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AFLP* linkage group assignment to the chromosomes of *Allium cepa* L. via monosomic addition lines

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Abstract Two complete sets of *Allium fistulosum* L. – *A. cepa* monosomic addition lines (2n=2x+1=17) together with an AFLP linkage map based on a cross between *A. cepa* and *A. roylei* Stearn were used to re-evaluate the eight *A. cepa* linkage groups identified in the mapping study. The linkage groups could be assigned to individual, physical chromosomes. The low level of molecular homology between *A. cepa* and *A. fistulosum* enabled the identification of 186 amplified fragment length polymorphisms (AFLP™ markers) present in *A. cepa* and not in *A. fistulosum* with ten different primer combinations. With the monosomic addition lines the distribution of the markers over the eight chromosomes of *A. cepa* could be determined. Of these 186 AFLP markers 51 were absent in *A. roylei* and consequently used as markers in the mapping study (*A. cepa* × *A. roylei* cross). Therefore, these 51 AFLP markers could be used to assign the eight *A. cepa* linkage groups identified in the mapping study to physical chromosomes. Seven isozyme and three CAPS markers were also included. Two of the linkage groups had to be split because they included two sets of markers corresponding to different chromosomes. A total of 20 (approx. 10%) of the *A. cepa*-specific AFLP markers were amplified in more than one type of the monosomic addition lines, suggesting unlinked duplications. The co-dominant isozyme and CAPS markers were used to identify the correspondence of linkage groups originating from *A. cepa* or from *A. roylei*.

Key words *Allium cepa* · Monosomic addition lines · AFLP™ · CAPS · Isozymes

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Introduction

Mapping studies in onion have thus far been scarce. The reasons for this are the biennial nature of onion, severe inbreeding depression and huge genome size (33.5 pg DNA per 1 C genome). Only recently King et al. (1998) presented a low-density genetic map of restriction fragment length polymorphisms (RFLPs) based on an intraspecific onion cross showing that the genomic organization of onion is complex and involves duplicated loci. Van Heusden et al. (1999) presented a genetic map based on the segregation of 505 polymorphic amplified fragment length polymorphisms (AFLPs) in an F_2 family from the interspecific cross *A. roylei* × *A. cepa*. The linkage groups based on markers originating from *A. cepa* and those based on *A. roylei* markers were not integrated because of the small size of the mapping population and the dominant character of the AFLP marker. The genetic linkage map based on *A. cepa* markers consisted of eight linkage groups with 262 markers covering 694 of the expected 800 cM. The map based on *A. roylei* markers comprised 15 linkage groups with 243 markers and had a length of 626 cM.

Complete sets of alien monosomic addition lines are valuable tools in genetic studies of plant genome organization (McGrath et al. 1990; Singh 1993). In wheat, tomato and sugi alien monosomic addition lines and other aneuploid lines have been used to assign linkage groups

to chromosomes (Chetelat et al. 1998; Kam Morgan et al. 1989; Suyama et al. 1996). Other uses of monosomic addition lines have been in deletion mapping (Werner et al. 1992) and the transfer of important genes of wild species by means of translocation (e.g. Heijbroek et al. 1988; Jung et al. 1992).

For *Allium*, eight monosomic addition lines, representing the eight different chromosomes of shallot (*A. cepa* L. Aggregatum group) in an *A. fistulosum* background have been established (Shigyo et al. 1996, 1998). The Japanese bunching onion (*A. fistulosum* L.) was used as diploid background because this perennial species allows an easy vegetative maintenance and propagation of the monosomic addition lines. A number of genetic analyses resulted in 27 chromosome-specific genetic markers (ten isozyme, one 5 S rDNA, and 16 RAPD markers (Shigyo et al. 1995b, c, 1997b) and the chromosomal locations of genes related to flavonoid and anthocyanin production (Shigyo et al. 1997c). Effects of the extra chromosomes on morphology and fertility were also investigated (Shigyo et al. 1997a).

We have combined molecular marker and monosomic addition line technology in an investigation with the following objectives: (1) integration of the physical chromosomes and the genetic linkage map and the identification of discrepancies between the genetic linkage map and localization on the chromosomes; (2) estimation of the level of unlinked duplications; (3) identification of any correspondence of paternal and maternal linkage groups with co-dominant isozyme and cleaved amplified polymorphic sequence (CAPS) markers.

Materials and methods

Plant material

Two complete sets of *Allium fistulosum* – shallot monosomic addition lines were available (2n=2x+1=17; Shigyo et al. 1996). The two sets were independently obtained. The sets were analysed together with the parents: shallot (*A. cepa* L. Aggregatum group) and Japanese bunching onion (*A. fistulosum* L. cv. Kujyo). An F_2 mapping population, derived from an interspecific cross between onion *A. cepa* (Jumbo, common onion group) and *A. roylei* C502 (van Heusden et al. 1999), was employed. This mapping population consisted originally of 65 F_2 plants; 22 plants of this population could not be maintained vegetatively and only 43 plants were available for isozyme analysis. All 65 F_2 plants could be used for polymerase chain reaction (PCR) analyses because of the availability of DNA.

Chromosome and linkage group nomenclature

The system of chromosome nomenclature for *A. cepa* was according to De Vries et al. (1990). In a previous study (Shigyo et al. 1996), a capital letter "A" was written after the chromosome rank number of shallot. However, the "C" used for onion chromosomes can also be used for shallot chromosomes (De Vries et al. 1990). Therefore in this study, the capital letter "C" is used to characterize both shallot and onion chromosomes. In order to avoid confusion the *A. cepa* linkage groups C1-C8 in Van Heusden et al. (1999) have been renamed in this study as L1-L8 (L=linkage group).

Table 1 Primer sequences based on sequences of four different onion cDNA probes. The sequences had been submitted to the GenBank Sequence Database of the National Center for Biotechnology Information under the accession numbers AA451563, AA451533, AA451571 and L48164, respectively

cDNA clone	Primer set	Sequence (5´–3´)
AJK265	AJK265a	AACATCACTTCCCAGGAACG
	AJK265b	TTCCAAGAACGGGGTCA
API73-2	API73-2a	CAGCATATGCATCAGTTTCACA
	API73-2b	TACTGCAAAAAGCATTTGGCT
AOB262	AOB262a	CCCTGACTGGTAAAACCATCA
	AOB262b	AGAACCAGGTGGAGGGTTG
alliinase	ALLa	TGGGTACTCCAATAGCCAGTG
	ALLb	TGCAACCTTCGGAGAACAG

DNA isolation

Total genomic DNA was isolated from frozen leaf tissue with the mini prep DNA-isolation method described by van Heusden et al. (1999).

AFLP analysis

AFLP analyses were performed using a modification of the protocol of Vos et al. (1995) as described by Van Heusden et al. (1999). Because of the huge genome of onion an extra selective basepair was added to the *Mse*I primer. Of the 13 *Eco*RI–*Mse*I primer combinations used in that study the 10 most informative were used to find chromosome specific markers in this study. AFLP fragments present in shallot, but not in *A. fistulosum*, were used for chromosomal assignment by identifying monosomic addition lines possessing that fragment. AFLP fragments present in shallot and onion, but not in *A. fistulosum* and *A. roylei*, were used for the alignment of chromosomes with the linkage groups established in the *A. cepa* × *A. roylei* F_2 population. Fragment sizes were calculated in number of nucleotides with a basepair ladder (SequaMark™).

CAPS markers

On the basis of the sequences of three onion cDNA clones (AJK265F, API73–2, AOB262F; King et al. 1998) and alliinase (Gilpin et al. 1995) primer sets were designed (Table 1). The sequences had been submitted to the GenBank Sequence Database of the National Center for Biotechnology Information under the accession numbers AA451563, AA451533, AA451571 and L48164, respectively. Primers were chosen based on their T_m (60°C) and length of the fragment to be amplified (400–600 bp). PCR amplification was done with 25 ng template DNA, 37.5 ng of each of the primers, 0.4 m*M* dNTPs, 1×super*Taq* buffer and 0.5 U of super*Taq* (SphaeroQ, The Netherlands) in a volume of 25 µl. Template DNA was initially denatured at 92°C for 5 min, followed by 30 cycles of PCR amplification (a 1-min denaturation at 92°C, a 1-min annealing at 60°C and a 2-min primer extension at 72°C) and a final 10-min incubation at 72°C. PCR products were separated on 1.5% agarose gels. If the PCR products were monomorphic they were digested with up to 31 restriction enzymes in order to find polymorphisms.

Isozyme analysis

A total of 11 enzyme systems were studied. Phosphoglucomutase (PGM, EC 5.4.2.2) and shikimate dehydrogenase (SKDH, EC 1.1.1.25) were used in addition to 9 previously reported enzyme systems (Shigyo et al. 1996). For the 2 new enzymes, the extrac-

tion and electrophoresis conditions were as described in Shigyo et al. (1995c). Staining conditions were according to Vallejos (1983). Segregation in the F_2 plants was assessed for those enzymes with a clearly detectable polymorphism between the parents of the mapping population.

Marker segregation and linkage analysis

The isozyme and CAPS marker segregation data were added to the AFLP marker segregation data (Van Heusden et al. 1999), and linkage maps were generated using JoinMap version 2.0 (Stam and van Ooijen 1995). A critical LOD value ≥2.5 was used to detect linkage between AFLPs and isozymes/CAPS.

Results

Chromosome-specific AFLP markers

The use of 10 *Eco*RI/*Mse*I primer combinations resulted in 186 chromosome-specific fragments that were distributed over all eight chromosomes (Table 2; Fig. 1). Each chromosome-specific AFLP fragment was only amplified in the two plants of the same monosomic addition line. Twenty AFLP fragments were amplified in more than one addition line and is most probably the result of duplicated regions or co-migration. The distribution of these duplications was random with two exceptions: for 5 out of the 9 putatively duplicated loci of which one copy was present on chromosome C2 there was another copy on C4; similarly, for 4 out of the 7 duplicated loci on C3 the other copy was on C6. These markers were not mapped and it was not possible to compare the order of the markers on the different linkage groups.

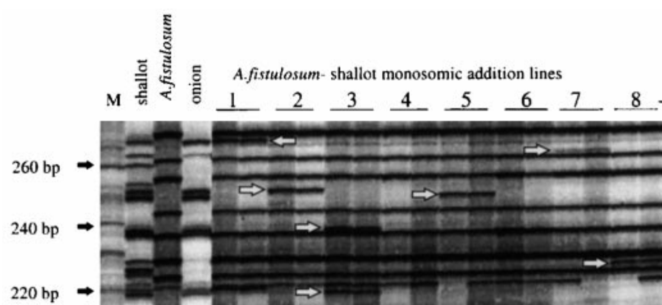

Fig. 1 Part of an AFLP pattern (220–260 bp) with primer combination E37M52 A. *M*=Molecular size marker, *lanes 1–8* the eight different monosomic addition lines of shallot in an *A. fistulosum* background, the *onion lane* shows the pattern of the the same onion plant used in the mapping population. *Arrows* indicate chromosome specific markers on chromosomes 1, 2, 3, 5, 7 and 8. The two lanes per monosomic addition line represent two different plants

Table 2 The number of chromosome-specific AFLP markers per shallot chromosome

Primer combination	Chromosome							
	C1	C2	C3	C4	C5	C6	C7	C8
E35M52A	0	2	3	6	5	2	2	0
E35M52C	2	2	1	1	5	1	1	3
E35M52T	2	3	4	4	3	1	4	1
E36M52A	1	3	3	6	3	4	4	2
E36M52C	1	3	3	1	3	3	2	4
E36M52G	5	3	1	3	1	4	1	1
E37M52A	2	6	2	2	4	0	4	1
E38M52A	5	0	2	2	2	0	3	1
E38M52G	1	0	1	1	0	1	0	3
E38M52T	3	4	3	4	1	1	3	1
Total	22	26	23	30	27	17	24	17

Chromosomal assignment of AFLP linkage groups using *A. fistulosum* – shallot monosomic addition lines

The onion and shallot accessions used in this study were genetically not very different. More than 90% of their AFLP fragments are equally sized in both accessions. This allows the assumption that most of the equally sized fragments must be homologous and there is no reason to assume that a large portion of them originates from accidently co-migrating fragments.

Of the 186 shallot chromosome-specific AFLP markers 51 had already been mapped (Van Heusden et al. 1999) assuming the homology of equally sized fragments. These markers were not present in the *A. roylei* parent. The 51 markers were distributed over all eight linkage groups, thereby allowing the assignment of these groups to chromosomes. In two cases there was a discrepancy between the results of the linkage analysis and the results of the screening with the monosomic addition lines (Fig. 2). Linkage group L1 consisted of two groups of markers: the first 20 markers span a region of 85 cM on the original linkage map and correspond to physical chromosome C6, and the second 37 AFLP markers (93 cM) correspond to chromosome C5. Three markers (E35M52T-4, P43M47–1 and P43M47–2) were the reason that the two groups were considered as one in the mapping study. These 3 markers had significant LOD scores with markers of both groups; e.g. P43M47–1 was linked to 3 markers of the first group and with 2 markers of the second group, all with LODs higher than 3.0. Linkage group L6 also contains markers belonging to two different chromosomes, the upper part (14 markers) corresponds with chromosome C2 and the lower part (9 markers) corresponds to chromosome C8. These sets of markers were not significantly linked to the other linkage groups (L4 and L8) known to correspond with chromosomes C2 and C8, respectively.

Isozyme and CAPS analysis

Two types of co-dominant markers were used in this study, cleaved amplified polymorphic sequences (CAPS) markers and isozymes. Based on the sequence of several RFLP probes (King et al. 1998) primers for CAPS markers were made (Table 1). Amplification with the primer

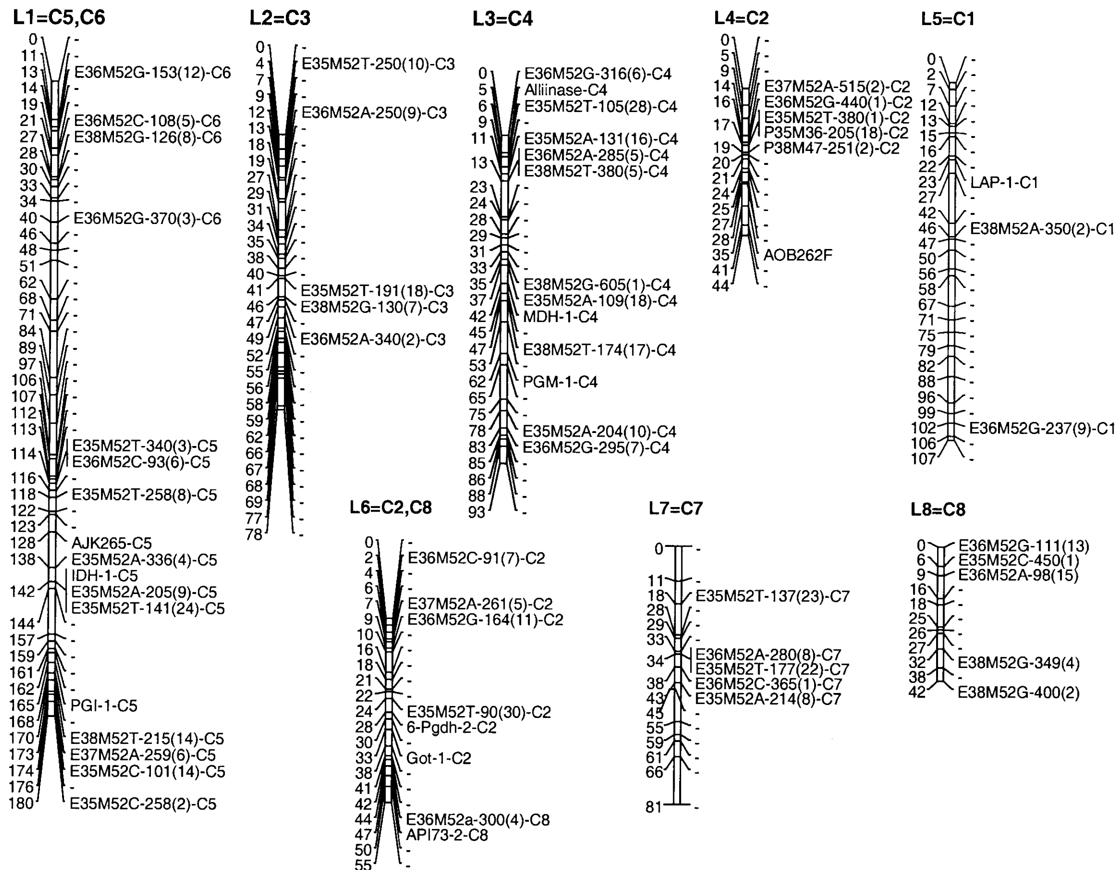

Fig. 2 Linkage map of *A. cepa* based on the AFLP linkage data from an interspecific cross between *A. cepa* and *A. roylei* (Van Heusden et al. 1999) and 11 new markers. Map distances were slightly changed due the incorporation of extra markers. The *number after* each AFLP marker gives the length of the fragment in base pairs, the *number between brackets* refers to the nomenclature as used previously. The other marker names are not shown, but a hyphen indicates that 1 or more markers were mapped at that position. *L1–L8* represent the eight linkage groups; *C1 behind* a marker name indicates that this marker was only present in the monosomic addition with chromosome C1. The linkage groups L1–L8 were assigned to physical chromosomes C1–C8, e.g. L2=C3

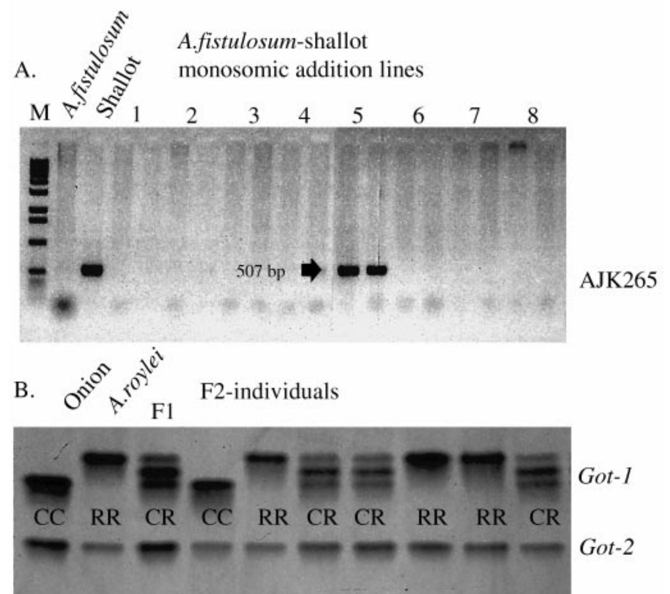

Fig. 3 **A** Amplification profiles for AJK265 in *A. fistulosum*, shallot, and the sets of *A. fistulosum* – shallot monosomic addition lines. *Lanes 1–8* the eight different monosomic addition lines of shallot in an *A. fistulosum* background, *M*=molecular size marker. **B** Zymograms of *Got-1* in part of the F_2 mapping population. *CC* is homozygous *A. cepa, CR* heterozygous *A. roylei*, *RR* homozygous *A. roylei*

sets of AJK265, API73–2 and alliinase amplified a product with shallot DNA but not with *A. fistulosum* DNA. The chromosomal locations of these markers could be determined with the *A. fistulosum* – shallot monosomic addition lines. The shallot-specific fragment of AJK265 was only amplified in both monosomic addition lines with chromosome C5 of shallot (Fig. 3). Similarly API-73 was assigned to chromosome C8 and the alliinase gene to chromosome C4. With the primers of AOB262, products were amplified both in shallot and *A. fistulosum.* Even after digestion with 31 restriction enzymes no polymorphisms were detected. Therefore this marker could not be assigned to a chromosome. Amplification with the primer sets of AJK265, API73–2 and AOB262 gave equally sized products in the parents of the mapping population. Differences between the parents could be visualized after digestion with *Hpa*II. These

Table 3 Correspondence of paternal and maternal linkage groups based on analyses with co-dominant isozyme and CAPS markers

Marker	Chromosome	*A. cepa* map[a]		*A. roylei* map[a]	
Lap-1	C1[b]	L5	(5.9)	R8	(2.8)
Got-1	C2[b]	L6	(3.5)	R1	(4.7)
6-Pgdh-2	C2[b]	L6	(5.4)	R1	(3.0)
AOB262	C2	L4	(13.2)	R1	(11.2)
Mdh-1	C4[b]	L3	(9.1)	R5	(4.3)
Pgm-1	C4	L3	(5.3)	R5	(2.6)
Idh-1	C5[b]	L1	(8.6)	R4	(4.1)
Pgi-1	C5[b]	L1	(6.2)	R3	(6.5)
AJK265	C5	L1	(9.9)	R4	(9.6)
API73-2	C8	L6	(8.7)	R12	(8.5)

[a] The chromosomal location of the marker. Data of isozyme markers are from Shigyo et al. 1996
[b] In parenthesis, the highest LOD score of the co-dominant marker with an AFLP marker of the *A. cepa*. resp. *A. roylei* map. Data on the AFLP markers are from Van Heusden et al. (1999)

differences allowed the use of these three markers as co-dominant markers. Therefore, these three CAPS markers function as a link between the paternal and maternal linkage groups (Table 3). With the primer set of the alliinase gene a product was amplified only with DNA of the *A. cepa* parent. Therefore this marker was of no use.

Polymorphisms between *A. cepa* and *A. roylei* were found for 7 isozyme gene loci. Shigyo et al. (1995a, b, 1996) had determined the chromosomal locations of 6 of these 7 isozyme gene loci in previous studies: *Lap*-1 on C1, *Got*-1 and *6-Pgdh-2* on C2, *Mdh*-1 on C4 and *Idh*-1 and *Pgi*-1 on C5. The chromosomal location of *Pgm*-1 was not known because there were no differences in isozyme patterns between shallot and *A. fistulosum.*

The segregation data collected for the 7 isozyme loci and the three CAPS-based markers were analysed with data from the eight maternal and 15 paternal linkage groups (originating from *A. cepa* and *A. roylei*, respectively). The chromosomal locations are shown in Table 3 and Fig. 2. The combination of chromosome-specific AFLP markers and segregation of *Pgm*-1 showed that this isozyme locus was located on chromosome C4. In a similar way it was possible to assign the RFLP probe AOB262 to chromosome C2. Due to the low number of co-dominant markers it was possible to find corresponding *A. cepa* and *A. roylei* linkage groups for only 6 out of the 15 *A. roylei* linkage groups already constructed by Van Heusden et al. (1999) (Table 3).

Discussion

Asssigning linkage groups to chromosomes

The onion and shallot accessions used in this study are genetically not very different. More than 90% of their AFLP fragments are equally sized in both accessions. This allows the assumption that most of the equally sized fragments must be homologous, and there is no reason to assume that a large portion of them originates from accidently co-migrating fragments. The availability of a complete set of *A. fistulosum* – shallot monosomic addition lines and the low level of molecular homology between *A. fistulosum* and shallot allowed the detection of 186 chromosome-specific AFLP markers. Many of these markers were also found in the onion parent (*A. cepa*) and not in the *A. roylei* parent of a mapping population used to construct an interspecific *Allium* AFLP map (van Heusden et al. 1999) Fifty-one of the chromosome-specific markers were part of the *A. cepa* genetic linkage map. These 51 markers with their known chromosomal positions, determined with the set of monosomic addition lines, were used to find any correspondence between the linkage groups and the physical chromosomes. This made a re-evaluation of the linkage map possible. Two discrepancies were found, two of the eight linkage groups consisted of two groups of markers from different chromosomes. So actually there should have been ten linkage groups, including two linkage groups each for chromosomes C2 and C8. Skewed segregation of several linkage groups, a small population size and the use of dominant markers cause seemingly significant linkage between markers, thereby resulting in the joining of linkage groups that are in fact unlinked. The assumption that there is a very low level of co-migrating fragments between onion and shallot was confirmed by the results. Co-migrating fragments can only give problems in assigning linkage groups to physical chromosomes if they originate from different chromosomes. This would result in 1 or more markers within a group of markers being assigned to other chromosome(s) than the majority of the markers in that group. This has never been detected. (Fig. 2). The availability of a complete set of monosomic addition lines therefore increases substantially the reliability of our *Allium* linkage map. In future *Allium* mapping studies monosomic addition lines will be incorporated, showing directly for many markers whether they belong to the same chromosome/linkage group or not. In several cases only 1 AFLP primer combination was sufficient to find chromosome-specific markers for all eight chromosomes (Table 2).

Integration of paternal and maternal linkage groups

Co-dominant isozyme and CAPS markers were incorporated in this map to find the correspondence of paternal and maternal linkage groups in the interspecific AFLP linkage map and to compare the results of AFLP data with other molecular marker data (CAPs and isozymes). The co-dominant markers made it possible to assign a number of paternal linkage groups (markers originating from *A. roylei*) to the maternal linkage groups (markers originating from *A. cepa*). Due to the lack of a sufficient number of co-dominant markers it was not possible to assign all 15 maternal linkage groups to the eight paternal linkage groups and, consequently, to the eight physical chromosomes. No discrepancies between the chromosomal location of AFLP linkage groups and known

chromosomal locations of the 8 co-dominant markers were detected (Fig. 3). The development of microsatellite markers in onion (Fischer and Bachmann 1998) and the availability of a large number of the sequences of onion cDNA probes used in the mapping study of King et al. (1998) will yield more co-dominant markers that can be used to assign all maternal linkage groups to chromosomes.

Duplicated regions

Of a total of 206 shallot-specific AFLP fragments 10% were amplified in more than one monosomic addition line. This is in agreement with results based on RFLP-analysis by King et al. (1998), who found a level of duplicated RFLP loci of 21%, of which 53% were unlinked (>30 cM). Amplification in more than one addition line can be caused by duplicated regions but can also be the result of non-linked co-migrating fragments. However, the latter is not likely because on average there were only 18 *A. cepa*-specific AFLP markers per primer combination, and such a low number minimizes the chance of co-migrating fragments. Indications were obtained for duplicated regions on chromosomes 2 and 4 (5 AFLP fragments were amplified on these two types of monosomic addition lines) and on chromosomes 3 and 6 (4 AFLP fragments were amplified on these two types of monosomic addition lines).

Future research

Markers based on the cDNA probes used in the mapping study of King et al. (1998) make it possible to look for correspondence between RFLP linkage groups, AFLP linkage groups and chromosomes. However, caution is necessary because due to the high level of duplications it is possible that RFLP markers and CAPS markers based on the same cDNA probe reveal polymorphisms at different loci on different chromosomes. We will analyse as many markers as possible per linkage group. The combination of both AFLP and RFLP linkage maps and the availability of a complete set of monosomic addition lines will result in an integrated *Allium* chromosome map which will be of great value for future research in *Allium.*

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