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The fate of chromosomes and alleles in an allohexaploid *Brassica* population

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SUMMARY

Production of stable allohexaploid (three-genome) hybrids in the agriculturally significant *Brassica* genus has been a goal of geneticists and breeders for 100 years. However, synthetic allohexaploids in *Brassica* are usually unstable and lose chromosomes from generation to generation. Why this happens is unknown, as naturally-occurring stable allopolyploid species are common in this genus. We used a combination of modern molecular marker and cytogenetics techniques to comprehensively assess karyotype stability and chromosome behaviour in allohexaploid *Brassica*. Our results shed light on possible factors contributing to polyploid formation in the *Brassica* genus, and suggest fruitful avenues for production of stable synthetic hybrids.

ABSTRACT

Production of allohexaploid *Brassica* (2n = AABBCC) is a promising goal for plant breeders, due to the potential for hybrid heterosis and useful allelic contributions from all three of the *Brassica* genomes present in the cultivated diploid (2n = AA, 2n = BB, 2n =CC) and allotetraploid (2n = AABB, 2n = AACC and 2n = BBCC) crop species (canola, cabbages, mustards). We used high-throughput single nucleotide polymorphism (SNP) molecular marker assays, flow cytometry and fluorescent in-situ hybridisation (FISH) to characterise a population of putative allohexaploids derived from self-pollination of a hybrid from the novel cross (*B. napus* × *B. carinata*) × *B. juncea*, to investigate whether fertile, stable allohexaploid *Brassica* can be produced. Allelic segregation in the A- and C-genomes generally followed Mendelian expectations for an F_2 population, with minimal non-homologous chromosome pairing. However, we detected no strong selection for complete 2n = AABBCC chromosome complements, with weak correlations between DNA content and fertility (r^2 = 0.11) and no correlation between missing chromosomes or chromosome segments and fertility. Investigation of next-generation progeny resulting from one highly-fertile F_2 plant using FISH revealed general maintenance of high chromosome numbers but severe distortions in karyotype, as evidenced by recombinant chromosomes and putative loss/duplication of A- and C-genome chromosome pairs. Our results show promise for the development of meiotically stable allohexaploid lines, but highlight the necessity of selection for 2n = AABBCC karyotypes.

INTRODUCTION

The *Brassica* genus contains the largest number of cultivated crop species of any plant genus (Dixon 2007). Six major crop species are *B. rapa* (Chinese cabbage, turnip), *B. oleracea* (cabbage, cauliflower, broccoli), *B. nigra* (black mustard), *B. napus* (canola, rapeseed), *B. juncea* (Indian mustard, leaf mustard) and *B. carinata* (Ethiopian mustard). These six species share a unique genomic relationship: progenitor diploid species *B. rapa* (2n = AA), *B. oleracea* (2n = CC) and *B. nigra* (2n = BB) gave rise to the allotetraploid species *B. juncea* (2n = AABB), *B. napus* (2n = AACC) and *B. carinata* (2n = BBCC) through pairwise crosses (Morinaga 1934; U 1935). However, despite the fact that each pair of genomes co-exists in an allotetraploid species, no naturally occurring allohexaploid species (2n = AABBCC) exists. In general, interspecific hybridisation and polyploidy in plants are potent evolutionary mechanisms, allowing formation of new species with adaptation to a wider range of climatic conditions and greater "hybrid vigour" than their progenitor species (Leitch and Leitch 2008; Otto and Whitton 2000). Hence, production of an artificial allohexaploid in the agriculturally important *Brassica* genus could potentially give rise to new crop types with greater inter-subgenomic heterosis (Zou *et al.* 2010) and tolerance of a wider range of environmental conditions than pre-existing *Brassica* crops (Chen *et al.* 2011).

Interest in producing an allohexaploid *Brassica* dates back to the 1960s, when induction of somatic chromosome doubling of triploid ABC interspecific hybrids to form AABBCC allohexaploids was first carried out (Iwasa 1964). However, mixed results have been reported for success of these synthetic allohexaploid *Brassica*. Howard (1942) reported relatively high fertility over several generations of self-pollination of 2n = AABBCC plants derived from the cross *B. rapa* × *B. carinata*. However, Iwasa (1964) found poor meiotic stability in the same species cross that failed to improve even up to the F_5 selfing generation, resulting in loss of fertility and hence usefulness of this crop. Allohexaploid *Brassica* has also been created as a bridge to transfer disease resistance between *B. nigra* and *B. napus* (Pradhan *et al.* 2010; Sjödin and Glimelius 1989), to produce yellow seeded *B. napus* by crosses between *B. rapa* and *B. carinata* (Meng *et al.* 1998; Rahman 2001), to transfer cytoplasmic male sterility from *B. oleracea* to *B. juncea* (Arumugam *et al.* 1996) and to resynthesise *B. napus* from *B. rapa* and *B. carinata* (Li *et al.* 2004). However, although these studies demonstrate the ease of production of allohexaploid *Brassica* from crosses between the diploid and allotetraploid species, meiotic stability and fertility was not assessed in these

allohexaploid types. Somewhat increased genomic stability in allohexaploids derived from genotypes of *B. rapa* × *B. carinata* crosses has recently been demonstrated after several successive generations of selfing and selection for 2*n* = 54 chromosome complements (Tian *et al.* 2010).

Mason et al. (2012) mimicked a natural evolutionary pathway for polyploid formation in *Brassica* by using unreduced gametes to produce an allohexaploid plant. *Brassica napus* and *B. carinata* were crossed in the first generation to create a hybrid with karyotype CCAB (Fig. 1). An unreduced gamete from this hybrid combined with a reduced gamete from *B. juncea* (n = AB) to form a near-hexaploid (AABBCC) plant with 2n = 49 chromosomes, 5 short of the full allohexaploid complement (Mason *et al.* 2012) (Fig. 1). Low-resolution molecular karyotyping using microsatellite markers indicated that the missing chromosomes were all monosomic deletions (that is, one copy of each of five different homologous chromosomes was retained in the allohexaploid plant (Mason *et al.* 2012)). This plant was highly fertile and provided plentiful self-pollinated progeny for the present study. We hypothesised that some lines produced from this allohexaploid may show 2n = 54, as well as demonstrating greater meiotic stability and fertility than lines produced through crosses between diploid and allotetraploid species followed by colchicine treatment to induce chromosome doubling.

MATERIALS AND METHODS

Generation and growth of plant material and experimental populations: Methods used in crossing *B. napus* × *B. carinata* to produce the first generation hybrid plant (2n = CCAB) are detailed in Mason et al. (2011). Crossing to generate the allohexaploid hybrid plant (hereafter referred to as "A1"; previously N1C1.J1-1) and preliminary cytological and molecular marker characterization of this plant is detailed in Mason et al. (2012) and further details are provided in Figure 1. The source of cytoplasm in A1 was *B. napus*, with *B. carinata* and *B. juncea* used as paternal parents in the formation of A1. The A genomes in *B. juncea* and *B. napus* are hereafter referred to as subgenomes "A^j" and "A^n" respectively, the B genomes in *B. juncea* and *B. carinata* are hereafter referred to as subgenomes "B^j" and "B^c" respectively and the C genomes in *B. napus* and *B. carinata* are hereafter referred to as subgenomes "C^n" and "C^c" respectively, following the naming conventions of Li et al. (2004).

Seeds from self-pollination of hybrid plant A1 were sown and are subsequently referred to as the SP population. Data collected from each experimental plant in the SP population are summarised in Supplementary Table 1. We characterised subsets of plants for total DNA content (flow cytometry), for A and C genome transmission (Illumina Infinium Brassica 60K SNP array), and for fertility (sporad observations, pollen viability and self-pollinated seed set). A set of second generation plants resulting from one of the most fertile SP progeny was then characterised by chromosome counts, and three plants with more than 50 chromosomes were tested for genome inheritance using fluorescent *in-situ* hybridisation (FISH).

The SP population was split into two groups. The first group (lines SP_001 to SP_050) were germinated in potting mix and grown to maturity in a controlled environmental room (16 h photoperiod with a light intensity of 200 μmol m^{-2} s^{-1} at 18º/10º C day/night) at The University of Western Australia (UWA), Perth, Australia. Lines SP_051 to SP_115 were germinated in potting mix and grown to maturity under air-conditioned glasshouse conditions at The University of Queensland (UQ), Brisbane, Australia. Self-pollinated seeds from plant SP_042 were germinated and grown under heated glasshouse conditions at INRA Le Rheu, France. DNA was extracted from leaf tissue of plants SP_001 to SP_050 and parent species controls using a Nucleon Phytopure Genomic DNA Extraction Kit (Illustra). DNA was extracted from leaf tissue of SP lines SP_051 to SP_115 using the Microprep method of Fulton et al. (1995).

Flow cytometry: Samples SP_001 to SP_050 and controls (parent species genotypes, CCAB hybrids and plant A1) were assessed for DNA content using flow cytometry. Young leaf tissue (5 mg) from each plant was individually sampled into 1.2 mL 8-strip tubes containing a 3 mm tungsten carbide bead on ice, following the methods of Cousin et al (2009). To each sample 500 μL of cold lysis buffer (0.1 M citric acid and 0.5% v/v Triton X-100 stored at 4ºC) was added. Samples were then placed at -20ºC for 10 minutes. Samples were shaken at 25 Hz for a total of 24 s in a TissueLyser (Qiagen), with the rack orientation reversed at 12 s to help evenly distribute the homogenisation. Homogenate (120 μL) was transferred by multichannel pipette to 96-well filter plates (800 μL Unifilter microplate 25-30 μm pore size with a long drip [Whatman]) sitting over collection plates, which were gently vacuumed to collect the filtrate.

A reading plate with 2 µL RNase A (3 mg/mL) per well was set up and 60 µL of the filtered samples were added to respective well locations. The reading plate was incubated at 37ºC for 30 minutes, and treated with 150 µL propidium iodide (PI) staining solution (Roberts 2007) following the methods of Cousin *et al* (2009). Nuclear DNA content was measured on a BD FACSCanto II (BD Biosciences) flow cytometer, using FACS Diva V6.1.1 operating software, at The University of Western Australia's Centre for Microscopy, Characterisation and Analysis. The PI dye was excited with a 488 nm (blue) laser and PI emission collected with a 585/42 (564-606 nm) bandpass filter for 60 seconds per sample or for 20000 nuclei events (whichever came sooner). Samples were repeated up to five times when no single clear peak was observed in the first run (26 SP lines). Lettuce (*Lactuca sativa* 'Grand Rapids') was used as the internal standard for nuclear DNA content, and *B. rapa*, *B. napus* and *B. carinata* as external standards. The *L. sativa* histogram peak was adjusted to fluorescence channel 200 at the beginning of each acquisition session. Experimental data were analysed and CV calculations performed using FlowJo V7.2.5 (Tree Star Inc.) FC analysis software to determine the CV and mean size of the gate, and means for the standard and sample peaks (Cousin *et al.* 2009). Sample 2C DNA was calculated by (mean of sample peak/mean of standard peak)*2C DNA amount of the standard species (*L. sativa*: 5.5 pg) (Zonneveld *et al.* 2005). Ploidy *x*-value was calculated by 2C value/ (0.97+2.36): (*B. rapa* AA-genome + *B. carinata* BBCC-genome, respectively).

Pollen viability: Flowers from all samples were collected and anthers were dissected from flowers and placed on microscope slides with a few drops of 1% acetic acid-carmine solution (1 g carmine powder in 45% glacial acetic acid solution). Pollen viability was assessed as described in Nelson et al. (2009): stained and swollen pollen grains (oval shape) were considered viable, and non-stained and shrivelled pollen grains were counted as nonviable. A minimum of 300 pollen grains were counted per plant.

Sporad observations: Buds were collected in 6:3:1 ethanol : chloroform : glacial acetic acid solution (Carnoy's II) in the morning, and incubated for 24 h at room temperature before being transferred to 50% ethanol at 4 ºC for storage. Anthers were dissected out from buds on glass microscope slides and gently squeezed to release sporads into 1% acetic acid-carmine solution. The number of sporads with one, two, three, four, five or six nuclei post-meiosis (monads, dyads, triads, tetrads, pentads and hexads, respectively) was counted for each of 58 individuals in the SP population, with at least 300 sporads observed per plant.

Chromosome counting: Twenty-five plants resulting from self-pollination of SP_042 (second generation self-pollinated plants) were assessed for chromosome number. Three to ten (mode five) counts were performed for each plant on different chromosome spreads. Fresh root tips were collected into glass bottles containing 0.04% 8-hydroxyquinoline solution, incubated for 2 hours in the dark at room temperature and then for another 2 hours at 4ºC. Samples were fixed in cold ethanol : glacial acetic acid (3:1) solution for 48 hours at room temperature, then stored in 70% ethanol at -20ºC until used. Fixed root tips were rinsed with deionised water for 2 × 10 minutes at room temperature to remove the fixative, then incubated in 0.01 M citrate solution (6 mM trisodium citrate-dihydrate, 4 mM citric acid monohydrate, pH 4.5) for 15 minutes at room temperature, then in 250 µL of enzyme solution (5% cellulase (Sigma-Aldrich) and 1% pectolyase (Sigma-Aldrich) in 5 ml citrate solution) for 30 minutes at 37 ºC. After incubation, the enzyme solution was removed with the aid of a Pasteur pipette, and roots were soaked in deionised water for 30 minutes. Washed meristem root tips were transferred to slides using a Pasteur pipette. One drop of 3:1 ethanol : acetic acid was added to the slides and root tips were scrambled with a pin. After the slides dried, one drop of DAPI (4',6 diamidino-2-phenylindole, 2 µg/ml in McIlvaines citric buffer, pH 7; blue emission) (Vector Laboratories) was added followed by a cover slide. Fluorescence images were captured using a CoolSnap HQ camera (Photometrics) on an Axioplan 2 microscope (Zeiss) and analysed using MetaVue^{TM} (Universal Imaging Corporation).

Fluorescent *In-situ* Hybridisation: Fluorescent *in-situ* hybridisation (FISH) was carried out according to protocols detailed in Mason et al. (2010), at INRA, Le Rheu, France. The B genome was detected using Texas-red-labelled *B. nigra* DNA as a probe, and the C genome using Alexa 488-labelled BAC BoB014O06 to highlight a C-genome-specific repetitive sequence distributed over the nine C genome chromosomes (Howell *et al.* 2002; Leflon *et al.* 2006). Three plants with chromosome counts of approximately 2*n* = 54 were chosen for FISH.

Molecular karyotyping using the Illumina Infinium Brassica 60K SNP chip: A SNP chip with 52 157 SNPs designed for *Brassica napus* (Illumina Infinium 60K bead array) was used to assess allele inheritance in SP_051 to SP_115, as well as parental controls (see Mason et al. (2014) for details of SNP data analysis). Chip hybridisation was carried out according to the manufacturer's instructions at The University of Queensland, Brisbane, Australia, and

chips were scanned using a HiScanSQ (Illumina). Data was visualised using the Genotyping module of Genome Studio V2011.1 (Illumina), and SNP locations were sorted with reference to the publically available sequenced *B. rapa* genome (Wang *et al.* 2011b) and *B. oleracea* genome (http://brassicadb.org/brad/).

Statistical analyses and genetic segregation hypotheses: R version 2.15.2 (The R project for statistical computing) base and "sm" packages were used to generate histograms and density distributions. Pearson's χ^2 test was used to assess whether allele inheritance followed Mendelian expectations for an F_2 selfing population. For each locus at which both an A^j and an A^n allele was present in the A1 allohexaploid plant (A^j/A^n), a 1 : 2 : 1 allelic segregation ratio was predicted for A^n/A^n : A^j/A^n : A^j/A^j in the progeny. Likewise, for each locus at which both a C^n and a C^c allele was present in the allohexaploid plant A1 (C^n/C^c), a 1 : 2 : 1 allelic segregation ratio of C^n/C^n : C^n/C^c : C^c/C^c was predicted in the segregating F_2 population. For univalent chromosomes A^j2, A^j6 and A^j9, where no *B. napus* (A^n) homologous chromosome partner was present in the A1 hybrid, gametes from A1 would be expected to carry each chromosome at a frequency of 50%. As two parent gametes united in a self-pollination event to produce the SP progeny, 75% of plants should have at least one copy of the univalent *B. juncea* alleles at each locus (1 : 2 : 1 ratio for two copies of A^j : one copy of A^j : no copies of A^j). Hence, a 3:1 ratio of presence : absence was expected for A^j alleles for chromosomes A^j2, A^j6 and A^j9.

RESULTS

Flow cytometry: Of the 44 plants assessed for DNA content using flow cytometry in the SP population (Supplementary Table 1), 43 had DNA contents ranging from 82% to 97% of the predicted DNA content for a 2*n* = AABBCC allohexaploid (average 88%) and formed a normal distribution about the A1 plant DNA content (Fig. 2). A single plant (SP_016) had a DNA content of 28% of the predicted allohexaploid DNA content, approximately the same as *B. rapa* (2n = AA).

Molecular karyotyping using the Illumina Brassica 60K SNP chip for the A and C genomes: A total of 7 651 out of the 52 157 A and C genome SNPs assessed were polymorphic between the *B. juncea* (A^j) and *B. napus* (A^n) A genomes or *B. napus* (C^n) and *B. carinata* (C^c) C genomes in the parent cultivars: 2 667 in the A genome (A^j/A^n SNPs) and

4 984 in the C genome (C^n/C^c SNPs). The number of SNPs per chromosome ranged from 123 to 1 283 (average 402 per chromosome). On the basis of the SNP molecular karyotyping, we determined if chromosomes from *B. juncea* (A^j alleles), *B. napus* (A^n and C^n alleles) and *B. carinata* (C^c alleles) were absent (all alleles from that subgenome absent for that chromosome), present (all alleles from that subgenome present for that chromosome) or partially present (some alleles from that subgenome absent and some present for that chromosome, such as would result from homologous recombination between A^n and A^j chromosomes or between C^n and C^c chromosomes). Absence of both parental alleles (i.e. both A^j and A^n or both C^n and C^c) was assumed to result from chromosome loss if a whole chromosome was missing, and to result from non-homologous recombination if only part of a chromosome was missing.

Brassica napus chromosomes A^n2, A^n6 and A^n9 were missing from the allohexaploid hybrid "A1": these *B. napus* chromosomes were not transmitted to A1 from the $C^nC^cA^jB^c$ parent, and hence were also absent in the SP population. A previously-characterised A^n7-C^n6 interstitial reciprocal translocation comprising about 1/3 of both chromosomes (Osborn *et al.* 2003) also existed within the *B. napus* cultivar but not in the *B. juncea*/*B. carinata* A^j and C^c genomes, as evidenced by haplotype block discontinuity and disrupted allelic inheritance in this region of the SP population. These events accounted for the vast majority of missing chromosome fragments in the 71 plants genotyped in the SP population (Fig. 3): chromosomes A7 and C6 showed 16% and 12% loss of both parental alleles respectively across the population. The next largest percentage loss of both parental alleles (1-2%) was observed for chromosomes A1 and C2 (Fig. 3). Part of chromosome A^n10 in the *B. napus* parent line exhibited two-locus amplification patterns across all SNPs (as assessed by SNP marker cluster plots) for a distance of approximately 5 Mb at the distal end of chromosome A10. Two-locus patterns were not observed in the *B. juncea* A^j10 chromosome in the same region. This putative duplication in A^n10 could not be readily matched to a haplotype pattern present in any other part of the genome.

Of the 71 SP progeny genotyped, 24 (32%) had complete complements of A and C genome alleles from at least one parent. For those chromosomes absent from the A1 hybrid (A^n2, A^n6 and A^n9), their homologue equivalents from the *B. juncea* parent (A^j2, A^j6 and A^j9) were present. In these cases, it was not possible to determine if one or two copies of each A^j2, A^j6 or A^j9 allele was present.

Allele transmission and genomic bias in the A and C genomes: Allelic inheritance for most chromosomes followed normal Mendelian expectations for segregation at heterozygous loci (Fig. 3). For loci that were heterozygous in the near-hexaploid parent, a 1 : 2 : 1 ratio of co-dominant parental alleles was expected in the self-progeny. For loci present as a single copy in the allohexaploid hybrid A1 (i.e. part of univalent chromosomes), a dominant-type ratio of 3:1 present/absent was expected in the self-progeny.

In total, 877 SNP loci were fixed to homozygosity for either *B. napus* or *B. carinata* parental alleles in the allohexaploid hybrid A1 (and subsequently in the SP population) due to homologous recombination between the C^n and C^c chromosomes during meiosis in the $C^nC^cA^nB^c$ hybrid (Fig.1). Regions of homozygosity fixed by homologous recombination in the *B. napus* × *B. carinata* hybrid were present on every chromosome. Of the remaining 6774 loci, 626 (9.2%) showed distorted segregation towards retention of either parental allele at $p<0.05$ (Pearson's χ^2 test for count data), with 28 loci (0.4%) significant at $p<0.001$.

All loci with significant distortion at $p<0.001$ were present in two clusters on chromosomes A7 and A10. The bottom part of *B. napus* A^n7 that represented the small telomeric region of the chromosome adjacent to the interstitial A7/C6 translocation region was retained more often than predicted by chance. *B. napus* alleles were also preferentially retained for the putative duplicated region on chromosome A^n10. In addition, 39% of the loci showing segregation distortion were present on *B. juncea* univalent chromosome A^j9. The univalent chromosome A^j9 from *B. juncea* was retained more often than predicted, although there was no such effect for the other univalent chromosomes A^j2 and A^j6 (Fig. 3).

Taking into account only loci segregating for C^n and C^c alleles, C genome allele inheritance included a slight bias towards inheritance of a section of *B. napus* C^n2, retention of *B. carinata* alleles in a section of C^c4, bias towards presence of either *B. napus* or *B. carinata* C7 alleles for different parts of the chromosome and bias towards presence of *B. carinata* alleles in part of C8. Sections of chromosomes A3, A4 and A5 showed bias towards retention of *B. juncea* alleles, and the top of chromosome A8 and the bottom of A10 showed bias towards retention of *B. napus* alleles (Pearson's χ^2 test, $p<0.05$). No genome-wide bias towards inheritance of either alleles from a particular subgenome or alleles from a particular species was observed.

Fertility of the SP generation: Pollen viability was assessed for 109 SP individuals. There was no significant difference in pollen viability between the two groups of the SP population grown at UWA and UQ (Student's t-test, $p>0.05$). Pollen viability ranged from 0 to 92% across the SP population, with an average of 39% (Fig. 4). A bimodal distribution for pollen viability was weakly suggested by the density distribution (Fig. 4). Twelve individuals out of the 109 were male-sterile (11%).

The number of seeds per plant harvested from 109 progeny in the SP population ranged from 0 to 583, averaging 68 and with a median of 28 (Fig. 5). Thirteen plants (12%) set no seeds, 34 plants (31%) set less than 10 seeds, and 28 plants (26%) set more than 100 seeds (Fig. 5). There were no obvious relationships between genomic structure, including missing chromosomes, and pollen viability or self-pollinated seed set in the SP population.

Sporad observations: Fifty eight individuals in the SP population were assessed for sporad production, and produced an average of 95.2% tetrads, 1.3% triads, 1.9% dyads and 1.5% monads. Tetrad production ranged from 61.3% to 99.7%, with a median of 96.7%. One (sterile, no self-seed produced) plant produced 36.2% dyads. The next highest dyad production was 8.8%, the median dyad production was 0.6%, and no dyads at all were observed in 13 plants. Triad production ranged from 0 – 6.9%, with a median of 0.8%, and seven plants did not produce triads. Monad production ranged from 0 – 8.7% with a median of 0.9%, and 16 plants did not produce monads. Only one individual produced hexads (0.5%), as well as pentads (0.3%), and an additional two individuals also produced pentads at low frequencies (0.3 – 0.6%).

Chromosome counts, fluorescent *in-situ* hybridisation and fertility in the second selfing generation: Individual SP_042 had high seed set (294), moderate pollen viability (62%) and DNA content 97% of a full hexaploid ($2n$ = AABBCC). A set of 25 second-generation self-pollinated progeny from SP_042 were characterised by chromosome counts. The average chromosome number was 50, ranging from 46 to 54 with a standard deviation of 2.6. Three plants with more than fifty chromosomes were selected for investigation using FISH, and are hereafter referred to as SP_042_01, SP_042_02 and SP_042_03 (Fig. 6).

SP42_01 had 53 chromosomes, compared with the expected chromosome complement for a allohexaploid plant of 54 (2n = AABBCC = 54). SP42_01 was missing four A-genome chromosomes and one C-genome chromosome, and had an additional four recombinant chromosomes (Table 1, Fig. 6). This plant produced 3 seeds from 27 self-pollinated flowers (Table 1). SP42_02 had an estimated 54 chromosomes, but was missing eleven A-genome chromosomes, and gained an additional six C-genome chromosomes and five recombinant chromosomes (Table 1). This plant set 2 seeds out of 18 self-pollinated flowers (Table 1). SP42-03 had an estimated 53 chromosomes, was missing seven A-genome chromosomes and three B genome chromosomes, and gained an additional four C-genome chromosomes and five recombinant chromosomes (Table 1). This plant was the most fertile of the three plants with a seed set of 124 seeds from 73 self-pollinated flowers (1.7 seeds/flower). Representative chromosome spreads for each of SP42-01, SP42-02 and SP42-03 are shown in Fig. 6 with chromosomes from each genome labelled by FISH, and recombinant chromosomes labelled.

Correlations between fertility measures and DNA content/allele inheritance: No significant association was observed between the loss of chromosome segments and seed set or pollen viability in the SP population (one-way ANOVA). A weak but significant correlation (one-way ANOVA, $p = 0.02$, $r^2 = 0.12$) existed between DNA content as estimated by flow cytometry and seed set, excluding the plant with abnormally low DNA content (Fig. 2). No significant association between DNA content and pollen viability was observed, although pollen viability and seed set in the subset of the SP population assessed for DNA content were significantly associated (one-way ANOVA, $p=0.01$, $r^2=0.17$).

DISCUSSION

In this study, we assessed fertility, chromosome transmission and genomic stability in self-pollinated progeny of a near-allohexaploid *Brassica* hybrid, using a combination of high-throughput molecular marker genotyping, flow cytometry, fertility measurements and cytogenetics techniques. Approximately one third of the self-pollinated progeny retained all parental A- and C-genome alleles, and unbiased allelic segregation was observed for more than 90% of the loci assessed. Surprisingly, this is higher than observed for previously-generated *B. napus* mapping populations. For example, 22 - 49% of alleles showed

segregation distortion in three well-characterised doubled-haploid-derived populations of *B. napus* (Wang *et al.* 2011a). This difference in segregation bias may be related to the use of microspore culture to produce these mapping populations, rather than fertilisation and embryo development *in planta*. Genomic regions conferring better microspore survival in culture may be selected for during the novel pressures of the culture process, resulting in allele segregation distortion. DNA content followed a normal distribution about the A1 plant mean, and several plants were highly self-fertile, setting hundreds of self-pollinated seeds. SNP data indicated that there was relatively little homoeologous recombination across most of the genome, and allele transmission followed patterns consistent with normal Mendelian segregation (Fig. 3). This represents an improvement over previously detected meiotic stability in synthetic *Brassica* types (Song *et al.* 1995; Szadkowski *et al.* 2010; Tian *et al.* 2010; Xiong *et al.* 2011; Zou *et al.* 2011). This effect may be related to the heterozygosity of the initial hexaploid hybrid used in this study (2n = ~$A^jA^nB^jB^cC^nC^c$), which could facilitate rapid allelic selection for meiotic stability and fertility in resulting progeny. Despite the confounding presence of the A^n7-C^n6 translocation and putative A10 duplication in the *B. napus* parental line used to generate this population, suggestive initial results for DNA content, allele inheritance and fertility were obtained. Taken together, these results suggested that several self-progeny of the interspecific hybrid had "normal" or near-normal, substantially un-rearranged chromosome complements. This is a promising result supporting the development of stable allohexaploid Brassica lines using this approach.

In our study, there was no relationship between missing chromosome fragments and fertility in the SP population, and only a weak predictive correlation (11%) between DNA content and self-seed set. Although Nicolas et al. (2012) observed significantly reduced fertility in progeny of *Brassica* allohaploids (AC) with increasing loss of chromosome segments, the higher genome redundancy of the allohexaploids (AABBCC) may have protected against this effect. Loss of A7 and C6 chromosome regions in the allohexaploid population was also not significantly different to expected proportions for progeny resulting from selfing of a translocation heterozygote (12.5%), suggesting rearrangement events were not discriminated against; similar results were observed by Nicolas et al. (2009) in allohaploid *B. napus*.

Surprisingly, there was no selection pressure for or against loss of univalent chromosomes A^j2 and A^j6 in the allohexaploid progeny, and univalent chromosome A^j9 was present more often than predicted by chance (Fig. 3). This contrasts with previous observations in *Brassica*

of reduced (<50%) transmission of monosomic C genome chromosomes in AA + C addition lines (Heneen *et al.* 2012), and biased transmission of univalents in AAC hybrid types (Leflon *et al.* 2006), and may be related to the higher ploidy level or genome structure of the allohexaploid hybrid types. Selection pressure to retain alleles conferring beneficial meiotic stability may also be operating to retain univalent chromosomes or chromosome regions. A number of QTL with effects on meiotic behaviour have been identified in *B. napus* (Liu *et al.* 2006), including a major locus *PrBn* (Jenczewski *et al.* 2003) with quantitative effects on crossover frequency between non-homologous chromosomes (Nicolas *et al.* 2009). Recently, presence of chromosomes C6 and C9 and retention of additional univalent chromosomes were also found to increase crossover frequency between homologous A-genome chromosomes in AA + C hybrids (Suay *et al.* 2014). Little is known about non-homologous chromosome pairing control in *B. juncea* and *B. carinata*, although some evidence for a genetic locus in *B. juncea* with a major effect on non-homologous pairing has been obtained (Prakash 1974). Preferential retention of A^j9 (and of univalents A^j2 and A^j6 relative to previous studies) in our population may be related to presence of alleles for meiotic stability or fertility on those chromosomes, or to the effect of additional univalent chromosomes on genome-wide meiotic behaviour.

Higher chromosome numbers in the three second-generation progeny did not correspond to euploid AABBCC chromosome complements (Table 1, Fig. 6). In fact, these progeny showed evidence for large-scale recombination between all three genomes (Table 1, Fig. 6), as well as loss of A genome chromosomes but putative retention of additional sets of putative C genome homoeologues (Fig. 6). The retention of four homeologous chromosomes for each set of homeologues but with loss/gain of homologous pairs (e.g. A1/A1/C1/C1 to A1/A1/A1/A1) was also reported by Szadkowski et al. (2010) and Xiong et al (2011) in resynthesised *B. napus*. Similarly, Nicolas et al. (2009) observed no discrimination against rearranged chromosome complements transmitted from haploid *B. napus*, supporting lack of selection pressure for euploid, unrearranged chromosome complements in *Brassica* hybrids. Therefore, even though several plants in the SP generation could hypothetically have had euploid 2n = AABBCC chromosome complements (Fig. 3), these plants may not have had a fertility advantage over their aneuploid counterparts: SP_042 was one of the most fertile of the SP generation, but gave rise to progeny with a standard deviation of ± 2.6 chromosomes. The SP42 plant is also predicted to have contained at least some chromosomal rearrangements and karyotype disturbances based on the commonality of the putatively rare

A-B recombinant chromosomes observed in all three progeny using FISH/GISH (Fig. 6), as well as the common loss of 2-4 pairs of A-genome chromosomes. A similar lack of correlation between fertility and aneuploidy, and even aneuploidy and regularity of meiotic behaviour, was also reported for *Brassica* hexaploids from the cross *B. carinata* × *B. rapa* (Iwasa 1964). The genome redundancy provided by the presence of three genomes may have reduced selection pressure for more normal chromosome complements, allowing retention of high frequencies of chromosome rearrangements, duplications and deletions in the progeny.

Generally regular gamete formation was observed at the sporad stage. Only 3 plants (5%) produced any pentads or hexads, and these were at very low frequency (0.3-0.6%), suggesting few laggard chromosomes were being lost due to formation of micronuclei during cytokinesis. This finding contrasts with Iwasa (1964), who found more than 4 nuclei in 5 – 18% of sporads in three 2n = AABBCC plants derived from the cross *B. carinata* × *B. rapa*, although no dyads, monads or triads were observed in these allohexapoid hybrid types. On average, frequencies of unreduced gametes produced by the SP population were higher than in the parent *Brassica* species, but lower than in the *B. napus* × *B. carinata* hybrid types (Mason *et al.* 2011). Genetic factors related to unreduced gamete production in the parent plants (Mason *et al.* 2011) may have been partially transmissible to the majority of (*B. napus* × *B. carinata*) × *B. juncea* SP progeny, based on comparison to the results of Iwasa et al. (1964). Several mutations resulting in high frequencies of unreduced gamete formation have been identified in *Arabidopsis* (D'Erfurth *et al.* 2008; De Storme and Geelen 2011) and genetic factors have been implicated in other species (Tavoletti *et al.* 1991). However, it is probable that univalent-induced meiotic restitution (the spread of univalents across the metaphase plate resulting in a restitution nucleus (Catcheside 1934; De Storme and Geelen 2013)) was responsible for the unreduced gamete that contributed to the allohexaploid plant A1. The absence of similarly high frequencies of unreduced gamete production in the SP population may support a mode of bivalent formation for most plants, as suggested by the molecular karyotyping results (Fig. 3).

We found that results from GISH in combination with molecular karyotyping were highly effective for determining detailed chromosome complements in cytogenetically complex hybrids, as has also been demonstrated in oats (Jellen *et al.* 1994), banana and sugarcane (D'Hont 2005). This analysis highlighted the dangers of using simple chromosome counts to assess meiotic stability of *Brassica* allohexaploids, as has been done in other studies (e.g.

(Howard 1942; Tian *et al.* 2010). In future, molecular karyotyping with molecular cytogenetics may allow identification of genotypic and species-specific variability for meiotic stability in higher-ploidy *Brassica*. With molecular karyotyping, we could identify chromosomes and genomic regions with regular or irregular A- and C-genome allele transmission, and GISH helped to identify recombinant chromosomes and chromosome copy number. The addition of high-throughput molecular markers for the *Brassica* B genome would also be beneficial in characterisation of *Brassica* allohexaploids to aid in the development of this new potential *Brassica* crop species. Production and characterisation of additional hexaploid lines from different genotypes of *B. napus*, *B. carinata* and *B. juncea*, and intercrossing between different sources of allohexaploids (Geng *et al.* 2013), may also help in elucidating the complex interplay of genetic and genomic factors contributing to *Brassica* meiotic stability, and contribute to the formation of stable, fertile allohexaploids.

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TABLE 1: NUMBER OF CHROMOSOMES INHERITED PER GENOME (A, B OR C) AS DETECTED BY FLUORESCENT IN SITU HYBRIDISATION IN THREE SECOND-GENERATION PLANTS DERIVED FROM A NEAR-ALLOHEXAPLOID 2N = AABBCC PLANT.

Plant	A	B	C	A/B	A/C	B/C	Total	Fertility
Expected for an AABBCC allohexaploid	20	16	18	0	0	0	54	
SP42_01	16	16	17	1	1	2	53	0.1 seeds/flower
SP42_02	9	16	24	2	1	2	54	0.1 seeds/flower
SP42_03	13	13	22	1	1	3	53	1.7 seeds/flower

FIGURES

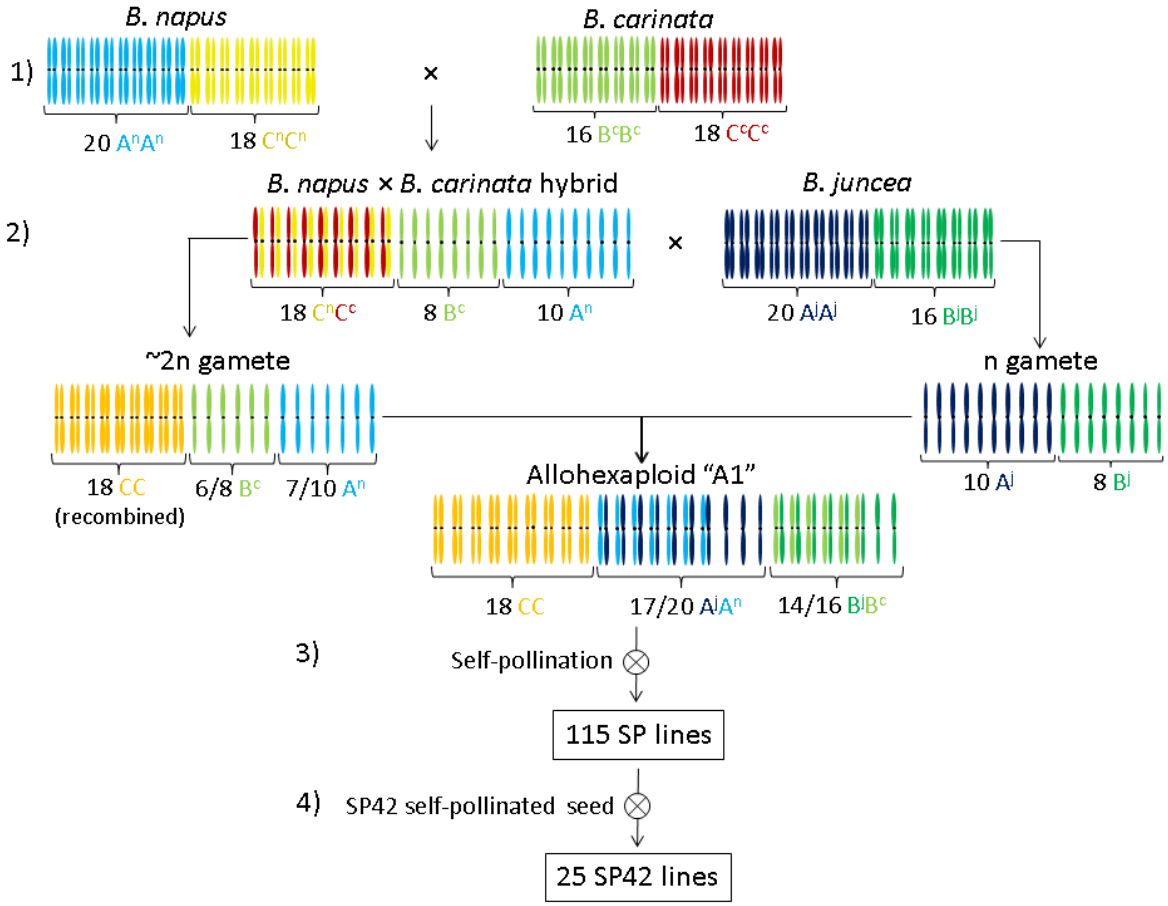

Figure 1: Generation of experimental *Brassica* populations. 1) Interspecific hybridisation between *B. napus* and *B. carinata* in the first generation, then 2) hybridisation between the *B. napus* × *B. carinata* hybrid and *B. juncea* in the second generation, with the *B. napus* × *B. carinata* hybrid producing an aneuploid unreduced gamete, then 3) self-pollination of the resulting plant A1 to give rise to the SP lines and 4) self-pollination of plant SP_042 to give rise to second generation SP42 progeny.

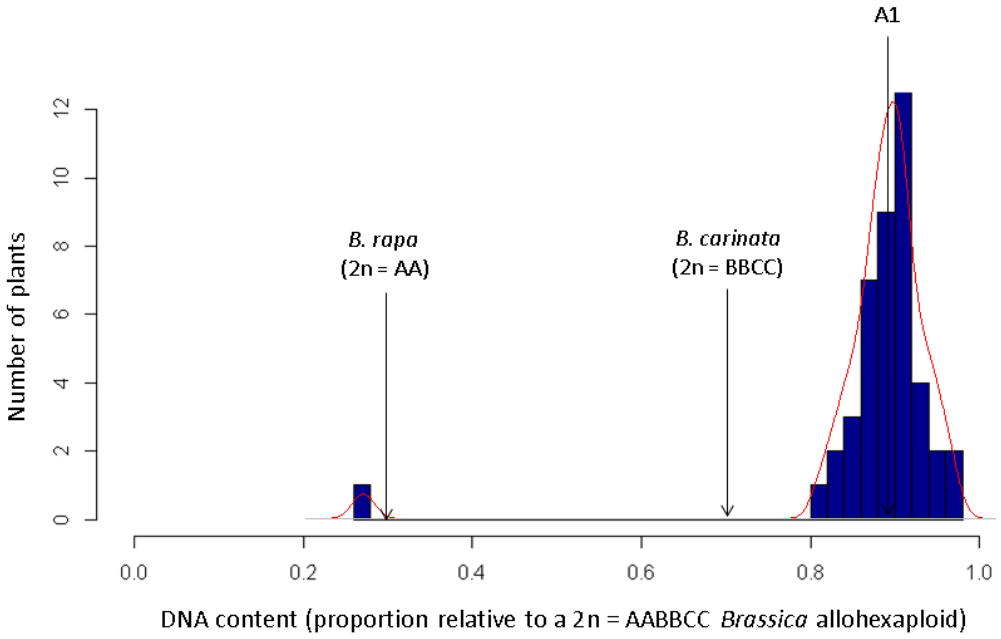

Figure 2: Approximate DNA content determined by flow cytometry in 44 self-pollinated (SP) plants derived from a plant (A1) resulting from the cross (*B. napus* × *B. carinata*) × *B. juncea*. A DNA content of 1.0 represents the expected value for a 2n = 6x = AABBCC allohexaploid, produced by summing values from *B. rapa* (2n = 2x = AA) and *B. carinata* (2n = 4x = BBCC).

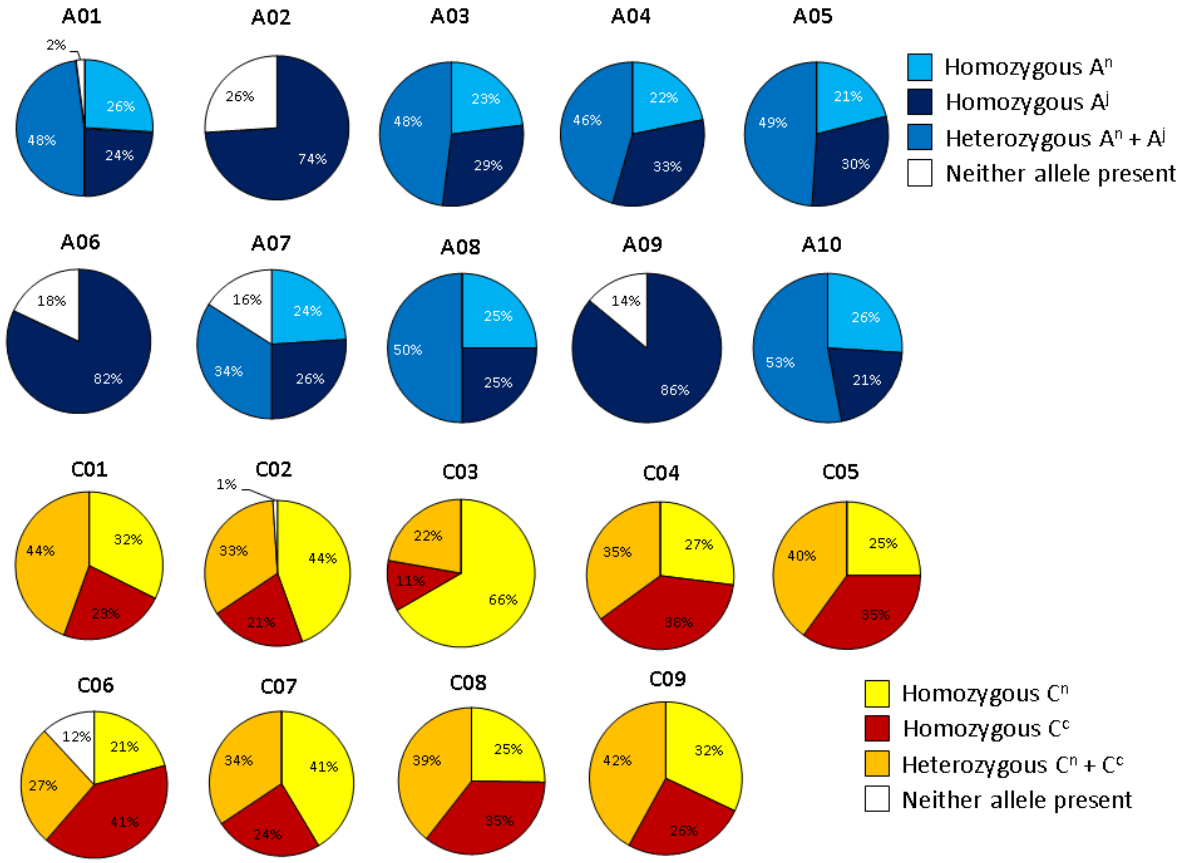

Figure 3: Inheritance of A genome alleles (A^j and A^n from *B. juncea* and *B. napus* respectively) and C genome alleles (C^c and C^n from *B. carinata* and *B. napus* respectively) in 71 self-pollinated progeny of a hybrid plant derived from the cross (*B. napus* × *B. carinata*) × *B. juncea.*

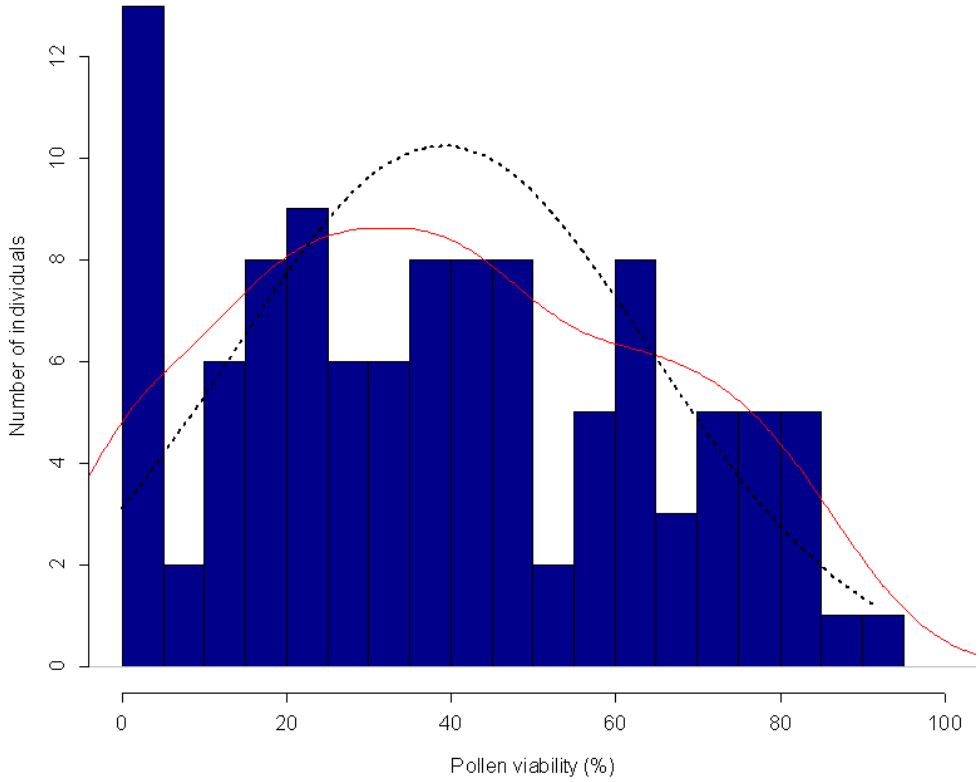

Figure 4: Distribution of pollen viability in 109 plants derived from self-pollination of a hybrid (A1) resulting from the cross (*B. napus* × *B. carinata*) × *B. juncea*, with overlaid density distribution (solid line) and normal distribution curve (dotted line).

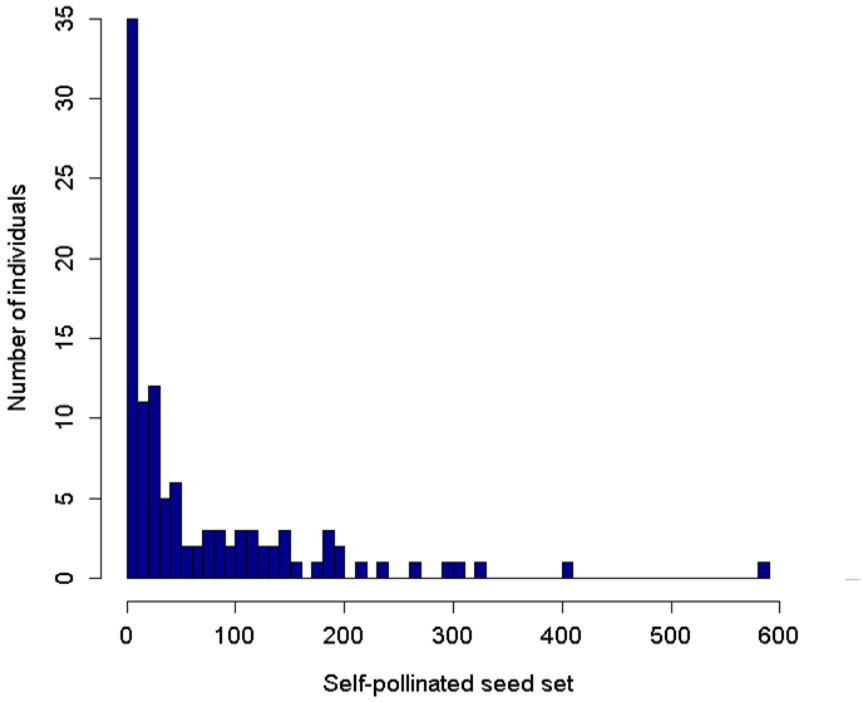

Figure 5: Distribution of self-pollinated seed set in 109 plants derived from self-pollination of a hybrid (A1) resulting from the cross (*B. napus* × *B. carinata*) × *B. juncea*, with overlaid density distribution (solid line).

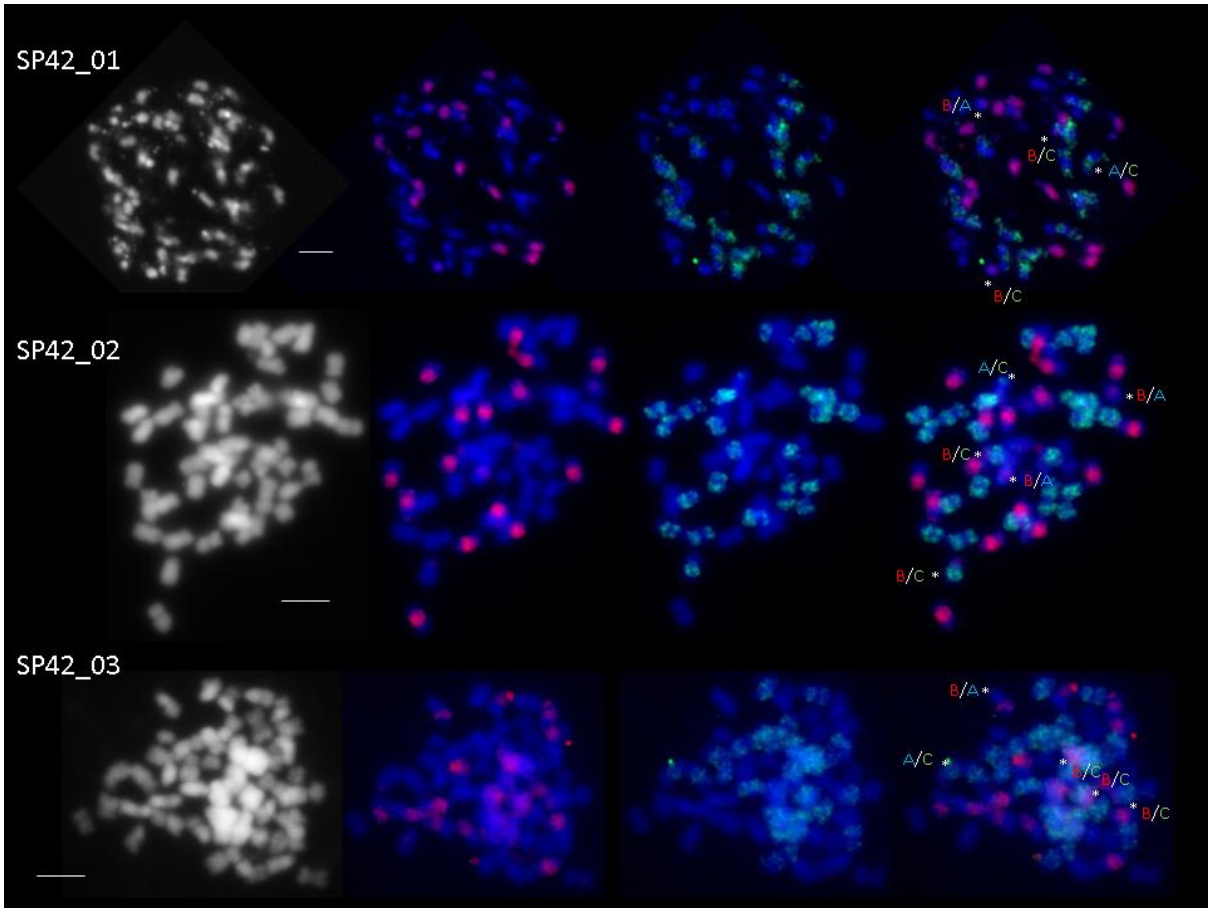

Figure 6: Fluorescent in-situ hybridisation using genome labels for chromosomes in second-generation progeny derived from a near-allohexaploid *Brassica* (2n = AABBCC) plant ((*B. napus* × *B. carinata*) × *B. juncea*): A-genome chromosomes are blue (DAPI, background stain), B-genome chromosomes are labelled red and C-genome chromosomes are labelled green. Recombinant chromosomes are labelled with an asterisk and the two parent genomes involved. Images were taken at 50 × magnification. Bar: ~5 µm

SUPPORTING INFORMATION

TABLE S1: Data collection for all populations used in this study. "NA" represents no data collected or available. Plants were grown in three locations: The University of Western Australia (UWA), Perth, Australia; The University of Queensland, Brisbane, Australia; and The French National Institute for Agricultural Research (INRA), Le Rheu, France.