids, odd-allotetraploids; and aneuploid lilies are partial female fertile regardless of their male sterility and thus produce aneuploid again. In addition, the most important for lily breeding: once good lines are selected from aneuploid lines, they may not only become cultivars through vegetative propagation, but also become parents to breed new aneuploids.

Declarations

Acknowledgments

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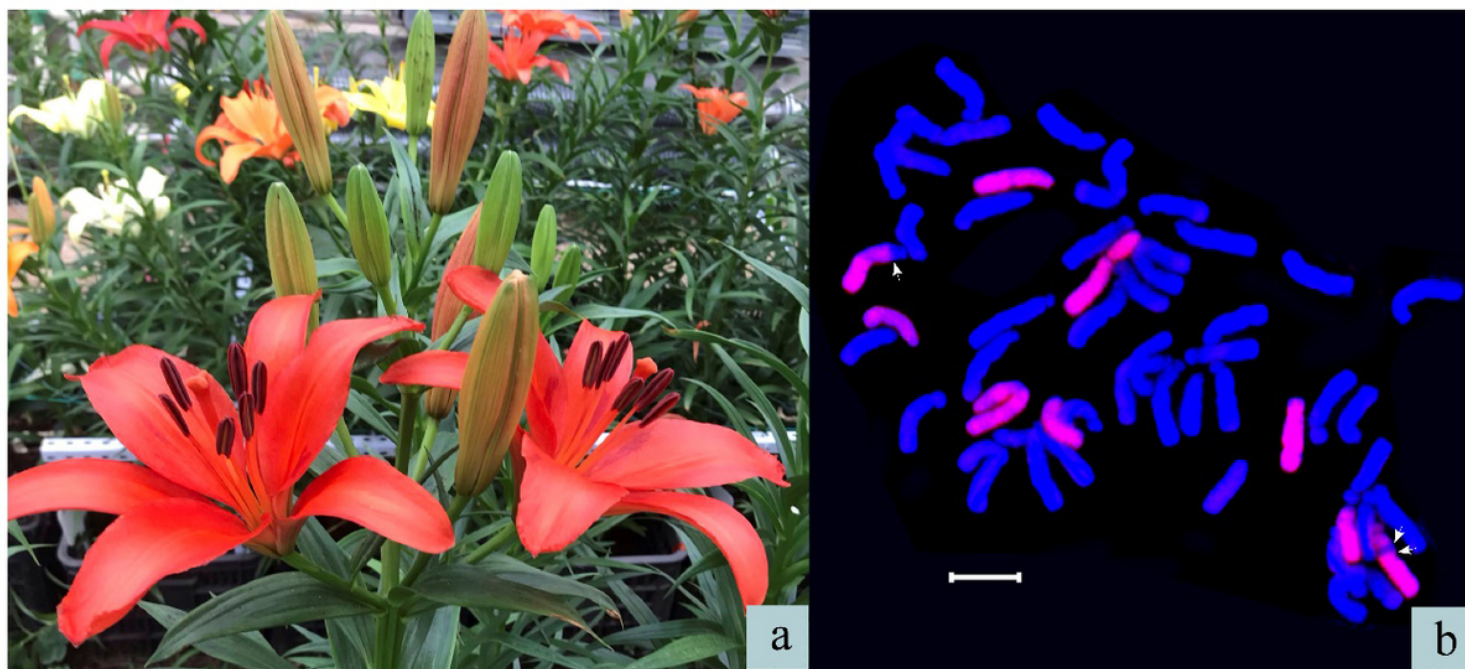
Figures

Figure 1

J1614: (a) flowers and (b) metaphase chromosomes, in which A-chromosomes are dyed blue with DAPI while L-chromosomes red with Cy3 using GISH. Arrows indicate break points of L/A-recombinant
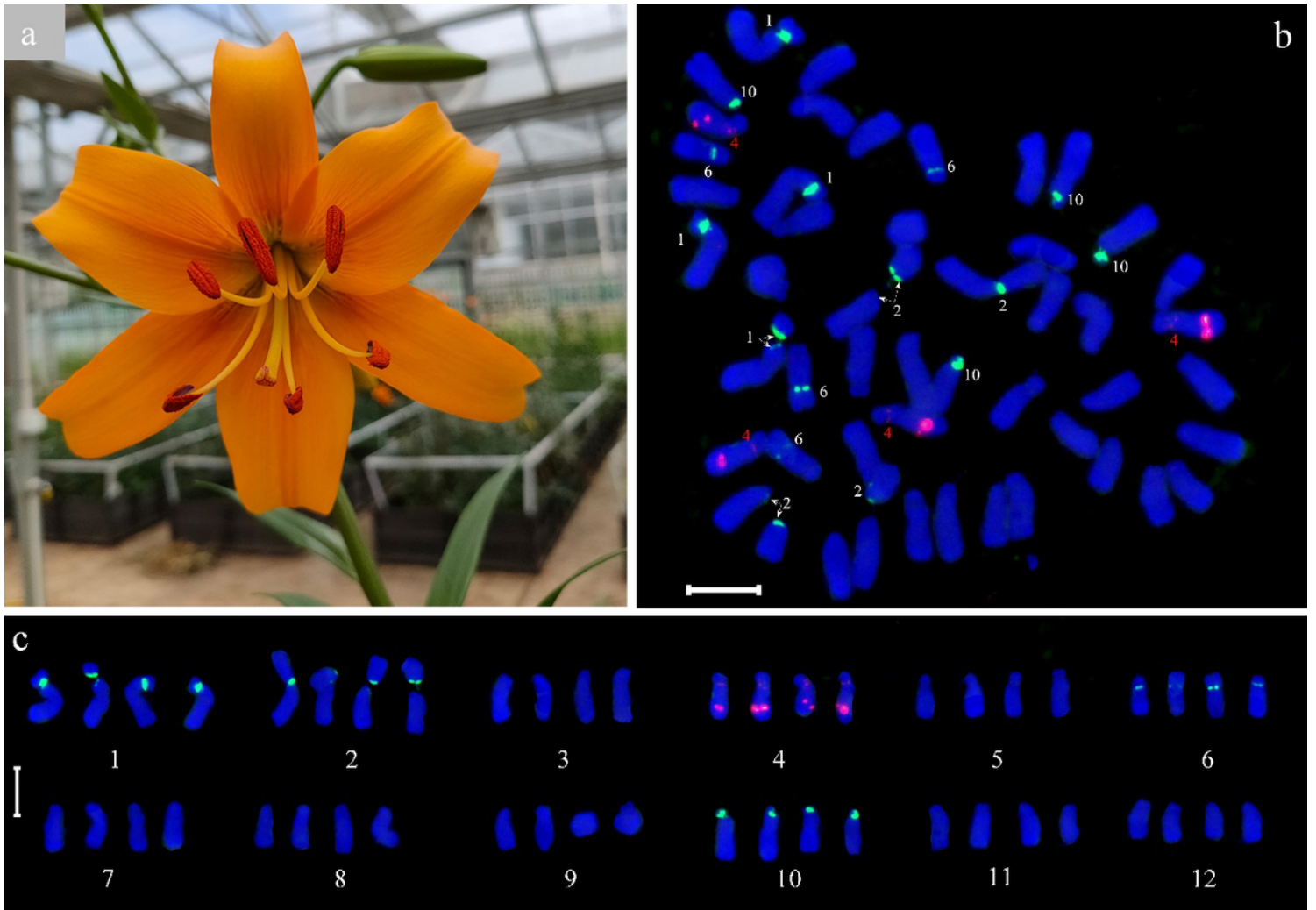
chromosomes. Bar = 20μm.

Figure 2

Asiatic lily 'Pearl Jason': (a) flower, (b) metaphase chromosomes of FISH painting with 5S rDNA (red) and 45S rDNA green), and c) FISH karyotype. Bar = 20μm.
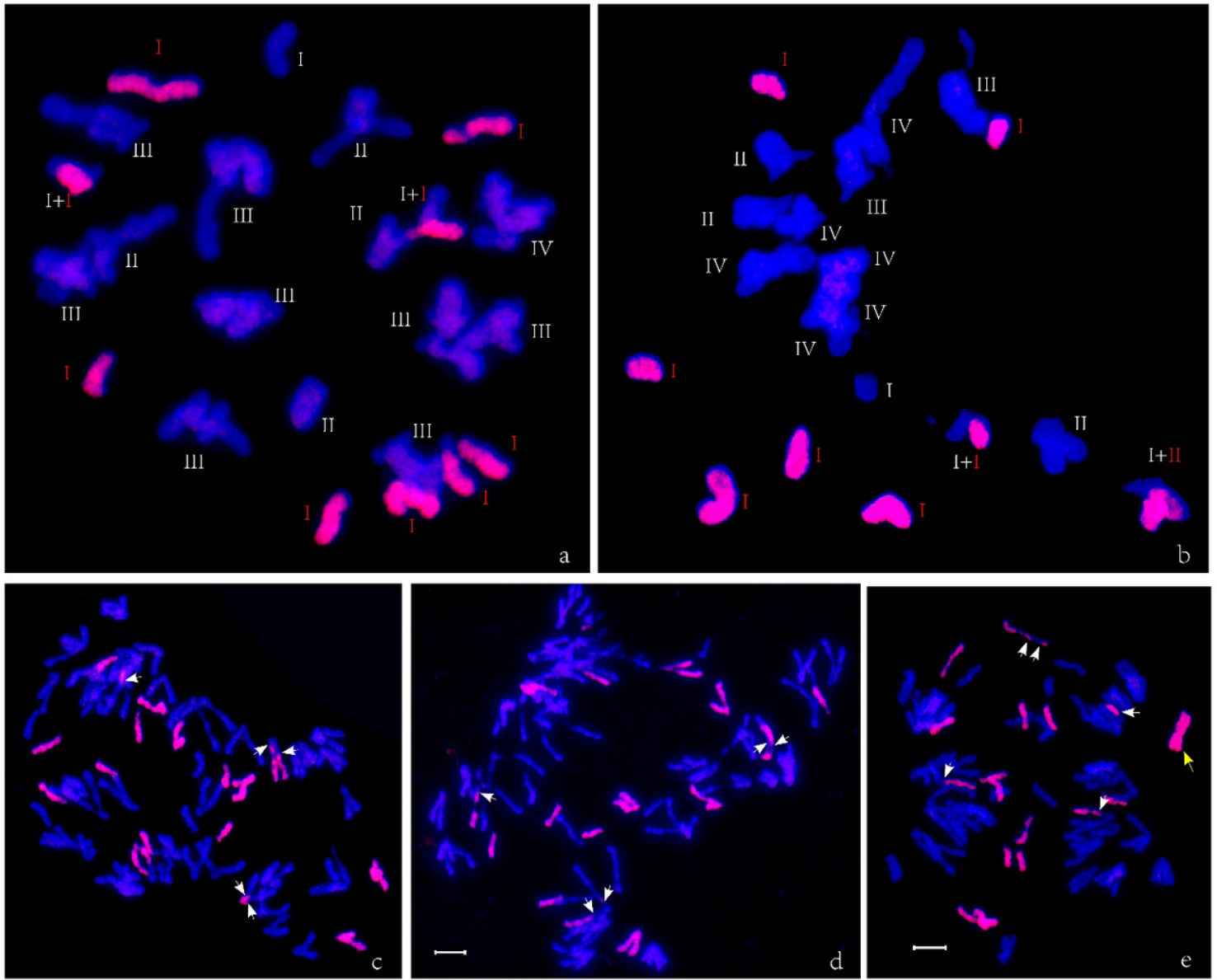
Figure 3

The microsporogenesis of J1614: (a-b) metaphase I, (c-e) anaphase I, in which A-chromosomes are blue and L-chromosomes are red; "I", "II", "III"and "IV"means univalent, bivalent, trivalent and tetravalent, respectively the arrow heads indicate break points of L/A recombinant chromosomes. Bar = 20μm.
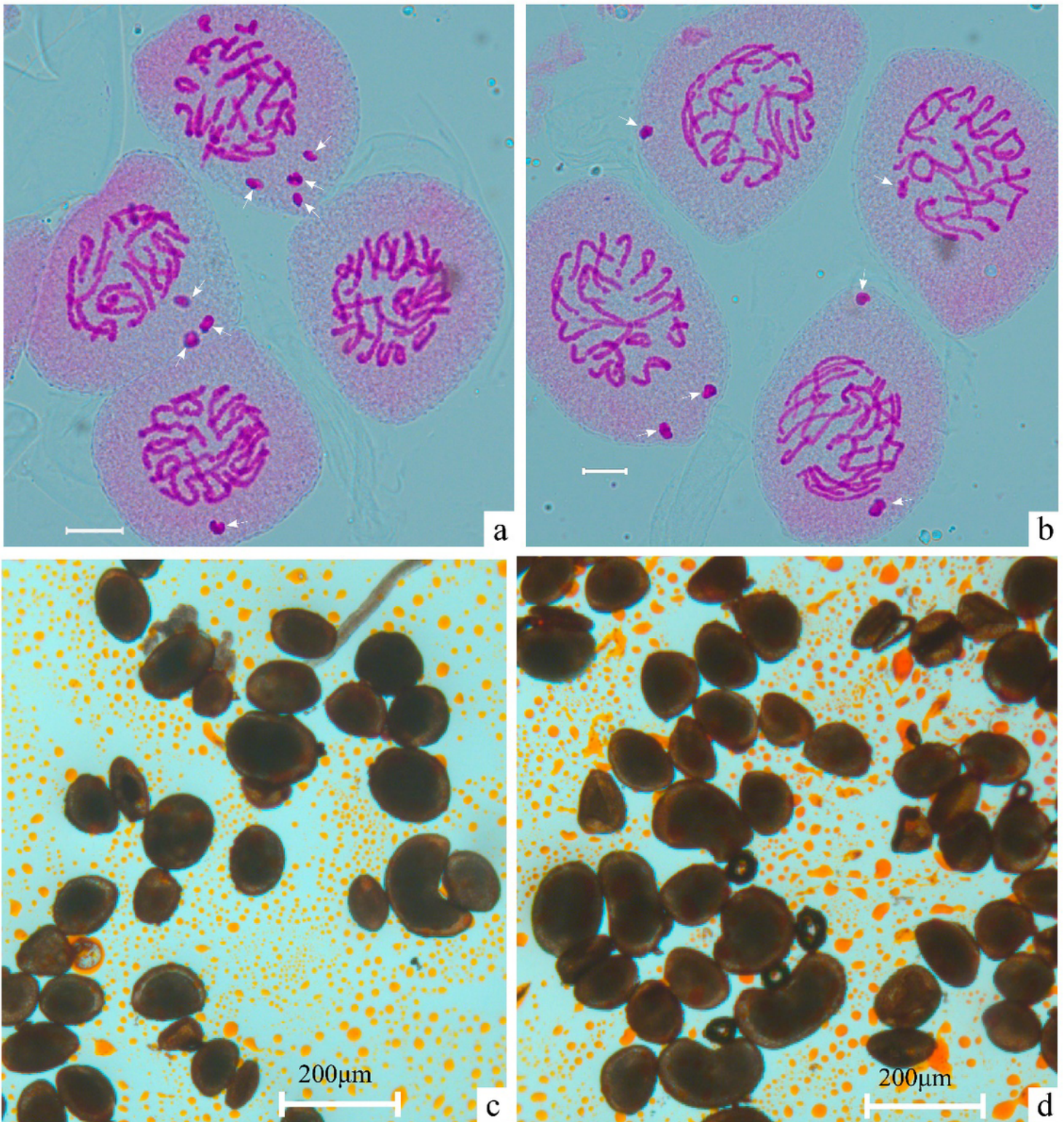
Figure 4

Microspores and pollen grains formed from abnormal meiosis. In (a) and (b), the arrows indicate micronuclei, and Bar = 20μm, and in (c) and (d), bar = 200μm.
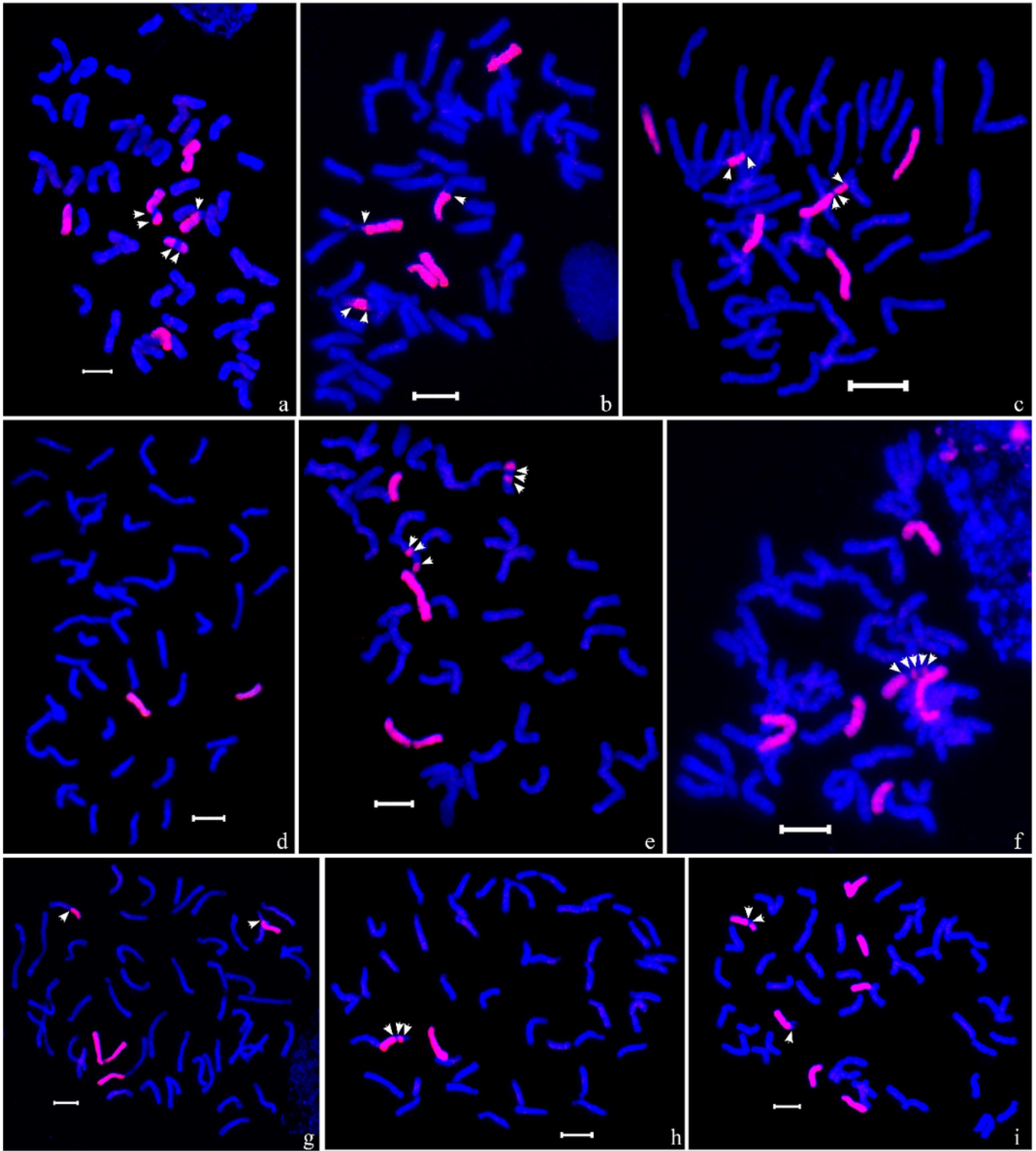
Figure 5

The metaphase chromosomes of root tips of 9 seedlings obtained from J1614 × AAAApainted with GISH: (a) 20106-1, (b) 20106-4, (c) 20106-5, (d) 20106-7, (e) 20106-8, (f) 20106-10, (g) 20106-13, (h) 20106-15, and (i) 20106-17. L-chromosomes are dyed red with Cy3 while A-chromosomes blue with DAPI. The arrow heads indicate the break points of L/A recombinant chromosomes. Bar = 20 μm.
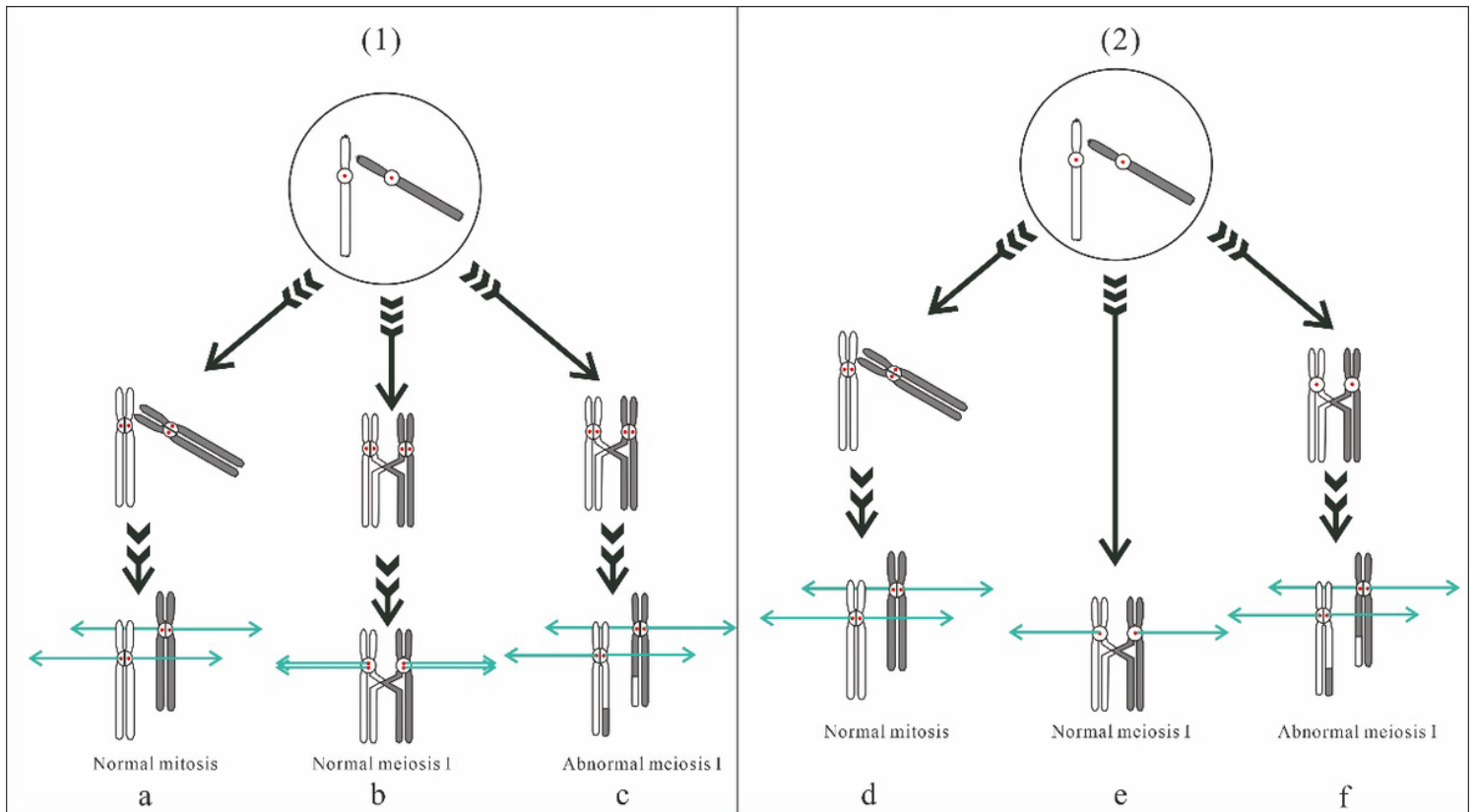
Figure 6

The differences of relationship of kinetochore and chromosome separation between Akera and Lampson (2016)'s mechanism and ours based on the findings in Lilium. (1) The diagram is redrawn referring to Akera and Lampson (2016): for all chromosomes, their kinetochores (red dots) are split or duplicate accompanying their DNA replicate; in normal mitosis (a) or in abnormal meiosis I (c) in which univalent are formed by premature separation of bivalents, the two kinetochores per chromosome attach to microtubules (blue arrows) in opposite direction and thus the two sister chromatids are pulled to two opposite poles; in normal meiosis I (b), the kinetochores on sister chromatids are fused together and become attached to microtubules from the same poles (double arrows) and then two homologous chromosomes (bivalents) are disjoined to the opposite poles. (2) one chromosome has one kinetochore (red dot) in its centromere, and the kinetochore is duplicated accompanying DNA replicates in mitosis (d); however, in normal meiosis I (e), kinetochore duplication is hindered by synapses and cohesion in bivalents; and in abnormal meiosis I (f), their kinetochores could replicate because no chiasmata or cohesion suppress kinetochore duplication.
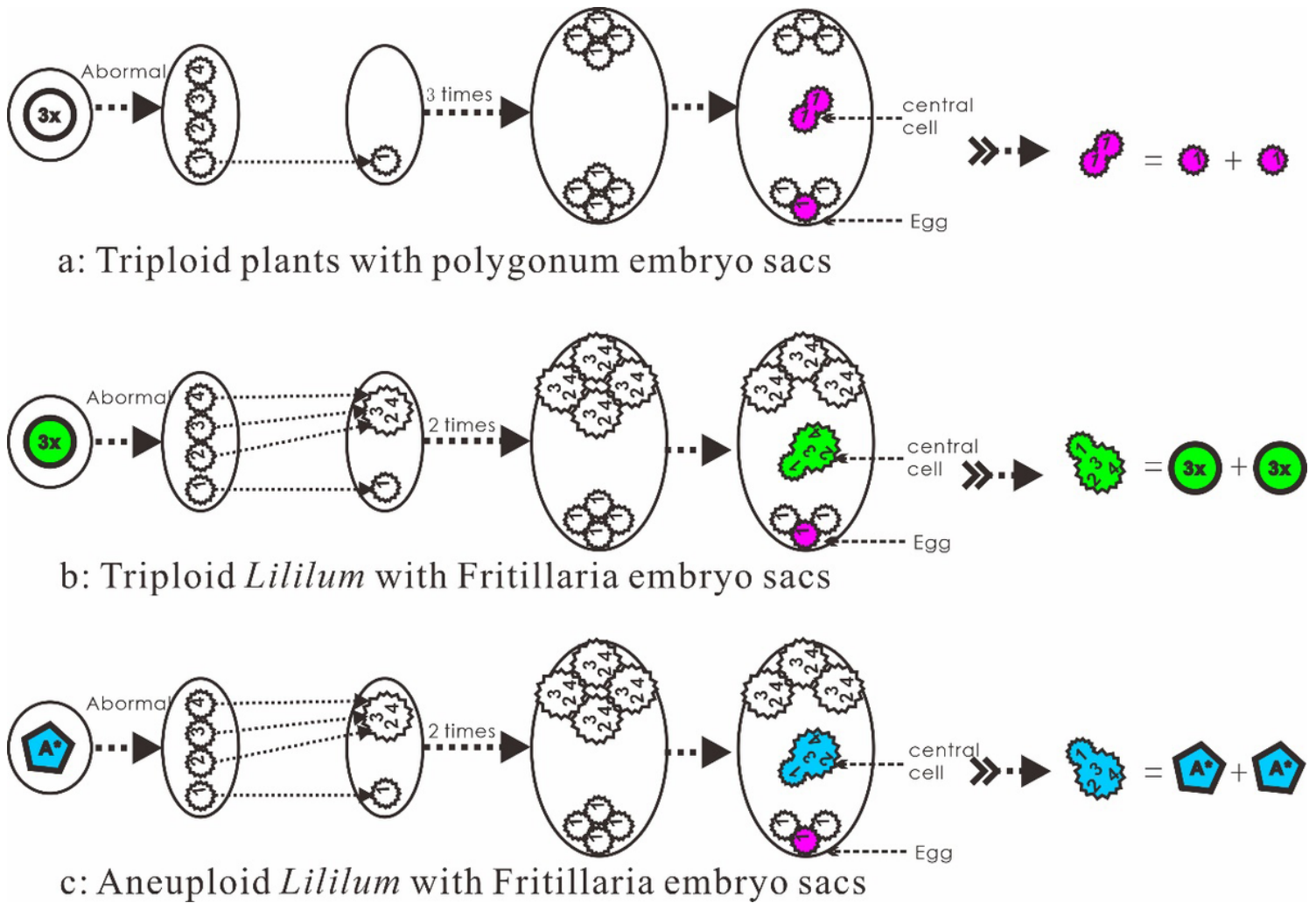
a: Triploid plants with polygonum embryo sacs

b: Triploid *Lililum* with Fritillaria embryo sacs

c: Aneuploid *Lililum* with Fritillaria embryo sacs

Figure 7

Comparison of megasporogenesis of triploid polygonum-type plants (3x), triploid (3x) and aneuploid (A*) Fritllaria-type *Lilium*. All of them are abnormal meiosis; However, in each embryo sac of polyogonum type plants (a), the nucleic DNA of its central cell is twice that of its egg cell, while in each embryo sac of Fritillaria-type plants (b & c), the nucleic DNA of its central cell is twice that of a somatic cell.