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Stable, fertile lines produced by hybridization between allotetraploids *Brassica juncea* (AABB) and *Brassica carinata* (BBCC) have merged the A and C genomes

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Summary

- Many flowering plant taxa contain allopolyploids that share one or more genomes in common. In the *Brassica* genus, crop species *Brassica juncea* and *Brassica carinata* share the B genome, with $2n$ = AABB and $2n$ = BBCC genome complements, respectively. Hybridization results in $2n$ = BBAC hybrids, but the fate of these hybrids over generations of self-pollination has never been reported.
- We produced and characterized *B. juncea* × *B. carinata* ($2n$ = BBAC) interspecific hybrids over six generations of self-pollination under selection for high fertility using a combination of genotyping, fertility phenotyping, and cytogenetics techniques.
- Meiotic pairing behaviour improved from 68% bivalents in the F_1 to 98% in the S_5/S_6 generations, and initially low hybrid fertility also increased to parent species levels. The S_5/S_6 hybrids contained an intact B genome (16 chromosomes) plus a new, stable A/C genome (18–20 chromosomes) resulting from recombination and restructuring of A and C-genome chromosomes.
- Our results provide the first experimental evidence that two genomes can come together to form a new, restructured genome in hybridization events between two allotetraploid species that share a common genome. This mechanism should be considered in interpreting phylogenies in taxa with multiple allopolyploid species.

Introduction

Polyploidy is defined as the presence of more than two complete sets of chromosomes within an organism (Ramsey & Schemske, 1998; Soltis & Soltis, 1999). Polyploidy is reported to occur in many animals (e.g. fish, insects, and amphibians) and plants (e.g. fern and mosses), but with a higher frequency in flowering plants, and hence most major crops (Leitch & Leitch, 2008), where it represents a major mechanism of adaptation and speciation (Ramsey & Schemske, 1998). Reports indicate that 30–80% of all extant flowering plants are polyploids, with all angiosperms having experienced at least one round of whole genome duplication (Jiao *et al.*, 2011). There are two major types of polyploids: autopolyploids, which arise within a population or species; and allopolyploids, which result from hybridization between two species.

In polyploid taxa, primary polyploids may also hybridize, leading to the formation of secondary polyploid hybrids (Rieseberg, 1997; Soltis & Soltis, 2009; Abbott *et al.*, 2013). In the *Aegilops* genera, it was found that allotetraploids that share one common genome hybridize easily (Zohary & Feldman, 1962; Dubovets & Sycheva, 2017). In hybridization between allotetraploids that share a common genome, it has been suggested that this common genome serves as a buffer, providing the opportunity for recombination between the differential genomes and leading to the formation of many new variants of the recombinant genome (Zohary & Feldman, 1962; Kimber & Yen, 1988; Badaeva *et al.*, 2002; Dubovets & Sycheva, 2017) (Fig. 1). Although never experimentally validated, polyploids with putatively recombinant genomes have been identified in the Triticeae tribe and in cereals (Wang *et al.*, 2000; Badaeva *et al.*, 2004; Molnár *et al.*, 2013). The molecular analysis of genomic changes that accompany polyploidy has led to a significant breakthrough in understanding how primary polyploids form new, stable genomes. However, how secondary polyploids may form stable, recombinant genomes is unknown. Elucidating this process will deepen our understanding of micro-evolutionary differentiation within families and may assist in phylogenetic reconstruction.

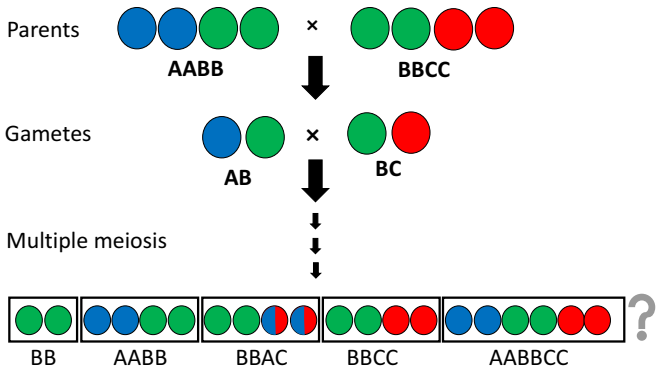

Fig. 1 Formation of a possible new, stable allopolyploid hybrid through hybridization between two allotetraploids that share one of two genomes in common. The different genome compositions BB, AABB, BBAC, BBCC, and AABBCC represent different possible karyotypes that could arise from this hybrid combination.

The *Brassica* genus is an important model for studying interspecific hybridization and polyploidy. It is a complex of related diploid and allopolyploid species containing the A, B, and C genomes, where the evolutionary relationship between six agriculturally important members of this genus was illustrated by U (1935). The 'triangle of U' consists of three diploid species (*Brassica rapa*, $2n = AA = 20$; *Brassica nigra*, $2n = BB = 16$; and *Brassica oleracea*, $2n = CC = 18$) and three allotetraploids (*Brassica juncea*, $2n = AABB = 36$, a product of hybridization between *B. rapa* and *B. nigra*; *Brassica carinata*, $BBCC = 34$, a hybrid between *B. nigra* and *B. oleracea*; and *Brassica napus*, $2n = AACC = 38$, a hybrid between *B. rapa* and *B. oleracea*). The ancestral relationship that exists between the *Brassica* A, B, and C genomes has been well elucidated (Attia & Röbbelen, 1986; Lagercrantz & Lydiate, 1996; Mason *et al.*, 2010; Chalhoub *et al.*, 2014), with the A and C genomes shown to be more closely related to each other than to the B genome. Although the B genome species separated from the A/C lineage some 6 Ma, comparison of the palaeopolyploid genomes reveal extensive conservation of gene content and sequence identity (Navabi *et al.*, 2013).

Brassica allotetraploids species can readily hybridize to produce trigenomic hybrids AABC, BBAC, and CCAB, with each hybrid combination having one of the subgenomes in a diploid state and the other two in a haploid state (Schelfhout *et al.*, 2008; Nelson *et al.*, 2009; Navabi *et al.*, 2010; Mason *et al.*, 2010). The chromosome pairing behaviour of trigenomic *Brassica* allotetraploid hybrids AABC, BBAC, and CCAB has previously been reported in the F_1 generation (Mason *et al.*, 2010). Although all types of allosyndesis (A–B, B–C, and A–C) are observed in all hybrids at varying frequencies, AABC and CCAB hybrid types show little pairing between chromosomes belonging to the haploid genomes, whereas BBAC hybrids show high frequencies of A–C pairing (Mason *et al.*, 2010). However, the fate of these hybrid lineages under self-pollination conditions in subsequent generations has never been reported. In this study, we aimed to determine the genome stability and fertility of BBAC hybrids across multiple generations, to see if stable, fertile hybrid offspring could be recovered in later generations, and if so by which mechanism(s).

Materials and Methods

Experimental plant material

Brassica trigenomic tetraploids with genome complement BBAC are the products of the cross between the two *Brassica* allotetraploid species *B. juncea* ($2n = AABB$) and *B. carinata* ($2n = BBCC$). The parental *B. juncea* genotype 'JN9-04', hereafter represented with the code J1, was crossed with two different *B. carinata* genotypes – '195923.3.2_01DH' and '94024.2_02DH', hereafter called C1 and C2, respectively – to generate two separate F_1 hybrid genotypes: J1C1 and J1C2 (Fig. 2; Supporting Information Dataset S1). In brief, self-pollination in each generation was encouraged by enclosing racemes in microperforated plastic bags, and the most fertile plants in each generation (two to five plants per genotype combination) were selected as parents for the next generation. The generations were labelled as 'F_1' for the initial BBAC hybrids, then S_1 to S_6 for the subsequent six self-pollination generations.

Molecular karyotyping using marker-based genotyping data

Leaf samples were collected in 2 ml microcentrifuge tubes and stored at −20°C until use. DNA was extracted for the S_3, S_5, and S_6 generation plants using the BioSprint 96 plant work station (Qiagen) according to the manufacturer's instructions (http://qiagen.com/), and for earlier generations (S_1 and S_2) using the Microprep method described in Fulton *et al.* (1995). Single-nucleotide polymorphism (SNP) genotyping was performed using the Illumina Infinium 90K *Brassica* SNP array (A, B and C

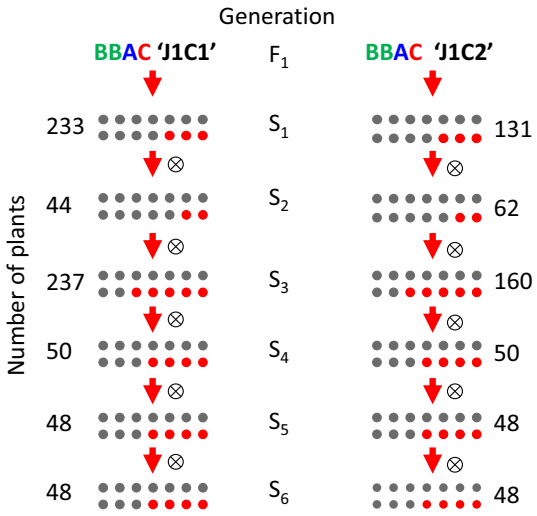

Fig. 2 Schema for generational selection of BBAC hybrid plants from the cross *Brassica juncea* × *Brassica carinata* based on highest numbers of self-pollinated seeds produced per plant. Red dots indicate the actual number of parent plants selected in each progeny generation.

genomes) for the S_3, S_5, and S_6 generations, and using the Illumina Infinium 60K *Brassica* SNP array (A and C genomes) for S_1 and S_2 generations. Hybridization was performed according to the manufacturer's instructions for all samples, and the genotyped data were visualized and exported using GENOME STUDIO v.2.0.4 software (Illumina Inc., San Diego, CA, USA).

A total of 41 441 SNPs were exported for the A and C genomes after application of the recommended cluster file (Clarke *et al.*, 2016) for S_5 and S_6 hybrids. A and C-genome SNPs were mapped to the Darmor-*bzh* v.8 reference sequence (Bayer *et al.*, 2017) via BLAST of the SNP probe sequences (Karlin & Altschul, 1990). We filtered out SNPs that were mapped to the A genome but which amplified in *B. carinata* and SNPs that were mapped to the C genome but which amplified in *B. juncea*. As no allelic segregation is expected within the A and C genomes in these populations because they had no homologous pairing partners, SNPs that were heterozygous within the A and the C genomes were also filtered out. SNPs that had a 'no call' in > 10% of individuals within a haplotype block ($r^2 = 1$) of called SNPs or that had a 'call' in > 10% of individuals within a haplotype block ($r^2 = 1$) of 'no-call' SNPs were removed, in addition to SNPs showing patterns of segregation inconsistent with determined genomic locations. Genotype calls were finally converted to presence/absence calls (1 for presence and 0 for absence). After filtering, 26 484 SNPs were retained for the J1C1 genotype: 10 773 in the A genome and 15 711 in the C genome (Dataset S2). For the J1C2 genotype, 26 523 SNP markers were retained: 10 717 for the A genome and 15 806 for the C genome (Dataset S3).

The B genome SNP array data comprised 25 101 SNPs for which genomic positions were supplied with the public Illumina Infinium *Brassica* 90K array for an assembled *B. nigra* genome (available under MTA from Isobel Parkin, Agriculture and AgriFood Canada). The data were filtered to retain only SNPs that were polymorphic between the B genome of *B. juncea* and the B genome of *B. carinata* for each genotype combination (Datasets S2, S3). For early generations of BBAC S_1 and S_2 hybrids, SNP genotyping was performed using the Illumina Infinium 60K *Brassica* array and mapped to the Darmor-bzh v.8.1 reference sequence (Chalhoub *et al.*, 2014). SNP filtering was performed as already reported herein (Datasets S4–S9). In summary, SNP genotyping and data analysis were performed for the S_1, S_2, S_3, S_5, and S_6 generations and for both the J1C1 and J1C2 lineages in each generation. The S_4 generation was not included because of a failure to collect leaf samples from S_4 hybrids.

The R package CHROMDRAW (Janečka & Lysak, 2016) was used to produce the karyotypes of these hybrids. The centromere locations for the A and C genomes were assessed using the populations and methods reported in Mason *et al.* (2016) for *B. napus* Darmor-*bzh* v.1 (Chalhoub *et al.*, 2014), remapped to the latest version of the *B. napus* cultivar Darmor-*bzh* v.8 reference genome sequence (Bayer *et al.*, 2017) (Table S1).

Cytological analysis

Root tips and young flower buds were collected and prepared according to the procedure of Snowdon *et al.* (1997), and Leflon *et al.* (2010). Mitosis slides were observed after 4′,6-diamidino-2-phenylindole staining to visualize chromosomes under ultraviolet excitation using a Leica fluorescence microscope (Leica DMR; Leica Microsystems, Wetzlar, Germany) and meiosis slides were stained with 1% acetic acid carmine solution and observed using a Leica microscope with phase contrast. Mitotic chromosome analysis was done for 34 plants in the S_4 generation, 60 plants in the S_5 generation, and 82 plants in the S_6 generation. Two plants from each progeny set (eight plants per genotype) were selected to assess meiotic pairing behaviour at metaphase I of meiosis in the S_5 and S_6 generations. A minimum of 20 (mode 40) pollen mother cells from two different buds were assessed per plant for which data could be collected (Table S2).

Bacterial artificial chromosome–fluorescence *in situ* hybridization and genomic *in situ* hybridization

Slide preparation followed by hybridization using bacterial artificial chromosome (BAC)–fluorescence *in situ* hybridization (using BAC clone BoB014O06 containing C-genome-specific dispersed repeat sequence *Bot1* (Alix *et al.*, 2008) labelled with Cy3) and genomic *in situ* hybridization (using DNA extracted from *B. nigra* labelled with fluorescein isothiocyanate) was carried out according to the procedures detailed in Leflon *et al.* (2006) and Mason *et al.* (2010). Images were captured using a Leica fluorescent microscope (Leica Microsystems).

Fertility data

Total seed set data was collected for all plants after encouraging self-pollination using micro perforated sleeves to enclose racemes. Newly opened flowers (at least two per plant) were collected when plants started flowering and pollen stained with 1% aceto-carmine solution before assessing viability using a Leica microscope. At least 300 pollen grains were counted per flower. Plants were then bagged to encourage self-fertilization, and total seed was counted after drying.

Results

Fertility in BBAC F_1 and S_1 hybrids

BBAC F_1 hybrids from two genotype combinations (produced between a homozygous inbred line of *B. juncea* with two doubled-haploid-derived lines of *B. carinata* – see (Mason *et al.* (2011b) for details) were grown under several different glasshouse and controlled-environment growth-room temperature conditions. The seed fertility under all conditions ranged from 0 to 333 seeds/plant with an average of 101 seeds/plant (Dataset S1; includes subset of plants from Mason *et al.*, 2011b). Pollen viability was collected for a subset of individuals: F_1 hybrids showed moderate pollen production (average 15%, range 3–59%).

A total of 44 BBAC S_1 plants (20 J1C1 and 24 J1C2) resulting from seeds produced by F_1 hybrid parents were grown under

glasshouse conditions, and a further 220 BBAC S_1 seeds (113 J1C1 and 107 J1C2) were grown in the field (200 direct sown, 20 germinated under glasshouse conditions and planted out at the four to six-leaf stage) (Table S3).

Forty of the 44 glasshouse-grown plants (four were not genotyped) were found to result from self-pollination of F_1 hybrids following genotyping with the Illumina Infinium *Brassica* 60K genotyping array, as expected. Fertility varied dramatically in the S_1 generation, with 9–96% pollen viability and 0–403 seeds per plant. Most field-grown BBAC S1 (J1C1 and J1C2 combined) plants (85%, 186/220) failed to produce any seeds, but most glasshouse-grown BBAC S_1 (73%, 29/40, as 4/44 plants were not SNP genotyped and were left out of the analysis) did produce seeds. Significant differences ($P < 0.05$, one-way ANOVA) were observed between the two genotypes of BBAC S_1 hybrid: only 6% of J1C1 plants (6/107 sown seeds) produced seeds under field conditions, as opposed to 25% of J1C2 plants (27/113). For glasshouse-grown plants, 10% of J1C1 (2/20) and 55% of J1C2 (11/20) with a combined average of 33% (13/40) failed to produce any seed.

Chromosome numbers, pairing behaviour, and genome constitutions in BBAC S_1 hybrids

Chromosome count data were obtained for 30 BBAC S_1 plants. An average of 33 chromosomes with a mode of 35 and a range from 25 to 36 chromosomes was observed. There were no significant differences between the two genotypes in terms of chromosome numbers of BBAC S_1 progeny (ANOVA, $P > 0.05$).

A high number of chromosomal rearrangements was observed in the A and C genomes, as assessed by deletions and duplications of parts of chromosomes based on SNP genotyping of 40 BBAC S_1 hybrids (two plants were discarded from the analysis because they were contaminated; Fig. 3). In the J1C2 population (20 plants), 53% of all A and C-genome chromosomes showed evidence of homoeologous recombination based on either absence or duplication of parts of chromosomes (0.53 events per chromosome per plant; Fig. 3d). More than one-third of A-genome and C-genome chromosomes were partially lost: 0.36 and 0.37 events per chromosome per plant for the A and C genomes, respectively. Complete loss of A and C genome chromosomes was relatively

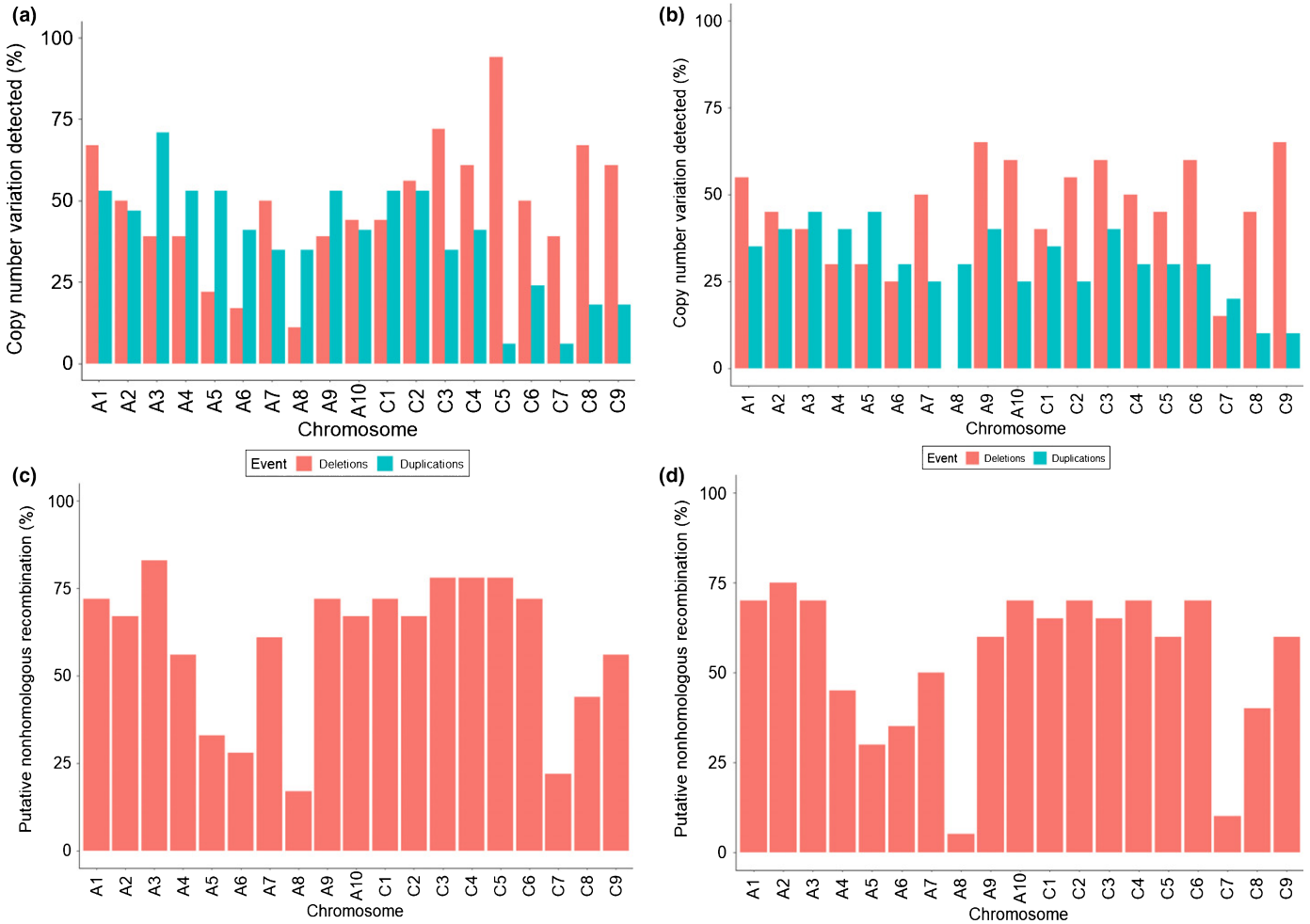

Fig. 3 Genetic changes in *Brassica juncea* × *Brassica carinata* self-pollinated S_1 interspecific hybrids as detected from Illumina Infinium *Brassica* 60K SNP array data. (a) Percentage of deletions/duplications (loss or gain of a whole or part of a chromosome) in the J1C1 population. (b) Percentage deletions/duplications in the J1C2 population for the different A and C chromosomes. (c, d) Percentage of individuals with recombinant chromosomes resulting from nonhomologous recombination events for each A and C-genome chromosome in the (c) J1C1 S_1 population and (d) J1C2 S_2 population.

rare: seven losses of complete A-genome chromosomes and 12 losses of complete C-genome chromosomes were detected across the 20 plants (with 10 A chromosomes and nine C chromosomes per plant in the F_1), giving an average loss of 0.035 per chromosome per plant for the A genome and 0.066 for the C genome (Fig. 3b). Similar patterns were observed for the J1C1 lineage (18 plants, two discarded as a result of contamination), with 55% of A and C-genome chromosomes undergoing homoeologous exchanges (0.60 per chromosome per plant; Fig. 3c), and with few chromosomes completely lost (six events in the A-genome, 0.03 per chromosome per plant; and 17 in the C genome, 0.10 per chromosome per plant; Fig. 3a).

Selection and fertility in the BBAC S_2 generation

Self-pollinated seed set ranged from 0 to 745 seeds per plant in the BBAC S_2 J1C1 and J1C2 lineages. J1C1 plants set an average of 81 seeds, with 11% (5/44 plants) failing to produce any seeds. J1C2 had an average of 40 seeds/plant with 16% (10/62 plants) failing to produce any seeds. Only one plant in the J1C1 population failed to produce viable pollen, with an average of 71% pollen viability (range 0–97%) across the two populations. This result was obtained from 106 BBAC S_2 plants that were grown to maturity from selected S_1 parents and confirmed as true self-pollinated progeny.

Chromosome numbers and genomic constitutions in the BBAC S_2 hybrids

Chromosome counts were obtained for 22 BBAC S_2 individuals (11 J1C1 plants and 11 J1C2 plants) and showed a wide distribution (24–37 chromosomes, average 31). The chromosome distribution was the same in both lineages, with an average of 31 chromosomes. There was no observed correlation between chromosome number, pollen viability, and seed set (ANOVA, $P > 0.05$).

Nonhomologous recombination events were very frequent, and were differentially distributed across the different A and C-genome chromosomes (Fig. S1) in the J1C1 plants and J1C2 plants in the S_2 generation genotyped using the Illumina Infinium 60K *Brassica* array for the A and C genomes. Out of the 44 J1C1 plants that were SNP genotyped, five were unsuccessful; and for the J1C2 plants, 2/62 were unsuccessful. SNP analysis was therefore done for 39 J1C1 plants and 60 J1C2 plants. The average number of deletions per chromosome per plant was 0.6 in the J1C2 and 0.5 in the J1C1 progeny. The average number of duplications (gain of a partial or complete chromosome copy) per chromosome per plant was 0.36 in the J1C1 progeny and 0.38 in the J1C2 progeny. Chromosomes A8 and C7 consistently had the lowest number of deletion and duplication events in the J1C1 and J1C2 populations (Fig. S1a,b).

In the J1C1 population, chromosome C8 was most frequently lost, followed by A10, C7 and A4. In the J1C2 population, A8 was most frequently lost, followed by C7, A10, and C4. Chromosomes that showed partial deletions and/or duplication events were considered to have undergone a nonhomologous recombination event, as this is the primary mechanism by which deletions or duplications of only a partial instead of a whole chromosome can be observed (Mason *et al.*, 2011b). Homoeology refers to chromosomes or chromosome segments that diverged from a common origin as a result of speciation and were brought back into the same genome by allopolyploidy, and which hence share sequence similarity. The sequence similarity between the A and C chromosomes, which is a measure of their homoeology, is reported in Chalhoub *et al.* (2014), Mason *et al.* (2014), and Lagercrantz & Lydiate (1996). The number of putative homoeologous exchanges in these S_2 hybrids were similar, especially for chromosomes with high homoeology between the subgenomes, and all chromosomes showed evidence of putative homoeologous exchange events. Chromosomes A6 and A8 had the lowest number of putative homoeologous exchanges in the J1C1 population, whereas A8 and C7 were lowest in the J1C2 population (Fig. S1c,d). Percentage genome presence was calculated using the number of present or missing SNPs for each chromosome in relation to the total number of SNPs for that chromosome.

Selection, fertility and genetic constitution of BBAC S_3-generation hybrids

Four BBAC S_2 plants (two from each of the J1C1 and J1C2 genotype combinations) were selected as parents of the BBAC S_3 generation. A total of 397 BBAC S_3 hybrid plants were grown and survived to maturity, with 100, 60, 93 and 144 plants from each BBAC S_2 parent. Of the total 397 plants, seed data were collected for 358 plants, with 39 being discarded due to heavy powdery mildew infestation. Seed production ranged from 0 to 1196 seeds/plant, with an average of 217 seeds/plant. Only 4% of plants (14/358) failed to produce self-pollinated seed (Dataset S1).

Of the total number of plants grown, 88 J1C1 plants and 92 J1C2 plants were SNP genotyped using the Illumina Infinium *Brassica* 90K SNP array (Table S3). Using the proportion of SNPs present and absent for each chromosome, we calculated the percentage of genome present for each chromosome. In comparison with the S_2 generation, the variation in the percentage of the genome present for the different chromosomes was reduced in the S_3 generation, as expected for increasing fixation of 'heterozygous' chromosome rearrangements. For example, chromosome A1 was present 30–100% of the time in the J1C1 S_2 generation and 60–75% of the time in the J1C1 S_3 generation, whereas chromosome A2 was present 0–100% of the time in the S_2 but from 0 to 25% of the time in the S_3. Although this type of variation was generally reduced in the S_3 generation compared with the S_2 generation, it was still high for some chromosomes, including A7, C3, C4, C6 and C7 of the J1C1 lineage. There was a significant difference in the percentage genome present between the chromosomes in both populations (ANOVA, $P < 0.05$; Fig. 4c,h).

There was no significant effect of any large-scale (> 0.5 Mbp) genomic rearrangements in the A and C genomes on fertility. However, stepwise regression analysis showed that the absence of

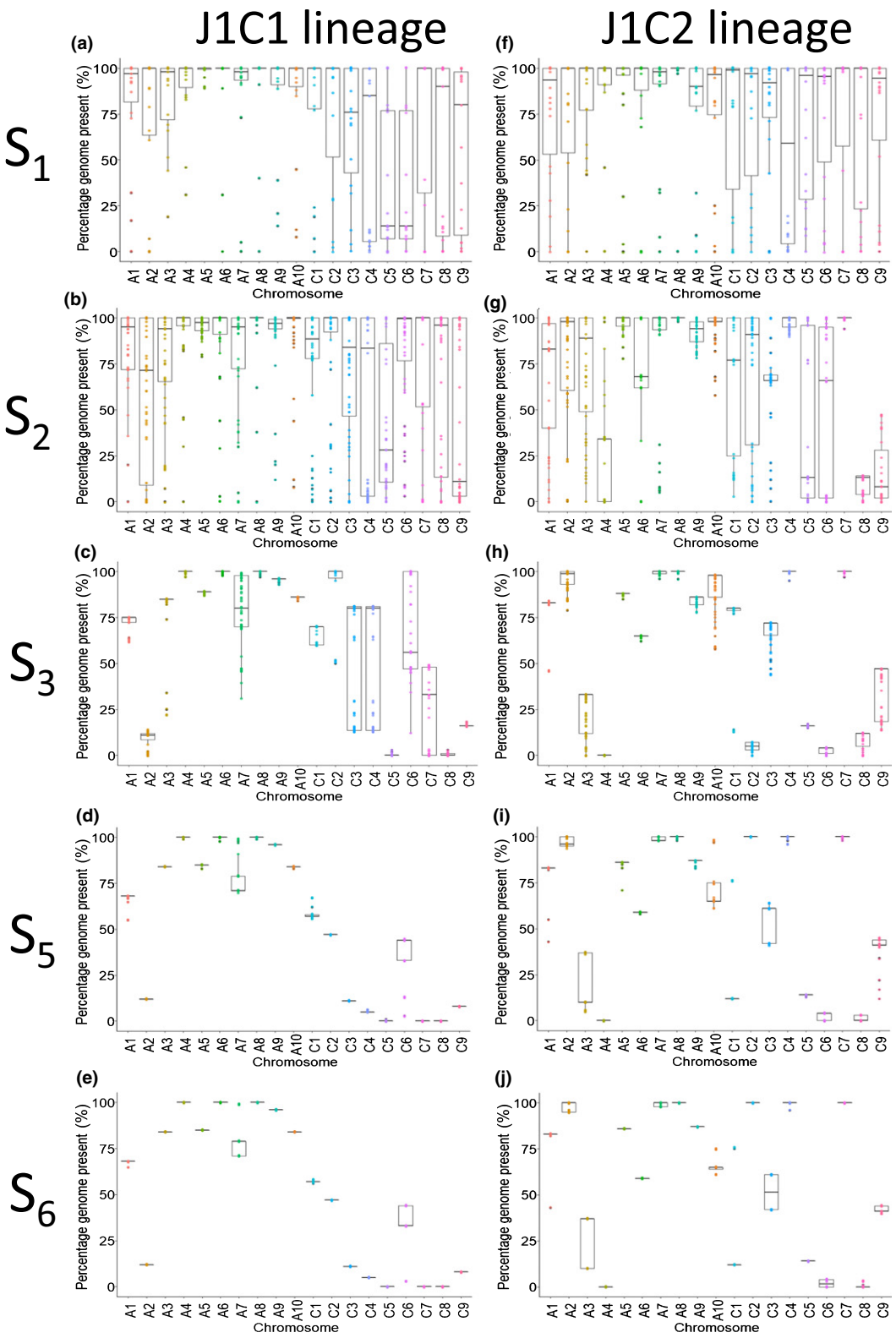

Fig. 4 Percentage of genome present per chromosome in *Brassica juncea* by *Brassica carinata* self-pollinated interspecific hybrids from the S_1 to S_6 generation (top to bottom) in (a–e) J1C1 hybrids and (f–j) J1C2 hybrids.

chromosome A3 in the J1C1 progeny and A1 in the J1C2 progeny reduced fertility ($P = 0.03$ for J1C1 and $P = 0.0122$ for J1C2). The B genome showed very limited genetic changes compared with the A and C genomes, indicating limited recombination between the B and A/C genomes. However, a 2.8 Mbp deletion at the top of chromosome B8 (loss of both copies),

which was present in 19% of J1C2 hybrid plants, caused a reduction in seed fertility ($P = 0.00085$, stepwise regression analysis).

Fertility and chromosome numbers in the BBAC S_4 and S_5 hybrids

By the S_4 generation, there was an increase in the pollen and seed fertility of these hybrids. The average pollen viability of the J1C1 and J1C2 populations was 51% (range 17–89) and 91% (range 67−98), respectively, with a significant difference between these two genotypes (ANOVA, $P < 0.05$). The seed fertility ranged from 274 to 1551 for J1C1 and 442 to 1884 for J1C2, with an average seed fertility of 809 seeds/plant for the J1C1 and 1127 seeds/plant for the J1C2 populations, with a significant difference between the two populations (ANOVA, $P < 0.05$). This was based on analysis of 100 plants: 50 J1C1 and 50 J1C2. The average J1C2 S_4 seed fertility was higher than that of the *B. carinata* C2 parent, but the seed fertility of the *B. juncea* and *B. carinata* C1 parent was higher than the average of both lineages. The chromosome numbers of these plants ranged from 32 to 36 in both genotypes, with an average number of 34. Seed fertility in the S_3, S_4 and S_5 hybrid plants was higher on average in the J1C2 plants than in the J1C1 plants (ANOVA, $P = 0.01$; Fig. S2). The average fertility of the S_5 was also less than that of S_4. This is likely the result of severe disease pressure in the glasshouse, as the fertility of the parent genotypes was also seriously affected.

Fertility of BBAC hybrids increased with generational selection

Hybrids generally became more fertile across generations with selection (in each generation the most fertile individuals were selected as parents of the next generation) (Dataset S1). The general increase in seed production was, however, reversed in the S_5 generation due to severe disease pressure in the glasshouse (Fig. S3). By the sixth generation of self-pollination, some hybrid progeny sets had restored equivalent seed fertility to their parent species, with a combined average of 1072 seeds per plant (Fig. 5c; Dataset S1). In the J1C2 lineage, there was an increase in pollen viability from the F_1 generation to the S_4 generation, after which (S_4–S_6 generations) pollen viability levels were close to those of the parental controls. Variation between plants in pollen viability also decreased across the generations (Fig. 5b). In the J1C1 lineage, pollen fertility also increased from the F_1 to S_6 generation, although the increase was less consistent across the generations compared with the J1C2 lineage. The variation observed between plants was also higher in the J1C1 lineage than in that of the J1C2 (Fig. 5a).

Chromosome number in later generation hybrids was highly conserved and restored regular meiosis

Chromosome number per plant was counted in the S_1, S_2, S_4, S_5 and S_6 generations (Fig. 5d; S_3 data were not obtained). In the F_1 generation, the chromosome number was $2n = \text{BBAC} = 35$, as expected from the union of haploid gametes from *B. juncea* (AB) and *B. carinata* (BC) (Mason *et al.*, 2010). Variation in chromosome number was higher in the first four self-pollinated generations (S_1–S_4) than in the last two generations (Fig. 5d). In the S_5 generation, 64 plants were analysed: 47 (74%) had 36 chromosomes, 4 (6%) had 35 chromosomes, and 13 (20%) had a chromosome number of 34. Of the total 82 plants (41 J1C1 and 41 J1C2) analysed in the S_6 generation, 64 (78 %) had 36 chromosomes, 13 (16%) had 34 chromosomes, and 5 (6%) had 35 chromosomes. Fewer J1C1 plants, 28/41 (69%), showed $2n = 36$ chromosomes compared with J1C2 plants 35/41 (86%).

Meiotic chromosome pairing behaviour was analysed in the F_1, S_4, S_5, and S_6 generations (Table S4), most comprehensively in the S_5 and S_6 (Table S2). The parent *B. juncea* and *B. carinata* genotypes showed 100% regular bivalent pairing (18 and 17 bivalents at metaphase I, respectively) with no univalent or multivalent chromosome pairing configurations observed (Table S2), as expected from established allopolyploid species. BBAC F_1 hybrids ($2n = 35$) from the J1C2 genotype combination were previously reported to show only 68% of chromosomes involved in regular bivalent chromosome pairing per cell on average (3.1I + 11.7II + 2.3III + 0.2IV; Mason *et al.*, 2010). In the S_4 generation (four plants assessed), 95% regular bivalent pairing was observed (Table S4). An average of 96% regular bivalent pairing was observed in the S_5 generation, and 99% regular bivalent pairing in the S_6 generation (Table S4). S_5-generation hybrids showed an average meiotic configuration of 0.34I + 17.16II + 0.14III averaged across progeny sets, with a maximum of four univalents and two multivalents per cell. S_6-generation hybrids showed a significant improvement over the S_5 generation in meiotic regularity as assessed by percentage bivalent formation across progeny sets ($P = 0.028$; Fig. 6b,d; Table S2). In the S_6 generation, hybrids showed average configurations of 0.2I + 17.8II + 0.03III averaged across progeny sets, with a maximum of two univalents and one trivalent observed per cell. Neither progeny set nor lineage in either generation significantly affected meiotic pairing configuration (average number of univalents, bivalents, and multivalents).

Highly rearranged karyotype structure in later generation *Brassica* BBAC S_5 and S_6 interspecific hybrids

High-quality SNPs from 96 S_5 and 96 S_6 plants were used to determine the karyotype structure of S_5/S_6 hybrids. From these marker data, karyotypes of the A, B and C genomes for the two lineages were produced (Figs 7, 8, S3, S4). Based on the SNP data, all 16 B-genome chromosomes were present and not recombined with any A or C genome chromosomes in the S_5 and S_6 generations (Fig. 7a,b). This result was also confirmed using genomic *in situ* hybridization on mitotic and meiotic chromosome preparations (Fig. 7c), where the expected eight bivalents resulting from the correct pairing of the 16 B chromosomes were always observed at metaphase I and these chromosomes segregated properly at anaphase I (with eight chromosomes on opposite poles) (Fig. 7d). The B genome was fixed for either *B juncea* or *B. carinata* alleles, with some regions of residual heterozygosity.

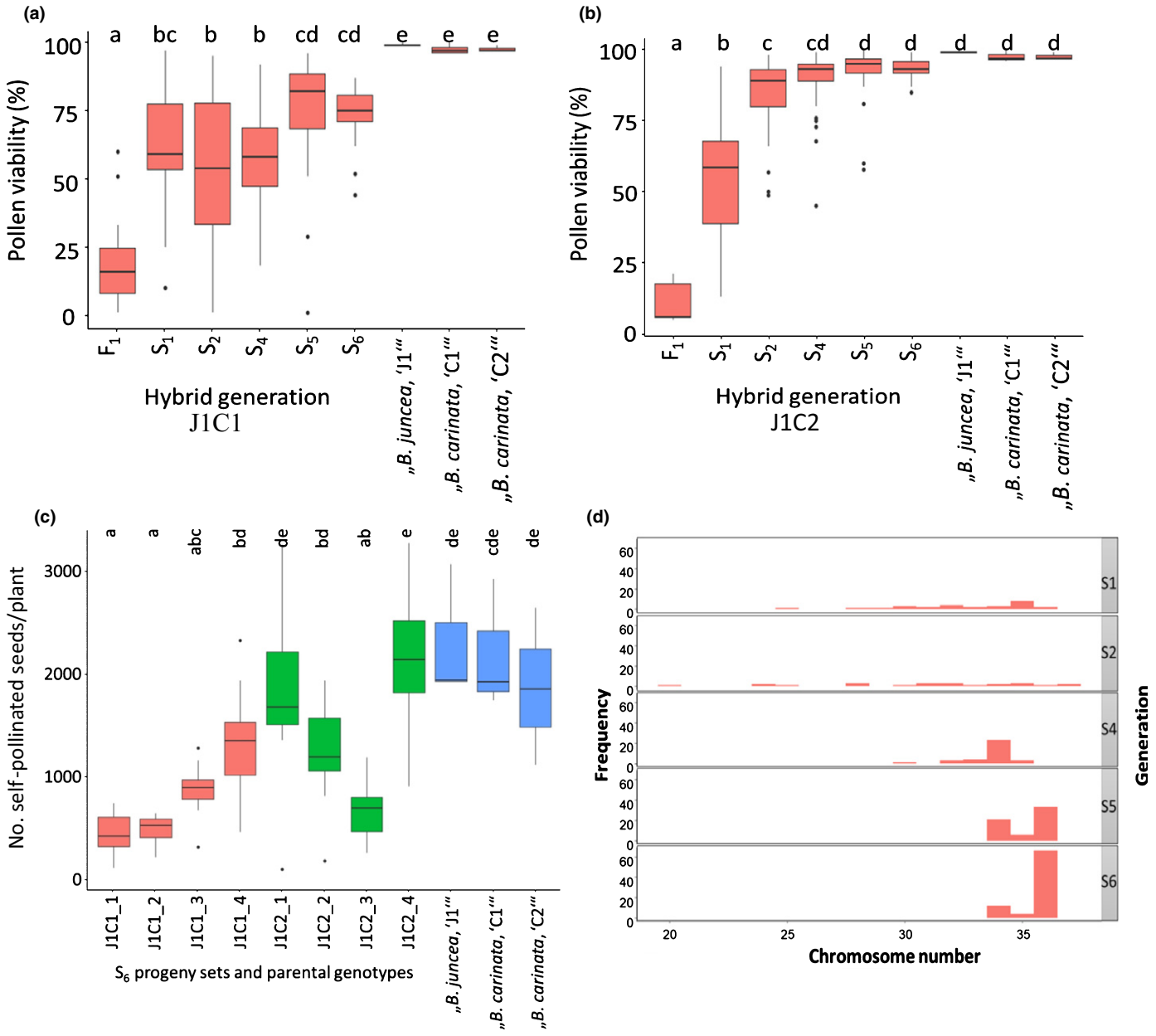

Fig. 5 Pollen, seed fertility, and chromosome number distribution of *Brassica juncea* × *Brassica carinata* interspecific hybrid progeny sets after six generations of self-pollination with selection for fertility relative to parental genotypes (*B. juncea* 'J1', *B. carinata* 'C1', and *B. carinata* 'C2'). (a, b) Pollen viability of (a) the J1C1 lineage and (b) the J1C2 lineage. Different letters indicate statistically significant differences. (c) Seed fertility of S_6 hybrids. Four progeny sets (12 plants per progeny set) are presented for each of two different genotypes J1C1 (green) and J1C2 (blue), along with the parental controls (red). (d) Distribution of chromosome number in self-pollinated progeny generations (S_1 to S_6) of *B. juncea* × *B. carinata* interspecific hybrids (F_1 = BBAC = 35 chromosomes) following selection for fertility in each generation.

By contrast, the A and C genomes were highly restructured, with chromosome losses and frequent translocations between the A and C genomes evident from the marker analyses. For the J1C1 lineage, 8/10 A-genome chromosomes showed genetic changes (deletions) based on SNP marker inheritance. whereas all C-genome chromosomes (9/9) showed genetic changes. Besides the loss of chromosome segments, there was no complete loss of any A-genome chromosome; for the C-genome, however, the three chromosomes C5, C7, and C8 were completely lost. These genetic changes were not different between the S_5 and S_6 generations. For the J1C2 lineage, 8/10 A-genome chromosomes were involved in rearrangements with a complete loss of chromosome A4 in the entire population, whereas 6/9 C-genome chromosomes were involved in rearrangements with a complete loss of chromosome C8. There was no clear selective pressure for particular chromosome segments or karyotype configuration: the genetic changes that occurred in the A and C genomes differed between the J1C1 and J1C2 lineages. For example, whereas in J1C1 all A-genome chromosomes were present, chromosome A4 was lost in J1C2.

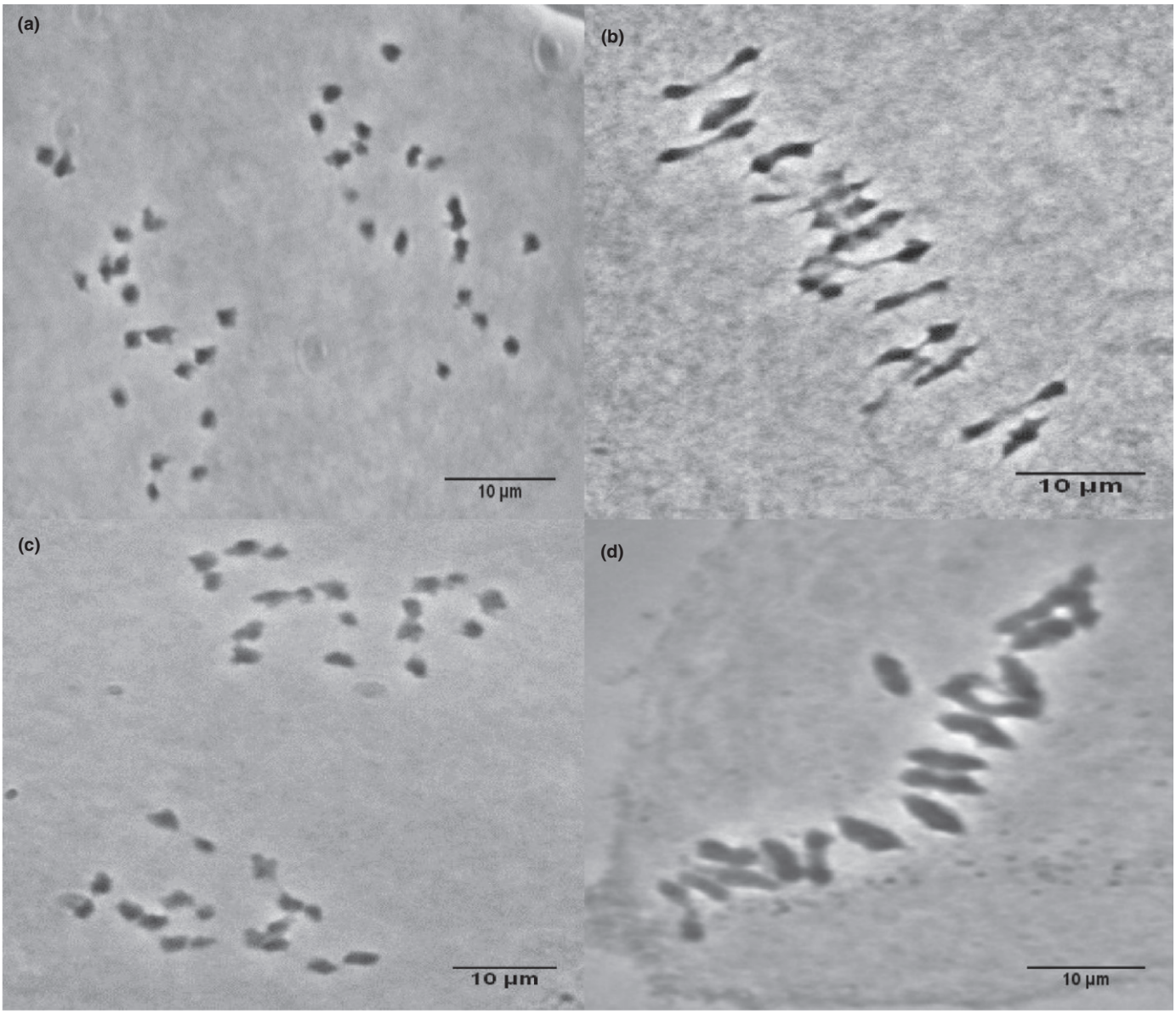

Fig. 6 Cytology of hybrids derived from the cross *Brassica juncea* × *Brassica carinata* followed by five or six generations of self-pollination with selection for high fertility. (a) Anaphase I in a BBAC S_5 hybrid; (b) metaphase I in a BBAC S_5 hybrid; (c) anaphase I in a BBAC S_6 hybrid; (d) metaphase I in a BBAC S_6 hybrid showing correct bivalent pairing and proper segregation.

Chromosome A5 recombined with chromosome C4 in J1C1, but with chromosome C5 in J1C2.

There was clear bias towards the retention of the A genome in both linages, with the C-genome homoeologues more frequently lost (Figs 4, 8). In the J1C1 population, 81–84% of the A-genome and 18–21% of the C genome was retained per progeny set by the S_6 generation, where all progeny sets had a common ancestor that retained 92% of the A genome and 58% of the C genome in the S_2 generation (Fig. S3). In the J1C2 population, 67–70% of the A genome and 42–51% of the C-genome was retained in the S_6 generation, where all progeny sets had an S_2-generation ancestor with 81% A genome and 75% C genome retention. Every homoeologous chromosome region was present in exactly one copy in the final modal A/C genome karyotypes in each lineage: either the A genome copy or the C genome copy of the homoeologous region was retained (Dataset S10). No homoeologous regions were observed in which both the A-genome and the C-genome copy were retained, or in which both the A-genome and the C-genome copy were lost.

Generational progression of chromosome changes

Most of the exchanges that took place between the A and C genomes occurred between the chromosomes with the highest degree of homoeology, such as A1/C1, A2/C2, A3/C3, A6/C7, A9/C9, and A10/C9 (Lagercrantz & Lydiate, 1996; Chalhoub *et al.*, 2014; Mason *et al.*, 2014). Most of the recombination events between the A and C genomes took place in the first hybrid

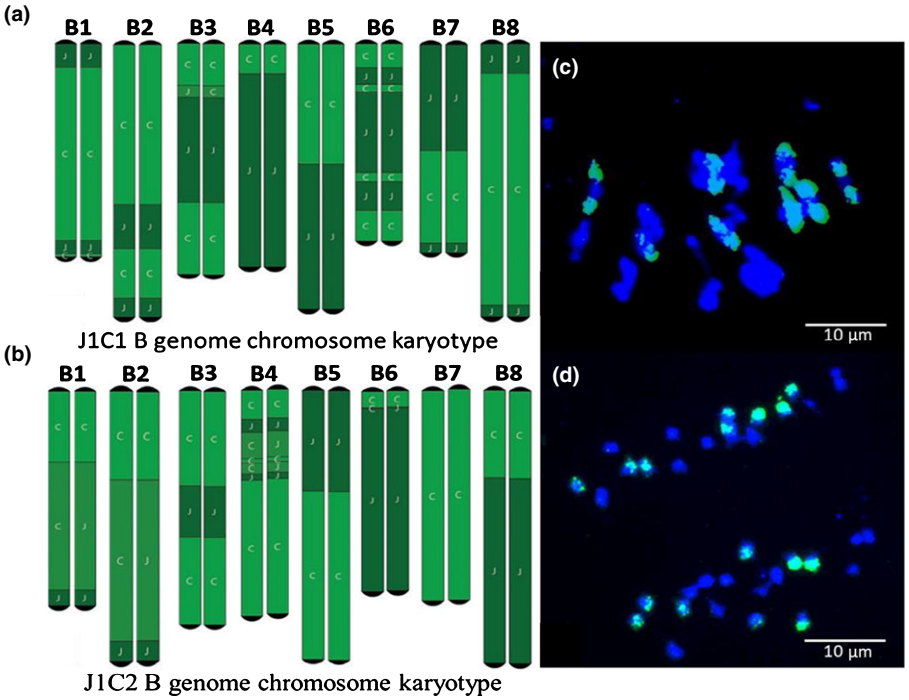

Fig. 7 Inheritance of B-genome alleles from *Brassica juncea* (J, dark green) and *Brassica carinata* (C, light green) based on single-nucleotide polymorphism marker genotyping in hybrids derived from the cross *B. juncea* × *B. carinata* following six generations of self-pollination (BBAC S_6) and selection for fertility: (a) J1C1 lineage and (b) J1C2 lineage. Forest green regions (CJ or JC) denote heterozygous regions with both *B. juncea* and *B. carinata* alleles. (c, d) Genomic *in situ* hybridization of (c) J1C1 chromosomes in metaphase I of meiosis with correct pairing of B genome (green) and (d) J1C1 chromosomes at anaphase I of meiosis showing proper segregation. Karyotypes of (a) and (b) produced using the R package CHROMDRAW.

meiosis (in F_1 plants), and hence were first observed in the S_1 generation in a heterozygous state (Figs S3, S4) before putatively being inherited and fixed in subsequent generations. Analysis of A-genome chromosome structure for J1C1 showed that genetic changes in chromosomes A1, A3, A5, A6, A7, and A9 remained the same between S_2 and S_6 generations. For the C genome, C4, C5, C6, and C7 did not undergo further changes between the S_2 and S_6 generations except for the loss of C8. Some of the karyotype rearrangements that were heterozygous in the J1C1 S_2 generation (e.g. involving A2, A3, and A7) appeared in the S_6 generation as homoeologous exchanges between A2/C2, A3/C3, and A7/C6 (Figs 8, S3, S4). Residual variation (presence of some individuals still segregating for chromosomal rearrangements or presence/absence) in karyotypes was observed in both lineages between progeny sets. In the J1C1 lineage, an A7/C6 translocation segregating in the S_5 generation was fixed in the S_6 with different variants between progeny sets, and a similar pattern was observed for an A2/C2 karyotype variant in the J1C2 lineage.

Discussion

In this study, we analysed the chromosome behaviour, stability, and fertility of *Brassica* trigenomic BBAC hybrids over six generations of self-pollination and selection for high fertility. Our results show that self-pollination and selection for fertility can lead to stable, fertile hybrids with novel karyotypes. Recombination and restructuring occurred between the A and C genomes in BBAC hybrids, whereas the B genome remained unchanged, and these A/C rearrangements appeared to be fixed by the $S_{5/6}$ generation, accompanied by a restoration of fertility and meiotic stability to produce 'true-breeding' progeny.

The ancestral relationship which exists between the *Brassica* A, B, and C genomes has been well-elucidated (Attia & Röbbelen, 1986; Lagercrantz & Lydiate, 1996; Ge & Li, 2007; Mason *et al.*, 2010; Chalhoub *et al.*, 2014), with the A and C genomes shown to be more closely related to each other than to the B genome. It has been predicted that the *B. nigra* (B) lineage diverged from the *B. rapa* and *B. oleracea* (A/C) lineage *c.* 7.9 million years ago (Mya) followed by the separation of the *B. rapa* (A) and *B. oleracea* (C) lineages *c.* 3.7 Ma (Inaba & Nishio, 2002; Panjabi *et al.*, 2008). As a result of this close relationship, the A and C genomes pair readily with each other in haploids (Nicolas *et al.*, 2009), AAC and CCA triploids (Leflon *et al.*, 2006), synthetic allotetraploids (Xiong *et al.*, 2011), and unbalanced AABC, BBAC, and CCAB tetraploid hybrids (Mason *et al.*, 2010), whereas A–B and B–C homoeologous pairing is less frequently observed (Chen *et al.*, 2005; Mason *et al.*, 2011a; Navabi *et al.*, 2010). We observed a complete lack of recombination between the B genome and the A genome and between the B genome and C genome in BBAC hybrids after six generations of self-pollination in our study. This observation is likely due to the fact that the B genome was present as homologous chromosome pairs, whereas the A and C genomes formed highly homoeologous pairing partners. Selection for fertility may also have selected against plants with homoeologous recombination events involving the B genome.

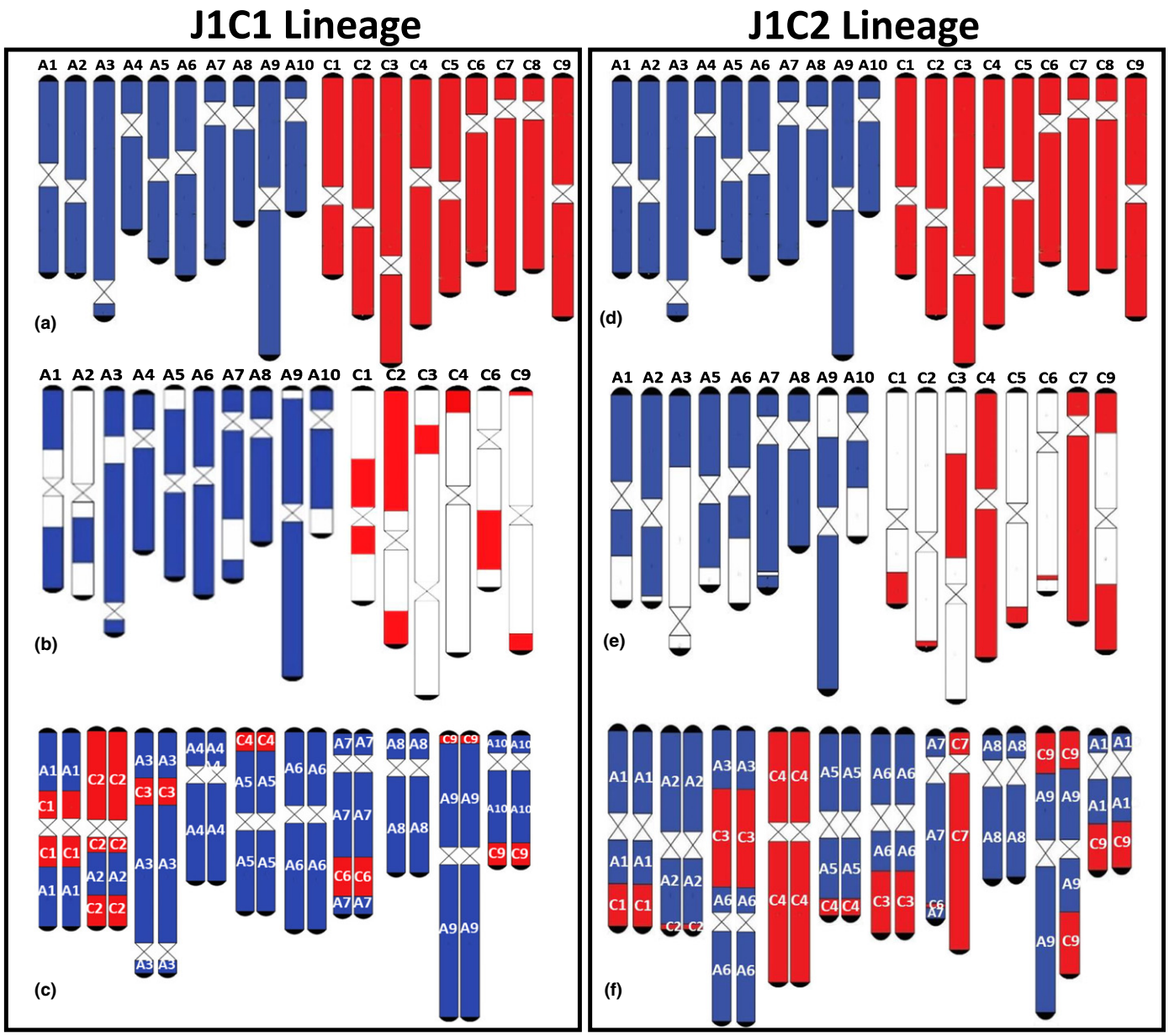

Fig. 8 Genetic changes and predicted recombined karyotypes in hybrids between *Brassica juncea* and *Brassica carinata* followed by six generations of self-pollination with selection for fertility (BBAC S_6) based on SNP marker genotyping: (a) A and C genome karyotype in the J1C1 BBAC F_1 hybrid with the expected 19 chromosomes given no recombination and segregation has occurred between the A and C chromosomes; (b) presence and absence of A and C genome chromosomes in the J1C1 lineage after six generations of self-pollination (S_6) based on SNP marker inheritance, where white represents absence of a chromosome segment: chromosomes C5, C7 and C8 are completely lost and therefore not represented; (c) the predicted modal recombined A–C chromosome karyotype for the J1C1 lineage after six generations of self-pollination (S_6) with a total of 20 chromosomes based on copy number analysis and chromosome counts; (d) A and C genome karyotype in the J1C2 BBAC F_1 hybrid with the expected 19 chromosomes because no recombination and segregation has occurred between the A and C chromosomes; (e) presence and absence of A- and C-genome chromosomes in the J1C2 lineage after six generations of self-pollination (S_6) based on SNP marker inheritance, where white represents absence of a chromosome segment: chromosomes A4 and C8 are completely lost and therefore not represented; and (f) the predicted modal recombined A–C chromosome karyotype for the J1C2 lineage after six generations of self-pollination (S_6) with a total of 20 chromosomes based on copy number analysis and chromosome counts.

Previously, self-pollination of a wheat (*Triticum aestivum*)–rye (*Secale cereale*) hybrid with $2n = $ RRAB up until the F_{17} generation revealed less frequent recombination between the A and B genomes and complete retention of the R genome in the early generations, but no restoration of genome stability (Dubovets & Sycheva, 2017). This is in contrast to our results of early fixation of karyotypes and restoration of genome stability by the $S_{5/6}$ generations. These contrasting results could be due to differences in the genetic control of meiosis between the wheat and *Brassica* genomes. The *Ph1* locus, located on chromosome 5B of bread wheat, is known to prevent homoeologous recombination between the wheat chromosomes almost entirely (Griffiths *et al.*,

2006). Hence, the presence of the *Ph1* locus could have decreased the ability of the A and B genomes to recombine and stabilize in early generations. By contrast, no strong control preventing homoeologous recombination between the *Brassica* A and C genomes is expected.

Most genetic changes took place in the S_1/S_2 generation meiosis rather than in later generation meiosis in both lineages, as has previously been reported in other synthetic *Brassica* types (Prakash, 1999; Szadkowski *et al.*, 2010). Interestingly, there was no clear selective pressure for particular chromosome segments or karyotype configurations between the two genotypes assessed. A similar lack of selection for particular chromosome segments has been observed in *Helianthus*, where the three homoploid hybrids *Helianthus anomalus*, *Helianthus deserticola*, and *Helianthus paradoxus* are all hybrids of two parent species *Helianthus petiolaris* and *Helianthus annuus*, but with different karyotypes in each hybrid species (Rieseberg, 2006).

The C genome was preferentially lost compared with the A genome in both BBAC lineages in our study. In allopolyploids, a phenomenon known as 'biased fractionation' is often observed over evolutionary time, whereby genes from one parental subgenome are preferentially lost (Bird *et al.*, 2018; Emery *et al.*, 2018). Biased fractionation has been reported in *Arabidopsis suecica* (Chang *et al.*, 2010; Novikova *et al.*, 2017), maize (*Zea mays*; Schnable *et al.*, 2011), *Arabidopsis thaliana* (Thomas *et al.*, 2006; Garsmeur *et al.*, 2014), *B. rapa* (Wang *et al.*, 2011), and cotton (Renny-Byfield *et al.*, 2015). Differences in transposable element density and methylation and the possibility that certain phenotypic traits may largely be under the control of one subgenome could be responsible for biased fractionation, or for the preferential expression of genes from one subgenome (subgenome dominance) which may lead to biased fractionation (Cheng *et al.*, 2016; Bird *et al.*, 2018; Wendel *et al.*, 2018). However, subgenome dominance does not seem to occur in all hybrid and polyploids; no subgenome dominance or evidence of biased fractionation has been observed in wheat, for example (Harper *et al.*, 2016). In *B. napus*, Chalhoub *et al.* (2014) did not find any significant bias in gene expression towards the A or C subgenome, despite the fact that the C genome has a higher transposable element density and more methylation than the A genome, and hence would be predicted to be similarly expressed (Wendel *et al.*, 2018). A more recent and comprehensive study by Wu *et al.* (2018) found a small but significant bias towards expression of genes from the A subgenome over the C subgenome (24% of gene pairs showed A > C compared with 15% showing C > A) in synthetic *B. napus*, but the generalizability of these results to natural *B. napus* is unknown. Interestingly, preferential loss of the C genome over the A genome has been observed frequently in different interspecific *Brassica* hybrid types, both for nonhomologous exchanges and whole chromosomes (Zhang *et al.*, 2016; Samans *et al.*, 2017), in line with our results. Possibly, the A genome contains more allelic variants responsible for improved fertility and viability than the C genome does, which would explain the retention of A-genome homeologues in our fertility-selected lines; more agriculturally significant quantitative trail loci also tend to be detected on the A genome relative to the C genome in natural *B. napus* (e.g. (Luo *et al.*, 2017; Zou *et al.*, 2018), supporting this interpretation.

Chromosome number was highly maintained within a narrow range from the BBAC F_1 hybrids ($2n$ = BBAC = 35) to the $S_{5/6}$ generation, by which generation almost all individuals showed between 34 and 36 chromosomes, accompanied by mostly regular meiosis. One important challenge that interspecific hybrids and neopolyploids encounter and must overcome to become established is the problem of incorrect meiotic pairing (Comai, 2005; Grusz *et al.*, 2017; Pelé *et al.*, 2018), specifically between homoeologous chromosomes and chromosome segments belonging to different subgenomes. This problem has been shown to persist for several generations following allopolyploid formation in synthetic *B. napus* (Xiong *et al.*, 2011), *Tragopogon* (Chester *et al.*, 2012), and synthetic wheat (Zhang *et al.*, 2013; Gou *et al.*, 2018). Homoeologous chromosome pairing can result in loss of chromosomes and chromosome segments important for fertility and viability, accompanied by loss of the ability to produce 'true-breeding' offspring. It has been proposed that selection for increased fertility should stabilize the genome and reduce the frequency of aneuploid offspring (Tian *et al.*, 2010). In initially unstable synthetic *Nicotiana* allotetraploid hybrids, the number of regular bivalents increased rapidly to > 99% after five generations of self-pollination (Ising, 1966). Similar observations have also been made in synthetic *Brassica* allotetraploids, albeit with genotype-specific variation (Song *et al.*, 1995; Prakash *et al.*, 2009). In our study, a combination of fertility-based selection and a high frequency of chromosome pairing between the homoeologous A and C-genome chromosomes may have interacted to retain viable chromosome complements.

A strong 'dosage compensation' effect was observed in the BBAC hybrids, as has previously been reported in *Brassica* (Xiong *et al.*, 2011) and *Tragopogon* (Chester *et al.*, 2012), where loss of A-genome chromosomes and homoeologous regions were compensated for by the retention of C-genome chromosomes and homoeologous regions and vice versa. Interestingly, we observed no instances in our S_6 generation hybrids where both the A and C genome homoeologue of a particular region were lost, or where both were retained. Only one copy (A or C) for each region of primary homoeology was detected for each of the modal karyotypes assessed in the J1C1 and J1C2 lines. This suggests that the negative effects of copy number variation (i.e. having an extra or missing copy of a homoeologous region relative to the normal dosage level of two copies (2A or 2C, as the third option for two copies of 1A + 1C is heterozygous/unstable and hence this was only observed in the early generations)) were extremely strong in these hybrids. As we applied very strong selection pressure for fertility in this project, we may have selected for lines with conserved dosages of A and C genomes. Aneuploidy can upset the expression levels of dosage-sensitive genes, resulting in lowered metabolic efficiency (Chester *et al.*, 2012), and has also previously been linked directly to lowered fertility in *Brassica* allohexaploid hybrids (Gaebelein *et al.*, 2019). Homoeologous chromosome copy numbers were also preferentially retained in self-pollinating lines of synthetic *B. napus*, suggesting that individuals with high deviation from chromosome balance had

reduced fertility and were selected against during generational advancement (Xiong *et al.*, 2011).

We conclude from this study that hybridization between *Brassica* allotetraploids sharing one of two genomes can lead to the formation of stable and fertile hybrids following self-pollination over a number of generations. Many authors have previously discussed the impact of homoploid and polyploid speciation and the various ecological, environmental, and genetic factors affecting their formation, maintenance, and diversification (Soltis & Soltis, 1999; Soltis *et al.*, 2003; Mallet, 2005, 2007; Leitch & Leitch, 2008; Levin & Soltis, 2018). However, relatively few experimental studies have demonstrated pathways for homoploid and polyploid hybrid speciation. Using randomly amplified polymorphic DNA/intersimple sequence repeat markers, James & Abbott (2005) and Brennan *et al.* (2012) showed that *Senecio squalidus* is a homoploid hybrid formed by hybridization of *Senecio aethnensis* and *Senecio chrysanthemifolius*. Studies in wild sunflower suggest the homoploid hybrid *Helianthus anomalus* arose rapidly (within fewer than 60 generations) by hybridization between *Helianthus annuus* and *Helianthus petiolaris* (Ungerer *et al.*, 1998). In allopolyploids, experimental studies have shown that the allotetraploids *Tragopogon mirus* and *Tragopogon miscellus* have formed repeatedly within the last 80 yr by hybridization of the three diploid species *Tragopogon dubius*, *Tragopogon pratensis* and *Tragopogon porrifolius* (Soltis *et al.*, 2004; Chester *et al.*, 2012; Lipman *et al.*, 2013). In our study, experimental *Brassica* hybrids rapidly recovered correct chromosome pairing and maintained chromosome number, and some plants even produced more seeds than the parents. However, the genetic relationship between the genomes seems to be the main contributing factor leading to this result. The haploid genomes of these hybrids were highly restructured and behaved as homologous chromosomes with high levels of chromosome rearrangements. These hybrids could serve as a potentially important genetic resource that could be exploited for breeding purposes through transfer of A-genome introgressions via backcrossing into *B. carinata* or C-genome introgressions via backcrossing into *B. juncea*, and also support previously theoretical mechanisms of hybrid speciation (Mirzaghaderi & Mason, 2017; Levin & Soltis, 2018).

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Author contributions

EK performed all experiments to generate S_4–S_6 generation data, performed all data analyses, produced the figures, and drafted the paper. RG performed all experiments to generate S_3 generation data, and PV-T, Y-TL, ZI and DN performed experiments to produce S_1 and S_2generation data. JB generated genotyping data and contributed to critical revisions of the manuscript. PV-T also contributed to critical revisions of the manuscript. ASM conceptualized the project, supervised EK, PV-T, Y-TL, ZI and DN, and contributed to critical revisions of the manuscript. All authors approved the manuscript version for submission.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Fertility data collected for BBAC hybrids from the cross *Brassica juncea* × *B. carinata* (genotypes J1C1 and J1C2) and their parent controls over six generations of self-pollination.

Dataset S2 Illumina Infinium *Brassica* 90K SNP genotyping array data for the *Brassica* A- B- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' × *Brassica carinata* 'C1' hybrids in the fifth (BBAC S_5) and sixth (BBAC S_6) selfing generation.

Dataset S3 Illumina Infinium *Brassica* 90K SNP genotyping array data for the Brassica A- B- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' × *Brassica carinata* 'C2' hybrids in the fifth (BBAC S_5) and sixth (BBAC S_6) selfing generation.

Dataset S4 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' × *Brassica carinata* 'C1' hybrids in the first (BBAC S1) selfing generation.

Dataset S5 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' × *Brassica carinata* 'C2' hybrids in the first (BBAC S1) selfing generation.

Dataset S6 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' × *Brassica carinata* 'C1' hybrids in the first (BBAC S_2) selfing generation.

Dataset S7 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for

Brassica juncea 'J1' × *Brassica carinata* 'C1' hybrids in the first (BBAC S_2) selfing generation.

Dataset S8 Illumina Infinium *Brassica* 90K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' × *Brassica carinata* 'C1' hybrids in the first (BBAC S_3) selfing generation.

Dataset S9 Illumina Infinium *Brassica* 60K SNP genotyping array data for the *Brassica* A- and C- genomes on the *Brassica napus* Darmor-bzh v8.1 and *B. nigra* reference genomes for *Brassica juncea* 'J1' × *Brassica carinata* 'C1' hybrids in the first (BBAC S_3) selfing generation.

Dataset S10 Inferred primary homoeologous regions between the *Brassica* A and C genomes based on Illumina Infinium array single nucleotide polymorphism (SNP) genotyping.

Fig. S1 Genetic changes in *Brassica juncea* × *Brassica carinata* self-pollinated S_2 interspecific hybrids as detected from Illumina Infinium *Brassica* 60K SNP array data.

Fig. S2 Fertility of *Brassica juncea* × *B. carinata* interspecific hybrids in S_3, S_4 and S_5 generations of self-pollination with selection for fertility relative to their parent genotypes (*B. juncea* 'J1', *B. carinata* 'C1' and *B. carinata* 'C2').

Fig. S3 A- and C- genome presence and absence based on SNP marker genotyping in hybrids derived from the cross *B. juncea* × *B. carinata* (J1C1 genotype) following two generations of self-pollination (BBAC S_2) and selection for fertility.

Fig. S4 A- and C- genome presence and absence based on SNP marker genotyping in hybrids derived from the cross *B. juncea* × *B. carinata* (J1C2 genotype) following two generations of self-pollination (BBAC S_2) and selection for fertility.

Fig. S5 Number of genetic changes in different generations of *B. juncea* × *B. carinata* J1C1 and J1C2 interspecific hybrids relative to the F_1 generation.

Table S1 Putative centromere locations for the *Brassica* A- and C- genomes on the Darmor-*bzh* v8.1 reference genome estimated using half-tetrad analysis of mapping populations of *Brassica juncea* × *Brassica napus* (AABC) and *Brassica carinata* × *Brassica napus* (CCAB) hybrids.

Table S2 Meiotic chromosome pairing configurations of hybrids produced from the cross *Brassica juncea* × *Brassica carinata* followed by six generations of self-pollination (BBAC S_6) for two genotypes 'J1C1' and 'J1C2'.

Table S3 Seed information for self-pollinated *B. juncea* × *B. carinata* hybrids from S_1 – S_6 showing the number of plants which were grown in each generation and under which condition and the number SNP genotyped.

Table S4 Meiotic pairing of F_1, S_4, S_5 and S_6 generation hybrid plants.

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