Construction of chromosomal recombination maps of three genomes of lilies (*Lilium*) based on GISH analysis

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Abstract: Chromosomal recombination maps were constructed for three genomes of lily (*Lilium*) using GISH analyses. For this purpose, the backcross (BC) progenies of two diploid ($2n = 2x = 24$) interspecific hybrids of lily, viz. Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA), were used. Mostly the BC progenies of LA hybrids consisted of both triploid ($2n = 3x = 36$) and diploid ($2n = 2x = 24$) with some aneuploid genotypes and those of OA hybrids consisted of triploid ($2n = 3x = 36$) and some aneuploid genotypes. In all cases, it was possible to identify the homoeologous recombinant chromosomes as well as accurately count the number of crossover points, which are called "recombination sites". Recombination sites were estimated in the BC progeny of 71 LA and 41 OA genotypes. In the case of BC progenies of LA hybrids, 248 recombination sites were cytologically localized on 12 different chromosomes of each genome (i.e., L and A). Similarly, 116 recombinant sites were localized on the 12 chromosomes each from the BC progenies of OA hybrids (O and A genomes). Cytological maps were constructed on the basis of the percentages of distances (micrometres) of the recombination sites from the centromeres. Since an Asiatic parent was involved in both hybrids, viz. LA and OA, two maps were constructed for the A genome that were indicated as Asiatic (L) and Asiatic (O). The other two maps were Longiflorum (A) and Oriental (A). Remarkably, the recombination sites were highly unevenly distributed among the different chromosomes of all four maps. Because the recombination sites can be unequivocally identified through GISH, they serve as reliable landmarks and pave the way for assigning molecular markers or desirable genes to chromosomes of *Lilium* and also monitor introgression of alien segments.

Key words: cytological maps, 2*n* gametes, genomic in situ hybridization (GISH), interspecific hybrids, *Lilium*, recombination sites.

Résumé : Des cartes de recombinaison chromosomique ont été produites pour les trois génomes du lys (*Lilium*) au moyen d'analyses GISH. Pour y arriver, les progénitures rétrocroisées (BC) de deux hybrides interspécifiques du lys ($2n = 2x = 24$), i.e. Longiflorum × Asiatic (LA) et Oriental × Asiatic (OA) ont été employées. Pour la plupart les descendants BC des hybrides LA étaient triploïdes ($2n = 3x = 36$) ou diploïdes ($2n = 2x = 24$) en plus des quelques génotypes aneuploïdes alors que ceux des hybrides OA étaient triploïdes ($2n = 3x = 36$) avec quelques génotypes aneuploïdes. Dans tous les cas, il a été possible d'identifier les chromosomes homéologues recombinants et de compter précisément le nombre d'enjambements, lesquels sont appelés des sites de recombinaison. Le nombre de sites de recombinaison a été estimé chez les progénitures BC des 71 génotypes LA et des 41 génotypes OA. En ce qui a trait aux progénitures BC des hybrides LA, 248 sites de recombinaison ont été localisés par examen cytologique sur les 12 chromosomes de chacun des génomes (L et A). De manière semblable, 116 sites de recombinaison ont été situés sur les 12 chromosomes de chaque génome (O et A) au sein des progénitures BC des hybrides OA. Des cartes cytologiques ont été produites sur la base du pourcentage des distances (micromètres) séparant les sites de recombinaison et les centromères. Puisqu'un parent Asiatic était impliqué dans les deux hybrides, LA et OA, deux cartes ont été construites pour le génome A et celles-ci ont été nommées Asiatic (L) et Asiatic (O). Les deux autres cartes ont été nommées Longiflorum (A) et Oriental (A). Remarquablement, les sites de recombinaison étaient répartis très inégalement sur les différents chromosomes sur les quatre cartes. Comme les sites de recombinaison sont identifiables sans équivoque par analyse GISH, ils peuvent servir de repères et ouvrent la voie à l'assignation de marqueurs moléculaires ou de gènes à des chromosomes chez le genre *Lilium* ainsi qu'au suivi de l'introgression de segments étrangers.

Received 2 July 2008. Accepted 16 November 2008. Published on the NRC Research Press Web site at genome.nrc.ca on 11 February 2009.

Corresponding Editor: G. Jenkins.

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Mots-clés : cartes cytologiques, gamètes 2*n*, hybridation génomique in situ (GISH), hybrides interspécifiques, *Lilium*, sites de recombinaison.

Introduction

Cytological maps represent the location and order of markers along the chromosomes in relation to structures such as centromeres, telomeres, secondary constrictions (if any), and knobs among others. Such maps are created by microscopic determination of the position of visible structures (or "markers") in fixed and stained chromosomes. These cytological maps are essential to relate genetic loci and molecular sequences to morphological features of chromosomes (Fransz et al. 2000; Cheng et al. 2001). In fact, cytological maps have been most useful in relating and orienting genetic linkage groups on individual chromosomes in crops such as maize (Burnham 1962), tomato (Khush and Rick 1968), and rice (Khush et al. 1984; Singh et al. 1996). Besides chromosome markers, the discovery of differential staining techniques, such as Giemsa C-banding, paved the way for using chromosome markers more extensively (Linde-Laursen 1988; Gill et al. 1991; Pedrosa et al. 2002). In addition, induced chromosome deletions and translocations that can be visualized cytologically have also been used for mapping genes in some cases, e.g., tomato (Khush and Rick 1968), wheat (Castilho et al. 1996; Gill et al. 1996; Sandhu and Gill 2002; Bhat et al. 2007), *Brassica* (Howell et al. 2002), and barley (Künzel et al. 2000).

Fluorescent in situ hybridization (FISH), a molecular cytogenetic technique, has opened up possibilities for localizing large numbers of cloned DNA sequences directly on chromosomes for mapping purposes. Nevertheless, chromosome maps or the so-called cytomolecular maps have been constructed in some of the plants with small as well as large chromosomes by FISH. Examples are *Arabidopsis* (Schmidt et al. 1995; Jackson et al. 1998), *Sorghum* (Islam-Faridi et al. 2002), legumes (Fuchs et al. 1998; Ohmido et al. 2007), and *Pinus* species (Hizume et al. 2002; Islam-Faridi et al. 2007). Since the cloned DNA sequences can be directly localized on chromosomes, this method is becoming increasingly important in plant molecular cytogenetics (Jiang and Gill 2006). In addition, although FISH is useful for the construction of physical maps and for the elucidation of molecular organization of chromosomes, it is less suitable for unravelling the process of crossing-over.

Unlike the FISH technique, genomic DNA in situ hybridization (GISH) can be most useful for analysing the process of intergenomic recombination as well as for the elucidation of chromosome organization. But GISH is restricted only to distant hybrids and their progenies for unravelling intergenomic recombination. This is because the parental genomes need to be sufficiently differentiated, as is the case for distant hybrids, to be able to distinguish the parental genomes in the hybrids. Using GISH, intergenomic recombination has been demonstrated to occur in the progenies of some of the intergeneric and interspecific hybrids such as *Gasteria* × *Aloe* (Takahashi et al. 1997), *Festuca* × *Lolium* (King et al. 2002*a*; Kosmala et al. 2006, 2007), *Alstroemeria aurea* × *Astroemeria inodora* (Kamstra et al. 1999), *Alstroemeria inodora* × *Alstroemeria pelegrina* (Ramanna et al. 2003), *Allium cepa* × (*Allium fistulosum* × *Allium roylei*) (Khrustaleva and Kik 1998), *Lilium longiflorum* × Asiatic hybrids (Karlov et al. 1999), Oriental × Asiatic lily hybrids (Barba-Gonzalez et al. 2004), and *Tulipa gesneriana* × *Tulipa fosteriana* (Marasek et al. 2006). In the case of distant hybrids, various strategies are required for backcrossing and analysing the progenies so that the number and positions of crossover points can be estimated. For example, in the case of *Festuca* × *Lolium*, triploid hybrids were successfully backcrossed to the *Lolium* parent using a disomic substitution for chromosome 3 of *Festuca pratensis* in *Lolium perenne* complement for physical mapping as well as establishing a 1:1 relationship between chiasma formation and crossing-over (King et al. 2002*a*, 2002*b*). In the case of *Allium*, the diploid hybrid *A. roylei* × *A. fistulosum* produced haploid (*n*) gametes that could be successfully used to cross with *A. cepa* and to obtain the so-called trispecific hybrid that was used for GISH analysis (Khrustaleva and Kik 1998). An advantage of using a trispecific hybrid for GISH analysis was that the recombinant segments of *A. roylei* × *A. fistulosum* could be directly visualized in the background of the chromosome complement of the *A. cepa* parent. Using such genotypes, the integrated recombination and physical maps of two chromosomes (chromosome 5 and 8) of the interspecific parent (i.e., *A. roylei* × *A. fistulosum*) were constructed (Khrustaleva et al. 2005). However, in these approaches, the cytogenetic map has been constructed for only one or two chromosomes of these plant species. As a rare exception, the intergeneric hybrid of *Gasteria* × *Aloe* produced haploid gametes that were used for the production of diploid backcross (BC) progenies so that the number and positions of recombinant break points were accurately estimated (Takahashi et al. 1997). Apart from these, some interspecific hybrids of *Alstroemeria* and *Lilium* were reported to produce either *n* or 2*n* gametes (Kamstra et al. 1999; Lim et al. 2001*b*; Ramanna et al. 2003; Barba-Gonzalez et al. 2004; Zhou 2007; Zhou et al. 2008). In the case of lilies, by using interspecific hybrids that produced both *n* and 2*n* gametes, it was possible to produce BC_1 progenies that were diploids as well triploids. Such progenies were used to identify a large number of homoeologous recombination break points, referred to as "recombination sites" in this article, through GISH analysis.

The species of the genus *Lilium* have probably the largest genomes among plants (Bennett and Smith 1976, 1991) and have been extensively used for cytological analysis in the past. For example, basic studies on chromosome identification and karyotype analysis (Stewart 1947; Noda 1991), chiasma formation and crossing-over (Mather 1940; Brown and Zohary 1955; Fogwill 1958), and time and duration of female meiosis (Bennett and Stern 1975) were conducted. Nevertheless, to our knowledge, no maps of any type are available for the genomes of lilies so far. In the present

study, we describe cytological maps of three complete genomes of lilies based on the recombination sites identified through GISH in the BC progeny populations of two interspecific hybrids. These involve hybrids between three main groups of diploid ($2n = 2x = 24$) cultivars, viz. Asiatic, Longiflorum, and Oriental lilies, that belong to three different taxonomic sections (Lim et al. 2000). The genomes are completely differentiated and suitable for GISH analysis (Lim et al. 2001*a*; Barba-Gonzalez et al. 2004). The distribution of crossover sites among different chromosomes within each as well as in different genomes is described below and their significance is discussed.

Materials and methods

Plant material

Diploid ($2n = 2x = 24$) cultivars of three groups of lilies (*Lilium*), Longiflorum (L), Asiatic (A), and Oriental (O), were used for producing F_1 hybrids and the BC progenies. Because all the cultivars of the three groups are interspecific hybrids of closely related species of complex origin, the species names are not mentioned. As the three groups belong to three different taxonomic sections, the cultivars of different sections could be hybridized, or backcrossed, only through special techniques such as cut-style pollination and embryo rescue (Lim et al. 2001*b*; Barba-Gonzalez et al. 2004). The two types of hybrids used were Longiflorum × Asiatic (LA) and Oriental × Asiatic (OA). For backcrossing, the LA hybrids were used as either female or male parent and crossed with 12 different Asiatic parents. An important feature of some of the LA hybrids was that they produced both *n* (haploid) and 2*n* gametes in both types of spore mother cells (Zhou 2007). Therefore, the BC_1 progenies resulting from LA × AA (LAA) or from the reciprocal AA × LA (ALA) consisted of both diploid or near-diploid ($2n = 2x = 24$) and triploid ($2n = 3x = 36$) progenies (Table 1). Besides BC_1, five BC_2 genotypes were also selected, three from reciprocal interploidy crosses between A and LAA and two from ALA × LA (Table 1). Similarly, five BC_3 plants were also analysed for chromosomal recombination mapping in LA hybrids (Table 1). In the case of OA hybrids, only those genotypes that produced 2*n* pollen were used for backcrossing with the Asiatic cultivars as female parents (i.e., AOA). In all cases, the progenies were triploid with two exceptions (Table 2). Similarly, 10 BC_2 progeny plants of OA hybrids resulting from a cross (AOA × AA) were also analysed (Table 2). All of the plant material is maintained vegetatively at Wageningen University and Research Plant Breeding.

Mitotic chromosome preparation

For mitotic metaphase chromosome analysis, root tips were collected early in the morning, incubated in 0.7 mmol/L cycloheximide solution for 4–6 h, fixed in an ethanol – acetic acid (3:1) solution for 12–24 h, and stored at 4 °C until use. The root tips after washing in distilled water were incubated in a pectolytic enzyme mixture containing 0.2% (*w*/*v*) pectyolase Y23, 0.2% (*w*/*v*) cellulose RS, and 0.2% (*w*/*v*) cytohelicase in 10 mmol/L citrate buffer (pH 4.5) at 37 °C for about 1 h. Squash preparations were made in a drop of 45% acetic acid and frozen in liquid nitrogen. The slides were then dehydrated in absolute ethanol and air dried.

GISH

Genomic DNA of Longiflorum cultivar 'White Fox' and genomic DNA of Oriental cultivar 'Sorbonne' were used as probes and labeled with either digoxigenin-11-dUTP or biotin-16-dUTP by a standard nick translation protocol (Roche Diagnostics GmbH, Mannheim, Germany). Total genomic DNA was extracted with the CTAB method (Rogers and Bendich 1988). The GISH procedure and mitotic chromosome spreads were performed as described by Lim et al. (2003) and Barba-Gonzalez et al. (2005*a*). Briefly, the hybridization mixture contained 50% (*v*/*v*) deionized formamide, 10% (*w*/*v*) sodium dextran sulfate, 2× SSC, 0.25% (*w*/*v*) sodium dodecyl sulfate, 1–1.5 ng/μL digoxigenin- or biotin-labeled DNA from the Longiflorum or Oriental cultivar, and 25–100 ng/μL block DNA of the Asiatic cultivar. The DNA was denatured by heating the hybridization mixture at 70 °C for 10 min followed by incubation in ice for 5 min. The hybridization mixture was then applied on each slide (40 μL). The preparations were denatured immediately prior to incubation at 80 °C for 5 min. After overnight hybridization at 37 °C in a humid chamber, the slides were washed at room temperature in 2× SSC for 15 min followed by stringent washing with 0.1× SSC for 30 min at 42 °C. Digoxigenin-labeled DNA was detected with antidigoxigenin–fluorescein raised in sheep (Boehringer, Mannheim, Germany) and amplified with fluorescein anti-sheep immunoglobulin raised in rabbit (Vector Laboratories, Burlingame, California). Biotin-labeled DNA was detected with CY-3-conjugated streptavidin and amplified with biotinylated goat-antistreptavidine (Vector Laboratories). Preparations were analysed using a Zeiss Axiophot epifluorescence microscope and photographed with a Canon digital camera. For each plant, the total number of chromosomes and the number of recombinant points were determined.

Chromosome identification

In all the three genomes, the chromosomes are arranged in sequence of decreasing short arm length according to Stewart (1947) taking into account the position of 45S rDNA hybridization signals in LL and OA hybrids (Lim et al. 2001*a*; Barba-Gonzalez et al. 2005*a*). Some of the chromosomes in the somatic karyotype could be identified on the basis of total length and arm ratios (e.g., 1, 2, 3 10, 11, and 12). In other cases, however, the differences in the lengths of short arms were used for identification. By using recombination sites as markers, the same chromosome could be accurately identified in 5–10 cells and compared with the previous and the succeeding chromosomes in the karyotype. Furthermore, the centromeric index (short arm length/(short arm length + long arm length)) and the relative chromosome length index (individual chromosome length/total length of all chromosomes) were determined for all genotypes (Barthes and Ricroch 2001) and used as an additional identification tool. The differences in size of the genomes of L, O, and A are less than 5% (Van Tuyl and Boon 1997), so this will have no influence on the calculations.

Measurements and mapping

Images of mitotic metaphase chromosomes from each genotype were collected and measured in micrometres using the computer program MicroMeasure (Reeves and Tear

Table 1. Genome composition, recombinant chromosomes, and recombination sites in the BC progenies of LA hybrids.

			Genome composition			
Cross	Genotype code	Ploidy level	L (L/A)	A (A/L)	No. of recombinant chromosomes	No. of recombination sites
LAA	041551	3*x*	12 (1)	24 (1)	2	2
LAA	041552	3*x*	12 (2)	24 (2)	4	4
LAA	041553	3*x*	12 (1)	24 (2)	3	3
LAA	041554	3*x*	12 (1)	24	1	1
LAA	041555	3*x* + 1	12 (1)	25	1	1
LAA	041569	3*x*	12 (1)	24 (1)	2	2
LAA	041571	3*x*	12 (2)	24 (2)	4	4
LAA	041572	3*x* + 1	13 (2)	24 (1)	3	3
LAA	041573	3*x*	12 (1)	24 (1)	2	2
LAA	041574	3*x*	12 (1)	24 (1)	2	2
LAA	041575	3*x*	12 (1)	24 (1)	2	2
LAA	041578	3*x*	12 (1)	24	1	1
LAA	041580	3*x*	12 (3)	24 (2)	5	5
LAA	041581	3*x*	12	24 (3)	3	3
LAA	041583	3*x*	12 (1)	24 (1)	2	2
LAA	044525-1	3*x*	12 (1)	24 (1)	2	2
LAA	044539-1	3*x*	12 (1)	24 (1)	2	2
LAA	044571-1	3*x*	12 (1)	24 (2)	3	4
LAA	062035-1	3*x*	12 (4)	24 (2)	6	9
LAA	062035-2	3*x*	12 (3)	24 (3)	6	7
LAA	062071-1	3*x*	12 (7)	24 (5)	12	23
LAA	062071-2	3*x*	13 (9)	23 (5)	14	42
LAA	062074-1	3*x*	12 (7)	24 (7)	14	29
LAA	062074-3	3*x*	12 (6)	24 (6)	12	20
LAA	066960-6	3*x*	11 (4)	25 (3)	7	11
LAA	066960-8	3*x*	12 (4)	24	4	6
LAA	066960-9	3*x*	12 (4)	24	4	8
LAA	066960-12	3*x*	12 (4)	24 (2)	6	8
LAA	066960-13	3*x*	12 (3)	24 (4)	7	9
LAA	066960-14	3*x*	12 (1)	24 (2)	3	5
LAA	066960-20	3*x*	11 (4)	25 (4)	8	11
LAA	066994-3	3*x*	13 (11)	23 (9)	20	49
LAA	066994-11	3*x*	12 (8)	24 (5)	13	23
LAA	066995-1	3*x*	12 (5)	24 (3)	8	8
LAA	066963-8	3*x*	12 (3)	24 (1)	4	4
A LA	044595-1	3*x*	10 (2)	26 (3)	5	5
A LA	044601-1	3*x*	12 (1)	24 (2)	3	3
A LA	044601-2	3*x*	10 (2)	26 (4)	6	6
A LA	044601-3	3*x* – 1	11	24 (1)	1	1
A LA	044601-4	3*x*	12 (1)	24 (1)	2	2
A LA	044601-5	3*x* – 1	11 (1)	24	1	1
A LA	044601-6	3*x*	12 (2)	24 (1)	3	3
A LA	044601-7	3*x*	12	24 (3)	3	3
A LA	044601-8	3*x* + 1	13 (2)	24 (1)	3	3
A LA	044638-1	3*x* – 1	11 (1)	24 (1)	2	2
A LA	044638-2	3*x*	10	26 (2)	2	2
A LA	044638-3	3*x*	10 (1)	26 (3)	4	4
LAA	044511-1	2*x*	3 (1)	21 (2)	3	3
LAA	044538-1	2*x*	7 (7)	17 (4)	11	23
LAA	044538-3	2*x*	4 (2)	20 (4)	6	10
LAA	044538-4	2*x*	5 (2)	19 (5)	7	14
LAA	062074-2	2*x*	5 (2)	19 (3)	5	6
LAA	066828-5	2*x*	4 (3)	20 (3)	6	8
LAA	066960-2	2*x*	9 (5)	15	5	5
LAA	066960-5	2*x*	9 (4)	15 (2)	6	9

Table 1 (*concluded*).

Cross	Genotype code	Ploidy level	Genome composition		No. of recombinant chromosomes	No. of recombination sites
			L (L/A)	A (A/L)		
LAA	066960-15	2*x* + 2	9 (3)	17	3	3
LAA	066960-17	2*x*	9 (4)	15 (1)	5	5
LAA	066963-2	2*x*	9 (6)	15 (3)	9	12
LAA	066966-2	2*x*	6 (3)	18 (3)	6	10
A LA	044602-2	2*x*	1 (1)	23 (2)	3	7
A LAA*	044529-2	2*x*	1 (1)	23	1	1
A LAA*	044530-1	2*x*	0	24 (1)	1	1
LAA A*	044634-1	2*x* + 1	1	24 (2)	2	2
ALA LA*	066836-13	5*x*	18 (5)	24 (4)	9	13
ALA LA*	066836-45	5*x* – 4	20 (5)	36 (1)	6	7
L ALA LA†	044501-1	3*x* + 1	12	25 (2)	2	2
L ALA LA†	044501-2	3*x*	12	24 (6)	6	6
A ALA LA†	044506-4	3*x*	12	24 (3)	3	4
A ALA LA†	044507-2	3*x*	12	24 (4)	4	7
A ALA LA†	044507-5	3*x*	12	24 (4)	4	11
A ALA LA†	044507-6	3*x*	12	24 (4)	4	5

Note: L (L/A), chromosome having a Longiflorum centromere with Asiatic recombination sites; A (A/L), chromosome having an Asiatic centromere with Longiflorum recombination sites.

*BC_2 LA hybrids.

†BC_3 LA hybrids.

2000). The centromere of each chromosome was taken as the starting point and recombination sites identified by GISH were used as markers for recombination mapping. Recombination sites were identified and their distances from the centromeres were measured and expressed as a percentage of the arm length (both short arm and long arm). After compiling the recombination data, the recombination distribution was determined on each chromosome based on its length in relation to the size of the whole genome in micrometres. To determine the number of expected recombination sites (based on random distribution) on each chromosome, the length of that chromosome was measured, divided by the total length of the genome, and multiplied by the total number of observed recombination sites in the whole genome. The calculated expected values were compared with the observed ones for each chromosome.

Statistical analysis

We applied a χ^2 test to check whether the number of observed recombination sites significantly deviates from the expected number of recombination sites among the chromosomes. Furthermore, by considering the distances between the centromeres and the recombination sites as ''fragments'', their distribution in the four cytological maps was tested against the expected distribution to verify whether the distribution of these recombination sites is exponential (Haldane 1919).

Results

To construct cytological maps, as a first step, the genome composition and recombinant chromosomes were identified in 71 BC progenies of LA hybrids and 41 BC progenies of OA hybrids. Subsequently, the frequencies and distribution of recombination sites in different chromosomes of three genomes were determined.

Genome constitution and recombinant chromosomes

In the BC_1 progenies of LA hybrids, there were triploid as well as diploid plants together with a few aneuploids (Table 1). Among triploids, 35 were derived from the LAA type of crosses and 12 from the reciprocal crosses (i.e., ALA). In both cases, the chromosome constitution was predominantly 12 Longiflorum and 24 Asiatic (Table 1). This was consistent with the expectation that either the 2*n* egg or the 2*n* pollen from the LA hybrid had contributed 12 L + 12 A chromosomes to the BC_1 progenies. There were, however, some deviations from the expected numbers that resulted from indeterminate meiotic nuclear restitution during meiosis (Lim et al. 2001*b*). The recombinant chromosomes could be clearly distinguished from the nonrecombinant chromosomes and there were two distinct types. Chromosomes with a Longiflorum centromere possessing an Asiatic recombinant segment were indicated as L/A, whereas chromosomes with an Asiatic centromere possessing a Longiflorum recombinant segment were indicated as A/L (Figs. 1*a* and 1*b*; Table 1). The number of these two types of recombinant chromosomes varied in different BC_1 genotypes, and the total ranged from 1 to 20 (Table 1). This variation was expected to occur in view of the disturbed homoeologous chromosome pairing during meiosis in the LA hybrids, which showed near absence to complete pairing, forming 12 bivalents (Lim et al. 2001*b*; Zhou 2007). A notable feature was that the 2*n* gametes had transmitted two parental genomes of the LA hybrid to the BC_1 progeny and the number of recombinant chromosomes that occurred was a direct reflection of the amount of crossovers that had occurred during the meiotic division that preceded restitution nucleus formation.

In the case of 14 diploid and two near-diploid BC_1 progenies (Table 1), the number of recombinant chromosomes also varied among different genotypes (Table 1; Fig. 1*b*) but the range was narrower (3–11) as compared with their

Table 2. Genome composition, recombinant chromosomes, and recombination sites in the BC progenies of OA hybrids.

Cross	Genotype code	Ploidy level	Genome composition		No. of recombinant chromosomes	No. of recombination sites
			O(O/A)	A(A/O)		
AOA	022538-1	3*x*	12 (4)	24 (3)	7	9
AOA	022538-3	3*x*	12 (4)	24 (2)	6	7
AOA	022538-7	3*x*	13 (3)	23 (3)	6	12
AOA	022538-8	3*x*	12 (2)	24 (2)	4	11
AOA	022538-9	3*x*	13 (2)	23 (2)	4	8
AOA	022538-15	3*x*	11 (1)	25 (3)	4	5
AOA	022538-16	3*x*	12 (5)	24 (4)	9	18
AOA	022538-17	3*x*	12 (1)	24 (1)	2	2
AOA	022605-2	3*x*	12 (1)	24 (1)	2	5
AOA	022605-3	4*x*	12 (4)	36 (3)	7	9
AOA	022605-5	3*x*	12 (1)	24 (1)	2	5
AOA	022605-8	3*x*	12 (2)	24 (1)	3	3
AOA	022605-9	3*x*	12 (4)	24 (3)	7	8
AOA	022605-10	4*x*	12	36	0	0
AOA	022605-11	3*x*	12 (1)	24 (1)	2	2
AOA	022605-12	3*x*	12 (1)	24 (1)	2	2
AOA	022605-18	3*x*	12 (2)	24 (2)	4	4
AOA	022605-20	3*x*	12 (4)	24 (2)	6	6
AOA	022605-21	3*x*	12 (4)	24 (2)	6	6
AOA	022605-22	3*x*	12 (1)	24 (1)	2	2
AOA	022605-24	3*x*	12 (3)	24 (2)	5	6
AOA	022605-25	3*x*	12 (3)	24 (2)	5	5
AOA	022605-27	3*x*	12 (3)	24 (2)	5	2
AOA	022605-28	3*x*	12 (1)	24	1	1
AOA	022605-30	3*x* + 1	12 (2)	25 (2)	4	8
AOA	022605-34	3*x*	12 (2)	24 (2)	2	4
AOA	022605-37	3*x*	12 (1)	24	1	1
AOA	022605-38	3*x*	12 (1)	24 (1)	2	2
AOA	022605-39	3*x*	12 (2)	24 (2)	4	3
AOA	022605-40	3*x*	12 (2)	24 (2)	4	4
A OA	022605-46	3*x*	12 (3)	24 (3)	6	8
AOA A*	042616-2	2*x* + 5	5 (1)	24 (2)	3	5
AOA A*	042620-3	3*x* – 1	11 (1)	24 (1)	2	2
AOA A*	042620-14	3*x* – 1	11 (2)	24 (1)	3	4
AOA A*	042627-1	2*x* + 1	1	24 (1)	1	1
AOA A*	042627-2	2*x* + 8	8 (1)	24 (1)	2	2
AOA A*	042627-3	2*x* + 7	7 (1)	24 (1)	2	2
AOA A*	042627-4	2*x* + 6	6 (1)	24 (1)	2	2
AOA A*	042627-5	2*x* + 5	5 (1)	24 (2)	3	4
AOA A*	042627-6	2*x* + 5	5 (1)	24 (2)	3	4
AOA A*	042627-7	2*x* + 8	8 (2)	24 (2)	4	4

Note: O (O/A), chromosome having an Oriental centromere with Asiatic recombination sites; A (A/O), chromosome having an Asiatic centromere with Oriental recombination sites.

*BC_2 OA hybrids.

triploid counterparts. This could be explained by the fact that when normal *n* (haploid) gametes arise due to normal meiosis, both chromosome and chromatid segregations occurred. As a result, the number of recombinant and nonrecombinant chromatids segregated randomly to four haploid spores and, therefore, the number of recombinant chromatids in each *n* gamete was expected to range only from 0 to 12. On the other hand, in restitutional meiosis leading to 2*n* gametes (and triploid BC_1 progenies), only chromatid segregation had occurred (chromosome segregation at anaphase I was avoided) and a large proportion of recombinant chromatids were included in one or the other of the two spores that resulted from each cell. Thus, 2*n* gametes were able to transmit more recombinant chromosomes to the progenies as compared with *n* gametes.

The 31 BC_1 progenies of OA hybrids (Table 2) were triploid with two exceptions. One was an aneuploid (3*x* + 1) and the other a tetraploid, which originated from 2*n* gametes of both parents. Nevertheless, the OA hybrid had contributed 12 O + 12 A chromosomes to the progeny in all cases (Table 2). The number of recombinant chromosomes varied from 0 to 9 among different genotypes (Table 2). Chromo-

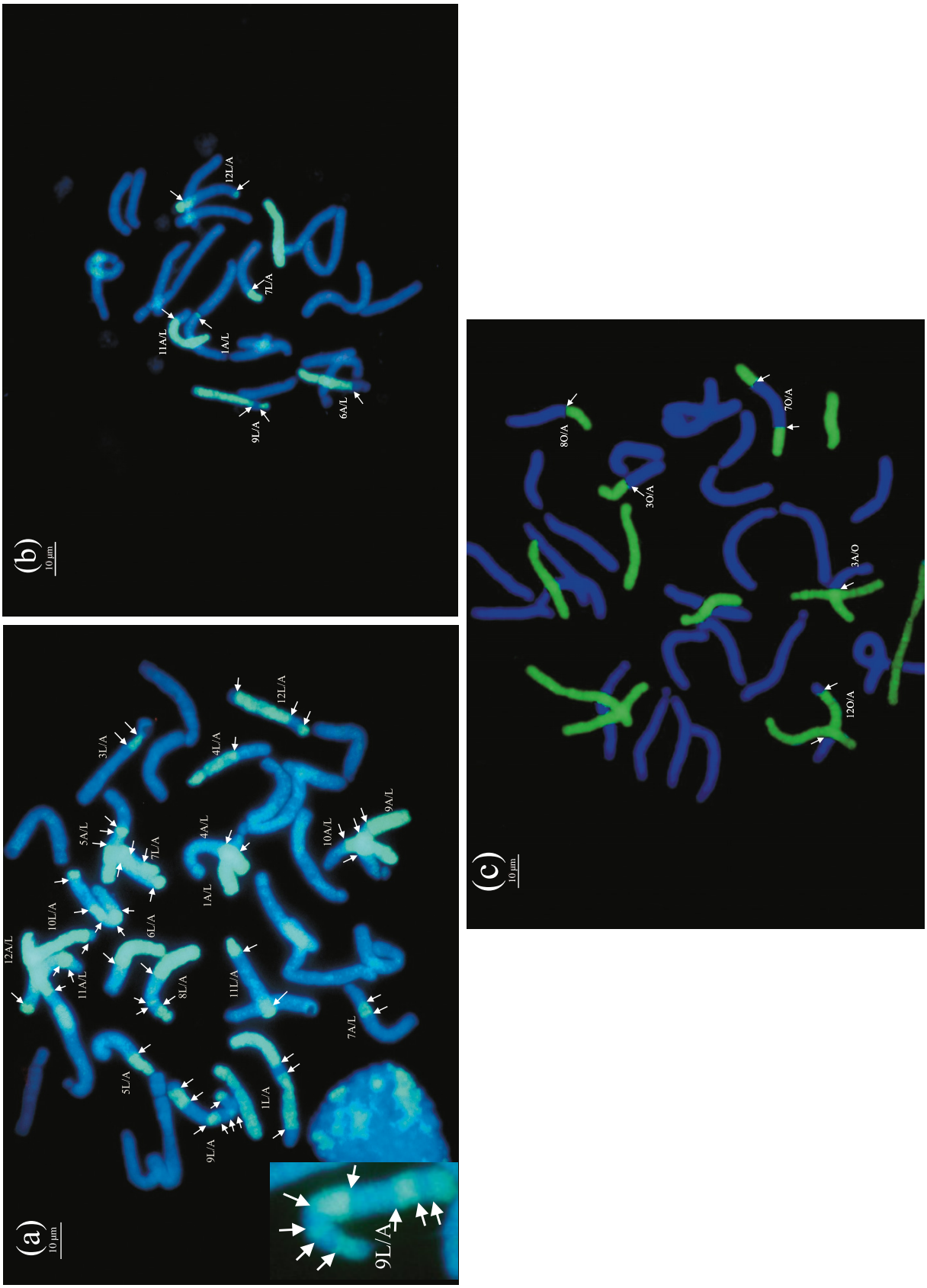


Fig. 1. (*a–c*) Somatic metaphase chromosomes of BC_1 progenies of LA and OA hybrids showing recombination sites on different chromosomes after GISH (green represents the L and O genomes and blue represents the A genome). (*a*) A triploid ($2n = 3x = 36$) BC_1 progeny of an LA hybrid (LAA, 066994-3) with 49 recombination sites (arrows). Inset: a recombinant chromosome showing eight recombination sites in a BC_1 LA hybrid (062071-2). (*b*) A diploid ($2n = 2x = 24$) BC_1 progeny of an LA hybrid (LAA, 066828-5) with eight recombination sites (arrows). (*c*) A triploid ($2n = 3x = 36$) BC_1 progeny of an OA hybrid (AOA, 022605-24) showing seven recombination sites (arrows).

somes with an Oriental centromere with a recombinant segment from an Asiatic were indicated as O/A and those with an Asiatic centromere with a recombinant segment from an Oriental as A/O (Table 2; Fig. 1*c*). As compared with the number of recombinant chromosomes in the triploid progenies of LA hybrids (maximum of 20), there were fewer recombinant chromosomes (maximum of 9) in the case of BC_1 progenies of OA hybrids. This implied that in LA hybrids, certain genotypes had much higher levels of homoeologous chromosome pairing and recombination as compared with those of OA hybrids (Barba-Gonzalez et al. 2005*b*). Some additional recombination sites have been found in both BC_2 and BC_3 progenies of LA and OA hybrids. As in BC_1 progenies, a higher rate of recombination sites was found in BC_2 and BC3 progenies of LA hybrids with 2–13 recombination sites per genotype as compared with BC_2 progenies of OA hybrids with 1–5 recombination sites per genotype (Tables 1 and 2).

Recombination sites and their distribution

One of the advantages of GISH analysis was that a direct and reliable assessment of the recombination sites on individual chromosomes could be made in the BC progenies of both LA and OA hybrids. The nonrecombinant and recombinant chromosomes of both genomes could be identified unambiguously and the number of recombination sites on individual chromosomes could be counted (Fig. 1). The number of recombination sites on individual chromosomes varied from one to eight (Fig. 1*a*, inset). The total number of recombination sites per genotype also varied from 1 to 49 in LAA and ALA and from 1 to 18 in AOA (Tables 1 and 2). The identification of two types of recombinant chromosomes and the recombination sites in the progenies of each of the hybrids, i.e., L/A, A/L in LAA or ALA, and O/A, A/O in AOA progenies, enabled a simultaneous mapping of recombination sites on all 12 individual chromosomes of the constituent genomes of both LA and OA hybrids. A total of 248 recombination sites were mapped on the L and A genomes and a total of 116 recombination sites were mapped on the O and A genomes. From the analysis of two hybrids, four maps were constructed; as the Asiatic parent was common to both hybrids, this resulted in two maps: Asiatic (L) and Asiatic (O). The remaining two were Longiflorum (A) and Oriental (A). For the construction of maps, the distances (micrometres) between the centromere and recombination site were expressed as percentages of the total length of the respective chromosome arms (Fig. 2). In some cases, two or more recombination sites on individual chromosomes mapped very close to each other and these were indicated by forked bars in the maps.

A remarkable feature was that the recombination sites were unevenly distributed on different chromosomes in all genomes (Table 3). In general, the number of recombination sites was not proportional to the size of the chromosomes. For example, the two largest chromosomes (chromosomes 1 and 2) possessed hardly any sites in both Asiatic (O) and Oriental (A) maps (Figs. 2*c* and 2d). A similar tendency, although not as pronounced, was also evident in the case of Asiatic (L) and Longiflorum (A) maps (Figs. 2*a* and 2*b*). In contrast, large numbers of recombination sites were concentrated on the long arms of chromosomes 7, 8, 9, 10, 11, and 12 in Longiflorum (A) and Asiatic (L) genomes. Although a similar concentration of recombination sites was evident in the case of Oriental (A) and Asiatic (O) genomes, the trend was less pronounced. It should be pointed out, however, that the numbers of recombination sites mapped in the latter (OA) are less than half of those mapped in the case of the progenies of LA hybrids. In general, very few recombination sites were found on the short arms of Oriental (A) and Asiatic (O) genomes but in the case of Longiflorum (A) and Asiatic (L) genomes sites were present on the short arms in several cases. Besides the uneven distribution of recombination sites among chromosomes, there were large gaps or even total absence of recombination sites in some cases, e.g., chromosome 6 of Asiatic (O) (Fig. 2*d*).

The distribution of recombination sites on all 12 chromosomes of four cytological maps (Longiflorum (A), Asiatic (L), Oriental (A) and Asiatic (O)) of three different genomes showed significant deviation from the expected number of recombination sites per chromosome at $P = 0.025$, <0.001, and 0.005, respectively. The contribution of individual chromosomes has been estimated and those chromosomes that contributed much to the χ^2 test are indicated in bold type (Table 3).

To statistically verify whether the distribution of recombination sites on different chromosomes significantly deviated from expectation, the distances between the centromere and the recombination site in each case was considered as a "fragment" in the statistical analysis. If it is assumed that recombination sites occur along the chromosome according to a Poisson distribution (Haldane 1919), then the distribution of the fragment sizes should be exponential. So we fitted an exponential distribution from these fragment sizes and then tested the empirical distribution of the fragment sizes against the expected exponential distribution at 95% confidence limits. There were clear deviations in all four maps (Fig. 3).

Discussion

The cytological maps constructed in the present investigation show that the entire genomes of lilies can be mapped through GISH. Inevitably, the BC progenies from distant hybrids could be used for mapping without which the constituent genomes and the recombinant sites could not be distinguished through GISH. Thus, it should be recognized that these maps are constructed from progenies derived from distant species hybrids, such as LA and OA, which show a failure of normal chromosome pairing during meiosis. Therefore, the question arises whether these maps are comparable with those that are constructed from the progeny analyses of intraspecific hybrids that are normally used in other plant species for mapping. In the case of BC_1 progenies of LA hybrids, there is convincing evidence that they have originated from the functioning of both *n* and 2*n* gametes following normal chromosome pairing in the spore mother cells at least in some cases. This is evident from the occurrence of a maximum of up to 20 recombinant chromosomes and 49 recombination sites in a genotype (Fig. 1*a*; Table 1) and indicates normal levels of chromosome pairing in some of the LA hybrids that contributed 2*n* gametes. Based on the argument that each recombination site repre-

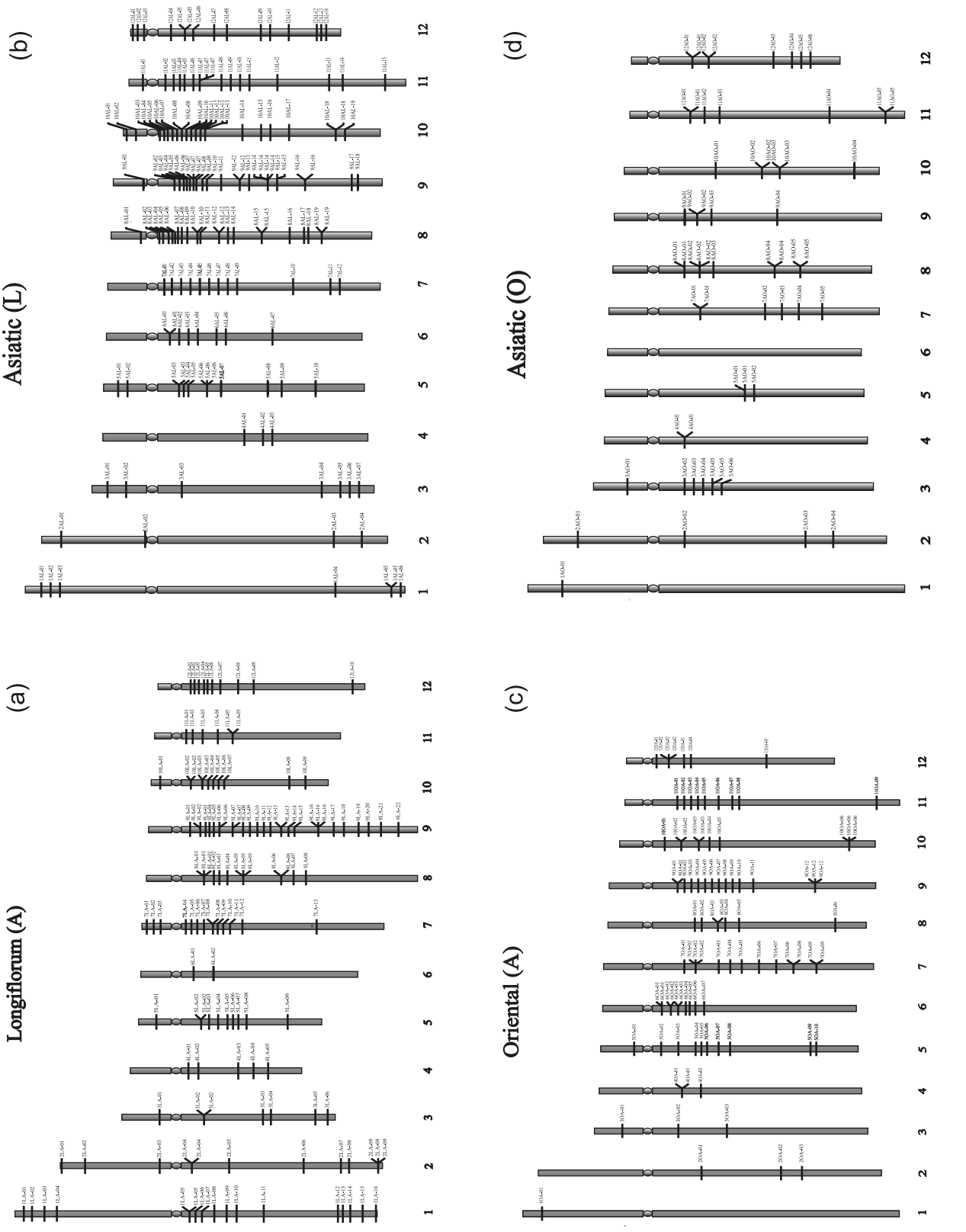


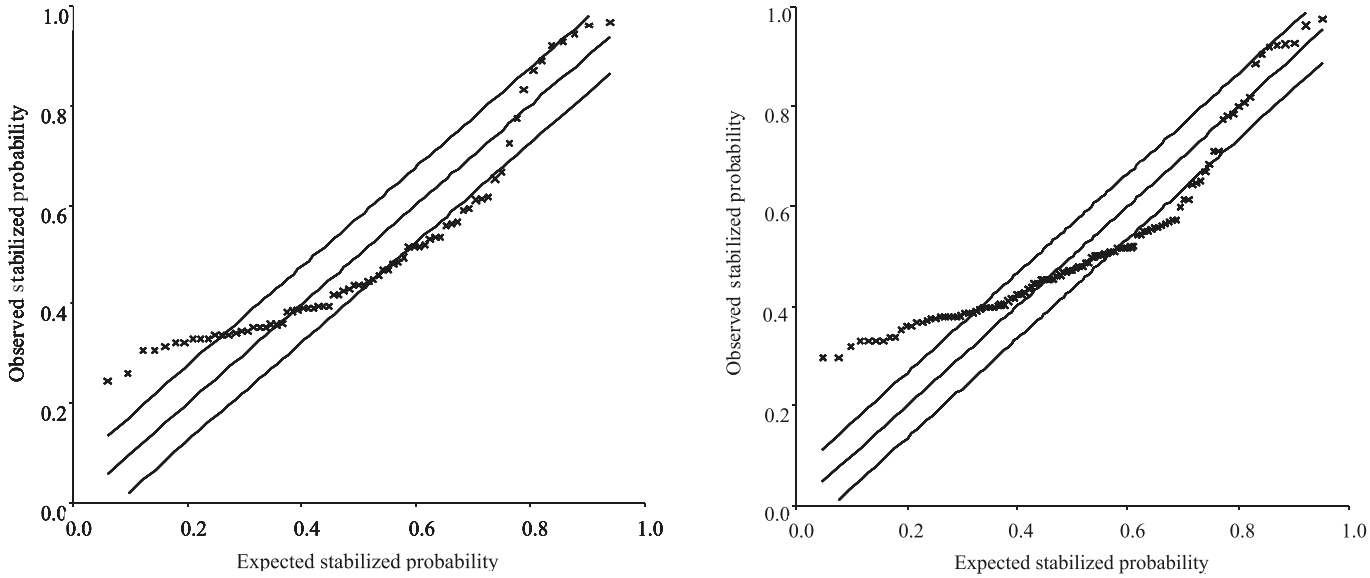
Fig. 2. Four chromosomal recombination maps resulting from the analysis of BC progenies of LA and OA hybrids. The genomes are (a) Longiflorum (A), (b) Asiatic (L), (c) Oriental (A), and (d) Asiatic (O); the recombination partner in each is given in parentheses. Note that recombination sites are asymmetrically distributed among chromosomes in all cases.

Table 3. Chromosome length, expected and observed recombination sites per chromosome, and their distribution in three genomes of *Lilium*.

Chromosome No.	Total length of chromosome (μm)	Distance to centromere (μm)		No. of expected breaking points	No. of observed breaking points	% of expected values	$(O - E)^2/E$
		Long arm	Short arm				
L/A							
1	36.76	20.6	16.15	14.81	16	108.03	0.09
2	33.08	21.58	11.5	13.38	9	67.27	1.43
3	21.2	15.64	5.26	8.39	6	71.51	0.68
4	18.25	13.9	4.33	7.22	5	69.25	0.68
5	18.56	15.16	3.4	7.34	9	68.11	0.37
6	21.5	18.26	3.25	8.51	2	23.50	**4.98**
7	24	21	3	9.5	13	136.84	1.28
8	27.06	24.41	2.65	10.71	8	74.69	0.68
9	26.75	24.4	2.36	10.58	22	198.48	**12.32**
10	17.2	15.16	2.03	6.8	9	132.35	0.71
11	18.2	16.41	1.78	7.2	5	69.44	0.67
12	20.36	19	1.36	8.05	10	111.80	0.47
							24.40*
A/L							
1	33.5	22.5	11	15.45	6	38.83	**5.78**
2	30.43	20.83	9.6	13.93	4	28.71	**7.07**
3	24.66	19.66	5	11.9	7	58.82	2.01
4	23.09	19.06	4.03	10.57	3	28.38	**5.42**
5	22.79	18.83	3.96	10.43	10	94.6	0.01
6	22.33	18.6	3.73	10.22	7	68.5	1.01
7	23.75	20.25	3.5	10.87	12	110.4	0.11
8	22.71	19.41	3.3	10.4	19	182.7	**7.11**
9	23.41	20.41	3	10.71	18	168.06	4.96
10	22.45	20.25	2.2	10.27	19	185	**7.42**
11	24.26	22.53	1.73	11.1	15	135.13	1.37
12	18.19	16.66	1.53	8.32	14	168.27	3.87
							46.14*
O/A							
1	39.86	26.8	13.06	8.36	1	11.96	**6.47**
2	36.59	25.23	11.36	7.69	3	39.01	2.86
3	28.76	23.46	5.3	6.05	3	49.58	1.53
4	27.46	22.7	4.76	5.77	2	34.66	2.46
5	26.96	22.33	4.63	5.67	10	176.36	3.30
6	26.56	22.16	4.4	5.58	7	123.45	0.36
7	28.33	24.03	4.3	5.96	9	151.00	1.55
8	27.06	23.26	3.8	5.69	6	105.44	0.01
9	27.83	24.23	3.6	5.85	12	205.12	**6.46**
10	26.84	24.21	2.63	5.64	6	106.38	0.02
11	29.22	27.26	1.96	6.14	9	146.57	1.33
12	21.51	19.75	1.76	4.52	5	110.61	0.05
							26.44*
A/O							
1	33.5	22.5	11	4.8	1	25	**3.00**
2	30.43	20.83	9.6	4.36	4	91.75	0.02
3	24.66	19.66	5	3.53	6	181.26	1.72
4	23.09	19.06	4.03	3.31	1	30.2	1.61
5	22.79	18.83	3.96	3.36	2	59.52	0.10
6	22.33	18.6	3.73	3.2	0	0	**3.2**
7	23.75	20.25	3.5	3.4	5	156.25	0.63
8	22.71	19.41	3.3	3.25	5	153.84	0.94
9	23.41	20.41	3	3.35	4	119.4	0.12
10	22.45	20.25	2.2	3.22	4	124.22	0.18
11	24.26	22.53	1.73	3.48	5	143.67	0.66
12	18.19	16.66	1.53	2.61	6	229.8	**4.40**
							16.22

Note: L/A, Longiflorum chromosome with Asiatic recombination sites; A/L, Asiatic chromosome with Longiflorum recombination sites; O/A, Oriental chromosome with Asiatic recombination sites; A/O, Asiatic chromosome with Oriental recombination sites. *Contribute significantly at $P = 0.025$, <0.001, and 0.005. Chromosomes that contributed much to the χ^2 test are indicated in bold type.

Fig. 3. Q–Q plots for the exponential stabilized distribution of "fragments" (distance between the centromere and the recombination sites) for observed and expected stabilized probabilities at 95% confidence limits among different chromosomes of two genomes: (*a*) Longiflorum (A) and (*b*) Oriental (A).



sents a chiasma that was formed in the spore mother cell, one should expect that 49 recombination sites in the genotype 066994-3 (Table 1) were the result of nearly as many chiasmata that had occurred in the megaspore mother cell. A comparable number of chiasmata were counted in the pollen mother cells of some six *Lilium* species and four hybrid derivatives (Fogwill 1958). However, it should be mentioned that the present cytological maps only indicate the pattern of recombination in the hybrids of species and provide an insight into the nature of differentiation.

There are some notable advantages of using 2*n* gametes rather than *n* gametes for mapping of recombination sites. (*i*) In most cases, distant hybrids do not produce *n* gametes because of abnormal meiosis, as in the case for OA hybrids, and the only method of mapping in such cases is through the use of 2*n* gametes. (*ii*) A large number of recombinant chromosomes and recombination sites can be recovered in the BC progenies when 2*n* gametes are used instead of *n* gametes. This is because almost always, (only) chromatid segregation occurs during meiosis that leads to restitution gametes. As a result, the probability of inclusion of crossover chromatids in each of the two unreduced spores that result from such meiosis is increased. On the contrary, when *n* gametes originate through normal meiosis, both chromosome and chromatid segregation occur and the four chromatids of each tetrad (of a bivalent) are distributed to four haploid spores. The differences in the number of recombinant chromosomes and recombination sites observed in the case of triploid and diploid progenies of LA hybrids (Table 1) clearly illustrate the efficiency of 2*n* gametes. (*iii*) Whereas *n* gametes are more likely subjected to selection during the process of fertilization or subsequent development, 2*n* gametes might be less susceptible for selection because they include both parental genomes nearly intact and are genetically more homogeneous.

Various methods have been used for the construction of cytological, cytogenetic, or cytomolecular maps (Khrustaleva et al. 2005). Maps constructed by using recombination sites have three important advantages: (*i*) they serve as permanent cytological landmarks on chromosomes that can be used for mapping molecular markers or genes of interest because of vegetative propagation in lilies, (*ii*) they mark crossing-over sites in the genomes, and (*iii*) they give a clear picture of whole-genome structure rather than concentrating on only one or two chromosomes that might not be representative of the whole genome.

The numbers of crossovers on different chromosomes are disproportionately distributed on all four maps. The largest chromosomes have very few recombination sites (e.g., chromosomes 1 and 2; Figs. 2*c* and 2*d*), whereas smaller chromosomes possess far more sites as in chromosomes 10 (Fig. 2*a*) and 8 and 9 (Fig. 2*b*). With regard to the positions of recombination sites, there is no regular pattern that can be consistently assigned to any set of chromosomes. There are some instances of clustering of recombination sites in the proximal parts for a number of chromosomes, i.e., near the centromere of chromosomes 1, 7, 10, and 12 (Fig. 2*a*), 6 and 7 (Fig. 2*b*), 6 and 12 (Fig. 2*c*), and 8 (Fig. 2*d*). But a similar pattern is not present in other chromosomes. There are fewer instances of clustering of recombination sites in the distal regions of the Longiflorum (A) genome, i.e., chromosomes 1, 2, and 9 (Fig. 2*a*), and the Asiatic (L) genome, i.e., chromosome 1, 3, and 12 (Fig. 2*b*). The results are contrary to those for the wheat genome in which the recombination was low in proximal chromosomal regions and very high towards the distal ends (Werner et al. 1992; Akhunov et al. 2003). The present study reveals that there is an increase in recombination events just next to the centromere in most cases (Fig. 2). Previously, it was also found that recombination increases with relative distance from the centromere in various large as well as small genome crops such as maize (Lukaszewski and Curtis 1993; Anderson et al. 2004). Similar behavior has been found in rice (Wu et al. 2003; Kao et al. 2006). A common feature is that all of

these species have less recombination just next to the telomere. However, in barley, it was found that the extreme distal regions of several chromosome arms have areas of increased recombination also (Künzel et al. 2000). Our investigation shows a gradient of recombination from one chromosome to another within a genome and also from one part to another on the same chromosome. A detailed cytological analysis of lily species genomes shows that distal recombination is not a general rule in *Lilium*.

One important feature that is common to all four cytological maps is that there are large gaps where there are no recombination sites at all, e.g., chromosome 6 of Asiatic (O) (Fig. 2*d*). An explanation for the occurrence of such gaps has to be deferred until more data become available. The most likely explanation for such gaps is the occurrence of structural differences between homoeologous chromosomes, especially heterozygous paracentric inversions. Indeed, paracentric inversions do occur in *Lilium* (Brown and Zohary 1955). If a chiasma occurs in the inverted segment, it leads to a dicentric bridge that might eliminate the chromatids concerned. The large numbers of gaps that are observed in the maps do indicate that constituent genomes of the two hybrids might be heterozygous for many paracentric inversions. This, however, needs to be confirmed.

Reduced recombination has been found between Oriental (A) and Asiatic (O) cytological maps. This might be attributed to larger genome divergence between A and O as compared with the L and A genomes in *Lilium*. We therefore conclude that there is more recombination between genomes in L and A than between those in O and A. Furthermore, chromosome pairing and crossing-over are genetically controlled and are thus genotype dependent.

An attractive feature of the cytological maps is that large numbers of recombination sites become available as physical landmarks on individual chromosomes. Using such sites or landmarks, molecular markers such as AFLPs and RFLPs can be assigned to specific positions on individual chromosomes, as has been done in the case of a *Festuca–Lolium* substitution line (King et al. 2002*a*) and *A. cepa* × (*A. roylei* × *A. fistulosum*) interspecific crosses (Khrustaleva et al. 2005). Mapping of individual chromosomes in these two aforementioned cases shows that proper integration of molecular maps with respective chromosomes can only be accomplished when reliable cytological markers are available. Moreover, molecular maps reported for several crop plants have either under- or overestimated the map lengths depending on the type of markers used or the type of software used for the analysis of the data (King et al. 2002*b*). To overcome such pitfalls, cytological maps of genomes such as the ones reported here can pave the way for the construction of more meaningful integrated maps.

Acknowledgment

We are thankful to Dr. Chris Maliepaard for help with the statistics.

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