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Seed colour loci, homoeology, and linkage groups of the C-genome chromosomes revealed in *Brassica rapa – B. oleracea* monosomic alien addition lines

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. *Background and Aims Brassica rapa* and *B. oleracea* are the progenitors of oilseed rape *B. napus*. The addition of each chromosome of *B. oleracea* to the chromosome complement of *B. rapa* results in a series of monosomic alien addition lines (MAALs). Analysis of MAALs determines which *B. oleracea* chromosomes carry genes controlling specific phenotypic traits, such as seed colour. Yellow-seeded oilseed rape is a desirable breeding goal both for food and livestock feed end uses that relate to oil, protein and fibre contents. Our aims included developing a missing MAAL to complement an available series, for studies on seed colour control, chromosome homoeology and assignment of linkage groups to *B. oleracea* chromosomes.

. *Methods* A new batch of *B. rapa – B. oleracea* aneuploids was produced to generate the missing MAAL. Seed colour and other plant morphological features relevant to differentiation of MAALs were recorded. For chromosome characterization Snow´s carmine, fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) were used.

. *Key Results* The final MAAL was developed. Morphological traits that differentiated the MAALs comprised cotyledon number, leaf morphology, flower colour and seed colour. Seed colour was controlled by major genes on two *B.oleracea* chromosomes and minor genes on five other chromosomes of this species. Homoeologous pairing was largely between chromosomes with similar centromeric positions. FISH, GISH and a parallel microsatellite marker analysis defined the chromosomes in terms of their linkage groups.

. *Conclusions* A complete set of MAALs is now available for genetic, genomic, evolutionary, and breeding perspectives. Defining chromosomes that carry specific genes, physical localization of DNA markers, and access to established genetic

linkage maps contributes to the integration of these approaches, manifested in the confirmed correspondence of linkage groups with specific chromosomes. Applications include marker assisted selection and breeding for yellow seeds.

Key words: *Brassica rapa* var. *trilocularis*, *B. oleracea* var. *alboglabra*, MAALs, characterization of C chromosomes, plant morphology, seed colour control, FISH, GISH, chromosome homoeology, chromosome structural changes, linkage groups, crop plant breeding

INTRODUCTION

The addition of single alien chromosomes or chromosome arms from a related species can add considerable value to the genetic stock of many common crops. This has often been achieved using defined crossing schemes where the dissected alien genome is represented by alien mono-, di- or telosomic additions to the chromosome complement of the host genome. Such interspecific or intergeneric addition lines are suitable materials for fundamental genetic and cytogenetic research, and for use in applied plant breeding (Chang and de Jong, 2005). Stocks of addition lines have been developed for a variety of crop plants. Wheat (*Triticum aestivum*) carrying barley (*Hordeum vulgare*) chromosomes has been useful for mapping genes to chromosome arms on the basis of transcript information (Bilgic *et al.*, 2007), and for studies of the morphology and stability of these lines (Szakács and Molnár-Láng, 2010). The contribution of alien chromosomes to phenotype has also been documented in a complete set of oat (*Avena sativa*) lines carrying maize (*Zea mays*) chromosomes (Rines *et al.*, 2009). Homoeology between alien and background chromosomes can be monitored by chromosome pairing and intergenomic recombination during meiosis, as documented in the Japanese bunching onion (*Allium fistulosum*) carrying chromosomes of *A. cepa* (Barthes and Ricroch, 2001), and in tomato (*Lycopersicon esculentum*) carrying chromosomes of the wild relative *Solanum lycopersicoides* (Ji and Chetelat, 2003). Transfer of desirable genes of relevance to plant breeding has been the goal after addition of chromosomes of wild relatives to sugar beet (Gao *et al.*, 2001), potato (Dong *et al.*, 2005) and rice (Khush, 2010).

Genome dissection of crop brassicas and related species through the development of monosomic alien addition lines (MAALs) and disomic addition lines (Budahn *et al.*, 2008) is summarized in reviews by Prakash *et al.* (2009) and Ziolkowski *et al.*

(2011). Assignment of genes to specific chromosomes and transfer of desirable genes have been the main objectives in these studies. Background genomes that hosted individual *Brassica oleracea* chromosomes (2n=18, genome CC) were those of *B. rapa* (2n=20, genome AA) (Quiros *et al.*, 1987; Chen *et al.*, 1992) and *Raphanus sativus* (2n=18) (Kaneko *et al.*, 1987; Budahn *et al.*, 2008). Up to seven out of the nine possible *B. rapa – B. oleracea* MAALs have been characterized by C genome specific isozyme and restriction fragment length polymorphism (RFLP) markers, and by documenting gene duplication, intergenomic recombination, alien chromosome transmission and occurrence of deletions (Quiros *et al.*, 1987; McGrath and Quiros, 1990; McGrath *et al.*, 1990; Hu and Quiros. 1991). Using a pair of parent lines distinct from those used by the Quiros group, our work has previously led to the development of eight *B. rapa* var. *trilocularis – B. oleracea* var. *alboglabra* MAALs with analysis focussed on cytological chromosome differentiation and identification, and use of flower colour, seed colour, isozyme and random amplified polymorphic DNA (RAPD) markers for genetic differentiation between lines (Chen *et al.*, 1992, 1997*a*, *b*; Cheng *et al.*, 1994*a*, *b*, 1995; Jørgensen *et al.*, 1996; Heneen and Brismar, 2001; Heneen and Jørgensen, 2001; Hasterok *et al*., 2005).

In this paper we report the development of the previously undeveloped MAAL, and making available all nine MAALs of *B. rapa* var. *trilocularis – B. oleracea* var. *alboglabra*. Although the C chromosome in one of the established lines is represented by only one chromosome arm, this does provide greater resolution for phenotypic traits determined by that arm. The use of C genome and C chromosome specific microsatellites (simple sequence repeats, SSRs) as molecular markers was decisive for the characterization of the available MAALs and development of the final MAAL (Geleta *et el*., 2012). Detailed documentation of seed coat colour for the different

MAALs provides grounds for dissection of the genetic factors that control this trait, which is of agronomic interest (Shirzadegan and Röbbelen, 1985; Slominski *et al.*, 1999). Behaviour of the C chromosome during diakinesis of pollen mother cells, and preference when pairing with background A chromosomes, was documented in all lines. The identification of the alien C chromosome was greatly facilitated by applying a new FISH technique that allows for integrating the cytogenetic and genetic maps (Xiong *et al.*, 2010, 2011; Xiong and Pires, 2011). Genomic *in situ* hybridization (GISH) also contributed to the identification of the alien C chromosome. The combined FISH, GISH and SSR data provide a platform for determination of the correspondence between the cytological and linkage group designations of the C chromosomes.

MATERIALS AND METHODS

Plant material

Eight available *B. rapa – B. oleracea* MAALs (Chen *et al.*, 1992, 1997*a*; Cheng *et al.*, 1994*a*; Heneen and Jørgensen, 2001) were derived from a cross between a yellow-flowered and yellow-seeded *B. rapa* var. *trilocularis* (yellow sarson, K-151), and a white-flowered and black-seeded *B. oleracea* var. *alboglabra* (No. 4003) (Fig. 1). This original cross gave rise to the resynthesized *B. napus* (No. 7406, genomes AACC) which after backcrossing to *B. rapa* produced sesquidiploids (genomes AAC). These were selfed or backcrossed to the AA parent and produced a progeny of expected aneuploids (AA + 1-9 C chromosomes) and parental AA plants. Aneuploids and their progenies were studied cytologically and with RAPD markers, for the detection and selection of monosomics that were carriers of the different C chromosomes (Fig. 1).

Chronologically, and applying the numerical designation system of Cheng *et al.* (1995), the extra C chromosome was defined as C4 (Cheng *et al*., 1995), C8 and C9 (Chen *et al.,* 1997*a*), C1 (Chen *et al*., 1997*b*), and C2, C3, C5 and C6/7 (referred to as chromosomes D, E, G and F, respectively; Heneen and Jørgensen, 2001; Heneen and Brismar, 2001). Chromosomes C5, C8 and C9 were later verified as rDNA carriers (Hasterok *et al.,* 2005). During the propagation of this material, C3 lost one chromosome arm and the maintained MAAL of C3 with this deletion is referred to as C3d. In addition to the maintained MAAL with a seemingly intact C4, a line was developed with a C4 that had a shorter short arm due to a deletion. This MAAL is referred to as C4d. Plants of the parental *B. rapa* var. *trilocularis* (K-151) and *B. oleracea* var. *alboglabra* (No. 4003) species and their resynthesized *B. napus* (No. 7406) were also raised. Backcrosses between *B. napus* and *B. rapa* were repeated in order to produce new batches of sesquidiploids and aneuploids in an effort to develop the missing MAAL.

Methods

Morphology. Morphological features of relevance for the differentiation of monosomics, representing the different C chromosomes were recorded. These primarily included the colour of sown seeds, number and size of cotyledons, even or puckered leaf surface, green or faint anthocyanin colouration of the stem, flower colour, plant and silique size as well as the seed coat colour of harvested seeds.

Chromosome number determination and chromosome nomenclature. Chromosome numbers were determined from meiotic analysis of pollen mother cells. Flower buds were fixed in ethanol – acetic acid (3:1) and stained in Snow´s carmine as previously

applied on this material (Chen *et al*., 1992; Cheng *et al*., 1994b; Heneen and Jørgensen, 2001). Emphasis was laid on studying early diakinesis since differences in chromatin condensation patterns between the A and C chromosomes at this stage were distinctive, permitting the differentiation between these chromosomes (Cheng *et al*., 1994*b*; Heneen *et al*., 1995; Heneen and Jørgensen, 2001).

The cytological numerical designation of the chromosomes of *B. rapa* (AA) and *B. oleracea* (CC) is according to our earlier reports (Cheng *et al.,* 1995; Heneen *et al.*, 1995). Identification of the C chromosomes in the developed MAALs was based on size, defined or inferred centromeric position, and the heterochromatic nature of the NOR (nucleolar organizer region) and its proximal chromatin. For the sake of simplicity in the following text, AA and CC will be used when referring to the parental genomes and the A-genome and C-genome chromosomes will be referred to as A1-A10 and C1-C9. Another commonly used, and internationally agreed designation system of the A and C chromosomes is based on molecular genetic linkage groups (Parkin *et al*., 2005; http://www.brassica.info/resource/maps/lg-assignments.php; Wang *et al.* 2011). This nomenclature system has been integrated with, and consolidated by FISH-based physical localization of DNA probes (Howell *et al.*, 2002; Xiong and Pires, 2011).

The nomenclature system of the C genome (Cheng *et al.*, 1995) is based on arranging the chromosomes in groups depending on centromeric position, and within groups according to size, and so differs from the system based solely on chromosome size applied by Armstrong *et. al.* (1998) and Howell *et al.* (2002). Similarly, it was not possible to ascertain the correspondence between all chromosomes when comparing the systems of Cheng *et al.* (1995) and Xiong and Pires (2011). Accordingly, we were not previously able to designate all the C chromosomes of the eight established

MAALs in terms of linkage groups. However, the current FISH and GISH physical mapping of the MAALs, and the molecular SSR characterization of these lines (M Geleta, 'pers. comm.') made it possible to determine the correspondence between the cytological hitherto used cytological designations (A1-A10 and C1-C9) according to Cheng *et al.* (1995) and the linkage group (LG) designations (*sensu* Parkin *et al.*, 2005) hereafter referred to as LG-A1 to LG-A10 and LG-C1 to LG-C9. Both cytological and linkage group designations will be used in the following text.

FISH: Multiple target. The use of immature buds as sources of mitotic cells as well as pollen mother cells, the choice of DNA probes, and the application of FISH were according to a new technique developed for the characterization and differentiation of *Brassica* A and C chromosomes (Xiong *et al.*, 2010, 2011; Xiong and Pires, 2011). In this method, genetically mapped bacterial artificial chromosome (BAC) probes of *B. rapa* were used to identify the locations of repetitive elements in both *B. rapa* and *B. oleracea*. A second hybridization was done on the same chromosome spreads using a C genome repeat, giving a result similar to a GISH experiment. The probes used in the first round comprised those specific to 5S and 45S rDNA, repeated DNA sequences in eight chromosome pairs of *B. rapa* by using BAC KBrB072L17, and repeated DNA sequences specific to two chromosome pairs of *B. rapa* by using BAC KBrH092N24. The probes used in the second round were those for the repetitive centromeric DNA sequences CentBr1 and CentBr2 and for repetitive DNA sequences that are C-genome specific by using BAC BNIH 123L05 which gives a GISH-like labelling. In addition, the BAC KBrH117M18 probe specific to LG-A3 and LG-C3, followed by the C genome specific probe BAC BNIH 123L05 were used.

GISH. Labelled C-genome DNA. GISH alone, and GISH following FISH using the BAC BoB004H11 probe specific to the LG-C4, was carried out by S. Armstrong and E. Howell at the University of Birmingham according to Howell *et al.* (2008).

RESULTS

Development of the missing MAAL

The backcross between the resynthesized *B. napus* (No. 7406) and the parental *B. rapa* (K-151), that led to the development of the available eight MAALs, was repeated to provide new batches of sesquidiploids and aneuploids, in an effort to develop the missing MAAL (Fig. 1). Pollination of 34 flowers of the AACC parent with pollen from the AA parent resulted in 35 seemingly viable well developed seeds expected to have the AAC constitution. Plants raised from these seeds were either selfed or backcrossed to the AA parent. Selfing of 160 flowers yielded 14 seeds, while backcrossing of 66 flowers resulted in 80 seeds. As expected, more viable zygotes and seeds were produced after pollination with balanced *B. rapa* pollen in the case of backcrossing than after fusion of frequently produced unbalanced gametes when the AAC plants were selfed. Plants raised from seeds obtained after backcrossing/selfing are largely aneuploids (AA + 1-9 C chromosomes) and to a minor extent parental euploids (AA).

Of the seeds obtained after selfing and backcrossing, and their progeny seeds, a total of 52 were sown, giving rise to 47 viable plants. Of these, 23 comparatively less vigorous plants, possibly representing aneuploids, were chosen for cytological characterization and SSR marker analysis (Geleta *et al*., 2012). Emphasis was laid, in the first instance, on finding plants labelled by C-genome specific SSR primer pairs that did not label monosomics of the available eight MAALs. Such SSRs, inferred to

be possibly specific for the undefined C chromosome, labelled six presumed aneuploid plants. Of these plants, two were sterile and did not yield any seeds. Meiotic chromosome number was estimated on three of the four remaining plants. Two plants had 2n=23 and one monosomic plant had 2n=21. The monosomic plant, monosomic progenies of this plant, and certain monosomic plants among the progeny of one of the plants with 2n=23, were shown by SSR analysis to be carriers of the undefined C chromosome (Geleta *et al*., 2012). This confirmed that we had successfully generated the starting material for the previously unavailable MAAL. The C chromosome in this line has been designated C7, and the previously available line C6/7 is now designated as C6 (see below).

Morphology

A general characteristic of plants carrying additional C chromosomes in all the MAALs studied is decreased vigour, reduced stature and decreased fertility compared to sibling euploid AA plants. This is also reflected in silique size being smaller in monosomics than in the euploid parent *B. rapa* (Fig. 2A). Other morphological features that discriminate between the parental species and between monosomics carrying different C chromosomes are listed in Table 1. These characters comprise number of cotyledons, anthocyanin colouration of the stem, appearance of the leaf surface, flower colour and colour of seed coat (Fig. 2B-H). Seed coat colour (henceforth referred to as seed colour) is the most valuable character for differentiation between MAALs and between carriers of C chromosomes and euploids in the progeny of the MAALs. Therefore, data on seed colour is presented in more detail (Fig. 3, Table 2).

Differences between the parental species regarding the characters mentioned above relate to the yellow flower colour (Fig. 2B) and yellow seed colour (Fig. 3A) specific to the AA parent, and anthocyanin colouration of stem, white flower colour (Fig. 2C) and black seed colour (Fig. 3B) specific to the CC parent. White flower colour and black seed colour are dominant characters. In accordance, resynthesized *B. napus* from these parental species produces a mixture of black, dark brown and dark grey seeds (Fig. 3C).

MAAL for C1. Dark brown seeds (Fig. 3D) were grown. The occurrence of three cotyledons in some seedlings (Fig. 2D) raised from these seeds has been established to be correlated with the presence of C1. These plants produce dark brown seeds. The chromosome number 2n=21 and the presence of C1 prevailed in 15 tested plants originating from tri-cotyledonous seedlings. Seedlings with two heteromorphic cotyledons, one almost double the size of the other (Fig. 2E), were commonly carriers of C1 as well. Plants from this line, regardless of the number or shape of cotyledons, produce either yellow seeds, similar to those of the *B. rapa* parent, or dark brown seeds (Fig. 3D). Cytological analysis has shown that all plants that produce brown seeds are carriers of C1, while plants producing yellow seeds are euploids with 2n=20 (Table 2). The fact that a C1-carrier plant produces only brown seeds irrespective of the euploid or monosomic nature of these seeds indicates that the maternal inheritance of the seed colour character is controlled by C1 (Heneen and Brismar, 2001). The frequency of progeny plants producing brown seeds reflects the transmission rate of C1, amounting to 30.3% (Table 2). The MAAL for C1 is the only line where plants produce either one of two homogenous types of seeds based on seed colour.

MAAL for C2. Seeds expected to be C2-carriers are yellow with brown spots/patches (Fig. 3E). Plants raised from these seeds produce either only yellow seeds or a mixture of yellow seeds and brown-spotted/patched yellow seeds. It was found that the brown-spotted/patched seeds (Fig. 3E) give rise to C2-carrier plants which in turn produce a mixture of seed types, while plants raised from pure yellow seeds are largely of the parental *B. rapa* type and produce just yellow seeds (Table 2). The frequency of the brown-spotted/patched seeds indicated the transmission rate of this chromosome (33.6%, Table 2).

MAAL for C3d. Selected yellow seeds with faint brown dots were grown. Raised plants from these seeds produce yellow seeds (Fig. 3F). It is difficult to detect differences between these yellow seeds by the naked eye, but with the aid of a stereo microscope some plants can be discerned as producers of a mixture of pure yellow and faint brown spotted seeds (33.8%, Table 2). The faint brown spotted seeds are largely producers of C3d-carrier plants that generate seed mixtures (Table 2).

MAALs for C4 and C4d. For the MAAL for C4, brown seeds with dark spots (Fig. 3G) were sown. Plants were observed to produce either yellow or white flowers (Fig. 2B and C). The white-flowered plants turned out to be carriers of C4, which harbours the dominant gene for white flower colour. The yellow-flowered plants were euploids. One C4-carrier offspring plant (2n=21) was found to have yellow flowers. Closer cytological examination of this plant showed that it had a deletion in the short arm of its C4 chromosome (see below). Apparently, the deletion carrying the gene for white flower colour allows the background gene for yellow colour to be expressed. This plant was the starting point of a daughter MAAL designated C4d (with a deleted

segment in the short arm of C4). Differences in plant size also characterized the yellow-flowered C4d carriers, being slightly smaller than the white-flowered C4-carriers (Fig. 2F). Seeds selected for sowing in the case of MAAL for C4d were faint brown with dark spots (Fig. 3H). In MAALs for C4 and C4d, plants produced either yellow seeds or a mixture of easily differentiable yellow seeds and brown seeds with dark spots (Fig. 2G, 3G and H). The brown seeds in both lines gave rise to C chromosome carrier plants which in turn produced a mixture of seeds (Table 2). From seed counts, the transmission frequencies of C4 and C4d amounted to 34.7% and 36.7%, respectively (Table 2).

MAALs for C5, C6 and C7. In plants that are carriers of C5, the leaves express a distinctive character by having a puckered appearance (Fig. 2H) from the seedling stage onwards and throughout plant growth and development. The puckered leaf surface is visually easily detectable and chromosome analysis of 15 plants with such leaves revealed that they had 2n=21 and were carriers of C5. Seeds selected for sowing from MAALs for C5-C7 are yellow with brown spots/patches (Fig. 3I-K). Plants raised from these seeds produced either only yellow seeds or a mixture of yellow seeds and brown-spotted/patched yellow seeds (Fig. 3I-K). In these lines, the brown spots/patches were less pronounced than in the MAAL for C2. Seeds with brown spots/patches largely gave rise to C-carrier plants that produced a mixture of seeds (Table 2). Accordingly, the frequencies of pigmented seeds in these lines (32.0-32.5%, Table 2) are approximate values of the transmission frequencies of these chromosomes.

MAALs for C8 and C9. Yellow seeds were sown and the resulting plants produced only yellow seeds (Fig. 3L, M), thus implying that these two chromosomes do not carry genes controlling or affecting seed colour.

Maintenance and availability of the MAALs

For the C1-carrier MAAL, plants solely producing brown seeds were propagated for the maintenance of the line. Seedlings with three cotyledons or two cotyledons that markedly differ in size are usually carriers of C1. In the case of MAALs for C2, C4, C4d and C5-C7, brown or brown-spotted/patched seeds, expected to be C-carriers, were separated from pure yellow seeds produced by C-carrier plants and propagated. For MAAL C3d, either bulk seeds, or selected seeds with faint brown dots, produced by C3d-carriers were propagated. At the plant level, puckered leaves are a reliable indicator of a C5-carrier state. For MAALs for C8 and C9 that produce only yellow seeds, bulk seeds produced by C-carriers were propagated. In the case of these two lines in particular, but also relevant for all other lines, cytological and molecular SSR analyses are required for verification of the C-carrier plants.

Limited amounts of bulk seeds of MAALs for C1, C3d, C8 and C9, as well as selected pigmented seeds of MAALs for C2, C4, C4d and C5-C7, together with the parental lines *B. rapa* var. *trilocularis* (K-151) and *B. oleracea* var. *alboglabra* (No. 4003) and their resynthesized *B. napus* (No. 7406) have been delivered to the gene bank NordGen (www.nordgen.org) in Alnarp, Sweden. Accession numbers of these materials and correspondence of the numerals of the C chromosomes in the MAAL to the linkage group designations (see below) are presented in Table S1 [**Supplementary Information**]. A description of the material and recommended method for propagation of the different MAALs will be supplied by the gene bank on request.

Cytology

Differentiation of A and C chromosomes. Differences in chromatin condensation patterns of the two parental AA and CC species observed during diakinesis in pollen mother cells often made it possible to differentiate between the chromosomes when present in the same cell. The chromosomes of *B. rapa* usually exhibit highly condensed heavily stained pericentric regions, and less condensed and less stained regions towards the chromosome ends (Fig. 4A and C). This is especially evident in chromosomes with median and submedian centromeres (Fig. 4C). The ten bivalents of *B. rapa* are identifiable as members of two groups depending on centromeric position, or as the nucleolar bivalent (Fig. 4C). The chromosomes of *B. oleracea*, on the other hand, at a corresponding stage of chromatin condensation exhibit no or slight differentiation of chromatin condensation (Fig. 4B). These species differences led to the distinction between the A and C chromosomes, as exemplified in diakinesis configurations of a sesquidiploid (Fig. S1A), an aneuploid (2n=22) with C6 and C8 (Fig. S1B), and MAALs with C5, C6 or C7 appearing as univalents or components of multivalents (Fig. S1C-F) [**Supplementary Information**].

Chromosome structural changes and substitution. Eight of the nine developed MAALs contain seemingly intact C chromosomes, judged by their size. C3 is represented by only one chromosome arm in the C3d line (see below). Another line with a deletion was the MAAL for C4d, which has a deletion in the short arm of C4 (see below). Further examples of structural chromosome changes in progeny plants from the MAAL for C4 are presented in Fig. S2A-E. In a *B. rapa* offspring plant (2n=20), one chromosome of the nucleolar pair had a deletion in the heterochromatic

short arm (Fig. S2A), and in a monosomic plant (2n=21), the C4 chromosome was exceptionally large, probably resulting from a duplication or translocation (Fig. S2B). Of interest is the finding of an additional mini-chromosome in a C4-carrier progeny plant (Fig. S2C-E). The mini-chromosome divided in a normal way (Fig. S2D, E) and was found in all analysable meiotic cells in this plant, indicating that it has a functional centromere. In addition to finding AA euploids as well as mono- and disomics with alien chromosomes (2n=21 and 22) among the progenies of monosomics, plants with a substitution (2n=20, 19 A chromosomes + 1 C chromosome) were also found (Fig. S2F) [**Supplementary Information]**.

Behaviour of the C chromosome during meiosis. By comparing the centromere position of the C chromosome in the newly developed MAAL with that of C6/7 (Cheng *et al.*, 1995)), the latter was designated C6 and the former as C7. A detailed chart showing examples of the most encountered patterns of behaviour for the C chromosomes in all developed MAALs is presented in Fig. 5. The C chromosomes appear as univalents, or components of bivalents, trivalents or pentavalents. The less condensed C univalents exhibit differential chromatin condensation generally portraying a more condensed state of the short arm. When the C chromosome pairs with a homoeologous A chromosome, they form a heteromorphic bivalent, while the unpaired A chromosome appears as a univalent. Thus, caution is necessary not always to infer univalents as representing the alien chromosome, without confirming their identity.

The frequencies with which the C chromosome remains as a univalent and when it pairs in different combinations with A chromosomes are given in Table 3. The frequency of univalent C chromosomes is highest (90.2%) in the MAAL for C3d. This

is apparently due to the fact that C3d is a short chromosome, representing only one arm. A seemingly intact C chromosome that generally remains unpaired as a univalent is C8 (82.5%), whereas the other C chromosomes appear as univalents in the range 38.9-68.1%. Chromosome pairing provides information on homoeological relationships between the extra C chromosome and the chromosomes of the AA background (Fig. 5). C1 and C2 with median centromeres and C3d which originates from a chromosome with a median/submedian centromere (C3) revealed their preference pairing with A chromosomes with a median/submedian centromere (Fig. 5, Table 3). Other C chromosomes with an affinity to pair with A chromosomes having a median/submedian centromere are C5, C7 and C9 (Fig. 5, Table 3). Homoeologous pairing with A chromosomes having a submedian/subterminal centromere occurs with C chromosomes (C5-C7) having submedian centromeres. Homoeologies to the nucleolar A9 chromosomes are expressed mainly by C4, C4d, C8 and C9 and to a minor degree by C6. Pentavalents resulting from pairing of the extra C chromosome with two types of A pairs are recorded in the MAALs for C5 and C9 (Fig. 5, Table 3).

FISH and GISH. The use of FISH with 5S and 25S rDNA probes in *B. oleracea* var. *alboglabra* has been useful for characterizing C5 (LG-C4) carrying 5S rDNA, as well as C8 (LG-C7) and C9 (LG-C8) carrying 25S rDNA, as reported earlier (Howell *et al.*, 2002; Hasterok *et al*., 2005). In addition, other FISH and GISH approaches have been applied to differentiate the C-chromosomes and determine their linkage groups. The FISH approaches comprised the use of genome-, chromosome- and LG-specific probes, and when applying GISH, labelled C-genome DNA was used. Application of the multiple target FISH technique (Xiong and Pires, 2011) led to the differentiation of all the A chromosomes in *B. rapa* (Fig. 6A, B) and identification of the C

chromosome in the five MAALs for C1, C3d, C4, C6 and C8 (Fig. 6B-F, Fig. S3A-F). In Fig. 6 and Fig. S3, the designations of the A and C chromosomes correspond to the linkage group numbers. For correspondence between linkage group and cytological numerical designations, see below (Table 4). Labelling with chromosome-specific probes is exemplified by the use of the LG-C3 specific BAC KBrH117M18 probe and the LG-C4 specific BAC BoB004H11 probe shown to be specific to chromosomes C4 and C5, respectively (Fig. 7A-C). Applying the 5S rDNA probe, followed by GISH, led to the detection and characterization of C5 (Fig. 7D, E). The use of GISH alone was enough for the detection of C chromosomes (Fig. 7F-H). In spite of labelling the pericentric regions of the A-chromosomes by GISH, the C-chromosomes are detectable being labelled along their whole length. Thus unlabelled segments of the C chromosomes, and labelled sites other than the pericentric regions of the A chromosomes, may denote intergenomic introgression (Fig. 7H).

DISCUSSION

The findings in the present work touch upon four topics that will be discussed in some detail. The first topic relates to the development and characterization of the MAALs. Of relevance here was the access to the newly produced aneuploid material and the use of C-genome and C-chromosome specific SSRs for distinguishing between lines and for developing the missing line. The second topic deals with the black/brown seed colour control by genes on the C chromosomes in view of the interest in developing yellow-seeded oilseed rape. Genes with major effects on seed colour occur on two chromosomes and genes with minor effect on five other chromosomes. The third topic covers the cytological identification of the C chromosomes and their homoeology to A chromosomes. Chromosome pairing at diakinesis and the use of FISH and GISH were

elucidative for this purpose. The fourth topic is on integrating cytological and genetic linkage data by defining the correspondence between these designation systems.

Development of the missing MAAL

Two approaches were crucial for the development of the missing MAAL. One was the development of a new batch of *B. rapa – B. oleracea* aneuploids as a source for the missing line. The second approach was the use of SSR markers for molecular characterization of the parental species, the available eight MAALs and the newly developed aneuploid material. SSR markers have been widely used for characterization of the C genome in *B. oleracea* and both the A and C genomes in *B. napus* (e.g. Lowe *et al.*, 2004; Piquemal *et al.*, 2005; Gao *et al.*, 2007; Iniguez-Luy *et al.*, 2008; Basunanda *et al.*, 2010; Wang *et al.*, 2011). Defining SSR markers that are specific to the currently used C genome and to each of the available eight MAALs led to the identification of SSR markers specific to the hitherto uncharacterized chromosome and discovering monosomics that carry this chromosome among the newly developed aneuploids (Geleta *et al.*, 2012). In this way, for the first time, access to all nine MAALs is now possible through the gene bank NordGen (www.nordgen.org) where limited amounts of seeds of the MAALs, the AA and CC parents and the resynthesized *B. napus* are deposited.

The fact that the MAAL for C3d carries only one chromosome arm might be restrictive for certain purposes, although resolution of phenotypic traits residing on this arm will be more precise. In the MAAL for C4d, plants that carry C4d are yellow flowered, while plants carrying the intact C4 are white flowered, indicating the localization of the flower gene in the deleted segment. The location of the white flower gene on C4 has been shown in earlier works (Chen *et al.*, 1992; Cheng *et al.*,

1995), and has been mapped to the corresponding LG-C3 (Ramsay *et al*., 1996). Comparative studies on MAALs for C4 and C4d would be informative as to gene localization and eventual position effects of the deletion. Access to MAALs for C3d and C4d emphasizes the value of establishing MAALs with structurally altered C chromosomes.

Morphological features other than the flower colour that discriminate plants carrying different C chromosomes have been defined. These comprise the occurrence of three cotyledons indicating the presence of C1 and a puckered leaf surface in plants carrying C5. A puckered leaf morphology has been observed in one of the seven *B. rapa* – *B. oleracea* MAALs developed by McGrath *et al.* (1990). Seed colour was also a reliable and valuable character for discrimination of plants or seeds that are carriers or non-carriers of C chromosome(s) in progenies of up to seven of the nine MAALs.

Seed colour control by major and minor genes in B. oleracea

Development of the current MAALs has been valuable for defining the chromosomes that control the black seed colour of *B. oleracea* var. *alboglabra* and to determine the mode of this control. This knowledge is a prerequisite if attempts are being made to develop yellow seeded oilseed rape from its progenitor species. Yellow seeded *B. rapa* are common while yellow seeded *B. oleracea* are scarce. The interest in developing yellow seeded oilseed rape (Chen *et al.*, 1988; Meng *et al.*, 1998; Rahman, 2001) relates to the fact that this character is associated with higher oil and protein contents and less fibre content which are desirable food and livestock feed agronomic goals (Shirzadegan and Röbbelen, 1985; Slominski *et al.* 1999). Findings in the present work indicate that seven out of the nine C chromosomes carry genes that affect seed colour. C1 and C4 carry major genes for dark seed colour that lead to

pigmentation of the entire seed coat. These genes are controlled maternally and through the embryo, respectively (Heneen and Brismar, 2001). In addition to these two major genes, biparental control through the embryo was further expressed as less pronounced pigmented spots/patches on a fraction of the yellow seeds produced by C2-, C3-, C5-, C6- and C7-carriers, apparently due to the presence of at least five minor quantitative genes on these chromosomes. This is in accordance with the indirect finding that the currently used *B. oleracea* var. *alboglabra* possibly contains two independently dominant genes with major and additive effect for black seed colour and that other genes with minor effects may also be present (Chen and Heneen, 1992). It remains to be determined if pigmentation expressed as spots or patches is due to incomplete penetrance, epigenetic factors, DNA transposon insertion or other factors. In most studies of seed colour inheritance in *B. napus*, it has been concluded that three or four genes control this character both maternally and through the embryo (Shirzadegan, 1986; Van Deynze and Pauls, 1994; Rahman *et al*., 2001, 2010).

Of interest is the finding that the extent of seed pigmentation and plant vigour are less pronounced in C4d-carriers compared to C4-carriers. This could be due to a position effect or due to the loss of minor quantitative genes for seed pigmentation and vigour in the deleted chromosome segment.

The interest in the seed colour character in oilseed rape has driven efforts to identify molecular markers that are closely linked to genes controlling this trait for use in marker assisted selection (Van Deynze *et al*., 1995; Somers *et al*., 2001; Badani *et al*., 2006; Liu *et al*., 2006; Fu *et al*., 2007; Xiao *et al*., 2007; Rahman *et al*., 2010; Zhang *et al*., 2011). In our work on the MAALs for C1, a RAPD marker relatively close to the major gene for dark seed colour on this chromosome was defined, and absence of this marker in a C1-carrier progeny plant was accompanied by production

of yellow seeds (Chen *et al*., 1997*a*). Progeny plants that lack the major genes for dark seed colour on C1 and C4 are of interest in breeding for yellow-seeded *B. oleracea* and *B. napus*. The manifestation of the seed colour character is also associated with seed coat thickness (Heneen and Brismar, 2007) and the accumulation of seed coat pigments. Transcriptome analysis and chemistry of seed coat pigmentation reveal a multitude of genetic factors that control pigmentation of the seed coat (Marles and Gruber, 2004; Akhov *et al*., 2009; Jiang and Deyholos, 2010).

Identification of alien C chromosomes and their homoeology to A chromosomes

Cytological characterization of the alien C chromosomes and the background A genome was based on comparisons with the previously described karyotypes of the parental species (Cheng *et al*., 1995) and diakinesis chromosomes of *B. rapa* (Cheng et al. 1994a; Heneen et al. 1995). When applying FISH on the MAALs, rDNA probes were used first, thus defining C5, C8 and C9 as carriers of 5S and 45S rDNA (Hasterok *et al*., 2005). This was expanded in the present work by using additional probes of other repetitive DNA sequences in multiple target FISH (Xiong and Pires, 2011), and using chromosome and LG specific probes, as well as GISH. This recent approach enabled the characterization of seven MAALs. A significant advantage of these different approaches was the identification of the C chromosomes in terms of their respective LGs. The use of FISH and GISH have enabled the distinction between the A and C genomes and identification of their individual chromosomes (Howell *et al*., 2008; Xiong and Pires, 2011). These approaches also provided evidence of what could be intergenomic introgression, as has been earlier inferred following RAPD characterization of the MAALs (Chen *et al*., 1992, 1997a, 1997b; Jørgensen *et al.*, 1996; Heneen and Jørgensen, 2001).

The pairing patterns at diakinesis marked the homoeologous relationships between the A and C chromosomes. These detailed data were restricted to the carmine stained preparations, thus defining only whether the A chromosomes belonged to groups with specific centromeric positions, or if they were the easily differentiable nucleolar chromosomes. Intergenomic pairing in the present material indicated that chromosome homoeology is maintained between C and A chromosomes with similar centromeric and karyotypic positions, in spite of the extensive duplications and structural chromosome changes shown or inferred to have occurred during *Brassica* evolution (Parkin *et al*., 2003; Lysak *et al*., 2007). The lowest frequency of pairing of a seemingly intact alien chromosome was manifested by C8. This might be a consequence of rearrangements of homoeologous regions in C8 relative to their counterparts in the A genome or vice versa.

The fact that many C chromosomes had more than one type of homoeological pairing partner among the A chromosomes most likely reflects the frequent presence of similar and homoeologous duplicate and triplicate sequences within and between the A and C genomes, as evidenced by molecular mapping and physical painting (McGrath *et al*., 1990; Parkin *et al*., 2003; Lysak *et al*., 2007), and more recently by whole genome analysis (The *Brassica rapa* Genome Sequencing Project Consortium, 2011; Wang *et al.*, 2011). An example relating to the frequent pairing between C4 and the nucleolar chromosomes of the A genome (Table 3), is the prevailing homoeology shown by FISH between the long arm of LG-C3 (cytological C4) and the long arm of the nucleolar A chromosome (LG-A3) (Xiong and Pires, 2011). The relatively high frequency of homoeologous pairing in the MAALs, and the occurrence of chromosome structural changes, certainly contribute to prevalence of variations in the make-up of plants carrying alien chromosomes within a MAAL. Changes are likely to

have happened in the background AA genome and in the alien chromosome during the development and propagation of MAALs. Such changes have been documented in resynthesized AACC oilseed rape (Song *et al.*, 1995; Pires *et al.*, 2004; Gaeta and Pires, 2010; Szadkowski *etal.*, 2010; Xiong *et al.*, 2011), in the sesquidiploid AAC (Nozaki *et al.*, 2000; Leflon *et al.*, 2006), and in the aneuploids and MAALs (Heneen and Jørgensen, 2001). Cytological differentiation of all *B. napus* chromosomes using FISH (Xiong and Pires, 2011) also made it possible to monitor the incidence of homoeologous chromosome compensation (replacement) following resynthesis and selfing. The highest rates of homoeologous pairing, reciprocal exchange, nonreciprocal transposition, and chromosome compensation occur between A and C chromosomes that are largely syntenic along their entire length (Parkin *et al*., 1995, 2005; Udall *et al*., 2005; Gaeta *et al*., 2007; Xiong *et al*., 2011). The observed relatively low frequency of intergenomic multivalents involving C2 (LG-C1) and C7 (LG-C2) known to share a high degree of homology with their counterparts in the A genome may relate to genetic changes that have occurred in these chromosomes.

Correspondence of the cytological and molecular linkage group nomenclatures

The identification of the alien C chromosomes of the MAALs in terms of LGs by different FISH and GISH approaches together with the SSR markers that are linked to specific LGs (Geleta *et al.*, 2012) were confirmative and complementary to each other as to the correspondence between seven of the cytological and LG designations (Table 4). The correspondence of C2 and C7 to LG-C1 and LG-C2, respectively is based on SSR evidence only. It is valuable for future users of the nine MAALs, to be able to refer to the C chromosomes in terms of LG designations commonly agreed upon in gene and molecular linkage mapping (Parkin *et al.*, 2005; see also

http://www.brassica.info/resource/maps/lg-assignments.php; Wang *et al*., 2011). Knowledge of the LGs represented in the MAALs adds to the benefits of using these cytological stocks for detailed studies on gene mapping, physical DNA probing, intergenomic gene transfer, and integration of these approaches (Armstrong *et al.*, 1998; Howell *et al*., 2002; Snowdon, 2007). Of interest is to compare the cytological karyotype (Cheng *et al*., 1995) with the idiogram of the linkage groups based on the FISH work by Xiong and Pires (2011). Putting these results together (Fig. S4) [**Supplementary Information**] permitted direct comparisons between these two modes. In general, chromosome size and centromeric positions in the karyotype coincided well with those in the idiogram. Only in the idiogram of LG-C2, the position of the centromere was more median than in its corresponding C7.

PROSPECTS

The available stocks of *B. rapa* var. *trilocularis* – *B. oleracea* var. *alboglabra* MAALs are suitable materials for cytological and genetic studies on the alien C chromosomes and the AA background. For refinement of physical mapping of the *B. oleracea* var. *alboglabra* chromosomes, painting of chromosome arms or whole chromosomes would be desirable. This can be achieved by multi-colour FISH of BAC contigs specific to the entire length of a C chromosome arm at a time, preferably at the pachytene stage, as applied on LG-A7 of *B. rapa* (Xiong *et al*., 2010). The advantage of applying this on the MAALs would be the presence of only one C chromosome in an AA background. Painting of the C chromosome will also be accompanied by partial painting of the homoeologous segments on the A chromosomes. This is valuable not only when the C chromosome is a univalent, but also when it pairs with A chromosomes, thus defining the exact identity of homoeologous chromosomes and the

order of pairing preferences, portraying the extent of homoeology between the C chromosome and different A chromosomes. Thus, a closer analysis of heteromorphic bivalents and multivalents is highly desirable.

To complement the array of seemingly intact and defined structurally changed alien C chromosomes represented in the available MAALs, it is desirable to place some effort towards developing a new MAAL with an intact C3. Having observed the advantages of maintaining MAALs with structurally changed C chromosomes, represented by MAALs for C3d and C4d, it is advisable to retain additional partial MAALs for detailed studies on gene and marker mapping, position effects and gene expression. Development of lines with stable mini-chromosomes could also be useful for gene mapping and gene transfer. Other materials that became available when working with the MAALs, and have not been discussed in the present work, are the euploid *B. rapa* plants which are siblings of the monosomics. They would be expected to carry introgressed C chromatin and are worth closer examination. Introgressed genetic material from the C genome might contain desirable genes for breeding improvement of *B. rapa.* Monosomics and their sibling *B. rapa* euploids, cytologically and molecularly monitored, could be valuable genetic resources.

Since a high number of A- and C-genome and -chromosome specific SSRs are defined in the reported literature, it would be highly desirable to determine their physical localization on chromosomes. Physical detection of SSRs and their mapping on chromosomes applying non-denaturing FISH (Cuadrado and Jouve, 2010), in comparison with SSR positions on linkage maps would be an appropriate approach to map meiotic recombination incidence along the chromosomes as well as approximate sites of SSR-linked genes of interest. This would also contribute to the integration of cytological, physical and genetic maps.

The MAALs are suitable for defining and mapping genes of agronomic interest on specific chromosomes. The application of next-generation sequencing on the MAALs by targeting the alien C chromosome or the background genome would enable the development of molecular markers for linkage mapping and marker assisted selection, the profiling of the transcriptome relating to the alien C chromosome, and monitoring of intergenomic introgression (Varshney *et al.*, 2009). The knowledge acquired on the maternal and embryonal control of seed colour character in *B. oleracea* var. *alboglabra* and the expression of this character sets the stage for a better understanding of seed colour inheritance, mapping of major and minor controlling genes and definition of linked molecular markers, as well as transcriptome analysis of pigmentation patterns and chemistry. MAALs proved useful for studies on other agronomic characters, such as control of seed size (Stoute, King, Scott, Kurup, unpublished).

Supplementary Information. Fig. S1 shows the differentiation between the A and C chromosomes at diakinesis of a sequidiploid AAC, a double MAAL with C6 and C8, and MAALs with C5, C6 and C7, stained by Snow´s carmine. Fig. S2 shows examples of chromosome structural changes and of a substitution found in progenies from MAALs for C4 and C5. Fig. S3 complements Fig. 6 by showing identification of the A and C chromosomes after applying two rounds of multiple target FISH on MAALs for C3d, C4 and C6. Fig. S4 is a comparison between the cytological karyotype of *B. oleracea* var. *alboglabra* (Cheng *et al.*, 1995) and the idiogram of *B. oleracea* (Xiong and Pires, 2011). Table S1 presents the accession numbers given by the gene bank NordGen (www.nordgen.org) to the deposited MAALs and their progenitor AA, CC and AACC materials.

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Table 1. Morphological features characteristic of the parental AA and CC species and of monosomics carrying different types of C chromosomes.

Material	No. of cotyledons	Stem anthocyanin	Leaf surface	Flower colour	Colour of seeds produced
AA	2	−	Even	Yellow	Yellow
CC	2	+	Even	White	Yellow
C1	3	+	Even	Yellow	Dark brown
C2	2	−	Even	Yellow	Yellow + Yellow with brown spots/patches
C3d	2	+	Even	Yellow	Yellow + Yellow with light brown dots
C4	2	+	Even	White	Yellow + Brown with dark dots
C4d	2	+	Even	Yellow	Yellow + Faint brown with dark dots
C5	2	−	Puckered	Yellow	Yellow + Yellow with brown spots/patches
C6	2	−	Even	Yellow	Yellow + Yellow with brown spots/patches
C7	2	−	Even	Yellow	Yellow + Yellow with brown spots/patches
C8	2	−	Even	Yellow	Yellow
C9	2	+	Even	Yellow	Yellow

Table 2. Frequency of plants producing pigmented seeds and of plants with alien chromosomes in relation to colour of seed of origin, and frequency of pigmented seeds in seed mixtures, in seven MAALs harbouring genes that affect seed colour

MAAL*		Number of sown seeds**				Number of raised plants producing seeds**				% of plants with pigmented seeds	Number of plants with chromosome numbers			% of mono- and disomics	Number of harvested seeds**				% of pigmented seeds
Cyt.	LG	B	S/P	S/P+Y	Y	B	B + Y	S/P+Y	Y		20	21	22		Total	B	S/P	Y	
C1	C9	122				37			85	30.3									
						9						9		100.0					
									37		37			0.0					
C2	C1		37					37		100.0					1899		639	1260	33.6
			20									17	3	100.0					
					15				15	0.0									
					24						23	1		4.2					
C3d	C5			65				22	43	33.8									
			10					10		100.0									
			7								1	5	1	85.7					
					10				10	0.0									
					9						8	1		11.1					
C4	C3	58					56		2	96.6					1832	636		1196	34.7
		51										45	6	100.0					
					30		1		29	3.3									
					41						41			0.0					
C4d	C3	28					26		2	92.9					2164	795		1369	36.7
		5										5		100.0					
C5	C4		22					22		100.0					1764		573	1191	32.5
			16								1	15		93.8					
					10				10	0.0									
					12						10	2		16.7					
C6	C6		29					20	9	69.0					2297		738	1559	32.1
			16								4	12		75.0					
C7	C2		30					19	11	63.3					3298		1055	2243	32.0
			8									8		100.0					
					4						4			0.0					

*Cytological (Cyt.) and corresponding linkage group (LG) designations according to Table 4. **Seeds are brown (B), brown spotted or patched (S/P), or yellow (Y).

Table 3. The frequency of diakinesis cells in which the C chromosome stays as a univalent (I), and when it pairs with an A chromosome forming a heteromorphic bivalent (II), and when it is part of a trivalent after pairing with a pair of A chromosomes with median/submedian centromeres (III^1), or submedian/subterminal centromeres (III^2), or when it pairs with the nucleolar chromosomes (III^3), and when it pairs with two pairs of A chromosomes forming a pentavalent (V).

MAAL*		No. of cells	% of cells with:					
Cyt.	LG		I	II	III^1	III^2	III^3	V
C1	C9	100	39.0		61.0			
C2	C1	193	59.1	8.8	32.1			
C3d	C5	441	90.2	0.7	9.1			
C4	C3	573	51.3	0.4			48.3	
C4d	C3	537	38.9	0.2			60.9	
C5	C4	124	44.3	6.5	12.9	25,0		11.3
C6	C6	348	68.1	1.2		29.0	1.7	
C7	C2	80	62.5	5.0	-------32.5-------			
C8	C7	343	82.5				17.5	
C9	C8	350	39.7	0.3	9.4		48.0	2.6
Average**			55.8	2.8	----------39.7----------			1.7

*Cytological (Cyt.) and corresponding linkage groups (LG) designations according to Table 4.

**Averages excluding the data for the structurally changed chromosomes C3d and C4d.

Table 4. Correspondence between cytological (Cyt.) and linkage group (LG) designations of the *Brassica oleracea* var. *alboglabra* chromosomes.

Cyt.	LG	Evidence		
		SSR*	FISH and GISH	Figures
C1	C9	BnGMS185	FISH: Multiple target	Fig. 6E and F
		BnGMS634		
C2	C1	CB10277	-	-
C3d	C5	Na10B08	FISH: Multiple target	Fig. S3C and D
C4	C3	BRAS068	FISH: Multiple target	Fig. S3A and B
		FITO-306	FISH: Specific BAC	Fig. 7A
		FITO-505		
C5	C4	BRAS003	FISH: Specific BAC	Fig. 7B and C
		BnGMS408	FISH: 5S rDNA and GISH	Fig. 7D and E
		BnGMS490	FISH: 5S and 25S rDNA**	
C6	C6	CB10010	FISH: Multiple target	Fig. S3E and F
		FITO-067		
C7	C2	BnGMS280	-	-
		BnGMS454		
		CB10026		
C8	C7	BRAS019	FISH: Multiple target	Fig. 6C and D
		Fito-472	FISH: 5S and 25S rDNA**	
		FITO-497		
		Na12-F03		
		Ol10-H04		
C9	C8	BnGMS336	FISH, 5S and 25S rDNA**	
		BNGMS439		
		BNGMS509		
		CB10139		
		CB10179		

* (Geleta *et al.*, 2012)

** C5, C8 and C9 characterized in the MAALs by Hasterok et al. (2005) and correspond to LG-C4, LG-C7 and LG-C8, respectively (Howell et al., 2002)

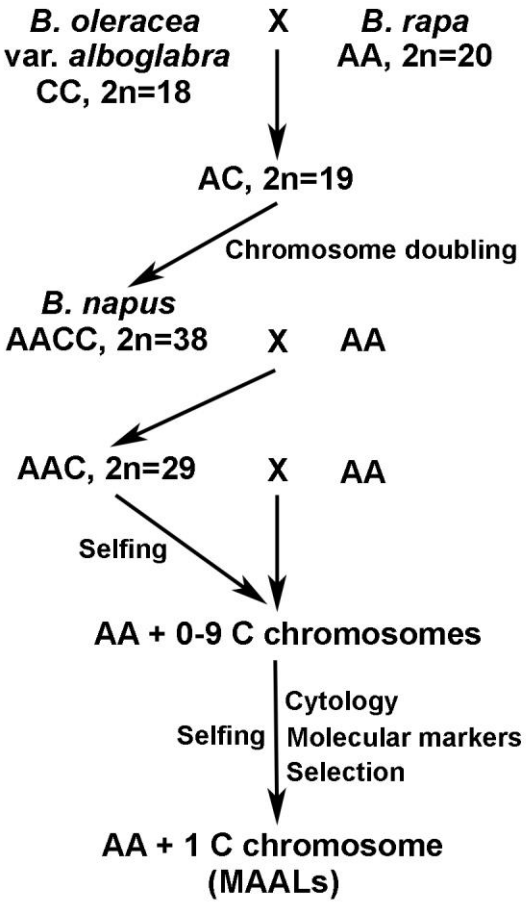

Fig. 1. Scheme showing the origin of the developed *Brassica* MAALs.

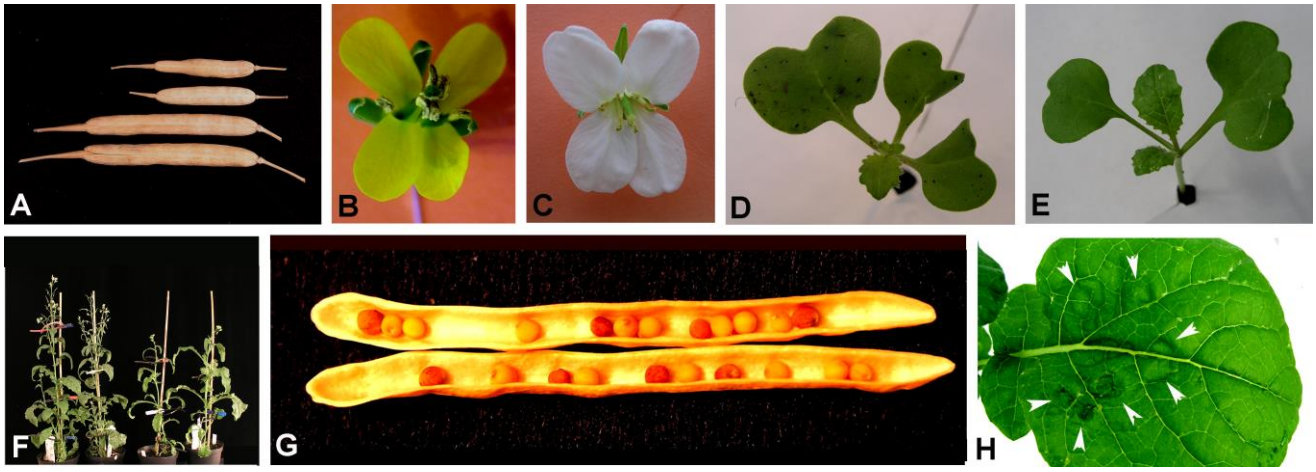

Fig. 2. Morphological features of relevance for the differentiation of plants that carry the alien C chromosome. (A) General small size of siliques exemplified by siliques of a C2-carrier plant and large siliques of the parental *Brassica rapa* species; (B) yellow flower of *B. rapa*, the colour characteristic of all MAALs except the MAAL for C4; (C) white flower colour characteristic of *B. oleracea* var. *alboglabra* and of C4-carriers; (D, E) C1-carriers; (D) three cotyledons; (E) two cotyledons, one almost double the size of the other; (F) two white-flowered C4-carriers to the left and two smaller yellow-flowered C4d-carriers to the right; (G) 8 brown seeds and 14 yellow seeds in a silique of a C4-carrier; (H) puckered surface of a leaf of a C5-carrier.

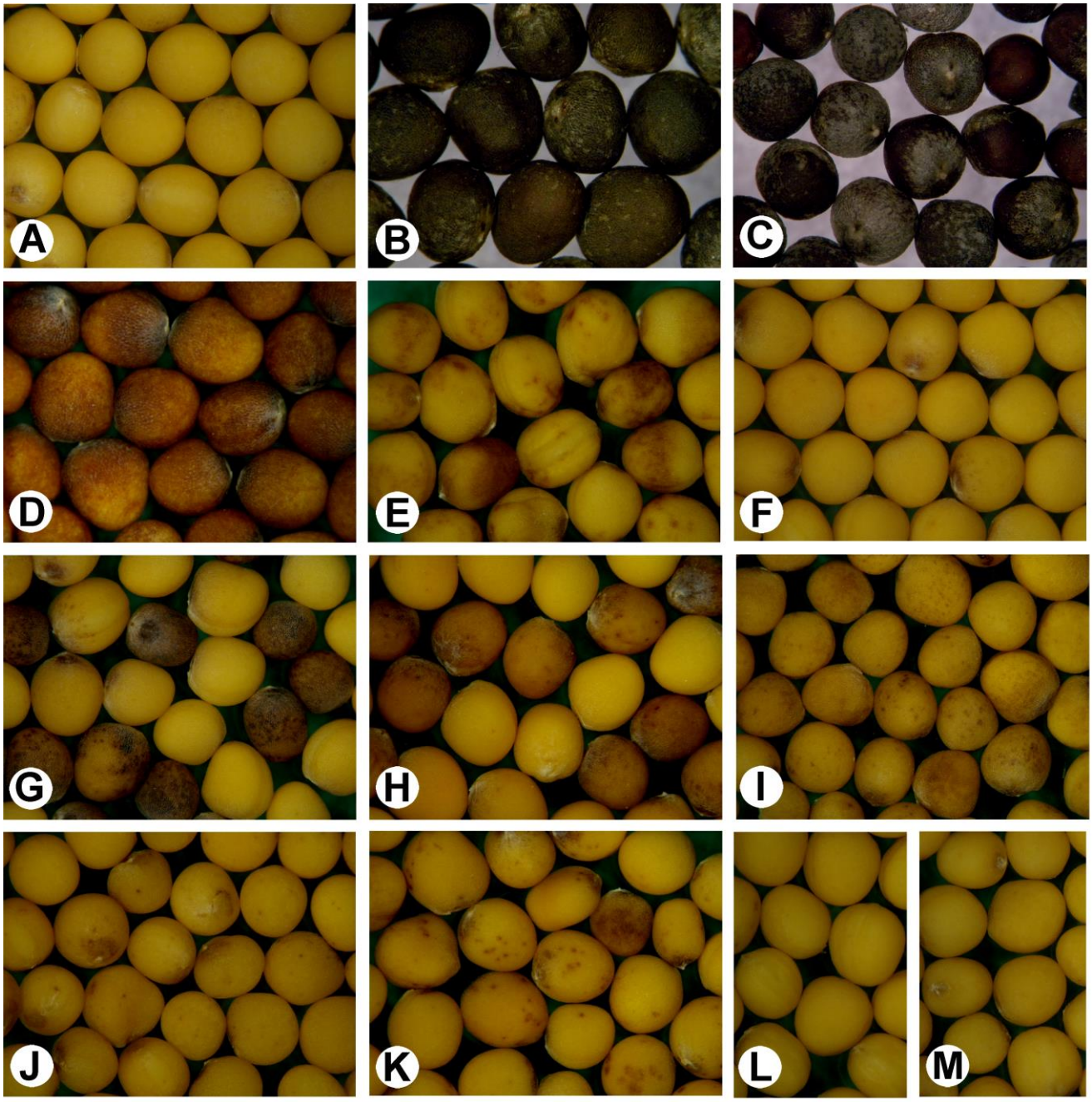

Fig. 3. Seed colour of the *Brassica* material studied. (A) Yellow in *B. rapa* var. *trilocularis* (K-151); (B) black in *B. oleracea* var. *alboglabra* (No. 4003); (C) black, dark grey and brown in resynthesized *B. napus* (No. 7406); (D) brown seeds harvested from a C1-carrier; (E) selected seeds with brown spots/patches from a C2-carrier; (F) bulk seeds harvested from a C3d-carrier, seeds with faint brown dots are difficult to

detect by the naked eye; (G, H) mixtures of easily distinguishable yellow and brown seeds originating from C4- and C4d- carriers, respectively, the brown seeds are slightly lighter in colour in the case of C4d; (I-K) selected seeds with brown spots-patches from plants carrying C5, C6 and C7, respectively; (L, M) yellow seeds harvested from plants with C8 and C9, respectively.

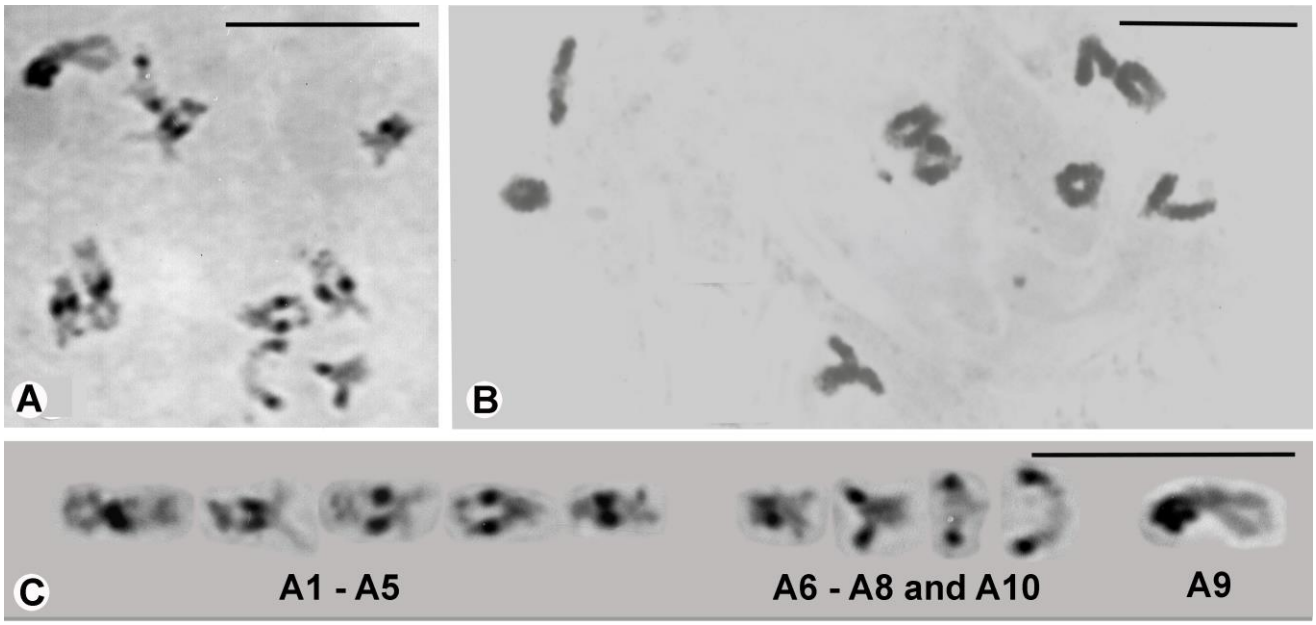

Fig. 4. Diakinesis chromosomes. (A, C) *Brassica rapa* (AA), differential condensation and staining marking the heavily stained heterochromatic pericentric regions, numeric designations of chromosomes with median/submedian centromeres (A1-A5), submedian/subterminal centromeres (A6-A8 and A10), and the nucleolar pair (A9) are according to Cheng et al. (1995); (B) *B. oleracea* var. *alboglabra* (CC), chromosomes are generally homogenously stained. Scale bar, 10 µm.

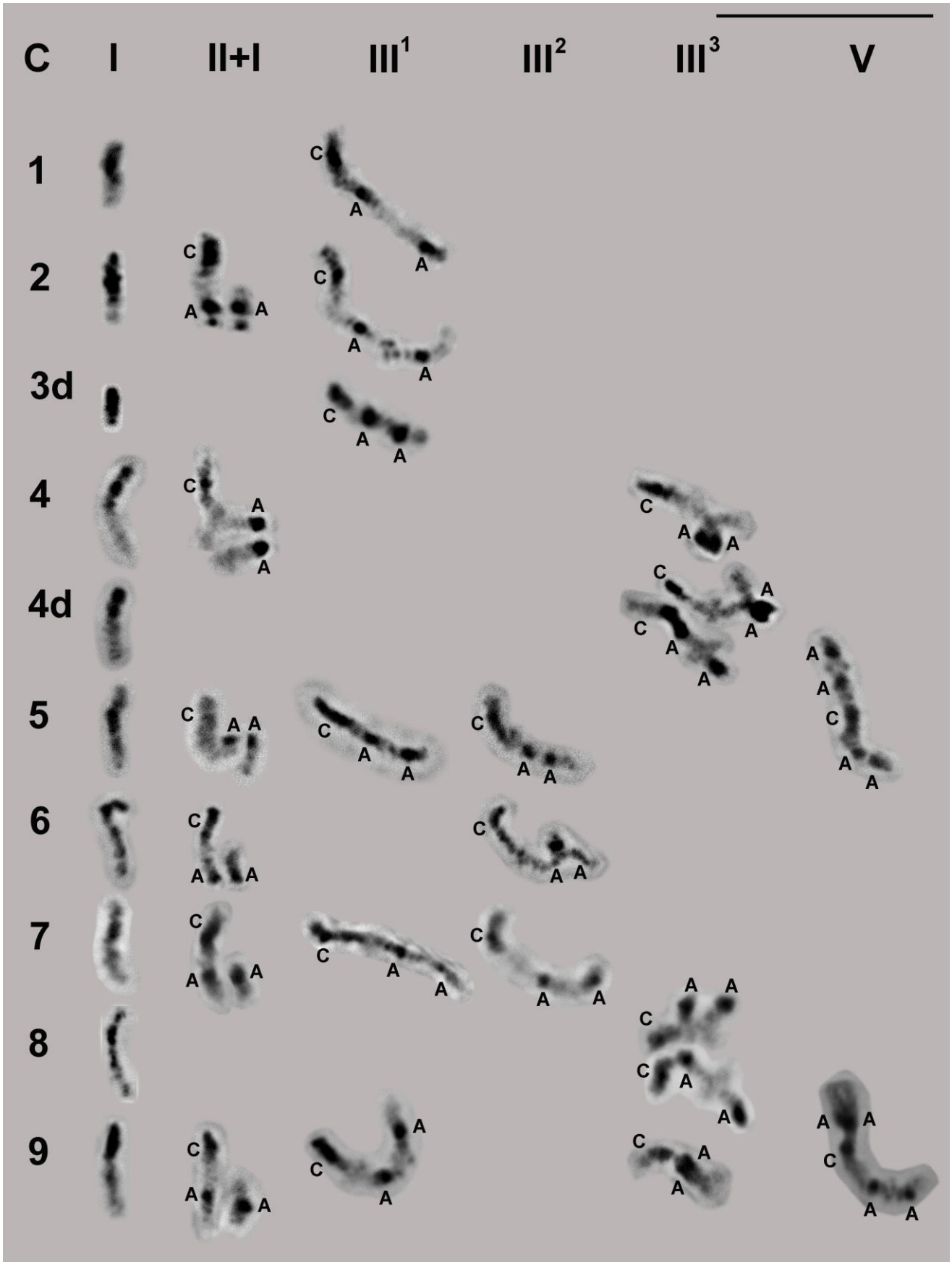

Fig. 5. The behaviour of the C chromosome during diakinesis in the MAALs. It

either remains unpaired as a univalent (I), or pairs with one A chromosome forming a heteromorphic bivalent, while the other A chromosome remains as a univalent (II+I), or appears as part of a trivalent when pairing with a pair of A chromosomes with median/submedian centromeres (III^1) or submedian/subterminal centromeres (III^2) or with the nucleolar organizer chromosomes (III^3), or as part of a pentavalent (V) when pairing with two pairs of A chromosomes. In the case of C4d and C8, two trivalents representing short and long arm associations of the C chromosome with the nucleolar A pair are depicted. Scale bar, 10 µm.

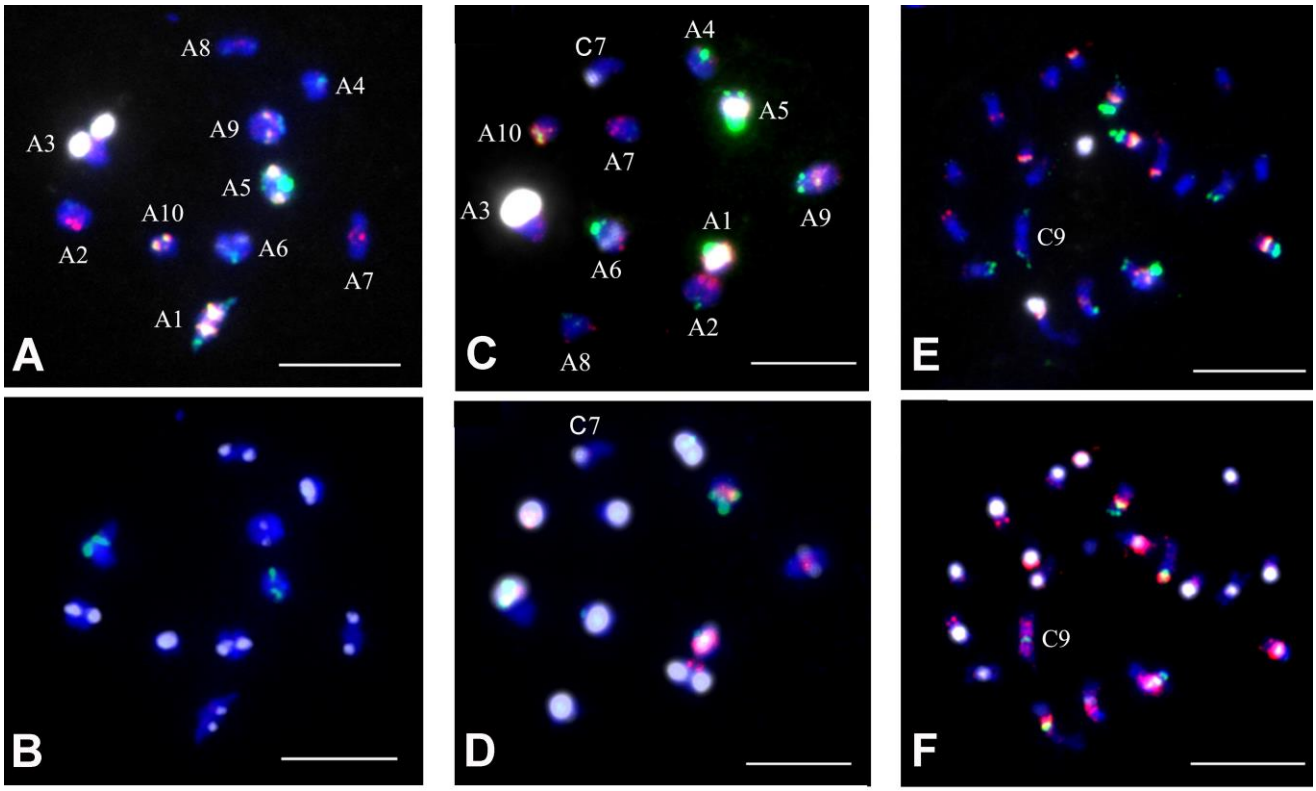

Fig. 6. Identification of the A and C chromosomes after applying two rounds of multiple target FISH according to Xiong and Pires (2011). (A, B) Diakinesis chromosomes of *B. rapa* (K-151), using in the first round (A) probes for 5S rDNA (yellow), 45S rDNA (white), repeated DNA sequences in eight chromosome pairs of *B. rapa* by using BAC KBrB072L17 (green), and repeated DNA sequences specific to two pairs of *B. rapa* by using BAC KBrH092N24 (red), and applying in the second round (B) probes for the repetitive centromeric DNA sequences CentBr1 (white) and CentBr2 (green) and for repetitive DNA sequences that are C-genome specific by using the BAC BNIH 123L05 (red); (C-F) identification of the C chromosome in MAALs; (C, D) diakinesis chromosomes with C8 (LG-C7); (E, F) mitotic chromosomes with C1 (LG-C9). Scale bar, 10 µm.

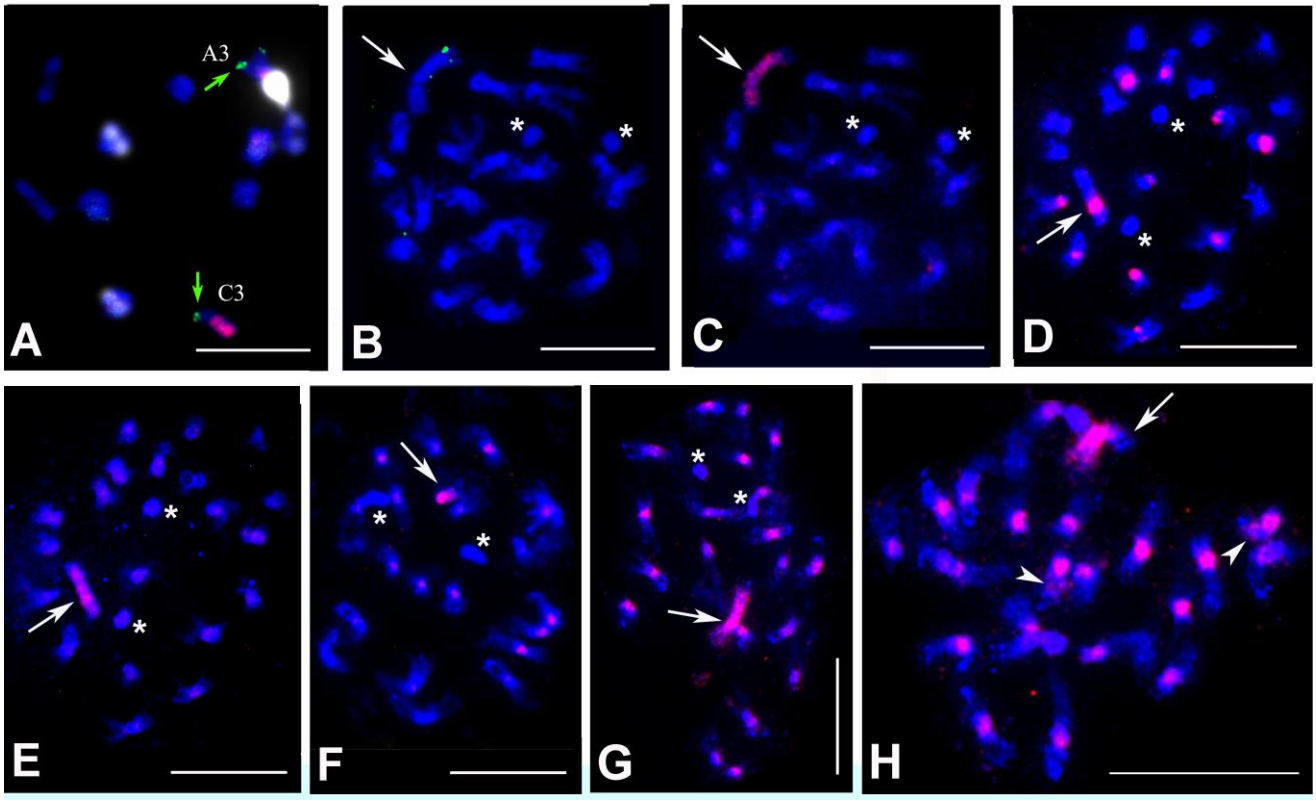

Fig. 7. Identification of the C chromosome in MAALs after applying chromosome-specific BAC probes (A-C), 5S rDNA probe followed by GISH with labelled C-genome DNA hybridizing to pericentric regions of A-chromosomes and to C-chromatin (D, E), or only GISH (F-H), and detection of possible intergenomic introgression (H). (A) Diakinesis chromosomes with C4 (LG-C3) labelled with BAC KBrH117M18 specific for LG-C3 and LG-A3 (green) and BAC BNIH 123L05 specific for C-genome chromosomes (red), and 45S rDNA probe (white); (B, C) mitotic chromosomes with C5 (LG-C4) labelled with BAC BoB004H11 probe (green) followed by GISH (red), arrows; (D, E) C5 labelled by 5S rDNA (red) and GISH (red) marked by arrows; (F-H) mitotic chromosomes; (F) C3d mainly composed of one arm (arrow); (G) C5 (arrow); (H) C4 (arrow) whose distal region of the short arm is unlabelled possibly denoting introgressed A-chromatin, also labelling of intercalary

sites in two A-chromosomes (arrowheads) possibly reflecting introgressed C-chromatin. Asterisks denote satellites of the nucleolar A pair. Scale bar, 10 µm.

Supplementary Table:

Table S1. Accession numbers of the *Brassica* material deposited in the gene bank NordGen in Alnarp, Sweden (www.nordgen.org) and corresponding linkage groups to the different C-genome chromosomes in the monosomic alien addition lines (MAALs)

Linkage group	Brassica material	Linkage group
NGB23151	MAAL for C1	C9
NGB23152	MAAL for C2	C1
NGB23153	MAAL for C3d	C5
NGB23154	MAAL for C4	C3
NGB23155	MAAL for C4d	C3
NGB23156	MAAL for C5	C4
NGB23157	MAAL for C6	C6
NGB23158	MAAL for C7	C2
NGB23159	MAAL for C8	C7
NGB23160	MAAL for C9	C8
NGB23161	*B. rapa* var. *trilocularis* (K-151)	
NGB23162	*B. oleracea* var. *alboglabra* (No. 4003)	
NGB23163	*B. napus* (No. 7406)	

Supplementary Figures 1-4:

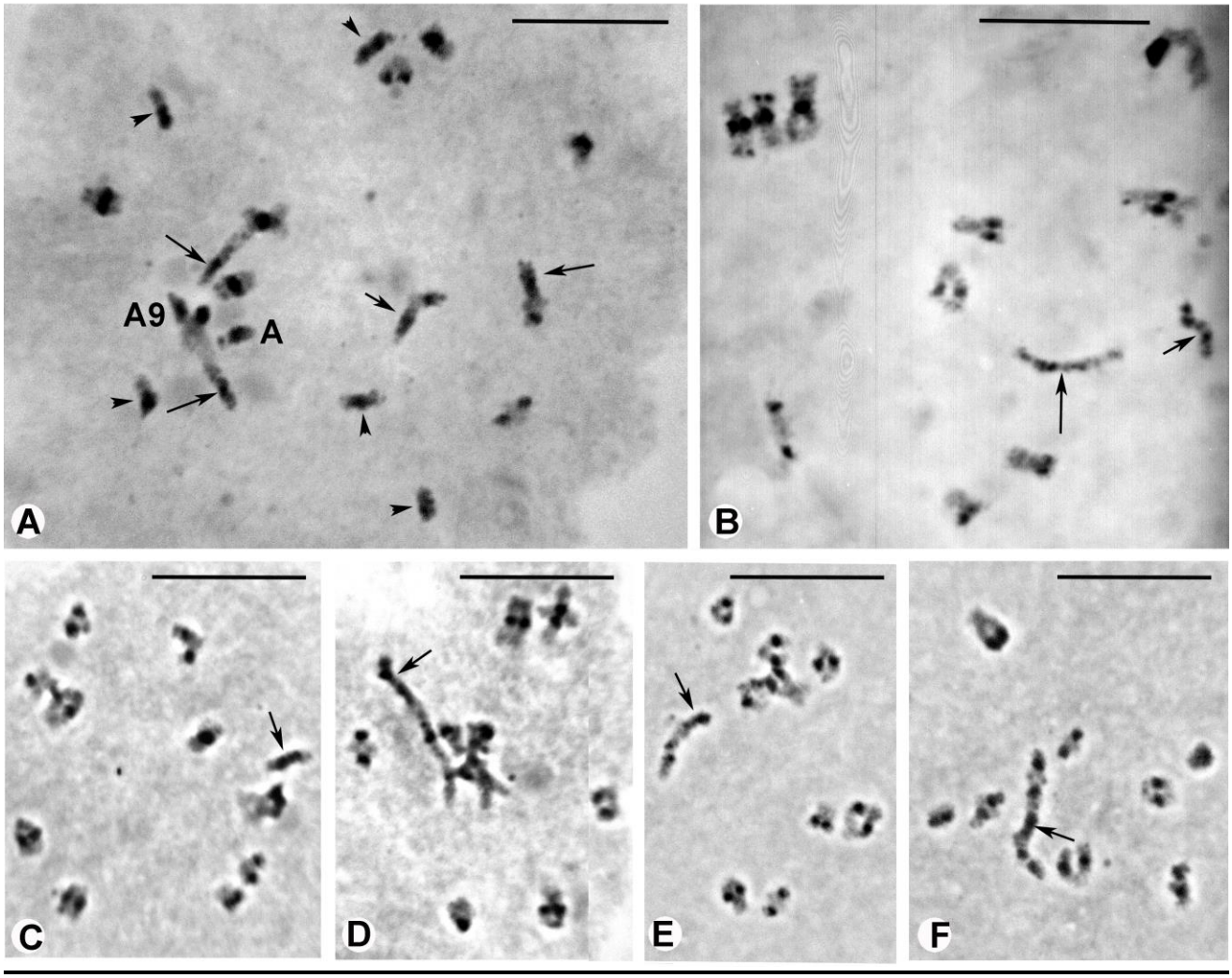

FIG. S1. Diakinesis chromosomes. (A) sesquidiploid (AAC, 2n=29), three C chromosomes are incorporated in three trivalents (long arrows pointing at the C chromosomes), one C chromosome is part of a heteromorphic bivalent (short arrow) while the other A chromosome is a univalent (A), and five C chromosomes are univalents (arrowheads); (B) double MAAL with C6 (short arrow) and C8 (long arrow); (C-F) MAALs; (C, D) carriers of C7 as a univalent (C, arrow) and as part of a trivalent involving two A chromosomes with median/submedian centromeres (D, arrow); (E) C6 (arrow) as part of a trivalent involving two A chromosomes with submedian/subterminal centromeres; (F) C5 (arrow) as part of a pentavalent involving

one pair of A chromosomes with median/submedian centromeres (upper) and one pair with submedian/subterminal centromeres (lower). Scale bar, 10 µm.

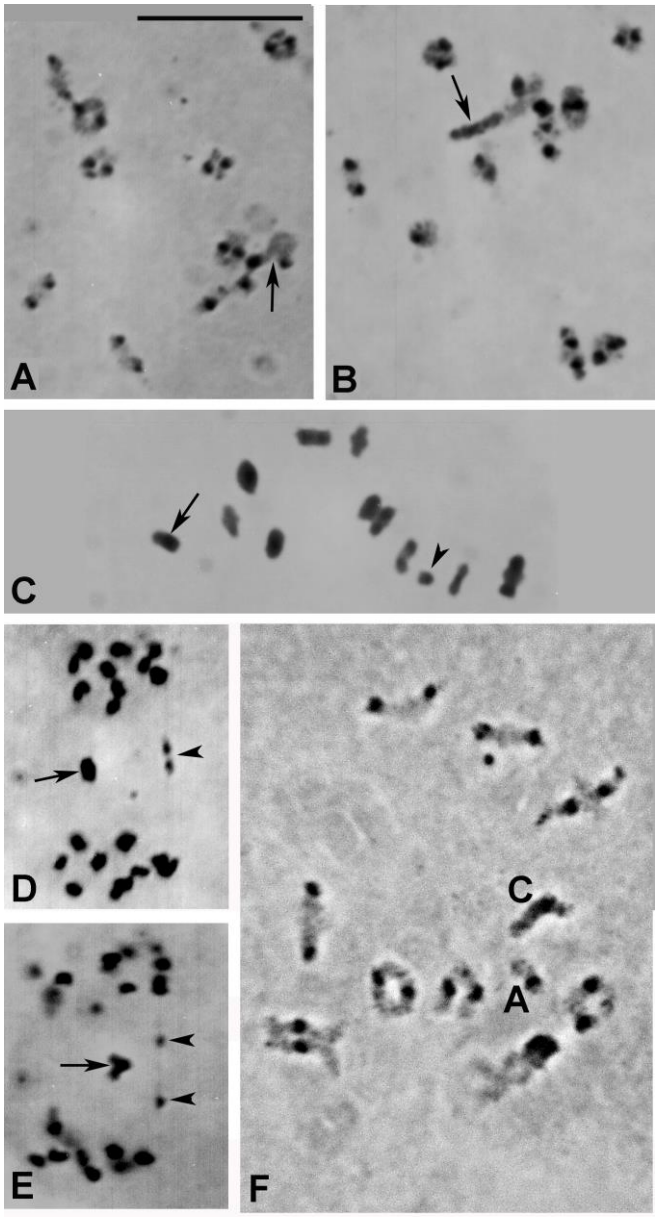

Fig. S2. Examples of chromosome structural changes found in progenies of C4-carrier plants (A-E) and of a substitution in a progeny plant of a C5-carrier (F). (A) Diakinesis of a euploid AA plant with 10 II, with a deleted major part of the short arm of one nucleolar chromosome in the heteromorphic bivalent (arrow); (B) diakinesis of a plant with a C4 larger than usual, apparently with extra duplicated or translocated C-genome chromosomal material, paired with the nucleolar chromosomes of the A-genome forming a trivalent (arrow); (C-E) meiosis of a

monosomic plant containing C4 and an additional mini-chromosome; (C) metaphase I with C4 as a univalent (arrow) and with the extra mini-chromosome (arrowhead); (D) anaphase I with a lagging C4 (arrow) and a divided mini-chromosome (arrowhead); (E) a later anaphase I stage with a dividing lagging C4 and separated daughter mini-chromosomes; (F) diakinesis of a progeny plant with 2n=20 containing a C5 and lacking the homologue to an A chromosome with a submedian/subterminal centromere. Scale bar, 10 μm.

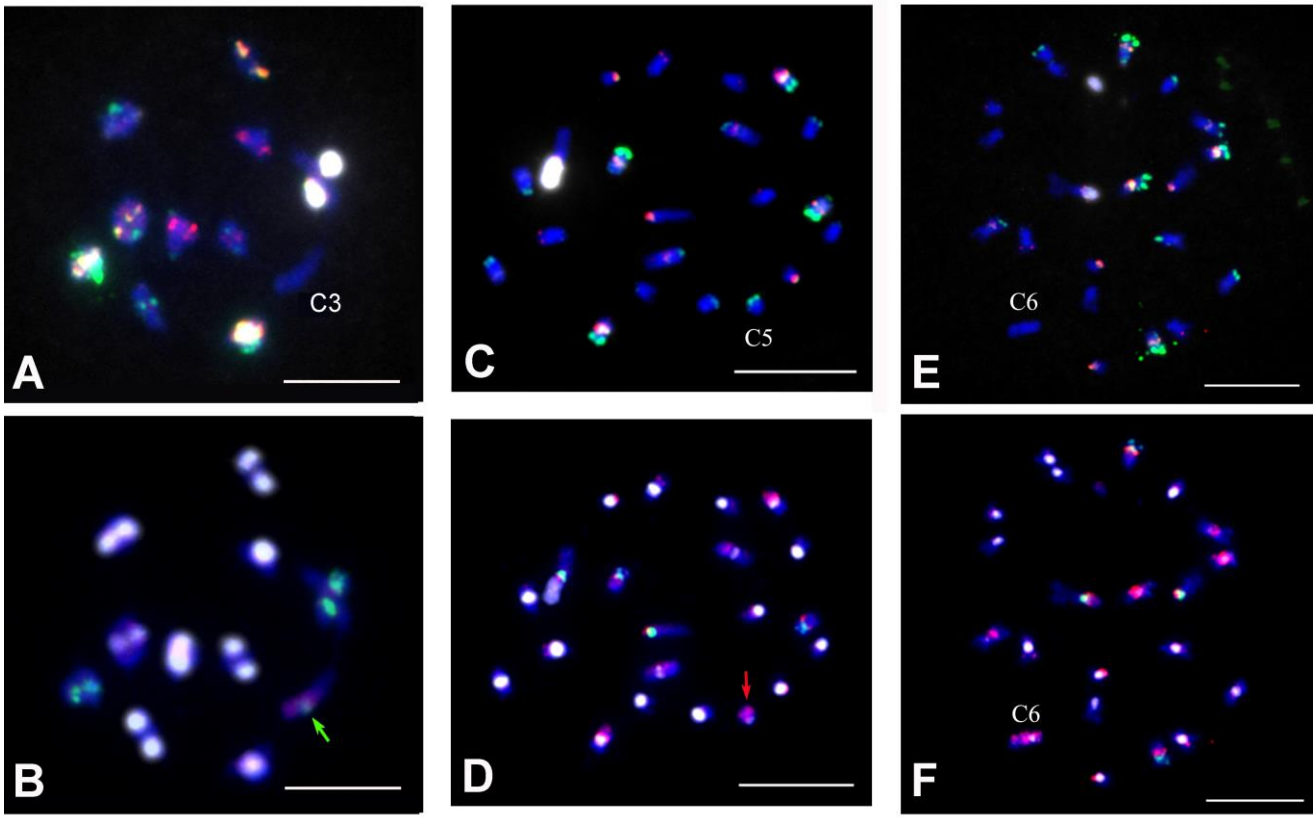

Fig. S3. Identification of the A and C chromosomes after applying two rounds of multiple target FISH (Xiong and Pires (2011), using in the first round (A, C, F) probes for 5S rDNA (yellow), 45S rDNA (white), repeated DNA sequences in eight chromosome pairs of *B. rapa* by using BAC KBrB072L17 (green), and repeated DNA sequences specific to two pairs of *B. rapa* by using BAC KBrH092N24 (red), and applying in the second round (B, D, F)) probes for the repetitive centromeric DNA sequences CentBr1 (white) and CentBr2 (green) and for repetitive DNA sequences that are C-genome specific by using the BAC BNIH 123L05 (red); (A, B) C4 (LG-C3) in a meiotic metaphase I; (C-F) mitotic metaphase; (C, D) C3d (LG-C5); (E, F) C6 (LG-C6). Scale bar, 10 µm.

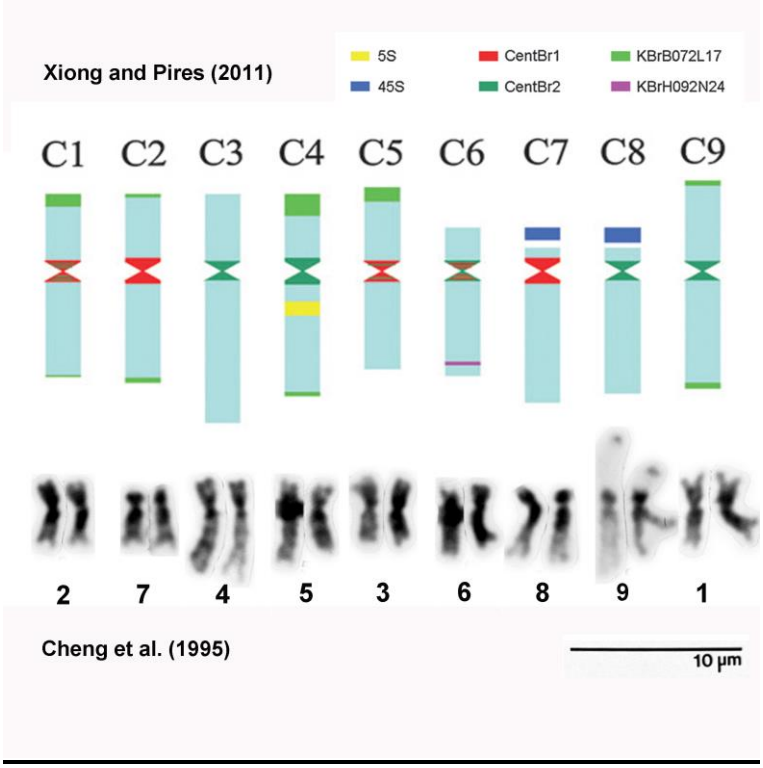

FIG. S4. Chromosomes of *Brassica oleracea* designated by linkage group numerals as depicted in an idiogram by Xiong and Pires (2011) and their corresponding chromosomes of *B. oleracea* var. *alboglabra* designated by cytological numerals in a karyotype by Cheng *et al.* (1995). There is a general correspondence regarding chromosome size and position of the centromere between the idiogram and the karyotype, with the exception of LG-C2 which has a more median centromere compared to its corresponding C7.