Chromosome Res (2011) 19:493–506
DOI 10.1007/s10577-011-9202-y

Comparative FISH mapping of *Daucus* species (Apiaceae family)

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Received: 7 December 2010 / Revised: 10 March 2011 / Accepted: 13 March 2011 / Published online: 6 May 2011
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Abstract The cytogenetic characterization of the carrot genome (*Daucus carota* L., 2*n*=18) has been limited so far, partly because of its somatic chromosome morphology and scant of chromosome markers. Here, we integrate the carrot linkage groups with pachytene chromosomes by fluorescent in situ hybridization (FISH) mapping genetically anchored bacterial artificial chromosomes (BACs). We isolated a satellite repeat from the centromeric regions of the carrot chromosomes, which facilitated the study of the pachytene-based karyotype and demonstrated that heterochromatic domains were mainly confined to the pericentromeric regions of each chromosome. Chromosome-specific BACs were used to: (1) physically locate genetically unanchored DNA sequences, (2) reveal relationships between genetic and physical distances, and (3) address chromosome evolution in *Daucus*. Most carrot BACs generated distinct FISH signals in 22-chromosome *Daucus* species, providing evidence for syntenic chromosome segments and rearrangements among them. These results provide a foundation for further cytogenetic characterization and chromosome evolution studies in *Daucus*.

Responsible Editor: Pat Heslop-Harrison.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no. HM565961.

Electronic supplementary material The online version of this article (doi:10.1007/s10577-011-9202-y) contains supplementary material, which is available to authorized users.

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Keywords Carrot · wild *Daucus* species · integration map · comparative FISH mapping · chromosome evolution

Abbreviations

BAC	Bacterial artificial chromosome
DAPI	4′,6-Diamidino-2-phenylindole
FISH	Fluorescent in situ hybridization
LG	Linkage group
STS	Sequence-tagged site marker
SSR	Simple sequence repeats
SCAR	Sequence-characterized amplified region

Introduction

The cultivated carrot (*Daucus carota* subsp. *sativus*) is the most economically important and extensively studied species of the Apiaceae family. It ranks among the top ten vegetable crops in the world (Rubatzky et al. 1999; Simon et al. 2008) and is an excellent source of dietary provitamin A carotenoids (Simon 2000). Carrot is a diploid outcrossing species like the majority of the approximately 20 *Daucus* species. However, carrot, *Daucus capillifolius*, *Daucus sahariensis*, and *Daucus syrticus* are the only members of the genus with $2n=18$ as most *Daucus* species are $2n=20$ or 22. The haploid genome size of carrot has been estimated at 473 Mbp (Arumuganathan and Earle 1991), which is similar to rice.

Several projects have been initiated to enhance genetic resources for carrot. In the last two decades, several carrot linkage maps have been developed, based on different types of mostly dominant markers (Simon et al. 2008). This effort was recently complemented by construction and end sequence evaluation of a deep-coverage bacterial artificial chromosome (BAC) library (Cavagnaro et al. 2009) and expressed sequence tags (EST) database development underway (Bowman et al. 2010). Despite these advances in the molecular analysis of the carrot genome, very limited work has been done on the cytological characterization of the carrot genome. Somatic chromosome identification in carrot has been hampered by their small sizes and similar morphology (Iovene et al. 2008a), and carrot chromosomes and linkage maps are not integrated.

The development of cytogenetic maps using a BAC fluorescent in situ hybridization (FISH) approach is a well-established procedure in plant species with small to medium genome sizes. These maps have played an important role in genome sequencing projects of several model and crop species and in comparative mapping studies (Jiang and Gill 2006; Figueroa and Bass 2010).

In this report, we describe a standard carrot karyotype and assign the carrot linkage groups to specific pachytene chromosomes by FISH mapping of 17 map-anchored BACs. We identified a tandem repeat that hybridized to the centromeric regions of all carrot chromosomes. Comparative FISH mapping of carrot BACs to two $2n=22$ different *Daucus* species (one from Europe and one from the Americas) established a framework of chromosomal synteny that provides insights into chromosome evolution in *Daucus* species with varying chromosome numbers.

Materials and methods

Plant materials and chromosome preparation

Seeds of *D. carota* L. var. *sativus* male-fertile inbred lines B2566 and B493 were germinated in Petri dishes to obtain root tips for mitotic chromosomes preparation, as previously described (Dong et al. 2000; Iovene et al. 2008a). Vernalized plants were also grown to flowering in the greenhouse to obtain meiotic samples, and immature umbels were fixed in 3:1 Carnoy's solution. In preliminary experiments, slides were prepared by squashing the whole flower bud. However, this resulted in poor preparations with relatively few pollen mother cells dispersed among many floral somatic cells. For this reason, the anthers were isolated under a dissecting microscope and digested in an enzyme mixture as previously described (Iovene et al. 2008b). After digestion, three to five anthers were transferred to a glass slide, macerated in a drop of 3:1 methanol/acetic acid solution with fine-pointed forceps and finally "flame-dried" over an alcohol flame. For comparative FISH mapping, mitotic chromosomes of the Mediterranean *Daucus crinitus* ($2n=2x=22$; Ames 26417) and the American *Daucus pusillus* ($2n=2x=22$; PI 511864; Sáenz Laín 1981) were prepared as previously described (Iovene et al. 2008a).

BAC library screening, probes, and FISH

Most BAC clones used for FISH mapping were identified by screening two carrot BAC libraries (Cavagnaro et al. 2009) with mapped sequences using a PCR-based approach (Table 1). A total of 17 mapped markers were used for screening. Of these, 15 markers were included in the carrot reference linkage map, constructed previously using an F_2 population derived from a cross between the dark orange inbred B493 and a wild white-rooted carrot Queen Anne's Lace (QAL) (Santos and Simon 2002, 2004; Grzebelus et al. 2007; Just et al. 2007; Cavagnaro et al. 2009, 2010). These markers were distributed across all nine linkage groups (Grzebelus et al. 2007; Just et al. 2007; Cavagnaro et al. 2009, 2010) and included eight STS markers (based on putative carotenoid biosynthetic gene sequences),

Table 1 Genetic and physical position of carrot BAC clones and rDNA on pachytene chromosomes of *Daucus carota* inbred line 2566B

Chr.	LG	BAC/plasmid	Marker/gene[b]	cM[c]	Genetic location%[d]		Physical location%[e]	*n*[f]
					Old	New		
1S	1	068M03[a]	GSSR-101	34.0	15	15	1.2±0.9	11
2L	4	095H14[a]	ZDS1	24.2	21	79	51.4±3.3	10
2L	4	012D08	Rs	0.0	0	100	95.6±0.7	6
3S	8	067N21	DCOR	71.8	69	31	20.1±2.4	8
3L	8	025M09[a]	GSSR-17	31.4	30	70	56.0±2.4	10
3L	8	032K14[a]	RFLP- DCG321	Dp	–	–	66.3±2.0	10
3L	8	238G06	PAL (from BES)	Nm	–	–	87.6±2.1	9
4S	6	pTa71	18S–25S rDNA	Nm	–	–	0.0±0.0	5
4L	6	019A16	IPI	Nm	–	–	36.9±3.0	8
4L	6	069N01	CRTISO	38.9	28	?	81.6±3.0	13
5S	2	A8N14[a]	PDS	142.8	100	0	0.3±0.6	11
5L	2	098O13	NCD2	110.4	77	23	43.5±3.6	6
5L	2	A6F01[a]	CHXE	102.8	72	28	46.8±3.0	11
6L	3	A9P11[a]	LCYB	57.5	52	?	82.6±3.1	11
7S	5	112G15	Random clone	Nm	–	–	2.3±0.9	5
7L	5	003E10[a]	ZEP	97.5	64	64	80.6±2.7	11
7L	5	004J17[a]	SCAR-Y2 mark	121.5	79	79	98.0±1.5	11
8S	9	064A04	GSSR107	76.0	48	52	11.5±1.5	7
8L	9	009K15	LCYE	72.0	45	55	30.3±4.7	4
8L	9	PTa794	5S rDNA	Nm	–	–	40.5±1.4	6
8L	9	002B20[a]	SCAR-Q1/800	Dp	–	–	64.5±3.1	11
8L	9	124B06	Inv Dc3 (from BES)	Nm	–	–	100±0.0	6
9S	7	113N07[a]	SSR-N6W93	63.8	44	?	33.7±3.7	11

IPI isopentenyl pyrophosphate isomerase, *Dp* mapped in a different population, *Nm* not mapped

[a] These clones were used in the probe cocktail in Fig. 4

[b] See CAVAGNARO et al. (2009) for a description of marker/gene. BES=BAC end sequence

[c] Map distances in cM refer to the B493×QAL population map (SANTOS and SIMON 2002; JUST et al. 2007; CAVAGNARO et al. 2010)

[d] Relative genetic position is calculated as (cMx/cMt)×100, where cMx is the cM value of a given marker on the linkage map and cMt is the total length (in cM) of the same linkage map. New relative genetic position is calculated considering the conventional north/south orientation of a linkage group according to the chromosome arm

[e] Relative physical position is calculated as $(S/T)\times 100$, where S=the distance (in μm) from the FISH site to the end of the short arm of the chromosome, and T=the total length of the chromosome (in μm).

[f] Number of measurements.

four simple sequence repeats (SSRs), one sequence-characterized amplified region (SCAR), and two PCR markers (for *Rs* and DCOR sequences; Table 1). In addition, two other markers from different carrot linkage maps were used to screen the BAC library and develop potential FISH probes. These markers were a SCAR (Q1/800) linked to the nematode resistance locus *Mj-1* (Boiteux et al. 2000), and an RFLP (DCG321) linked to a phenotypic locus conditioning purple root pigmentation (*P1*; Vivek and Simon 1999). Because their linkage was established in genetic background unrelated to the B493×QAL cross, and since no common markers among these linkage groups were available, the linkage relationships between these two markers and those previously mentioned were initially unknown. Finally, four BAC clones not genetically anchored to any

linkage map were also included. Of these non-anchored clones, BACs 124B06 and 238G06 were selected because their ends contained gene sequences (invertase and phenyl alanine ammonia-lyase) potentially associated with important carrot root traits (i.e., reduced sugar content and anthocyanin pigmentation, respectively); another clone (019A16) was identified in the library using the primers for isopentenyl pyrophosphate isomerase gene, which could not be mapped by Just et al. (2007) due to lack of polymorphism in the B493× QAL F2; and BAC clone 112G15 was randomly selected. Other DNA clones were pTa71 and pTa794 containing the 18S–25S rDNA unit and the 5S rDNA unit, respectively (Gerlach and Bedrock 1979; Gerlach and Dyer 1980). Overall, a total of 23 FISH probes were used for physical mapping in carrot, and—from these—a subset of 16 and 13 carrot BAC probes were used for comparative FISH mapping in *D. crinitus* and *D. pusillus*, respectively.

BAC DNA was isolated using the QIAGEN plasmid midi-kit and labeled with either biotin-16-UTP or digoxigenin-11-dUTP (Roche Diagnostic, Indianapolis, IN) by standard nick translation reaction. The FISH procedure was applied to both mitotic and meiotic chromosomes according to published protocols (Dong et al. 2000; Iovene et al. 2008b). Most BAC probes required small or moderate amounts of carrot-sheared genomic DNA in the hybridization mixture to reduce background signal (approximately a 20:1 ratio of sheared genomic DNA/BAC probe was used). A few BACs (for example, BAC 002B20, 009K15, and 098O13) required a larger amount of blocking DNA in the hybridization mixture (ratio, ~100:1) to produce a clear signal. To obtain more consistent measurements, high-quality pachytene chromosomes were used for repeated probing up to four times using the procedure described by Cheng et al. (2001a). Alternatively, FISH cocktails consisting of up to ten different BAC probes were applied. The carrot cot-1 DNA fraction was prepared according to Zwick et al. (1997) and used as a FISH probe along with BAC 004H08 (a clone containing a satellite repeat associated with all carrot centromeres; see below).

Biotin- and digoxigenin-labeled probes were detected by fluorescein isothiocyanate-conjugated anti-biotin antibody and rhodamine-conjugated anti-digoxigenin antibody (Roche Diagnostic), respectively. Chromosomes were counterstained by 4′, 6-diamidino-2-phenylindole (DAPI) in antifade solution Vector-Shield (Vector Laboratories, Burlingame, CA).

Cytological measurements and analysis

All images were captured using a SenSys CCD camera attached to an Olympus BX60 epifluorescence microscope, and the IPLab Spectrum v3.1 software (Signal Analytics, Vienna, VA) on a Macintosh computer. Final image adjustments were done with Adobe Photoshop software v6.0. Measurements were made on digital images using IPLab software. For karyotype description, all chromosomes in 24 pachytene cells were measured. The position of each BAC clone on a specific chromosome is given as a relative distance in percent, calculated as the distance of the FISH signal from the end of the short-arm relative to the total length (in micrometer) of the chromosome.

DNA sequencing and assembly

BAC 004H08 was sequenced by shotgun Sanger DNA sequencing essentially as described in Zhu et al. (2008) with the modifications of shotgun insert size range of 2–6 kbp and assembly with TIGR Assembler (Sutton et al. 1995). The low pass sequence resulted in 35 pieces and the Phase I BAC sequence is available in GenBank under the accession number HM565961.

Results and discussion

Identification of a satellite repeat associated with the centromeres of *D. carota* chromosomes

BAC clone 004H08 was initially identified by screening the library for the phytoene synthase 1 gene (*PSY1*; Cavagnaro et al. 2009). FISH analysis revealed that BAC 004H08 hybridized to the centromeric regions of all nine pairs of the carrot chromosomes and not elsewhere. The intensity of the FISH signals differed among chromosomes and overlapped with the FISH signals of the carrot cot-1 DNA fraction, suggesting that this BAC contains a dominant repeat associated with the centromere (Supplementary Fig. S1). The partial sequence of the BAC clone 004H08 revealed a tandem repeat with a typical length of ~159 bp, which we named Cent-Dc. This monomer is composed of a variable number of subrepeats,

typically four (Table 2, Supplementary File S1). These subrepeats vary slightly in sequence, each of

Table 2 The most frequent nucleotide motif of the 40 bp subrepeat of Cent-Dc based on approximately 345 subrepeat sequences

Position	Nucleotide	Frequency (%)
1	C	46.3
2	C	92.1
3	A	76.7
4	A	71.0
5	C	51.3
6	T	70.1
7	T	95.1
8	C	88.9
9	A	96.5
10	A	98.0
11	A	80.3
12	C	97.4
13	G	63.9
14	A	98.8
15	G	86.8
16	T	89.6
17	C	86.7
18	A	56.8
19	A	65.7
20	G	60.9
21	A	98.0
22	A	98.3
23	T	99.7
24	G	92.2
25	A	96.0
26	A	98.0
27	G	68.7
28	C	97.7
29	T	98.0
30	A	98.9
31	C	98.9
32	A	98.3
33	A	93.7
34	G	86.2
35	T	85.8
36	T	95.1
37	G	96.0
38	T	73.1
39	T	95.4
40	T	97.7

which has a period size of 39–40 bp. Cent-Dc has 91% similarity to DCRE20, a carrot-specific repeat element previously identified in the BAC end sequences of the same library (Cavagnaro et al. 2009). Considering a period size of 40 bp, these authors estimated the carrot genome to contain> 27,000 copies of DCRE20 (Cavagnaro et al. 2009).

A 1,816 bp subclone (named K11) containing approximately ten Cent-Dc repeats was labeled and hybridized to mitotic and meiotic chromosomes of carrot (Fig. 1a–c). At metaphase I, FISH signals were consistently detected in one discrete region only at the most poleward position of each bivalent chromosome, suggesting that Cent-Dc is located at the chromosomal region associated with the kinetochore complex (Fig. 1b). Centromeric repeats have been isolated in a number of plant species (Jiang et al. 2003). The Cent-Dc repeat has mononucleosomic size that is typical for previously characterized centromeric satellite repeats. The structure and the cytological location of Cent-Dc suggest this repeat is likely associated with the functional centromeres of carrot chromosomes. Therefore, this subclone was used to anchor the centromere position of the carrot pachytene chromosomes (see below, Fig. 1c).

A pachytene chromosome-based karyotype of carrot

Pachytene chromosome-based karyotypes are superior to those based on somatic metaphase chromosomes and have been developed in a number of species with small to medium size genomes (*Arabidopsis thaliana*: Fransz et al. 1998; cucumber: Koo et al. 2005; *Lotus japonicus*: Ohmido et al. 2010; *Medicago truncatula*: Kulikova et al. 2001; potato: Iovene et al. 2008b; Tang et al. 2009; rice: Cheng et al. 2001b; sorghum: Islam-Faridi et al. 2002; Kim et al. 2005).

The somatic chromosomes of carrot are only 2–4 μm in length and morphologically similar (Sharma and Ghosh 1954; Schrader et al. 2003; Iovene et al. 2008a), thus they are difficult to identify. We developed a pachytene chromosome-based karyotype of carrot from inbred line B2566. The chromosome length and arm ratio data were collected from all chromosomes in 24 pollen mother cells (Table 3). The pachytene chromosomes are ordered from 1 to 9 according to their descending length. On average, the length of the carrot pachytene complement is ~193±18 μm, which is only seven times longer than its mitotic

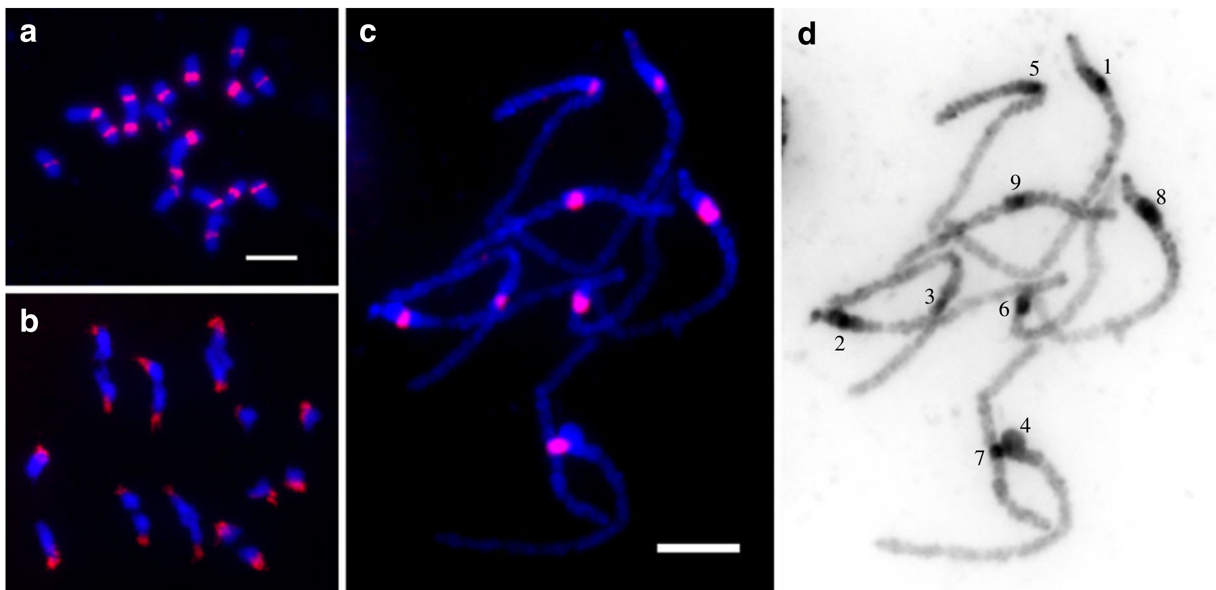

Fig. 1 FISH mapping of Cent-Dc repeat to the centromeric regions of all carrot chromosomes and distribution of euchromatin and heterochromatin in the carrot genome. **a** Carrot somatic chromosomes probed with the Cent-Dc repeat (*red signal*). **b** Cent-Dc detected on carrot chromosomes at anaphase I and **c** at pachythene. **d** DAPI-stained chromosomes in (**c**) are converted to a black-and-white image to enhance the visualization of distribution of euchromatin and heterochromatin along the pachytene chromosomes. Chromosomes are numbered according their decreasing length. *Bar scale*=5 μm

counterpart. In plant species with a wide range of genome sizes, including *Arabidopsis* (Fransz et al. 1998), cotton (Wang et al. 2009), cucumber (Koo et al. 2005), maize (Wang et al. 2006), *M. truncatula* (Kulikova et al. 2001), potato (Tang et al. 2009), and rice (Cheng et al. 2001b), the pachytene chromosomes are approximately 15–50 times longer than the somatic metaphase chromosomes. The relatively short length of the carrot pachytene chromosomes may decrease the spatial resolution of FISH mapping in this species compared with other plant taxa. However, preparation of super-stretched pachytene chromosomes as demonstrated in maize (Koo and Jiang 2009) may overcome this limitation.

Carrot pachytene chromosomes could be individually recognized based on chromosome length, arm ratio and the DAPI staining pattern. In general, heterochromatic regions represented a small frac-

Table 3 Karyotype analysis in the male-fertile carrot inbred line B2566: absolute length (μm), relative length (%), and arm ratio of the carrot pachytene chromosome and their corresponding linkage group

Chromosome no.	Linkage group	Total length (μm)	Short arm, μm	Long arm, μm	Relative chr. length,% [a]	Arm ratio L/S	*n*[b]
1	1	27.0±2.7	4.6±0.6	22.4±2.4	14.0±0.8	4.9±0.7	24
2	4	24.8±3.0	2.7±0.5	22.1±2.7	12.8±0.5	8.3±1.2	“
3	8	24.5±3.1	9.9±1.4	14.6±1.9	12.6±0.8	1.5±0.1	“
4	6	22.7±2.5	2.0±0.4	20.7±2.4	11.8±0.9	10.6 ±2.4	“
5	2	21.4±2.5	8.3±1.1	13.1±1.7	11.1±0.6	1.6±0.2	“
6	3	21.4±2.5	3.9±0.5	17.5±2.2	11.1±0.6	4.5±0.5	“
7	5	19.4±2.5	8.2±1.1	11.1±1.6	10.0±0.6	1.3±0.1	“
8	9	16.5±2.1	3.3±0.6	13.1±1.8	8.5±0.7	4.0±0.8	“
9	7	15.5±1.2	7.2±0.7	8.3±0.7	8.0±0.5	1.2±0.1	“

[a] Relative chromosome length is in percentage of the karyotype length.

[b] Number of measurements.

tion of all the chromosomes and were mainly located in the pericentric regions (Figs. 1d and 2). Centromeric regions of carrot pachytene chromosomes did not have obvious primary constrictions and distinct differential staining as has been observed in the pachytene chromosomes of tomato and maize (Barton 1950; Dempsey 1994). Thus, Cent-Dc was useful to highlight the centromeric regions of each pachytene chromosome (Fig. 1) allowing us to perform more accurate measurements. Overall, the arm ratios of the carrot pachytene chromosomes ranged from 1.2 to 10.6 (although for most chromosomes, it ranged from 1.2 to 4.9; Table 3) compared with ~1 to ~4 for mitotic chromosomes (Iovene et al. 2008a). This discrepancy may reflect an unequal condensation between the chromosome arms. Based on the position of the centromere, carrot pachytene chromosomes were divided into the following three groups:

A group of three chromosomes (1, 6, and 8) with subterminal centromeres: pairwise *t* tests indicated that chromosomes 1, 6, and 8 varied significantly in length ($P<0.01$). Thus, these chromosomes could be distinguished based on their length and DAPI staining pattern. In addition to being the longest chromosome, chromosome 1 had a small heterochromatic knob that was quite consistently detected on the long arm, at ~39% from the end of the short arm (based on 18 measurements out of the 24 pollen mother cells observed). This structure is potentially diagnostic to distinguish between chromosomes 1 and 6.

A group of two chromosomes (2 and 4) with terminal centromeres had similar lengths and were distinguishable based on the heterochromatin patterns on their short arms. The short arm of chromosome 2 is mostly heterochromatic (Fig. 2); whereas the short arm of chromosome 4 is occupied by the Nucleolar Organizer Region (NOR).

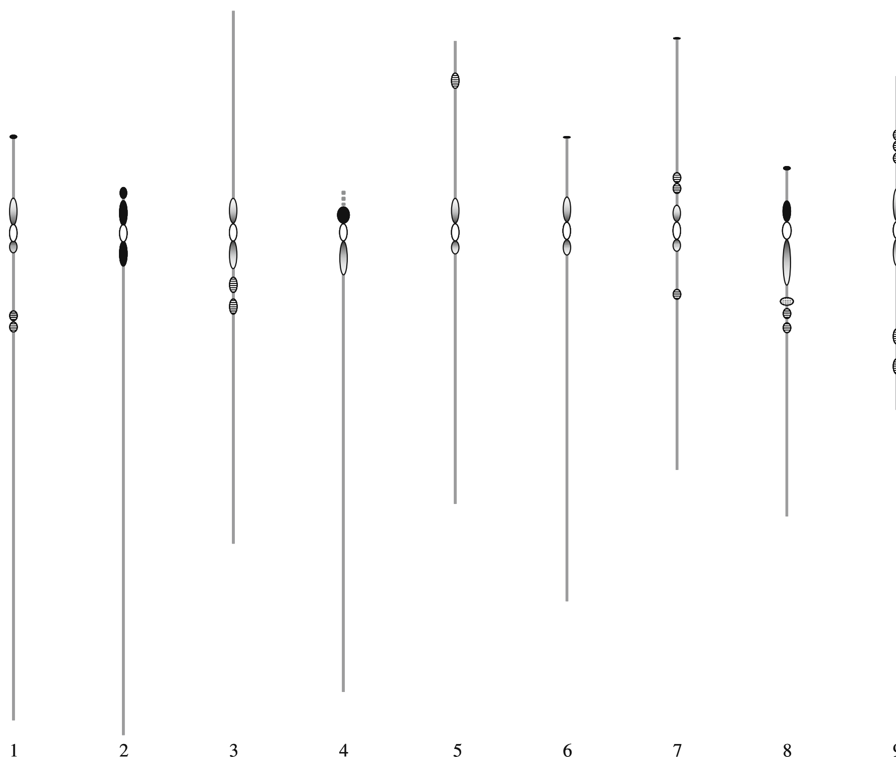

Fig. 2 Ideogram of the distribution of heterochromatin on the pachytene chromosomes of the carrot line B2566. *Solid/shaded* thickenings represent the heterochromatic regions. *Shaded* thickenings indicate regions that were less stained by DAPI than the regions marked by *solid* thickenings. *Hatched* thickenings indicate DAPI-stained regions that were not consistently observed in each pollen mother cell and/or were sometimes separated into multiple smaller regions. *Open circles* represent the location of the centromeres, based on the hybridization to Cent-Dc. The *dashed line* on the top of chromosome 4 represents the 18S–25S rDNA; the *dotted oval* on the long arm of chromosome 8 represents the 5S rDNA. The relative length of each chromosome and the arm ratio were drawn based on data presented in Table 3

A group of four chromosomes (3, 5, 7, and 9) with nearly median centromeres had significantly different lengths as indicated by *t* tests ($P<0.01$), allowing them to be distinguished on this basis.

Integration of linkage map with pachytene chromosomes in carrot

The assignment of linkage groups to specific chromosomes using a BAC-FISH approach is a well-established procedure in plant species with small and medium size genomes (Dong et al. 2000; Cheng et al. 2001b; Kulikova et al. 2001; Islam-Faridi et al. 2002; Kim et al. 2005; Zhang et al. 2005; Walling et al. 2006; Pedrosa-Harand et al. 2009; Tang et al. 2009; Fonsêca et al. 2010). Integration of linkage maps with pachytene chromosomes has been useful in BAC-by-BAC-based genome sequencing efforts, to validate contig order, resolve the order of tightly linked markers located in recombination-suppressed chromosomal domains, evaluate the size of regions with low map coverage or underrepresented in the BAC library, estimate the euchromatin-heterochromatin boundaries, characterize and integrate complex chromosomal regions, such as the pericentromeric regions, in the linkage maps (Jiang and Gill 2006; Figueroa and Bass 2010).

To integrate the carrot linkage maps with individual chromosomes, 15 marker-anchored BACs were hybridized to specific carrot pachytene chromosomes (Table 1, Fig. 3). The markers anchoring the BACs were mapped on the nine linkage groups of carrot with one to three markers for each linkage group. This enabled us to associate all linkage groups with chromosomes and to orientate six of the nine carrot linkage groups (with the exception of LG 3, LG 6, and LG 7, for which a single anchored BAC was used) according to the short/long arm of the corresponding chromosomes. Using the convention that the short chromosome arm is oriented north and the long arm south, the north/south orientation of the current carrot genetic linkage groups 1 and 5 match with the short/long arm; however, the orientation of linkage groups 2, 4, 8, and 9 should be reversed relative to the order of molecular markers published. Furthermore, all linkage groups should be renamed according to the chromosome numbering.

FISH mapping of additional DNA sequences

The developed chromosome-specific carrot BACs provided us a tool to localize additional DNA sequences that were either not mapped or mapped in genetic backgrounds unrelated to the B493×QAL population used to construct the reference linkage map (Santos and Simon 2002, 2004; Grzebelus et al. 2007; Just et al. 2007; Cavagnaro et al. 2010). Thus, we were able to assign chromosomal positions to the rDNA gene clusters, and to six BAC probes (four unanchored and

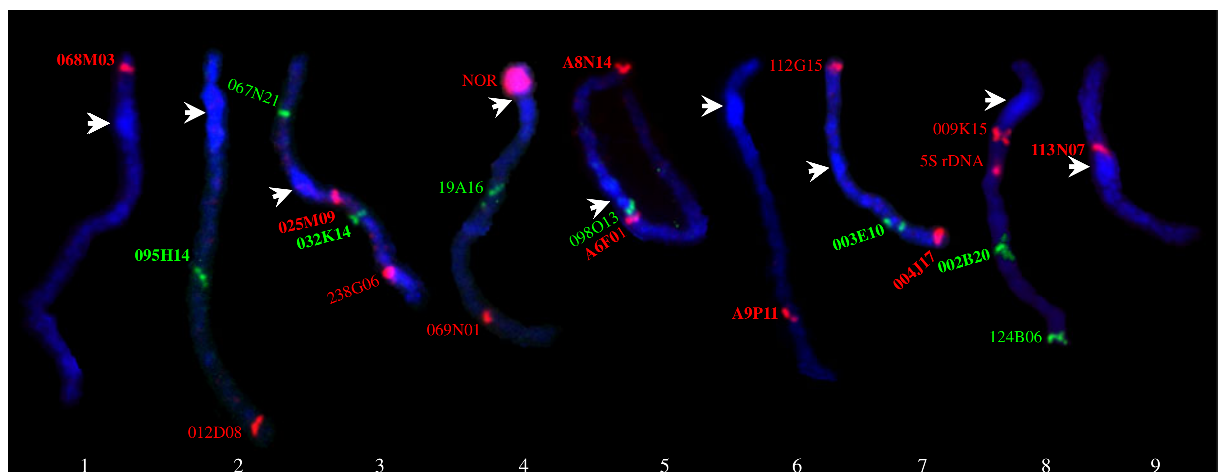

Fig. 3 A standard carrot karyotype using the chromosome-specific BACs (*red and green signals*) listed in Table 1. Only BAC clone 064A04 on the short arm of chromosome 8 is not shown in this image. BAC names are indicated for each chromosome. BAC names reported in bold were used in the probe cocktail in Fig. 4. *Arrows* indicate the centromeric regions based upon hybridization to Cent-Dc

two mapped in unrelated genetic backgrounds) relative to the chromosome-specific BACs described above (Table 1, Figs. 2 and 3).

For example, two BACs (BAC 002B20 and BAC 032K14) were selected using markers mapped in two different unrelated populations, thus the genetic relation between these and the previous markers was unknown. FISH analysis of these two clones revealed that BAC 002B20, harboring a SCAR marker linked to the nematode resistance locus *Mj-1* (Boiteux et al. 2000), is located on the long arm of chromosome 8 (LG 9), distal to BAC 009K15 and the 5S rDNA; whereas BAC 032K14, carrying a RFLP marker linked to *P1*, a phenotypic locus conditioning purple root pigmentation (Vivek and Simon 1999), is located on the long arm of chromosome 3 (LG 8) (Fig. 3). The same chromosome bears 067N21, a carrot BAC containing sequences homologous to the cauliflower *Or* gene (Lu et al. 2006; Cavagnaro et al. 2009).

Altogether, we have mapped 21 BACs on the nine carrot pachytene chromosomes. Using two-color FISH, a subset of these clones consisting of ten BACs hybridizing eight chromosomes provided a probe cocktail to reliably identify carrot pachytene chromosomes (Fig. 4).

Relationship between genetic and chromosomal distances in carrot

Cytological, genetic, and molecular studies have demonstrated that recombination events are not evenly distributed along the chromosomes (for a review, see Mézard 2006; Li et al. 2007), and significant discrepancies in relative genetic and physical distances have been reported in a number of plant species. Such discrepancies limit the use of linkage maps to guide genome sequence assembly or for map-based cloning. Pachytene FISH provides an efficient tool to visualize such disparities (Cheng et al. 2001a).

Although there were relatively few genetically anchored BACs placed on each chromosome, the available FISH data shed light on the relationship between genetic and physical distances on carrot chromosomes. First, all pairs of linked molecular markers were also physically linked (Table 1, Figs. 3 and 5, Supplementary Fig. S2). Second, the order of three genes on the map of linkage group 2 was consistent with the physical order of three corresponding BACs on chromosome 5. Third, comparing the ends of specific linkage groups to the ends of the corresponding chromosomes, all of the BACs used as anchor markers to map the end of a linkage group were indeed located at the chromosome ends (Table 1, Fig. 5, Supplementary Fig. S2), suggesting a good coverage for these chromosome arms. For example, BAC 012D08 was located ~4% away from the end of the long arm of chromosome 2 based on physical distance. This BAC clone was anchored at 0.0 cM on the map of LG 4 of the carrot inbred line B493 (Cavagnaro et al. 2010).

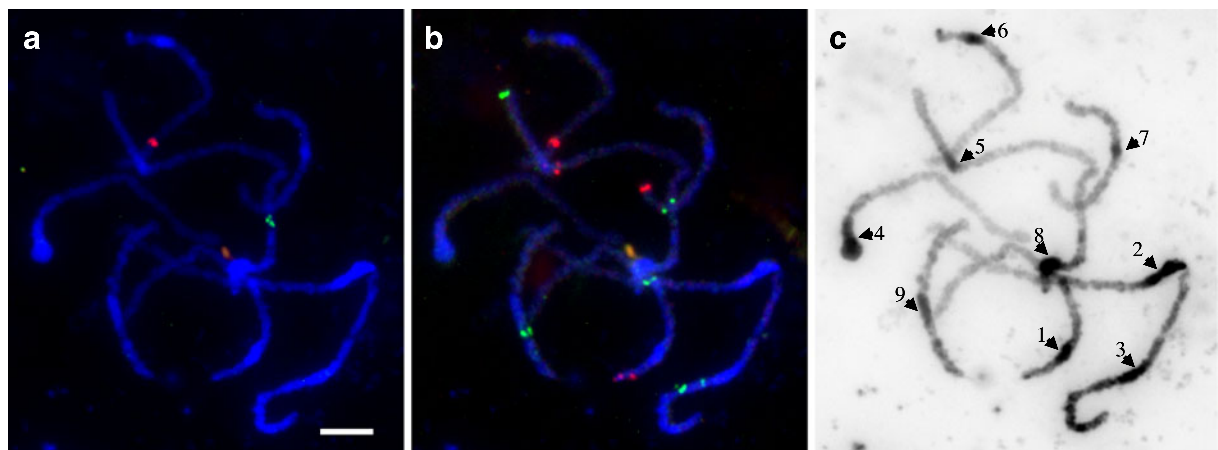

Fig. 4 Utilization of a probe cocktail consisting of ten chromosome-specific carrot BACs for straight forward chromosome identification in carrot. **a** Carrot pachytene chromosomes probed with two BACs specific to chromosomes 6 (*red signal*) and 8 (*green signal*). **b** The same preparation probed with a cocktail of eight BACs specific to six chromosomes. **c** The DAPI-stained chromosomes are converted to a black-and-white image. Arrows point to the centromeric region of each chromosome. *Bar scale*=5 μm

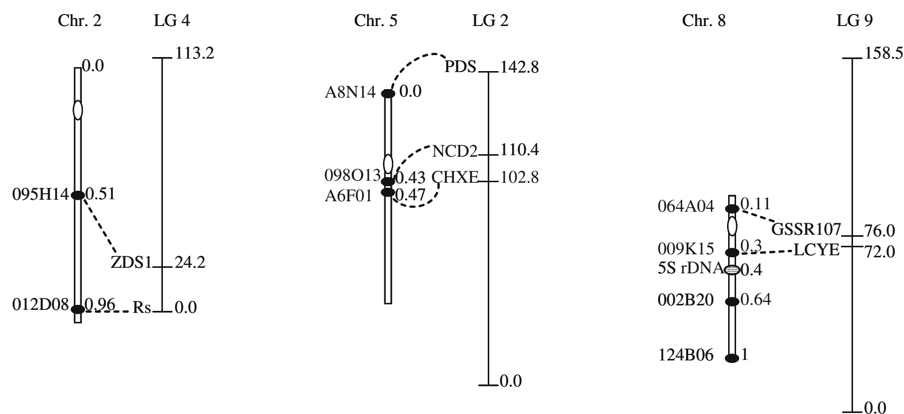

Fig. 5 Relationships between BAC-FISH signals on pachytene chromosomes 2, 5, and 8 and the corresponding genetic linkage groups 4, 2, and 9 in carrot. For each chromosome, BACs are placed according to their relative distance from the end of the short arm. Linkage groups and genetic distances (in cM) refers to the *D. carota* B493 map (Santos and Simon 2002; Just et al. 2007; Cavagnaro et al. 2010). Dashed lines connecting cytogenetic and genetic maps are drawn only between the map-anchored BAC clones and the corresponding genetic markers. Solid circles represent BAC clones, open circles represent centromeres. The orientation of the linkage groups was inverted to agree with the conventional north/south orientation of a linkage group according to the chromosome arm

Discrepancies between the genetic and physical distance of anchored BAC clones were observed from the available data set. For example, BAC clone 064A04 was physically detected ~11% away from the end of the short arm of chromosome 8 (corresponding to LG 9). The corresponding anchor marker, GSSR107 was mapped at 76 cM, which is a relative genetic distance from the end of the linkage group 9 of about 52%. The discrepancy indicated a genetic map distance five times longer than the physical map in this region. Another disproportion on chromosome 8 (LG 9) was detected between BACs 064A04 and 009K15, which are located close to flanking boundaries of the pericentromeric region on the short and long arm of the chromosome, respectively. These two BACs span 20% of the physical length of chromosome 8 but genetically they are separated by only 4 cM (Table 1, Fig. 5). The drop in recombination rate in the pericentromeric regions is a genetic feature common to all eukaryotes. However the size of these recombination-suppressed regions varies among species. In rice, which has a genome size similar to carrot, and in *Arabidopsis*, these regions contain few megabases of DNA (Yan et al. 2005, 2006), but in large grass genomes up to tens of megabases are involved (Mézard 2006).

Comparative FISH mapping in *Daucus* species with 2*n*=22

The genus *Daucus* contains about 20 species, mainly diploid with *n*=9, 10, and 11. It has been hypothesized that the basic chromosome number in the Apioideae is *x*=11 because this is the most common base number in this subfamily of the Apiaceae (Moore 1971). According to this hypothesis, *n*=9 and 10 should be considered derived karyotypes. However, phylogenetic analysis of combined morphological and the internal transcribed spacer region of the 18S–25S nuclear rDNA sequence data has suggested that *Daucus* is not monophyletic (Lee and Downie 1999; Lee et al. 2001). Those studies did not include all the species and subspecies, thus the current classification of the genus *Daucus* is unresolved.

We performed comparative FISH mapping using a subset of carrot BAC probes in *D. crinitus* and *D. pusillus* (both 2*n*=2*x*=22) which are both in section *Daucus* of the genus (Sáenz Laín 1981). The probes used (16 for *D. crinitus* and 13 for *D. pusillus*) included BACs mapped to carrot chromosomes 3 (LG 8), 4 (LG 6; used only in *D. crinitus*), 5 (LG 2), 7 (LG 5), and 8 (LG 9). All the clones (with the exception of BACs 098O13 and 009K15) generated distinct FISH signals in both species. Overall, our analysis suggested

that *D. crinitus* and *D. pusillus* chromosomes shared synteny (i.e., no evidence for translocation) with carrot chromosomes 7 and 8; whereas carrot chromosomes 3 and 5 had been involved in more extensive reshuffling due to putative translocations (Fig. 6, Supplementary Figs. S3, S4, and S5). In particular, the four clones mapped to carrot chromosome 3 (067N21, 025M09, 032K14, and 238G06) hybridized to two different chromosome pairs in *D. crinitus* (Fig. 6b, Supplementary Fig. S3a–c). Moreover, the relative order of BACs 025M09 and 032K14 was inverted in *D. crinitus* compared with carrot (Supplemental Fig. S3d). These FISH data suggest that carrot chromosome 3 has been involved in at least one translocation and one inversion. A similar hybridization pattern was detected in *D. pusillus* (not shown). However, due to the lack of plant material, BAC 025M09 was not used in *D. pusillus*, thus the aforementioned inversion could not be confirmed in this species. Since BAC clone 238G06 (mapped on carrot chromosome 3) was detected on the NOR chromosome in *D. crinitus* (Fig. 6b), we wanted to investigate whether this chromosome is partially syntenic to the NOR chromosome in carrot (carrot chromosome 4). Clones 019A16 and 069N01, both physically linked to the NOR on carrot chromosome 4, were also linked in *D. crinitus*, but they were not located on the NOR chromosome and thus, they were not linked to clone 238G06 (Fig. 6c).

FISH data suggested the involvement of carrot chromosome 5 in another putative translocation, since

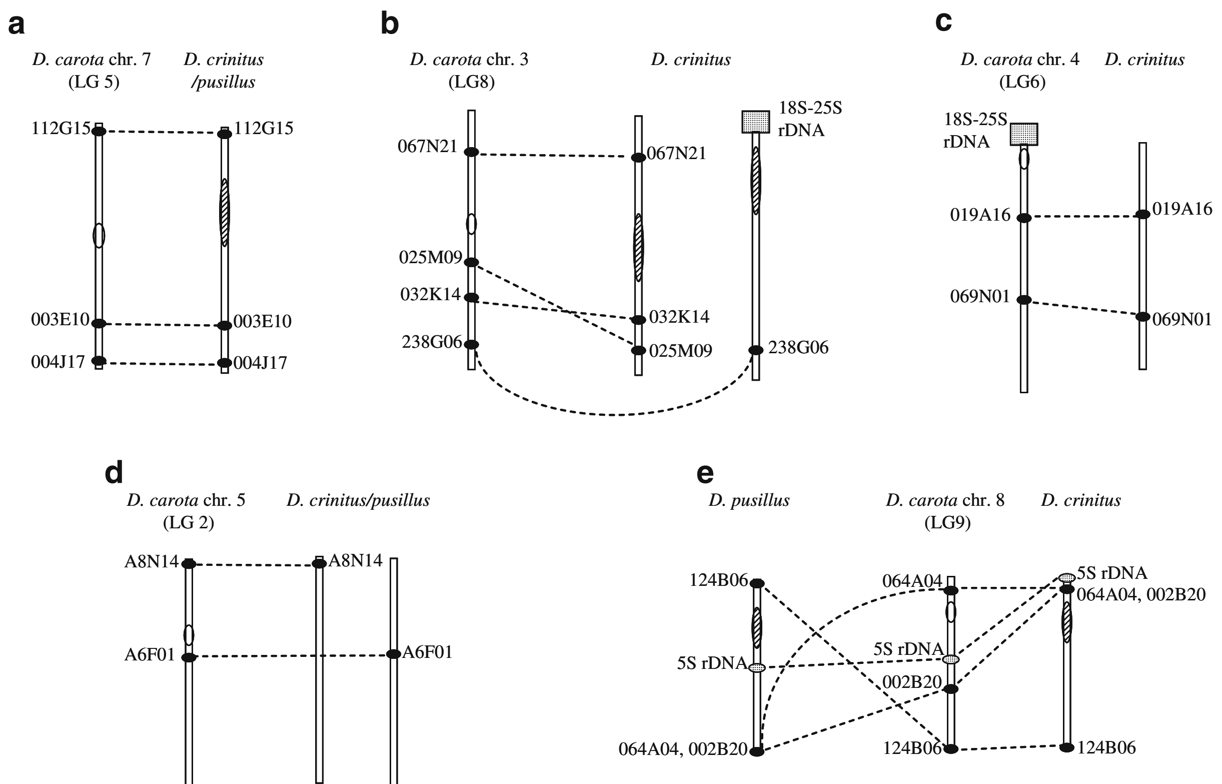

Fig. 6 Schematic drawings of selected chromosomes of carrot, *D. crinitus* and *D. pusillus* summarizing the results of the comparative FISH mapping of carrot-specific-chromosome BACs (*solid circles*). Open circles represent carrot centromeres. Striped circles (in **a**, **b**, and **e**) indicate the approximate location of the centromeric regions in *D. crinitus* and *D. pusillus*. **a** The linkage and relative order of the three clones mapped to carrot chromosome 7 are conserved in both *D. crinitus* and *D. pusillus*. **B**–**c** Carrot chromosome 3 has been involved in at least one translocation and one inversion relative to *D. crinitus* and *D. pusillus*. BAC 238G06 is located on the NOR chromosome in *D. crinitus,* but not in carrot. Two BAC clones located on the NOR chromosome in carrot hybridized to a different chromosome pair (not bearing the 18S–25S rDNA) in *D. crinitus*. **d** The linkage between two clones specific to carrot chromosome 5 is disrupted in both *D. crinitus* and *D. pusillus*. **e** BAC clones and 5S rDNA located on chromosome 8 in carrot are also linked in both *D. crinitus* and *D. pusillus*. Their relative order is different among the three species and might be the result of multiple inversions

the two clones located on carrot chromosome 5 hybridized to two different chromosome pairs in both *D. crinitus* and *D. pusillus* (Fig. 6d, Supplementary Figs. S4a, b). Identification of additional clones associated with carrot chromosome 5 (LG 2) may provide conclusive evidence that this is, perhaps, a key chromosome associated with variation in chromosome number in *Daucus*.

A previous survey on the cytological location of the ribosomal gene cluster in several *Daucus* species indicated that the 5S rDNA is located interstitially on the long arm of one chromosome pair in all the species analyzed with the exception of *D. crinitus* (Iovene et al. 2008a). In this species, the 5S rDNA locus was located distally on the short arm of one chromosome pair. Ribosomal DNA may move without the involvement of chromosomal rearrangements, and without altering the colinearity of other markers (Dubcovsky and Dvořák 1995). Thus, we used the BACs located on carrot chromosome 8 to assess synteny between carrot, *D. crinitus* and *D. pusillus*, and to test the hyposthesis that a chromosome rearrangement accounted for the repositioning of the 5S rDNA in *Daucus*. We found that 5S rDNA and BACs 002B20, 064A04, and 124B06 were also linked in both *D. crinitus* and *D. pusillus*. However, their relative order was different in any pairwise comparison among these three species (Fig. 6e, Supplementary Fig. S5) and compatible with the involvement of one or more inversions. To draw more certain conclusions, analysis of pachytene chromosomes of *D. pusillus* and *D. crinitus*, as well as of other 22- and 20-chromosome *Daucus* and the use of additional BAC probes, is highly desirable. Moreover, analysis of other *Daucus* and *Daucus*-related species and a better phylogenetic framework are also necessary to understand the direction of the chromosomal rearrangements.

The most common comparative FISH approach is mapping a common set of DNA clones in related species. Recent applications of this approach in plant species have yielded exciting results, including the first evidence for a centromere repositioning event in plants (Han et al. 2009) and the discovery of chromosomal inversions among *Solanum* species that diverged up to 12 Ma (Lou et al. 2010). The results obtained in our study further demonstrate the power of comparative FISH mapping to study synteny and chromosome rearrangements among related plant species lacking saturated genetic linkage maps and the availability of appropriate mapping populations (Lou et al. 2010; Lysak et al. 2006; Mandakova and Lysak 2008).

This study begins to reveal the translocations, inversions, and fissions/fusions that shaped the evolution of the genome for several species of *Daucus*. Whole genome sequencing of 18-, 20-, and 22-chromosome *Daucus* will be important to confirm and expand upon these observations.

In conclusion, we have described a standard carrot karyotype and established the relationship between carrot linkage groups and specific pachytene chromosomes using a set of map-anchored BACs. We have demonstrated that comparative FISH mapping is a useful approach to study synteny among *Daucus* species. The power of this strategy will be fully exploited once a more accurate phylogeny of the genus is established.

Acknowledgment We thank Dr. Jiri Macas for his invaluable help in the analysis of the BAC sequences generated in this work.

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