CARYOLOGIA Vol. 56, no. 2: 149-154, 2003

Karyoptype analysis of *Daucus carota* L. using Giemsa C-Banding and FISH of 5S and 18S/25S rRNA specific genes

O. SCHRADER[1, *], R. AHNE[1] and J. FUCHS[2]

[1] Bundesanstalt für Züchtungsforschung an Kulturpflanzen, 06484 Quedlinburg, Germany.
[2] Institut für Pflanzengenetik und Kulturpflanzenforschung, 06466 Gatersleben, Germany. Present address: Institut für Botanik, Universität Wien, A-1030 Wien, Austria.

Abstract - In *Daucus carota* L. subsp. *sativus* (Hoffm.) Arc. sixteen ideally extended mitotic pro-metaphases with total genomic length variation of 9% were used for karyotype analysis after Giemsa C-banding. The C-bands were localized exclusively in centromeric positions of all chromosome types (D1 – D9). After computer-aided measurements of the chromosomes with respect to their length and arm ratios the karyotype could be subdivided into two groups: one with five longer subtelocentric chromosome pairs (including the satellite chromosome pair) and one with four shorter submetacentric chromosome pairs. A t-test confirmed that most of the arm length differences between the chromosomes of the same group were significant, except for the short arms within the subtelocentric group and the very similar short arms of D5 and D8 in the submetacentric group. Using fluorescence *in situ* hybridization the physical localization of rRNA genes was studied. The 18S/25S rRNA gene locus was found on the short arm of the longest subtelocentric satellite chromosome D1 and the 5S rRNA locus on the longer arm of the submetacentric chromosome D5. Based on statistical analysis of 16 pro-metaphases all nine chromosome pairs of the carrot karyotype were distinguishable.

Key words: Karyotype analysis, *Daucus*, Giemsa C-banding, 18S/25S and 5S rRNA gene loci, physical mapping, computer-aided karyotyping.

INTRODUCTION

The carrot with a world-wide production area of around 850 000 ha (FAO 1998) is besides the potato the second most popular vegetable in the world. The genetic characterization of agronomically important traits succeeded using isozyme-, RAPD-, RFLP-, AFLP- or SAMPL-markers (SCHULZ *et al.* 1994; VIVEK and SIMSON 1999; NOTHNAGEL and STRAKA 2000). The resulting maps consist of maximally 11 linkage groups. In order to assign these linkage groups to individual chromosome types detailed knowledge about the carrot karyotype is necessary.

Karyotype analysis of the short chromosomic carrot (*Daucus carota* L., $2n = 2x = 18$) have been reported only rarely and were mainly restricted to the use of highly condensed metaphase chromosomes which do not show significant differences in their chromosome lengths (KUMAR and WIDHOLM 1984; ESSAD 1985). Analysis of such chromosomes revealed only minimal differences in the length of the individual chromosomes without any statistical relevance. Here we report on our efforts to establish a reliable karyotype of *D. carota* L. subsp. *sativus* (Hoffm.) Arc. by means of Giemsa C-banding, chromosomal localization of 18S/25S and 5S rRNA genes by fluorescence *in situ* hybridization (FISH) and computer-aided chromosome analysis on longer pro-metaphase chromosomes.

* Corresponding author: fax +03946 47579; e-mail: o.schrader@bafz.de

MATERIAL AND METHODS

Plant material and chromosome preparation

Root tips of young seedlings of *D. carota* L. subsp. *sativus* (Hoffm.) Arc. variety 'Lange rote Stumpfe' of a length of about 0.5 cm were synchronized with 1.25mM hydroxyurea for 16-18h at 25°C (modified after Pan et al. 1993), treated with 2mM 8-hydroxyquinoline for 2.5 h at 24°C, fixed in ethanol-glacial acetic acid (3:1) and stored in 70% ethanol at –20°C. After digestion (4% cellulase 'Onozuka R-10' (Serva) and 1% pectolyase Y-23 (Seishin Pharmaceutical) in 75 mM KCl and 7.5 mM Na-EDTA, pH 4.0 (Kakeda *et al.* 1991) at 37°C for 20 min) the root tips were squashed in 45% acetic acid, frozen, air-dried for 5-7 days and for longer storage transferred to –20°C.

Giemsa C-banding procedure

The Giemsa C-banding procedure was performed according to DE PUTTER and VAN DE VOOREN (1988) with some minor modifications. Slides were washed for 30 min in one-fifteenth M Sörensen phosphate buffer (SPB), pH 7.0; incubated in 45% acetic acid for 15 min at 60°C; rinsed in water for 12 min; incubated in 5% $Ba(OH)_2$ for 12 min at room temperature; rinsed in water for 30 min; incubated in 2 x SSC (pH 7.0) for 2.5 h at 62 °C; dipped for 5-10 sec in SPB; stained in 4% Giemsa (Gurr's improved R 66) for 5-15 min; rinsed in SPB and destilled water; air-dried and mounted in Entellan (Merk).

Fluorescence in situ hybridization (FISH)

A 25S rRNA gene fragment (220 bp) was amplified and simultaneously labelled with biotin-16-dUTP (Roche Diagnostics) from genomic DNA of *D. carota sativus* (Hoffm.) via PCR using gene-specific primers (YOKATA *et al.* 1989). As 5S rRNA gene-specific probe a 117 bp fragment was PCR-amplified using primers designed according to the gene sequence of Glycine (GOTTLOB-MYHUGH *et al.* 1990). *In situ* hybridization was carried out as described in FUCHS and SCHUBERT (1995) with 100ng labelled probe per slide. Digoxigenin was detected with anti-digoxigenin-fluorescein (Roche Diagnostics) and biotin with streptavidin-Cy3 (Dianova) and if necessary amplified as described (SCHRADER *et al.* 2000). Chromosomes were counterstained with DAPI and mounted with Vectashield antifade solution (Vector Laboratories). Each fluorochrome was captured separately using a computer-assisted CCD camera system (Photometrics), pseudocoloured (Gene Join) and merged (Photoshop).

Chromosome measurements

The analyses of 78 chromosome sets were done with the computer-aided program UNICHRO (AHNE *et al.* 1989). This program measures the length of chromosomes, their corresponding arms and the position and size of possible satellites. To estimate the minimal sample number (n) of pro-metaphase plates that had to be analysed, the normal distribution of all measured lengths was tested with the program CADEMO 3.0 (Biorat GmbH, Rostock). Sixteen prometaphases ($n = 16$, $\alpha = 5\%$) were selected for statistical analysis. The total genomic lengths of the diploid chromosome sets ranged between 70 and 77 µm, corresponding to a limit of total length variation of maximally 9%. For a better comparability, each chromo-

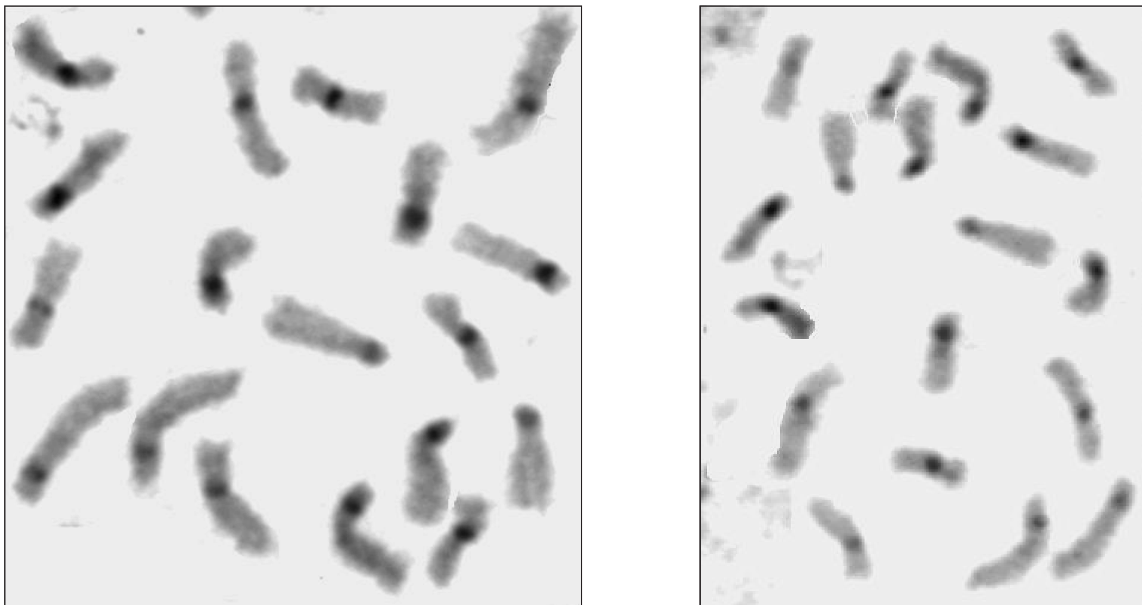

Fig. 1 – Two examples of pro-metaphases of *D. carota* L. with total genomic length of 77 (1a: left) and 70 (1b: right) µm after Giemsa C-banding.

some length was calculated in percent of total genomic length of the corresponding diploid chromosome set. The classified lengths of homologous chromosomes and their respective arms were combined to mean lengths of each of the 9 chromosome types (D1 – D9) per chromosome set and finally over all 16 prometaphases including the standard deviation (±) of chromosome arms.

RESULTS AND DISCUSSION

Giemsa C-banding on relatively long prometaphase chromosomes, which are more or less twice as long as the corresponding condensed metaphase chromosomes, resulted in the localization of C-bands at or in the vicinity of the centromeres of all 18 carrot chromosomes (Figs. 1, 2). In addition to the staining of the centromeres the satellites of the SAT-chromosome pair were intensly stained. No further bands on the chromosome arms were detectable.

Since the size and the intensity of the centromeric staining varied between different preparations as well as between pro-metaphases on the same slide, these data could not be used as chromosome-specific markers to distinguish between the individual carrot chromosomes. Though ESSAD (1984) tried to include variations in band sizes and intensities into a schematic drawing of the carrot karyotype, he admitted that the variations in band intensities do not interfere with the classification of the chromosomes.

The chromosomes of sixteen selected prometaphases (which were free of distorted and overlapped chromosomes) were measured with respect to their length and arm ratios by means of the computer-aided program UNICHRO. Bent and twisted chromosomes were automatically stretched (Fig. 2). The most intense gray values were defined as the position of centromeres. The homologous chromosomes were combined to mean values per haploid set first, and second in average over the 16 chromosome complements resulting in a mean total genomic length of the haploid set of 37.22 µm (Tab. 1 and Fig. 4). According to the measured data the karyotype of *D. carota* L. can be divided into two main groups, one with subtelocentric chromosome pairs (D1, D2, D4, D6, D7) and one with four submetacentric chromosome pairs (D3, D5, D8, D9). These two karyotypic groups were also described in three Giemsa stained metaphases by ESSAD (1984). Contradictory results were reported by KUMAR and WIDHOLM (1984) who examined the chromosome lengths and centromeric indices of 15 metaphases after staining with carbol fuchsin in haploid cell cultures of carrot. They divided the karyotype into three main groups with 3 acrocentric, 5 submetacentric and 1 metacentric chromosomes. Besides discrepancies between the presented

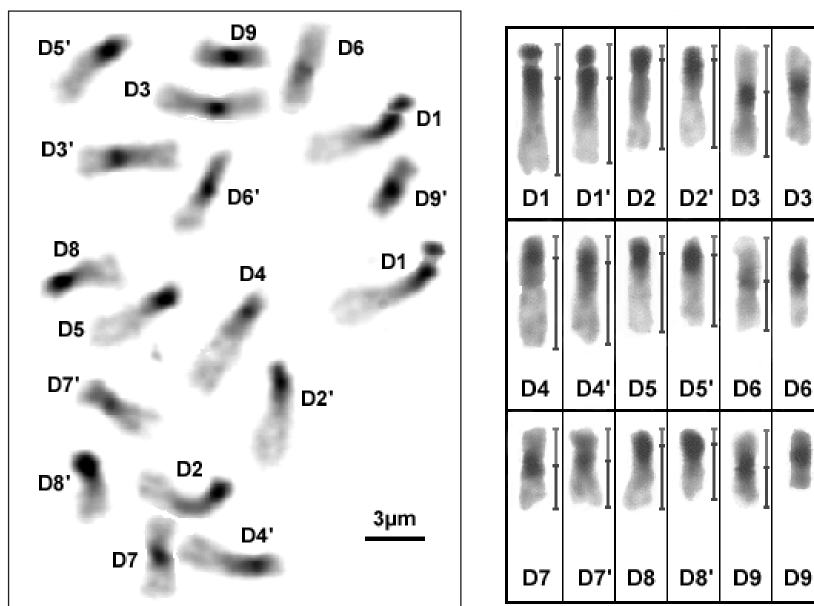

Fig. 2 – Pro-metaphase of *D. carota* L. after Giemsa C-banding (left) and image analysis (right). The bars right to the arranged chromosomes indicate the measured lengths and the centromere positions.

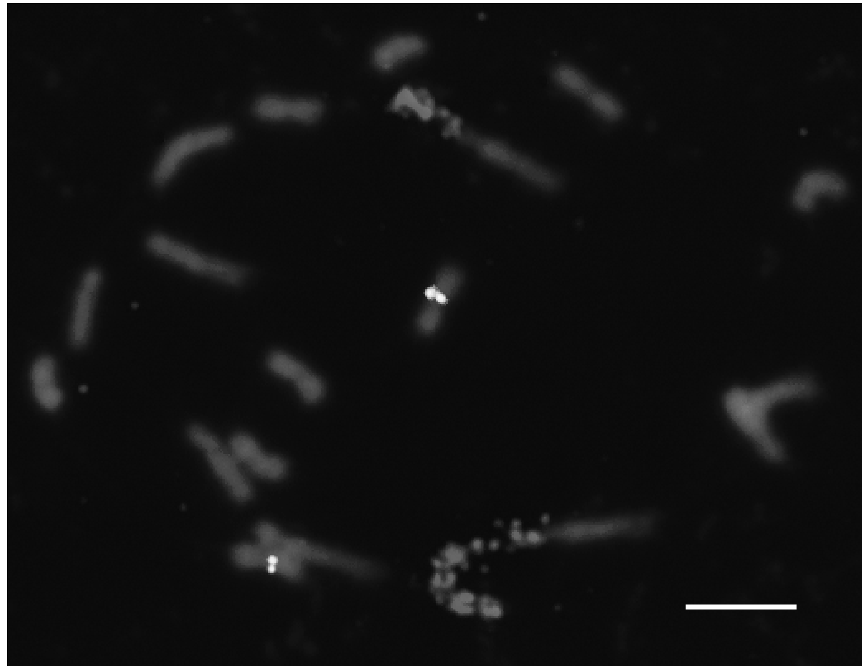

Fig. 3 – DAPI-stained pro-metaphase chromosomes (blue) of *D. carota* L. after fluorescence *in situ* hybridization with probes specific for 5S (yellow) and 18S/25S (red) rRNA genes. (Scale bar = 5 µm).

images and the table, their chromosome classification (no. 1-9) deviated also from our data, especially in the positions of centromeres (see Table 1). This could probably be explained by the fact that the used HA cell line (*D. carota* L. var. Juwart) differs from other carrot cell lines (initiated from var. Danvers Half Long) in terms of chromosomal rearrangements (KUMAR and WIDHOLM 1984) and possibly also from var. 'Lange rote Stumpfe' we have used in our experiments.

Double-FISH experiments with probes derived from 18S/25S and 5S rRNA genes produced clear and reproducible signals on two chromosome pairs. Whereas the 18S/25S rDNA locus was found in median position on the short arm of the longest subtelocentric chromosome D1 (50% of the whole arm length away from the centromere), the 5S rRNA genes were localized in proximal position on the long arm (25% of the whole arm length away from the centromere) of the medium sized submetacentric chromosome D5 (Fig. 3 and 4). No further minor loci could be detected by repeated double or single FISH experiments. Earlier FISH experiments with a probe specific for the *Arabidpsis*-type telomeric sequence repeat resulted in signals of similar intensities at the ends of all chromosomes (FUCHS *et al.* 1995). Due to the absence of signals at interstitial positions, the telomeric sequence can not be used as an additional marker for chromosome discrimination.

Table 1 – Average length of the chromosomes of *D. carota* L., calculated in percent of the mean haploid genome length of 16 pro-metaphases (37.22 µm). (LA: long arm; SA: short arm; CI: centromeric index; ±: standard deviation).

Type	LA %	SA %	LA+SA %	LA+SA (µm)	CI
D1	5.87 ± 0.54	1.31 ± 0.25	7.18	5.35	0.18
D2	5.20 ± 0.48	1.15 ± 0.23	6.35	4.72	0.18
D3	3.76 ± 0.31	2.53 ± 0.24	6.29	4.68	0.40
D4	4.77 ± 0.35	1.07 ± 0.15	5.84	4.35	0.18
D5	3.15 ± 0.28	2.18 ± 0.20	5.33	3.97	0.41
D6	4.11 ± 0.26	1.11 ± 0.20	5.22	3.88	0.21
D7	3.62 ± 0.30	1.19 ± 0.23	4.81	3.58	0.25
D8	2.63 ± 0.16	2.13 ± 0.18	4.76	3.51	0.45
D9	2.29 ± 0.27	1.95 ± 0.26	4.24	3.16	0.46

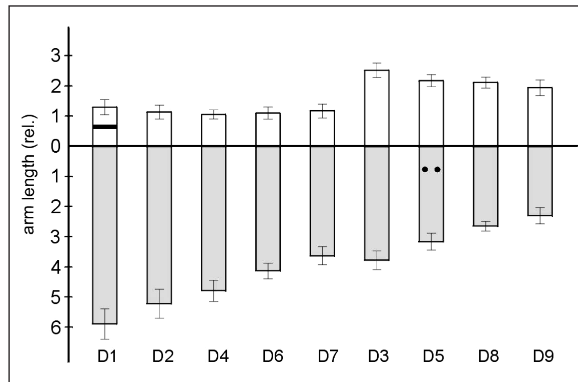

Fig. 4 – Idiogram of the haploid chromosome complement of *D. carota* L. derived from 16 analysed pro-metaphases. (18S/25S rRNA gene locus, black bar; 5S rRNA gene locus, black dots; standard deviations of chromosome arms as bars).

Based on the measurement of the 16 selected pro-metaphases a t-test (α=5%*, α=1%** and α=0.1%***) was performed to estimate the significance of the length differences between the individual chromosome arms. In the submetacentric group significant differences exist between the long arms of D3 and D5 (t=5.8***), D5 and D8 (t=6.5***) and D8 and D9 (t=4.3***) as well as between the short arms of D3 and D5 (t=4.5***) and D8 and D9 (t=2.3*). However, in the subtelocentric group significant differences only exist between the long arms of D4 and D6 (t=6.1***), D6 and D7 (t=3.7***), D2 and D4 (t=2.9**) and D1 and D2 (t=3.7***).

For a more reliable karyotyping of *Daucus* further cytological markers like chromosome-specific BACs or YACs (currently not available), and appropriate repetitive or single copy sequences would be desirable. Since first steps of construction of a genetic map with molecular markers in carrot are undertaken (SCHULZ *et al.* 1994; VIVEK and SIMON 1999; NOTHNAGEL and STRAKA 2000) the development of suitable hybridization probes will be only a question of time. The successful use of AFLP-, RAPD- and RFLP-sequences as hybridization probes for chromosome identification has been demonstrated for *Asparagus* (REAMON-BÜTTNER *et al.* 1999), *Petunia* (BENABDELMOUNA *et al.* 1999) and *Zea* (SADDER *et al.* 2000; SADDER and WEBER 2002). This can exemplary demonstrate the way how to continue the molecular karyotyping in *Daucus* in the future.

Acknowledgements – We would like to thank Miss. E. Koblitz (†) and Miss K. Maier for exellent technical assistance.

REFERENCES

AHNE R., HUHN P., LAUMER N. and ZERNEKE F., 1989 – *Interaktive rechnergestützte Chromosomenanalyse. Ein Grundsoftwarepaket.* Arch. Züchtungsforschung, 19: 133-140.

ANONYM, 1998 – *FAO Production Yearbook, 1997.* Vol. 51, FAO, Rome.

BENABDELMOUNA A., PELTIER D., HUMBERT C. and ABIRACHEDDARMENCY M., 1999 – *Southern and fluorescent in situ hybridization detect three RAPD-generated PCR products useful as introgression markers in Petunia*. Theor. Appl. Genet., 98: 10-17.

ESSAD S., 1985 – *Banding C et biometrie appliques a l'analyse du caryotype de carotte* (Daucus carota *L.).* Agronomie, 5: 871-876.

FUCHS J., BRANDES A. AND SCHUBERT I., 1995 – *Telomere sequence localization and karyotype evolution in higher plants*. Plant Syst. Evol., 196: 227-241.

FUCHS J. and SCHUBERT I., 1995 – *Localization of seed protein genes on metaphase chromosomes of* Vicia faba *via fluorescence* in situ *hybridization*. Chrom. Res., 3: 94-100.

GOTTLOB-MCHUGH S.G., LEVESQUE M., MACKENZIE K., OLSON M., YAROSH O. and JOHNSON D.A., 1990 – *Organization of the 5S rRNA genes in the soybean* Glycine max *(L.) Merrill and conservation of the 5S rDNA repeat structure in higher plants*. Genome, 33: 486-494.

KAKEDA K., FUKUI K. and YAMAGATA H., 1991 – *Heterochromatic differentiation in barley chromosomes revealed by C- and N-banding.* Theor. Appl. Genet., 81: 144-150.

KUMAR P. and WIDHOLM J.M., 1984 – *Techniques for chromosome analysis of carrot culture cells.* Plant Molecular Biology Reporter, 2: 37-42.

NOTHNAGEL T. and STRAKA P., 2000 – *Genetic likage map of carrot using morphological and molecular markers.* Vortr. Pflanzenzüchtg., 47: 99.

PAN W.H., HOUBEN A. and SCHLEGEL R., 1993 – *Highly effective cell synchronization in plant roots by hydroxyurea and amiprophos-methyl or colchicine.* Genome, 36: 387-390.

DE PUTTER M. and VAN DE VOOREN J.G., 1988 – *Identification of* Allium cepa *L. cultivars by means of statistical analysis of C-banded chromosomes.* Euphytica, 39: 153-160.

REAMON-BÜTTNER S.T., SCHMIDT T. and JUNG C., 1999 – *AFLPs represent highly repetitive sequences in* Asparagus officinalis *L.* Chrom. Res., 7: 297-304.

SADDER M.T., PONELIES N., BORN U. and WEBER G., 2000 – *Physical localization of single-copy sequences on pachytene chromosomes in maize*

(Zea mays L.) by chromosome in situ *hybridization.* Genome, 43: 1081-1083.

SADDER M.T. and WEBER G., 2002 – *Comparison between genetic and physical maps in* Zea mays *L. of molecular markers linked to resistance against* Diatraea *ssp.* Theor. Appl. Genet., 104: 908-915.

SCHRADER O., BUDAHN H. and AHNE R., 2000 – *Detection of 5S and 25S rRNA genes in* Sinapis alba, Raphanus sativus *and* Brassica napus *by double fluorescence in situ hybridization.* Theor. Appl. Genet., 100: 665-669.

SCHULZ B., WESTPHAL L. and WRICKE G., 1994 – *Linkage groups of isozymes, RFLP and RAPD markers in carrot* (Daucus carota *L.* sativus). Euphytica, 74: 67-76.

VIVEK B.S. and SIMON P.W., 1999 – *Linkage relationships among molecular markers and starage root traits of carrot (*Daucus carota *L. ssp.* sativus*).* Theor. Appl. Genet., 99: 58-64.

YOKATA Y., KAWATA T., IIDA Y., KATO A. and TANIFUJI S., 1989 – *Nucleotide sequence of the 5.8S rRNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA.* J. Mol. Evolution, 29: 294-301.

Received July 8, 2002; accepted October 10, 2002