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ORIGINAL ARTICLE

Identification of all homoeologous chromosomes of newly synthetic allotetraploid *Cucumis* × *hytivus* and its wild parent reveals stable subgenome structure

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Abstract Allopolyploidy and homoeologous recombination are two important processes in reshaping genomes and generating evolutionary novelties. Newly formed allopolyploids usually display chromosomal perturbations as a result of pairing errors at meiosis. To understand mechanisms of stabilization of allopolyploid species derived from distant chromosome bases, we investigated mitotic stability of a synthetic *Cucumis* allotetraploid species in relation to meiosis chromosome behavior. The *Cucumis* × *hytivus* is an allotetraploid synthesized from interspecific hybridization between cucumber (*Cucumis sativus*, 2n = 14) and its wild relative *Cucumis hystrix* (2n = 24) followed by spontaneous chromosome doubling. In the present study, we analyzed the wild parent *C. hystrix* and the latest generation of *C. hytivus* using GISH (genomic in situ hybridization) and cross-species FISH (fluorescence in situ hybridization). The karyotype of *C. hystrix* was constructed with two methods using cucumber fosmid clones and repetitive sequences. Using repeat-element probe mix in two successive hybridizations allowed for routine identification of all 19 homoeologous chromosomes of allotetraploid *C. hytivus*. No aneuploids were identified in any *C. hytivus* individuals that were characterized, and no large-scale chromosomal rearrangements were identified in this synthetic allotetraploid. Meiotic irregularities, such as homoeologous pairing, were frequently observed, resulting in univalent and intergenomic multivalent formation. The relatively stable chromosome structure of the synthetic *Cucumis* allotetraploid may be explained by more deleterious chromosomal viable gametes compared with other allopolyploids. The knowledge of genetic and genomic information of *Cucumis* allotetraploid species could provide novel insights into the establishment of allopolyploids with different chromosome bases.

Keywords *Cucumis* allopolyploid · Karyotype · Chromosomal variations · Meiosis behavior · Pollen fertility

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Introduction

Polyploidy, which is also known as whole genome duplication (WGD), is a widespread and important evolutionary process in the evolution of all eukaryotic lineages especially in plants (Ohno 2013; Adams and Wendel 2005; Leitch and Leitch 2008; Soltis and Soltis 2009; Buggs et al. 2011). Evidence shows that all seed plants have experienced at least one round of WGD in their ancestry, and recurrent WGD events are particularly common in angiosperms (Bowers et al. 2003; Blanc and Wolfe 2004; Jaillon et al. 2007; Soltis and Soltis 2009; Jiao et al. 2011; Leitch and Leitch 2012). Allopolyploidy combines two or more divergent genomes in a common nucleus through interspecific hybridization followed by genome doubling. Changes in DNA methylation, gene expression, chromatin remodeling, and chromosomal rearrangements are generally associated with allopolyploids, which lay the foundation for morphological and physiological novelty and diversification (Comai 2005; Chen 2007; Gaeta et al. 2007; Leitch and Leitch 2008; Soltis and Soltis 2009; Flagel and Wendel 2010; Matsushita et al. 2012).

Cytogenetic studies on synthetic and naturally formed allopolyploids have reported various types of chromosomal variations, including intergenomic translocations, aneuploidy, ribosomal DNA changes, and the loss of repetitive sequences (Pontes et al. 2004; Skalická et al. 2005; Mestiri et al. 2010; Xiong and Pires 2011; Xiong et al. 2011; Chester et al. 2012). In natural *Tragopogon miscellus* populations (~40 generations old), 31% of the plants analyzed are euploid, and the remaining 69% of the plants had one or more aneuploid chromosomes (Chester et al. 2012). However, in natural populations of *Tragopogon mirus*, 62% individuals had additive karyotypes, and 38% had aneuploid compositions (Chester et al. 2015). Chromosomal rearrangements, such as translocations, were detected in both *Tragopogon* populations. In resynthesized allohexaploid wheats, whole chromosome aneuploidy occurred ubiquitously in early generations, whereas other types of structural variations were minimal (Zhang et al. 2013). This chromosomal instability is typically caused by meiotic irregularities, such as homoeologous pairing, generating multivalents or univalents that lead to unbalanced segregation. In contrast, the synthesized *Arabidopsis* allotetraploids are meiotically stable, with relatively low frequencies of aneuploidy and chromosome abnormalities (Chen et al. 2004; Comai et al. 2000). In the case of experimental *Nicotiana* allopolyploids, meiosis regularity increased rapidly after five selfed generations and displayed diploid-like bivalent pairing and >99% stainable pollen (Ising 1966). Chromosomal changes could reduce viability, because duplication and deficiency gametes may be produced (Gaeta and Pires 2010). In resynthesized *Brassica napus*, reduced pollen viability and seed set were observed with increasing chromosome dosage changes (Xiong et al. 2011). To date, most previous studies were conducted in allopolyploids with the same or a close chromosome base number, whereas little is known about the stability of homoeologous chromosomes and meiotic behavior in relation to genetic structure in allopolyploids derived from a distant chromosome base.

The allotetraploid *Cucumis* species, named as *Cucumis* × *hytivus* (HHCC, 2n = 4x = 38), was synthesized through interspecific hybridization between cucumber (*Cucumis sativus*, CC, 2n =2x = 14) and *Cucumis hystrix* (HH, 2n = 2x = 24) followed by chromosome doubling (Chen and Kirkbride 2000; Chen et al. 1997, 1998). *Cucumis* × *hytivus* (*C. hytivus*, the "×" represents synthetic species) is an experimentally synthesized new species and has no natural form. It provides a unique system for revealing the complicated and "mysterious" processes during polyploid speciation and evolution. Molecular evidence from plastid and nuclear markers suggests that *C. hystrix* is the closest wild relative of cucumber, diverging approximately 4.6 million years ago (Sebastian et al. 2010). Given its clear genetic background and small genome size, the *Cucumis* allotetraploid could serve as an excellent system for studying immediate consequences following allopolyploidization. A better understanding of *C. hytivus* would also facilitate cucumber improvement using beneficial wild alleles. Genetic and cytological studies in early generations of *C. hytivus* (S_1–S_3) reported rapid elimination of DNA sequences and large-scale chromosome lagging, conglutination, and multivalents (Chen et al. 2003); nonetheless, genetic structural stability in relation to meiotic behavior and reproduction fitness in allotetraploid *C. hytivus* remains unknown.

Cucumber cytogenetics has been extensively studied using fluorescence in situ hybridization (FISH) with various types of probes, including tandem DNA repeats (Type I/II, Type III, and Type IV), ribosomal DNA (45S and 5S), fosmid clones, and single copy genes (Ren et al. 2009; Zhao et al. 2011; Han et al. 2011, 2015; Lou et al. 2013, 2014; Sun et al. 2013). In contrast, cytological landmarks to distinguish *C. hystrix* chromosomes are comparatively limited. Comparative mapping studies have reported sequence conservation and synteny between *C. hystrix* and cucumber; 53 *C. hystrix* syntenic blocks were orientated on seven cucumber chromosomes (Yang et al. 2013). These achievements provide an opportunity for karyotyping in *C. hystrix* by cross-species fosmid-FISH and further promote our understanding of genetic structure and chromosomal stability of *C. hytivus*. In the present study, we identified all homoeologous chromosomes of allopolyploid *C. hytivus* by genomic in situ hybridization (GISH) and FISH using repetitive sequences. Distribution patterns of cucumber major repeats, 45S and 5S ribosomal DNA (rDNA), and a set of 29 cucumber fosmid clones were investigated to characterize the chromosomal structure of *C. hytivus*. No aneuploids were identified in any *C. hytivus* individuals that were characterized, and no large-scale chromosomal

rearrangements were identified, indicating a relatively stable chromosomal structure. Finally, we observed meiotic chromosome behavior, which plays a key role in the genetic stability of allopolyploids. Meiotic irregularities, such as homoeologous pairing, univalents, and intergenomic multivalents, were frequently observed. Analysis of chromosomal stability of *Cucumis* allotetraploid species could provide novel insights into the establishment of allopolyploids derived from a distant chromosome base.

Materials and methods

Plant materials

The synthetic *Cucumis* allotetraploid *C. hytivus* was derived from interspecific hybridization between cucumber and its wild relative *C. hystrix* followed by chromosome doubling (Chen et al. 1998; Chen and Kirkbride 2000) and 13 generations of selfing. There has been no naturally occurred *Cucumis* allopolyploid that existed. No intentional experiments regarding chromosome number selection or other traits were performed on *C. hytivus*. Fertility selection may be unconsciously performed (see the "Discussion" part). For mitotic karyotype analysis and genomic DNA used in GISH experiments, two diploid parents (cucumber cultivar "CC3" and *C. hystrix*) were grown. Fifteen individuals of *C. hytivus* from the S_{13} generation were grown for mitotic and meiotic analysis. Seeds were germinated at 28 °C and grown in a greenhouse at the Jiangpu Cucumber Research Station of Nanjing Agricultural University (JCRSNAU), Nanjing, China. The soil media was 25% peat + 25% cinder + 50% perlite. After 2 months, genomic DNA was extracted from CC3 and *C. hystrix*. Root tips, young flower buds, and flowers from *C. hytivus* and/or *C. hystrix* were harvested.

Chromosome preparation

Root tips of the allotetraploid *C. hytivus* and *C. hystrix* were harvested and fixed in fixative solution methanol/acetic (3:1 *v*/*v*) at 4 °C for at least 12 h. For meiosis chromosome preparation, young flower buds 1.0 to 3.5 mm in size were harvested and fixed in ethanol/acetic acid (3:1 *v*/*v*) solution for at least 1 day. The procedure for mitotic and meiotic chromosome preparations was the same as described by Lou et al. (2013). The root tips and anthers at different meiosis stages were digested with enzyme mixtures containing 4% (*w*/*v*) cellulose R-10 (Yakult), 2% (*w*/*v*) pectinase (Sigma-Aldrich), and 0.1% (*w*/*v*) pectolase (Yakult) in 1× PBS buffer, pH 5.5, at 37 °C for 40 min and 1.5 h, respectively. The digested root tips and anthers were fixed in fixative solutions described previously. Slides with well-spread pachytene, meiosis I metaphase, and anaphase chromosomes were obtained following the description by Lou et al. (2014). Cell suspension of the digested anthers was smeared onto slides in 60% acetic acid solution, macerated using the pipette tip, and then air dried.

Fosmids and repeat sequence clones

Genomic DNA (gDNA) was extracted from young healthy leaves of cucumber and *C. hystrix* following the cetyltrimethyl ammonium bromide (CTAB) method described by Murray and Thompson (1980). DNA concentration and quality were evaluated by electrophoresis in 1% agarose gel with standard lambda DNA and ultraviolet spectrophotometer. Approximately 1 μg gDNA of each species was used for probe labeling. Fosmid clones and repetitive sequence clones used in FISH experiment were obtained from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences. Different types of satellite DNA sequences, including Type III and 45S rDNA (Han et al. 2008), were used for the identification of mitotic chromosomes. Fosmid DNA was isolated using the QIAGEN (Valencia, CA) Plasmid Midi Kit.

Fluorescence and genomic in situ hybridization

Genomic DNA, fosmids, and repeat clones were labeled with either biotin-16-dUTP or digoxigenin-11-dUTP using nick translation, and these labels were subsequently detected with a fluorescein isothiocyanate-conjugated anti-biotin antibody and a rhodamine-conjugated anti-digoxigenin antibody (Roche Diagnostics, Indianapolis, IN), respectively. The experimental procedure for FISH was performed as previously described (Lou et al. 2013). Chromosomes were counterstained with DAPI in an anti-fade solution Vectashield (Vector Laboratories, Burlingame, CA), and images were captured with a SenSys CCD camera attached to an Olympus BX51 microscope (http://www.olympus-global.com). The CCD camera was controlled using the Applied Spectral Imaging FISH view 5.5 software (Applied Spectral Imaging, Inc., USA). FISH images were processed with Adobe Photoshop 6.0 (Adobe Systems, http://www.adobe.com).

Meiotic behavior analysis and pollen viability

At the metaphase stage of meiosis I, the main types of chromosome associations are as described by Mestiri et al. (2010): (1) univalent, where the chromosome is not paired with either a homologous or a homoeologous chromosome; (2) bivalent, where two homologous or homoeologous chromosomes are paired at both arm-ends (ring bivalents) or at one arm-end (rod bivalent); (3) tri- and/or tetravalent, where three or four chromosomes are associated; (4) multivalent, where greater than two chromosomes are associated. At least 50 pollen mother cells (PMCs) were analyzed, and the mean configuration was presented.

Pollen grains from three male flowers of the allotetraploid *C. hytivus* were collected and stained with 1% acetocarmine (Momotaz et al. 1998); more than 1000 pollen grains were observed under the microscope. The percentage of stained pollen grains was calculated to represent the pollen viability of *C. hytivus*.

Results

Selection of fosmid clones used for cross-species FISH

Sequence conservation and homoeology between *C. hystrix* and cucumber is evident as revealed by comparative genomic studies; each of the cucumber chromosomes generally corresponded to one or more chromosomes in *C. hystrix* (Yang et al. 2013). Thus, cross-species FISH was performed using the cucumber fosmid library (Ren et al. 2009) to screen for fosmids to identify 12 homologous chromosomes of *C. hystrix*. In total, 29 fosmids that generated distinctive FISH signals on *C. hystrix* were selected for further analysis (Table 1, data for hybridizations on individual chromosomes is not shown). The locations of the 29 cucumber fosmids on *C. hystrix* chromosomes were predicted by chromosome synteny and comparative mapping between cucumber and *C. hystrix* (reviewed in Fig. 5 from Yang et al. (2013)) and verified by FISH. However, due to lack of centromeric probes of *C. hystrix*, the locations of fosmids on *C. hystrix* chromosome arms could not be verified.

Specifically, *C. hystrix* chromosomes H08, H09, and H12 were mapped with one cucumber fosmid for each chromosome. H01, H05, H10, and H11 were assigned with eight cucumber fosmids (two fosmids for each chromosome). For chromosomes H02, H03, and H07, four fosmids were assigned two each chromosome, whereas H04 was mapped with three fosmids from cucumber chromosome 3. Details of fosmid codes, anchored markers, physical and genetic position, and predicted chromosome arms are provided in Table 1. The locations of these 29 fosmids were verified by performing multiple rounds of FISH hybridizations on mitotic chromosomes of *C. hystrix*. In addition, sequence alignment of 20 anchored simple sequence repeat (SSR) markers was performed with the markers that were used to anchor fosmids in Yang et al. (2013). Most of the positions of fosmids used in the present study were consistent with those from Yang et al. (2013), except for chr3-74, which was predicted in H07 but mapped to H06 by FISH (Table S1, Fig. S1). This is most likely due to genome sequencing or assembling errors instead of rearrangement.

Development of a standard karyotype for *C. hystrix*

GISH signals of *C. hystrix* were located at either terminal end of each chromosome, except for two chromosomes where signals were located at both terminal ends (Fig. S2). The strength of GISH signals on relative chromosomes could be classified as follows: very strong (three pairs), strong (four pairs), medium (one pair), and weak (four pairs) (Xiong and Pires 2011; Zhang et al. 2015). On the basis of the above results for 29 fosmids, 12 fosmid clones (Table 1, indicated with asterisk) were chosen to hybridize with genomic DNA (gDNA) from *C. hystrix* (hy-gDNA) to identify 12 chromosome pairs in dual-color FISH on mitotic chromosomes (Fig. 1). Using the distribution of pattern and strength of signals, all chromosome pairs of *C. hystrix* could be unambiguously identified (Fig. 2a). To investigate the location and pattern of 45S, fosmids were hybridized with the 45S probe. Results revealed three pairs of 45S signals. The strong signals of 45S loci are located on H08, a moderate pair of signals is located on H10, and the weak signals are on H12 (Fig. S3). When screening for probes to identify *C. hystrix* chromosomes, the bacterial artificial chromosome (BAC) library of *C. hystrix* were also used. Given that the length of BAC clones is generally larger than fosmids, only one BAC clone (457-F11) is found to hybridize near to the centromeric region of all *C. hystrix* chromosomes. The BAC probe generated two pairs of very strong signals, three pairs of strong signals, three pairs of medium signals, three pairs of weak signals, and one pair of faint signals.

A probe mix that contained repeat elements *C. hystrix*-gDNA and 457-F11 was used to rapidly distinguish *C. hystrix* chromosomes; this probe mix was quicker and more convenient than fosmid probes in karyotype analysis of *C. hystrix* (Fig. 2b). To obtain consistent chromosome numbering with the previous fosmid-FISH method, the repeat-element contained probe mix was hybridized to the same set of chromosome preparations used in Fig. 1 in 12 hybridizations (Fig. S4). An ideogram that summarized distribution patterns on individual chromosomes of *C. hystrix* is presented in Fig. 3. Descriptions of the probes that hybridized to each chromosome of *C. hystrix* are summarized as follows.

Chromosome H01: Very strong signals from *C. hystrix*-gDNA are at one tip of the chromosome arm. The fosmid from cucumber chromosome 7 (chr7–80) is located at the predicted long arm of H01. 457-F11 generates strong signals around the middle of the chromosome.

Chromosome H02: Strong signals of *C. hystrix*-gDNA are localized at the tip of the chromosome arm. The fosmid chr6-60 is near the end on the long arm of H02. Strong 457-F11 signals are found to disperse along the chromosome arm without gDNA signal. The 457-F11 FISH signals are more dispersed on H02 than H01.

Chromosome H03: Weak signals of *C. hystrix*-gDNA are at the chromosome tip. The fosmid chr6-45 localized at the proposed short arm of H03 and near signals of

Table 1 Information of the 29 selected cucumber fosmid clones; yellow boxes were the fosmids used in karyotype construction of *C. hystrix*

	Code	Marker	Position (cM)	Genome Position (bp)	Fosmid clone	
H01	chr7-78	SSR01898	50.9	15641671-15673977	gcfbd0_0343_F07.ab1	short arm
	chr7-80	—	—	—	gcfbe0_0497A02	long arm
H02	chr1-20	SSR14445	73.029	21848915- 21879630	gcfba0_0007_C04.ab1	long arm
	chr1-70	SSR23757	4.9	1966203- 2006434	gcfbd0_0464_C11.ab1	short arm
	chr6-35	—	—	—	gcfbe0_0180_C09.ab1	—
	chr6-60	SSR02906	84.5	24183018-24219723	rgcfbe0_0257_E12.ab1	long arm
H03	chr2-7	SSR23732	61.5	16344196-16377413	rgcfbd0_0252_E04.ab1	long arm
	chr2-23	SSR20045	74	17928507-17965119	gcfbd0_1142_B06.ab1	long arm
	chr2-69	SSR30665	94.3	21968920-22001624	gcfbd0_0304_A09.ab1	short arm
	chr6-45	SSR11219	6.2	3346943-3384961	gcfbe0_0187_G09.ab1	short arm
H04	chr3-**10**	SSR21454	92.4	32147183- 32188786	gcfbe0_0104_H02.ab1	long arm
	chr3-43	—	—	—	gcfbe0_0072_E12.ab1	long arm
	chr3-44	SSR23517	105.1	38050645- 38088342	gcfbe0_0001_D11.ab1	short arm
H05	chr2-41	SSR11952	5.9	1374855-1409777	gcfbd0_1078_H03.ab1	long arm
	chr2-54	SSR00184	0	142789-184478	gcfbe0_0022_B06.ab1	long arm
H06	chr3-56	SSR22514	31	10059164- 10101377	gcfbe0_0207_F03.ab1	long arm
	chr3-72	CSWGATT01B	49.4	16484183- 16510660	gcfbd0_0554_G09.ab1	long arm
	chr3-74	SSR03049	0	1121698- 1155015	rgcfbe0_0472_B12.ab1	—
H07	chr4-18	—	—	10327544- 10359112	gcfbd0_0108G02	—
	chr4-37	—	—	—	gcfbd0_0802_E07.ab1	long arm
	chr4-55	—	—	19347769- 19383016	gcfbe0_0098_F07.ab1	long arm
	chr4-95	—	—	351- 43490	gcfbe0_0315F11	short arm
H08	chr4-58	SSR23826	7.5	11480614- 11510697	gcfbe0_0243_E12.ab1	short arm
H09	chr5-40	—	—	3813355- 3851250	gcfbd0_0986F03	short arm
H10	chr5-36	SSR17975	39	24499019- 24533838	gcfba0_0066_B05.ab1	long arm
	chr5-52	—	—	11041510- 11076720	gcfbe0_0314B09	long arm
H11	chr5-59	SSR21918	26.5	22654705- 22698074	rgcfbe0_0257_D06.ab1	short arm
	chr6-57	SSR01903	10.8	5816959-5853441	gcfbe0_0459_C08.ab1	long arm
H12	chr1-30	SSR12070	33.502	7698959- 7732033	gcfbe0_0544_G05.ab1	short arm

C. hystrix-gDNA. FISH signals of 457-F11 (faint) are the weakest of all chromosomes.

Chromosome H04: *C. hystrix*-gDNA signals are strong at both chromosome terminals. The fosmid chr3-10 localized at the proposed long arm of H04. 457-F11 generates medium signals.

Chromosome H05: Weak signals of *C. hystrix*-gDNA are at the chromosome tip. The fosmid chr2-41 is located near the signals of gDNA. Weak signals of 457-F11 are near gDNA signals.

Chromosome H06: *C. hystrix*-gDNA signals are strong at one chromosome end but weak at the other end. The fosmid chr3-56 is located near the strong signals of gDNA. 457-F11 signals are weak.

Chromosome H07: Weak signals of *C. hystrix*-gDNA are located at the chromosome tip. The fosmid chr4-95 is located at the distal end of H07 and in gDNA signals. 457-F11 signals are weaker than H05.

Chromosome H08: Medium signals of *C. hystrix*-gDNA are located at the chromosome tip. The fosmid chr4-58 is localized at the proposed long arm of H08 and near signals of *C. hystrix*-gDNA. 457-F11 signals are very strong. The largest 45S signals are on the pericentromeric region of its short arm.

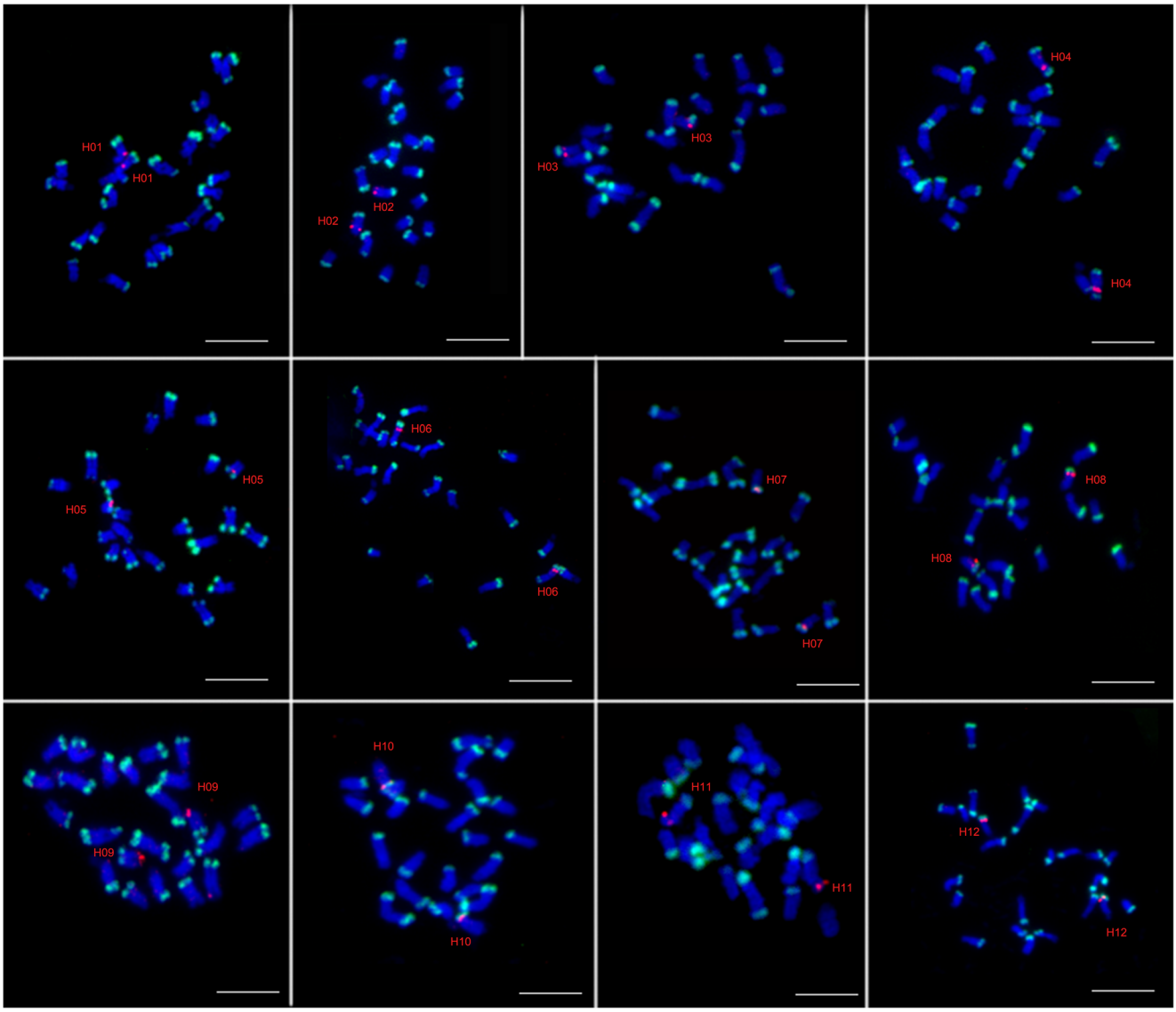

Fig. 1 FISH signals of 12 cucumber fosmid clones (*red*) and hy-gDNA (*green*) on mitotic metaphase chromosome spreads of *C. hystrix* that could distinguish 12 homologous chromosomes. Fosmids for identifying chromosomes H01–H12 are chr7-80, chr6-60, chr6-45, chr3-10, chr2-41, chr3-56, chr4-95, chr4-58, chr5-40, chr5-36, chr6-57, and chr1-30. *Bars* = 5 μm

Chromosome H09: Strong signals of *C. hystrix*-gDNA are at the chromosome tip. The fosmid chr5-40 is localized at the opposite end of gDNA signals on H09. 457-F11 generates very weak signals (weaker than H05 and H07).

Chromosome H10: Very strong signals of *C. hystrix*-gDNA are located at the chromosome tip. The fosmid chr5-36 is localized on the proposed long arm of H10 and near gDNA signals. 457-F11 signals are very strong. The second largest 45S signals and the 5S signals are located at the pericentromeric region.

Chromosome H11: Weak signals of *C. hystrix*-gDNA are located at the chromosome tip. The fosmid chr6-57 is localized on the proposed long arm of H11 and opposite with gDNA signals. 457-F11 signals are weak at the chromosome end without gDNA signals, but are stronger than that on H05.

Chromosome H12: Signals of *C. hystrix*-gDNA are very strong at the chromosome tip. The fosmid chr1-30 localized on the proposed short arm and near gDNA signals. 457-F11 signals are strong. The weakest 45S signals are located at the distal region of H12.

Karyotype of the allotetraploid *C. hytivus*

The cucumber gDNA produces FISH signals at subtelomeric and/or pericentromeric regions of chromosomes. Type III

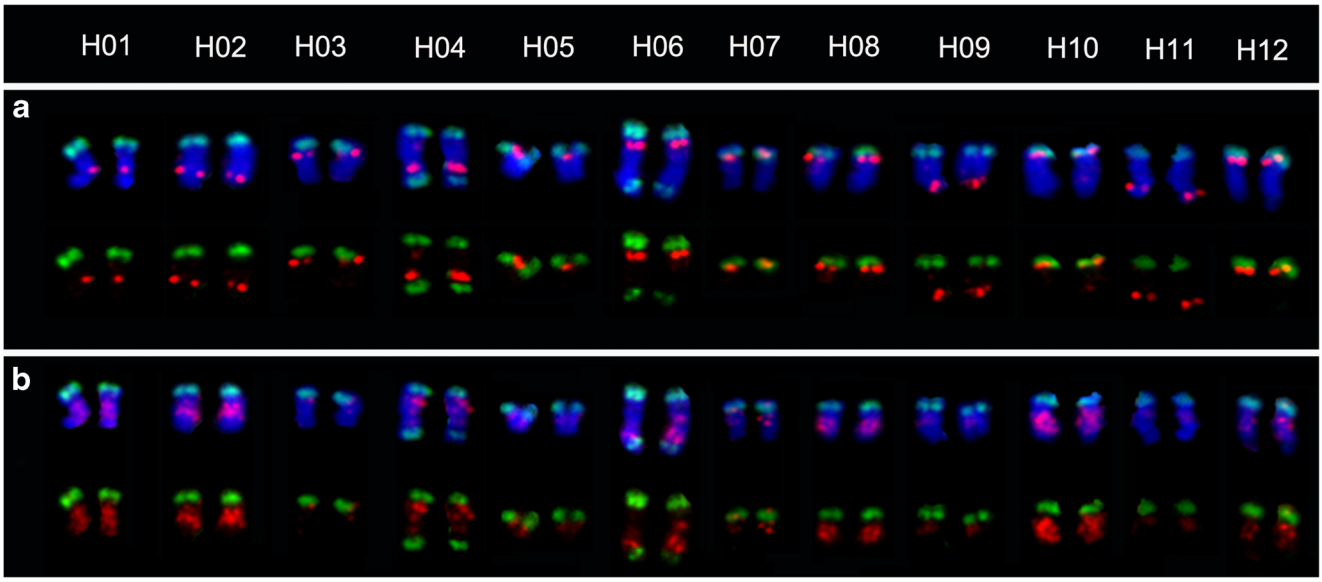

Fig. 2 Karyotype of mitotic metaphase chromosomes of *Cucumis hystrix*. H01~H12 represent chromosomes 1~12 of *C. hystrix*. **a** Karyotype constructed using 12 cucumber fosmids (*red*) and hy-gDNA (*green*). **b** Karyotype constructed with BAC clone 457-F11 (*red*) and hy-gDNA (*green*)

repeat is highly specific to the cucumber centromeres which hybridized exclusively to the centromeric regions of chromosomes. According to GISH patterns of cu-gDNA (Zhang et al. 2015) and Type III FISH signals (Han et al. 2008), cucumber mitotic metaphase chromosomes could be identified. Thus, two successive FISH experiments were performed on the same set of mitotic metaphase chromosomes of allotetraploid *C. hytivus* (Fig. 4a) using repeat-element probes (gDNA from diploid progenitors, BAC clone 457-F11, and Type III). The strength of cucumber gDNA signals on relative chromosomes could be classified as follows: very strong (three pairs), strong (two pairs), medium (one pair), and weak (one pair) (Xiong and Pires 2011; Zhang et al. 2015). The cucumber centromeric Type III sequences generated two pairs of very strong signals, one pair of strong signals, three pairs of medium signals, and one pair of weak signals. Based on the karyotype of *C. hystrix* constructed by BAC clone and hy-gNDA and distribution patterns of cu-gDNA and Type III repeats, all 19 homoeologous chromosomes of allotetraploid *C. hytivus* could be identified (Fig. 4b). Fifteen individuals of the S13 generation were karyotyped to examine the presence of aneuploid plants. However, statistical analysis indicated that all S13 individuals were euploids.

Twenty-six cucumber fosmids that generated distinguishing signals on *C. hystrix* (Table 1) were hybridized to mitotic metaphase chromosomes of *C. hytivus*. Seven FISH experiments were performed, and fosmid clones from the same cucumber chromosomes were hybridized

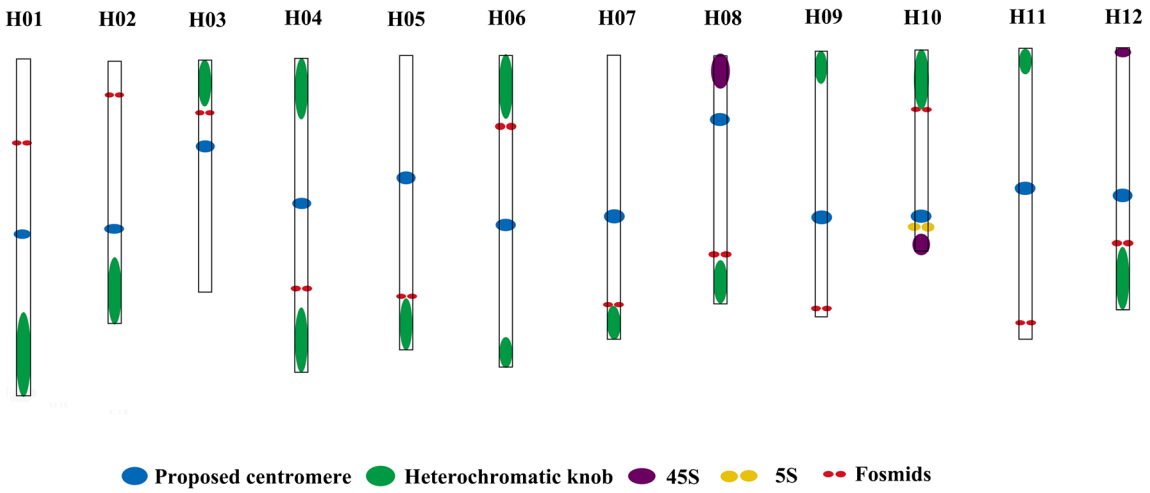

Fig. 3 An ideogram that summarizes the karyotype of *C. hystrix*. Fosmids used to identify the corresponding homologous chromosomes are listed in Table 1

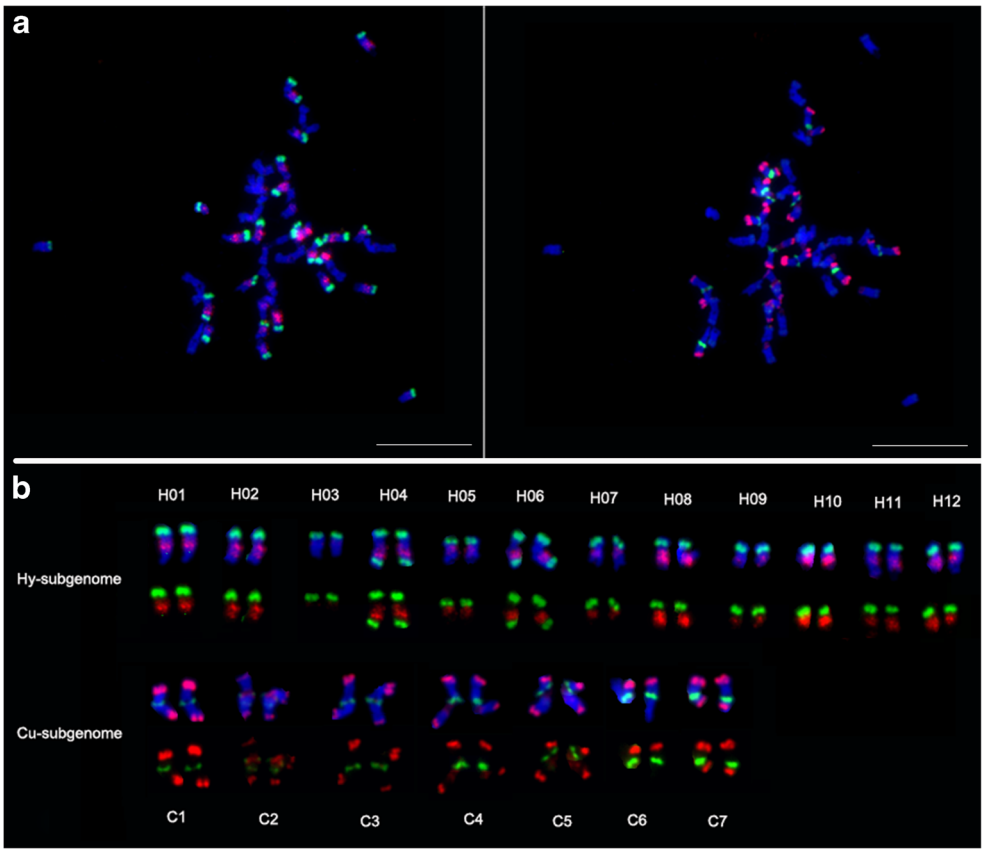

Fig. 4 Somatic chromosome karyotype of the allotetraploid *Cucumis hytivus* using the FISH mixture: cu-gDNA (*red*), Type III (*green*), BAC clone 457-F11 (red), and hy-gDNA (*green*) that could distinguish 19 homoeologous chromosomes. **a** Signal patterns of cu-gDNA (*red*) and Type III (*green*), BAC clone 457-F11 (*red*), and hy-gDNA (*green*) in two successive hybridizations on the same chromosome preparation. **b** Karyotype of *C. hytivus*, the cu-genome, and hy-genome chromosomes numbered following genetic linkage groups of the two parents. *Bars* = 10 μm

simultaneously to the same *C. hytivus* chromosome spreads (Fig. S5). As shown in Fig. 5, the distribution of pattern of the fosmids along cucumber chromosomes was unchanged compared with previous studies, except for two fosmids on chromosome 2 (chr2-54 and chr2-41). An intragenomic inversion occurred between these two fosmids (Fig. 5, indicated with asterisk). Cucumber chromosome 1 appears to be homoeologous to two *C. hystrix* chromosomes, H02 and H12. Similarly, C2 is homoeologous to H03 and H05. Cucumber chromosome 3 corresponds to H04 and H06. C4 is homoeologous to H07 and H08. C5 corresponds to H09 and H10. Cucumber chromosome 6 is related to four *C. hystrix* chromosomes, H02, H03, H08, and H11, whereas C7 is homoeologous to H01. In addition, fosmids from cucumber chromosomes 1 and 4 exhibited unexpected additional FISH signals on one and three chromosomes, respectively (Fig. S5a, d, indicated with arrows). These additional signals might represent small-scale chromosome segment transferring that caused by recombination events. Descriptions of signal patterns of repetitive sequences that hybridized to each chromosome of *C. hytivus* are summarized as follows.

Chromosome C1: Very strong signals from cucumber-gDNA are at the terminals of both chromosomes. Type III repeats generate medium signals at the centromeric region.

Chromosome C2: Weak signals from cucumber-gDNA appear at both chromosome ends and centromere regions. FISH signals of Type III sequences are weakest among seven chromosomes from cucumber subgenome.

Chromosome C3: Cucumber-gDNA signals are moderate at chromosome terminals. Type III generates medium signals.

Chromosome C4: Strong signals from cucumber-gDNA are located at both chromosome ends and pericentromeric regions. Strong signals of Type III sequences are detected.

Chromosome C5: Signals of cucumber-gDNA are strong at the terminals of both chromosomes and pericentromeric regions. Type III sequences generate medium signals.

Chromosome C6: Very strong signals from cucumber-gDNA are detected on the short arm. Type III generates very strong signals.

Chromosome C7: Very strong signals from cucumber-gDNA are located at both chromosome ends. FISH signals of Type III sequences are very strong.

Chromosome H01: Very strong signals from *C. hystrix*-gDNA are located at one tip of the chromosome arm. 457-

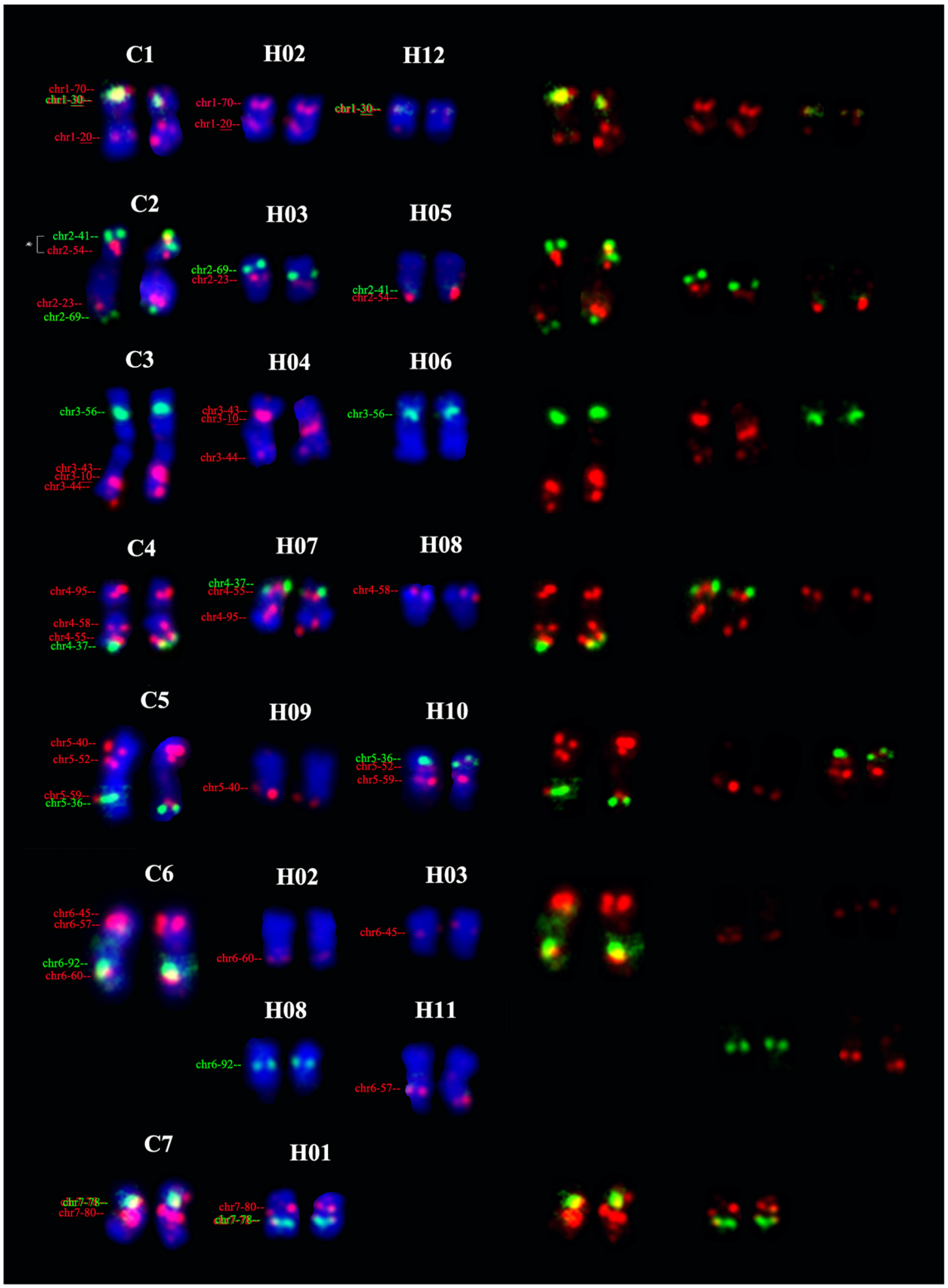

Fig. 5 FISH patterns of 29 fosmid clones from 7 cucumber chromosomes (*red* or *green*, listed in Table 1) on mitotic metaphase chromosome spreads of the allotetraploid *C. hytivus* that could distinguish 19 homoeologous chromosomes

F11 generates strong signals around the middle of the chromosome.

Chromosome H02: Strong signals of *C. hystrix*-gDNA are localized to the tip of the chromosome arm. Strong

457-F11 signals are observed to disperse along the chromosome arm without gDNA signal.
Chromosome H03: Weak signals of *C. hystrix*-gDNA are located at the chromosome tip. FISH signals of 457-F11 (faint) are the weakest of all chromosomes.
Chromosome H04: *C. hystrix*-gDNA signals are strong at both chromosome terminals. 457-F11 generates medium signals.
Chromosome H05: Weak signals of *C. hystrix*-gDNA are located at the chromosome tip. Weak signals of 457-F11 are near gDNA signals.
Chromosome H06: *C. hystrix*-gDNA signals are strong at one chromosome end but weak at the other end. 457-F11 signals are weak.
Chromosome H07: Weak signals of *C. hystrix*-gDNA are located at the chromosome tip. 457-F11 signals are weaker than H05.
Chromosome H08: Medium signals of *C. hystrix*-gDNA are located at the chromosome tip. 457-F11 signals are very strong.
Chromosome H09: Strong signals of *C. hystrix*-gDNA are located at the chromosome tip. 457-F11 generates very weak signals (weaker than H05 and H07).
Chromosome H10: Very strong signals of *C. hystrix*-gDNA are located at the chromosome tip. 457-F11 signals are very strong.
Chromosome H11: Weak signals of *C. hystrix*-gDNA are located at the chromosome tip. 457-F11 signals are weak at the chromosome end without gDNA signals.
Chromosome H12: Signals of *C. hystrix*-gDNA are very strong at the chromosome tip. 457-F11 signals are strong.

Distribution patterns of major repetitive sequences on *C. hytivus* chromosomes

Distribution of major satellite repetitive sequences (Type I/II, Type III, and Type IV), 45S and 5S rDNA, and the *Arabidopsis* telomeric repeats were investigated on mitotic metaphase chromosomes of *C. hytivus*. In the cucumber genome, Type I/II and Type IV sequences were located in telomeric heterochromatin regions. Type III sequences were cucumber centromere-specific repeats. As expected, Type III sequences were hybridized to centromeric regions on all 14 cucumber chromosomes in *C. hytivus* (Fig. 6c). The *Arabidopsis* telomeric repeats were detected on all heterochromatic region of each chromosome ends (Fig. 6c). Type I/II and Type IV repeats were exclusively detected and exhibited the same signal patterns on cu-subgenome of *C. hytivus* as described in cucumber (Fig. 6a, b; Han et al. 2008; Zhang et al. 2015). In addition, 45S rDNA generates three pairs of strong signals and two pairs of weak signals on cucumber chromosomes 1, 2, 3, 4, and 7; 5S has one pair of signals located on cucumber chromosome 5. In *C. hystrix*, previous studies reported three pairs of 45S signals and one pair of 5S signals. When 45S and 5S were hybridized to the allotetraploid *C. hytivus*, four pairs of 45S were clearly detected on cu-subgenome, whereas the weakest pair was barely observed. The strength and distribution of three pairs of 45S on *C. hystrix* chromosomes remained intact (Fig. 6d). The strength of 5S signals on H10 (indicated with yellow arrows) was much stronger compared with that on C5 (indicated with purple arrows).

Meiotic chromosome behavior and pollen viability

To associate mitotic stability with meiotic behavior, chromosome pairing at different stages of meiosis I in 60 PMCs from allotetraploid *C. hytivus* was examined. Genomic DNA from cucumber and *C. hystrix* was labeled with either digoxin-dUTP (red) or biotin-dUTP (green) to distinguish and record the presence and relationship of the parental chromosome sets in GISH experiments. The results showed that chromosome pairing was mostly presented as bivalents (II), with a mean frequency of 11.21 (7~14) per cell. A low frequency of trivalents (III, average 0.97) and quadrivalents (IV, average 2.12 per cell) was observed. Unpaired univalents (I) were prevalent and observed in 84.5% cells with an average of 2.7 per cell. The mean chromosome configurations for *C. hytivus* were 2.7 I + 11.21 II + 0.97 III + 2.12 IV. Notably, chromosome pairing did not strictly occur among homologous chromosomes; intergenomic bivalents and multivalents were frequently observed (Fig. 7a, b). As shown in Fig. 7b, two types of intergenomic trivalents were presented: cu/cu/hy and hy/hy/cu (indicated with white arrows). In addition, chromosome lagging and conglutination were also observed in some cells (Fig. 7c, d). Meiosis pachytene chromosomes exhibited unpaired chromosome strings that might be structural heterozygotes caused by homoeologous recombination (Fig. 7e, f), which were not detected in mitotic metaphase chromosomes using fosmid-FISH.

Mature pollen was stained using 1% acetocarmine to calculate pollen viability. The round pollen grains that stained were regarded as viable, and the non-stained and lightly stained pollen grains with irregular sizes were considered aborted. The stainability of *C. hytivus* was 41.84%. This relatively low pollen viability reflected the impact of meiosis irregularities. However, whether those chromosomally viable gametes could produce progeny needs further analysis.

Discussion

Newly formed allotetraploids typically undergo chromosome-level perturbations, appearing as various types of chromosome

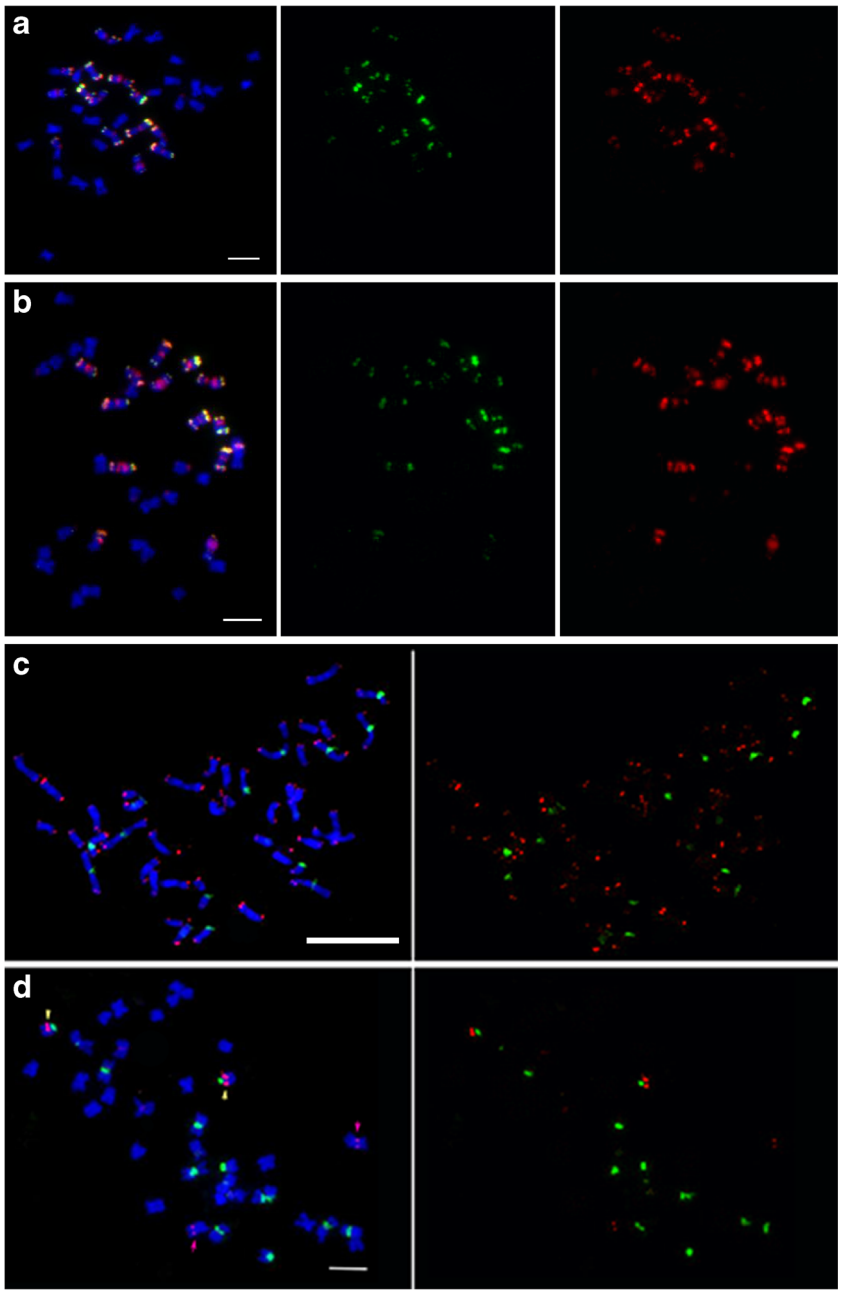

Fig. 6 FISH patterns of the repeat sequences on mitotic metaphase chromosome spreads of the allotetraploid *Cucumis hytivus*. **a** Type I/II (*green*) and cu-gDNA (*red*). **b**:Type IV (*green*) and the cu-gDNA (*red*). **c** *Arabidopsis* telomere sequences (*red*) and Type III (*green*). **d** 45S (*green*) and 5S rDNA (*red*); *yellow arrows* indicate the 5S loci on *C. hystrix* chromosomes, and *purple arrows* indicate the 5S loci on cucumber chromosomes. *Bars* = 5 μm

rearrangements and/or aneuploidy that may lead to destabilization of allotetraploid genomes. GISH and FISH experiments in synthetic allopolyploid wheat, *Brassica*, and naturally occurring allotetraploid *Tragopogon* using repetitive sequences, such as rDNA, centromeric and subtelomeric repeats, and BAC clones, suggest that chromosomal rearrangements and aneuploidy occur frequently in some plant species (Xiong and Pires 2011; Xiong et al. 2011; Chester et al. 2012, 2015; Zhang et al. 2013). To understand chromosomal stability of allopolyploid species derived from distant chromosome bases, we analyzed the karyotype of the latest generation of *Cucumis* allotetraploid *C. hytivus* and its wild parent *C. hystrix* and investigated mitotic stability in relation to meiotic behavior.

Karyotype analysis of the wild parent *C. hystrix* using cross-species FISH

Numerous molecular cytogenetic studies have reported karyotypes in cucumber on mitotic metaphase and meiotic pachytene chromosomes (Han et al. 2008, 2011; Zhao et al. 2011; Lou et al. 2013; Sun et al. 2013); whereas land marks to distinguish 12 pairs of *C. hystrix* chromosomes are

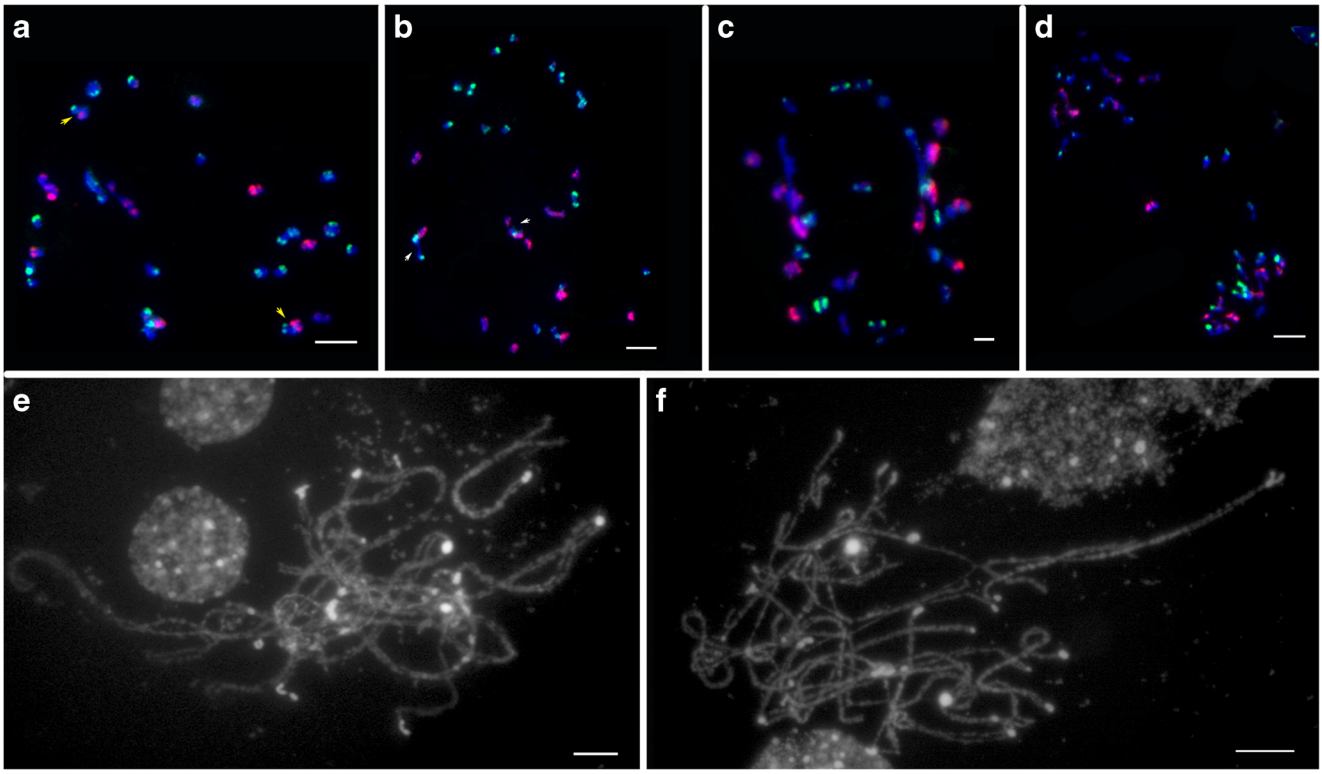

Fig. 7 GISH signals of cu-gDNA (*red*) and hy-gDNA (*green*) on meiosis I chromosome spreads of the allotetraploid *Cucumis hytivus*. **a** Chromosome pairing at meiosis I pre-metaphase chromosomes; *yellow arrows* indicate homoeologous bivalents. **b** Chromosome pairing at meiosis I pre-metaphase chromosomes; *white arrows* indicate two homoeologous trivalents. **c** Meiosis I post-metaphase chromosomes. **d** Meiosis I anaphase chromosomes; chromosome laggings are common. **e**, **f** Meiosis pachytene chromosomes of *C. hytivus*, which depicts heterozygosis chromosome strings. *Bars* = 5 μm

comparatively limited. In this study, the karyotype of *C. hystrix* was constructed using cross-species FISH with the cucumber fosmid library developed by Ren et al. (2009). In cucumber, 78 fosmid clones were used to construct cytogenetic maps for 7 cucumber chromosomes (Sun et al. 2013; Lou et al. 2013). Apart from those that lacked fosmid codes, 29 fosmids with distinctive FISH signals on *C. hystrix* chromosomes were selected (Table 1).

Cucumber fosmids used in comparative FISH mapping from Yang et al. (2013) were selected from a different fosmid library. To associate the 29 fosmids used in the present study with those from Yang et al. (2013), we conducted sequence blast of anchored molecular markers. However, due to lack of anchored markers for some fosmids in the Ren et al. (2009) fosmid library and blast misses between the two libraries, only 20 fosmids could be correlated (Table S1). Positions of all selected fosmids on *C. hystrix* chromosomes were verified by performing multiple FISH hybridizations. Most of the positions of fosmids on *C. hystrix* chromosomes used in the present study were consistent with those in Yang et al. (2013), except for chr3-74, which was predicted in H07 but mapped to H06 by FISH (Table S1, Fig. S1). This is probably the consequence of genome sequencing or assembling errors instead of rearrangement. The SSRs used to anchor fosmids in cucumber fosmid library construction are developed from first version of cucumber draft sequence assemblies (Ren et al. 2009). The anchored SSR of chr3-74 is aligned to cucumber chromosome 4 (Table S1) using the latest version of cucumber reference genome which has been revised for several times. A standard karyotype was constructed with 12 fosmids and hy-gDNA which could distinguish 12 chromosomes of *C. hystrix* (Table 1, Fig. 2a). Nonetheless, due to lack of centromere-specific probes of *C. hystrix*, the positions of selected fosmids on specific chromosome arms could not be directly inferred. Proposed chromosome arms in Table 1 were inferred from comparative mapping between *C. hystrix* and cucumber (Yang et al. 2013).

We found a BAC, 457-F11, which contains repeated sequences at centromeric region of *C. hystrix* chromosomes. A second karyotype of *C. hystrix* chromosomes was constructed using the probe mix that contained repeat elements hy-gDNA and 457-F11 (Fig. 2b). Although this repeat-element probe mix is not as precise as fosmids, it is fast and convenient, and could be combined with fosmid-FISH to unambiguously identify all the chromosomes. These cytological markers serve as excellent cytological tools for identifying *C. hystrix* chromosomes in alien additional lines and chromosomal stability between the cu-subgenome and hy-subgenome in

allotetraploid *C. hytivus*. Other types of probes especially *C. hystrix*-specific probes such as single copy probes (Lou et al. 2014) or oligonucleotides (Han et al. 2015) could help to trace each chromosome at meiosis in further investigation.

Genomic additivity of two parents in the allotetraploid *C. hytivus* genome

Cytogenetic studies in several species revealed the prevalence of structural changes in newly formed allopolyploid species. Most of the studied allopolyploids were derived from progenitors with either the same or similar chromosome base number (such as cotton, wheat, and *Brassica*). Whether these changes could occur in allopolyploids with distinct chromosome base remains to be examined. We analyzed for the first time the synthetic *Cucumis* allotetraploid, which was derived from interspecific hybridization between cucumber (n = 7) and the wild *C. hystrix* (n = 12) and selfed for 13 generations, to investigate genomic composition and stability. In the present study, a probe mix that contained gDNA from two parental species, Type III cucumber centromeric-specific sequences and *C. hystrix* BAC clone 457-F11, was used in FISH experiments to distinguish the 19 homoeologous chromosomes of the allotetraploid *C. hytivus*. Given the limited number of *C. hytivus* seeds, 15 individuals of the S13 generation were karyotyped to examine the presence of aneuploid plants. However, statistical analysis of all S13 individuals indicated that no aneuploid plants were recovered, i.e., all of the 15 *C. hytivus* plants had 19 pairs of chromosomes. There are several possible explanations, either *C. hytivus* does not exhibit aneuploidy or the number of individuals is insufficient. Another possibility is that *C. hytivus* may have undergone fertility selection during the selfing process throughout 13 generations. Only fully fertile seeds that are in good shape and could successfully germinate are selected to produce progenies. The fertility of chromosomal viable individuals (aneuploids) may be lower than euploids, which makes the grown plants all euploids. The morphology such as leaf shape and color and fruit shape varies slightly among individuals, which may result from other mechanisms rather than karyotype variation. However, future work should analyze a larger population to ascertain whether any aneuploids exist in allotetraploid *Cucumis* species.

The self-GISH patterns of gDNA from two parents on their own chromosomes in the allotetraploid did not differ from that hybridized separately in the parental species (Fig. 4, for self-GISH patterns of two progenitors see Zhang et al. (2015)). The repeated sequences in gDNA are preferentially distributed at subtelomeric and pericentromeric regions in cucumber and subtelomeric regions in *C. hystrix*. The unevenly distribution of gDNA on the chromosomes may be explained by relatively small genome sizes and low content of repetitive sequences. In contrast to the plant species with large genomes such as wheat (~17 Gb composing of approximately 80% repeats), the genome sizes of cucumber (~367 Mb) and *C. hystrix* (~400 Mb, unpublished data) are very small (Paux et al. 2008; Huang et al. 2009; Brenchley et al. 2012). Highly repeated DNA of cucumber accounted for only 20~30% of the total nuclear DNA (Han et al. 2008). High frequencies of chromosome rearrangements were detected using GISH in resynthesized *B. napus* and two naturally occurring *Tragopogon* populations (Xiong et al. 2011; Chester et al. 2012, 2015). However, no structural changes were uncovered using GISH in *C. hytivus* probably because the gDNA did not switch on chromosomes. No apparent chromosomal rearrangements were detected in resynthesized *Triticum* allohexaploids (Zhang et al. 2013). Moreover, gDNA of cucumber could also generate FISH signals on *C. hystrix* chromosomes and vice versa according to a previous study (Zhang et al. 2015). However, in *C. hytivus* where two genomes combine into one nucleus, signals of gDNA from one parent on chromosomes of the other parent were minimally observed (Fig. 4). One possible cause for this phenomenon is exposure, i.e., the signals of cu-gDNA (or hy-gDNA) on cucumber (*C. hystrix*) chromosomes were much stronger than that on *C. hystrix* (cucumber), which subsequently makes the signals on *C. hystrix* (cucumber) even weaker and filtered as background.

We also investigated the distribution of major satellite repeats (Type I/II, Type III, and Type IV), 45S and 5S rDNA, and the *Arabidopsis* telomeric repeats on *C. hytivus* chromosomes. Most of the strength and location of repetitive sequences exhibited no difference, except for changes observed at NOR (45S and 5S) loci. However, a similar exposure phenomenon as observed in gDNA was also present when hybridizing Type I/II and Type IV sequences on *C. hytivus* (Fig. 6a, b). Cytogenetic analyses of synthetic *C. hytivus* revealed seven pairs of 45S and two pairs of 5S gene loci (Fig. 6d). The signal patterns of 45S and 5S loci differ in size and location, allowing easy identification. The loci carrying faint 45S rDNA signals on cucumber chromosomes were lost, whereas the rDNA loci on *C. hystrix* chromosomes were stable. No significant enlargement of rDNA signals on existing loci existed. Cytogenetic analysis using 26 cucumber fosmids (Table 1) indicated that small-scale chromosome rearrangements and/or transferred segments occurred in *C. hytivus* (Fig. S5). An intragenomic inversion occurred on cucumber chromosome 2 between two fosmids (chr2-54 and chr2-41). Their locations on *C. hystrix* (H05) were stable, but the signal strength of chr2-41 was weakened (Fig. 5). Novel signals on one and three chromosomes were observed for fosmids from cucumber chromosomes 1 and 4, respectively (Fig. S5). The

existence of novel signals was possibly due to chromosomal recombination events during meiosis pairing.

Impact of homoeologous pairing on mitotic stability and male fertility in *C. hytivus*

The stabilization and establishment of a polyploid species depend on the regularity of chromosome pairing during meiosis. For the maintenance of an allopolyploid genome, it is essential to rapidly restore a diploid-like meiotic behavior (Gaeta and Pires 2010). The synthetic allohexaploids may exhibit homologous pairing at metaphase I of meiosis to form bivalents, such as observed in synthetic *Arabidopsis* allopolyploids without significant structural reorganization of homoeologous chromosomes (Comai et al. 2003). Some newly formed allopolyploids typically produce chromosomal variable gametes due to meiosis errors, such as homoeologous chromosome pairing and unequal segregation (Gottschalk 1978; Ramsey and Schemske 2002), which cause whole chromosome aneuploidy and structural changes as observed in synthesized allotetraploid *B. napus*, allohexaploid wheat, and natural *Tragopogon* populations (Xiong et al. 2011; Chester et al. 2012, 2015; Zhang et al. 2013; Zhou et al. 2016). We observed frequent homoeologous pairing, which leads to the formation of multivalents. The *C. hytivus* individuals also exhibited extensive unpaired univalents (84.5%, 2.7 per cell on average) and multivalents (3.24 on average). Homoeologous pairing in bivalents, trivalents, and quadrivalents was frequently observed (Fig. 7). The prevalence of intergenomic chromosome pairing may explain the chromosomal rearrangements observed by fosmid signals and pachytene chromosomes. However, karyotype analysis revealed that the allotetraploid *C. hytivus* was mitotically stable without displaying any type of aneuploidy in any individuals analyzed, which is different from previous reports (Xiong et al. 2011; Chester et al. 2012, 2015; Zhang et al. 2013). The karyotype stability of *C. hytivus* observed in the present study may be attributed to several reasons: first is the limited number of individuals used for karyotype analysis. Because *C. hytivus* has relatively low fertility, the seeds for each generation are very limited, and there could be aneuploids that were not detected in the present study. Second, gametes where there are fewer than 19 chromosomes may not survive or form zygotes; third, possible artificial selection against fertility. For each generation, only those seeds that are round in shape and could successfully germinate were grown to produce selfing progeny. The seeds of chromosomal viable plants may not be selected to grow due to irregular shape, or failed to germinate/develop; thus for each generation, most of the *C. hytivus* individuals grown are euploids. Because the chromosome bases of two parental species of *C. hytivus* are quite distant, the abortion rate for gametes with fewer than 19 chromosomes might be higher than those allopolyploids derived from progenitors with the same or similar chromosome bases, reducing the chances for stable inheritance of aneuploids. Indeed, male fertility, as reflected by stainability of mature pollen, is relatively low (41.84%). After self-pollination for 13 generations, there are still over half pollen grains aborted. Nonetheless, whether all gametes that have less than 19 chromosomes were aborted during gametogenesis and caused the lack of aneuploidy must be examined in more *C. hytivus* plants. In further investigation, cross-generation screening for the presence of aneuploids versus euploids should be conduct to provide a comprehensive demonstration of chromosomal viability across different generations of *C. hytivus*. The information presented here is of value for further characterization and understanding of the stability of homoeologous chromosomes and meiotic behavior in relation to genetic structure in allopolyploids derived from a distant chromosome base.

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Author contributions JFC and QFL conceived of the study and designed the experiments. YZW and QZZ conceived of the study, participated in experimental design, performed data analysis, and drafted the manuscript. XDQ, SQY, ZAL, and JL helped with synthesis of materials, statistics collection, and analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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