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Assignment of oat linkage groups to microdissected *Avena strigosa* chromosomes

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Abstract Microdissection of metaphase chromosome preparations of diploid oat *Avena strigosa* (2n = 14) allowed isolation of the three individual chromosomes with distinct morphologies, numbers 2, 3 and 7. Using a PCR approach based on the DNA of microdissected chromosomes, STS derivatives of RFLP markers, genetically mapped in *Avena* spp. linkage maps, have been physically assigned to these three chromosomes. Based on either two or four RFLP-derived STS markers, the *A. strigosa* chromosomes 2 and 3 were found to be homoeologous to the oat linkage groups C and E, respectively. With the DNA of chromosome 7, four RFLP-derived STS markers located within the central part of linkage group F and two distal ends of linkage group G were amplified. Accordingly, chromosome 7 corresponds to linkage group F and, most probably, is involved in an *A. strigosa*-specific chromosomal translocation relative to the diploid species *Avena atlantica* and *Avena hirtula*, of which the cross progeny was used for linkage mapping of the tested RFLP clones.

Keywords *A. strigosa* · RFLP markers · physical mapping · microdissected chromosomes · translocations

Introduction

Avena strigosa Schreb. (2n = 2*x* = 14, AA) is the only diploid *Avena* species with a history of cultivation as a forage or grain crop (Murphy and Hoffman 1992). Karyotype analyses, including C-banding and the localization of rDNA sequences (Rajhathy and Thomas 1974; Fominaya et al. 1988; Linares et al. 1992, 1996; Jellen et al. 1993), and studies of chromosome pairing in *A. strigosa* × hexaploid hybrids (Thomas 1992), have revealed a marked chromosome affinity between the genome of *A. strigosa* and the A genome of *Avena sativa* (2n = 6*x* = 42, AACCDD). Recent molecular cytogenetic studies, such as in situ hybridization using total genomic DNA (Chen and Armstrong 1994; Jellen et al. 1994; Leggett and Markhand 1995) or an A-genome specific DNA sequence (Linares et al. 1998) as a probe, have further supported the hypothesis that the *A. strigosa* genome is involved in the origin of cultivated oats. Nevertheless, the genetic research of this species is still rather limited.

Restriction fragment length polymorphism (RFLP) maps have been constructed for the A-genome of various diploid *Avena* species. O'Donoughue et al. (1992) published the first linkage map in *Avena atlantica* × *Avena hirtula*, which consisted of 194 markers assigned to eight linkage groups (A to H). Van Deynze et al. (1995) extended this diploid oat linkage map to 367 markers, allowing the joining of the linkage group H to that of D. Another diploid linkage map, this one with 210 RFLP loci in 11 linkage segments based on a population from the cross *A. strigosa* × *Avena wiestii*, was reported by Rayapati et al. (1994). Recently, Yu and Wise (2000) and Kremer et al. (2001) have introduced a larger number of markers into the *A. strigosa* × *A. wiestii* map. This resulted in some rearranged linkage blocks as compared with the map reported by Rayapati et al. (1994). By comparison of the *A. strigosa* × *A. wiestii* map with RFLP maps of diploid and hexaploid oats, Portyanko et al. (2001) suggested that linkage groups B and F should be combined into one (AswBF group).

Information on the genetic and physical locations of markers related to agronomically important genes is useful for map-based cloning and marker-assisted plant breeding. Linkage group A of diploid oats contains *Pca*, a cluster of loci conferring resistance to the crown rust

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Table 1 Summary of linkage groups, probe names, primer sequences, fragment sizes, and PCR results obtained with DNA of microdissected *A. strigosa* chromosomes 2 (chrom 2), 3 (chrom 3) and 7 (chrom 7) and nuclei (n), and with genomic DNA (gDNA). The presence/absence of probe-specific PCR products is indicated by +/-

Linkage group	Probe name	Primers	Fragment size (bp)	gDNA	n	Chrom2	Chrom3	Chrom7
	NOR-specificrDNA (ITS1)	5′-CGTGACCCTGACCAAAACA-3′ 5′-ATATCCGTTGCCGAGAGTC-3′	200	+	+	+	+	–
	5S rDNA	5′-GAGAGTAGTACATCGATGGG-3′ 5′-GGAGTTCTGACGGGATCCGG-3′	300	+	+	–	+	–
A	CDO98	5′-GATGGCCATCTCAATTTACA-3′ 5′-CTACAGCCCCTTACAGATTC-3′	553	+	+	–	–	–
B	CDO385	5′-CTGTGTAGACACCGCTGATCC-3′ 5′-GGACCTCAACTTATGCGCC-3′	179	+	+	–	–	–
C	CDO460	5′-GACTGCTGAGTTCAGATCC-3′ 5′-CCTCTCTTCAATGGTGGAC-3′	113	+	+	+	–	–
C	CDO 1479	5′-ACAAAAGGGATTACACAGCCC-3′ 5′-CCCGTCTGAATAAACATGGC-3′	310	+	+	+	–	–
D	CDO89	5′-CTGATCAGACTGACAAGCTG-3′ 5′-CTGTATCGCTGACGAAGTTC-3′	900	+	+	–	–	–
E	CDO57	5′-TTCGGCCTGATCTGCATAG-3′ 5′-AACCATCCAACAGCAGTCAG-3′	246	+	+	–	+	–
E	CDO241A	5′-CTCAAGCCACTTGTTCTACC-3′ 5′-CAGCAAGGTGTGTAAGAGAC-3′	750	+	+	–	+	–
E	CDO241B	5′-CTCAAGCCACTTGTTCTACC-3′ 5′-CAGCAAGGTGTGTAAGAGAC-3′	300	+	+	–	+	–
E	CDO457	5′-TACCTCGTAGGAAGCCATG-3′ 5′-TTCGGCCTGATCTGCATAG-3′	253	+	+	–	+	–
F	CDO64	5′-GCCCAACTGCAATCAGGATAC-3′ 5′-TGGCGAGAAGCTAACCGAC-3′	324	+	+	–	–	+
F, B	CDO680	5′-AGAACACCAGGGCGCTCAAC-3′ 5′-TGCTTGGCTTTATGCTTATGCC-3′	600	+	+	–	–	+
F	CDO749	5′-GGTGTTCTAGAACTGGTTGC-3′ 5′-TGCATGCTGACAGTATCTCC-3′	197	+	+	–	–	+
F	CDO938	5′-CCACAAAACAGTGTACGCAC-3′ 5′-AGGCTGGAGATGGAGAAGAC-3′	650	+	+	–	–	+
G	CDO105	5′-TCCAAGCTGACCATAGACAG-3′ 5′-GGGTGATGGTGATAAAGGTC-3′	146	+	+	–	–	–
G	CDO497	5′-CAGCATCCCAACAGAGTTCG-3′ 5′-ACCGAGGTTTGCAGGAAAGC-3′	380	+	+	–	–	+
G	CDO507	5′-GTCTTGACTCATTCGATCCG-3′ 5′-ACTCCGATGGTTGTTGTAGG-3′	104	+	+	–	–	+

fungus *Puccinia coronata* (Rayapati et al. 1994; Yu et al. 1996), and *Avn*, a prolamin seed-storage protein gene (Rayapati et al. 1994; Yu et al. 1996). One satellited chromosome pair corresponding to linkage group 22 of hexaploid oats contains a dwarfing locus (*Dw 7*) (Milach et al. 1997). However, the correspondence of individual *A. strigosa* chromosomes to these linkage groups or loci is still unknown.

A direct approach for assigning linkage groups to individual chromosomes would be to hybridize genetically mapped RFLP probes to chromosomes. In plants, many efforts have been made to improve the sensitivity of fluorescence in situ hybridization (FISH). These include primed in situ hybridization (PRINS, Abbo et al. 1993), coupling PRINS and thermocycling (PCR-PRINS, Shi et al. 1996), and competitive in situ suppression (Sadder et al. 2000). However, in situ detection of single-copy sequences of less than 1-kb length, i.e. the size of DNA fragments that detect RFLPs, is not yet routinely feasible in plant chromosome spreads. An alternative approach is to directly amplify sequence-tagged site (STS) markers, derived from genetically mapped RFLP clones, in the DNA of microdissected chromosomes. Using defined locations of translocation breakpoints as chromosomal landmarks, this approach has resulted in cytogenetically integrated RFLP maps for all chromosomes of the barley genome (Künzel et al. 2000).

The aims of this study were: (1) to establish a protocol for isolation of individual *A. strigosa* chromosomes by microdissection, (2) to amplify RFLP-derived STS markers with the DNA of microdissected chromosomes as a template, and (3) to identify homoeologous relationships of microdissected *A. strigosa* chromosomes with existing diploid and hexaploid oat linkage groups.

Materials and methods

Plant material and chromosome preparation

Seeds of *A. strigosa*, PI 258729 and Av 96, obtained from the John Innes Center (Norwich, U.K.) and the IPK (Gatersleben, Germany) respectively, were germinated on moist filter paper for 48 h at 22 °C. To synchronize cell divisions and accumulate metaphases, the germinated seeds were subsequently exposed to 1.8 °C for 48 h followed by 6 h at 22 °C (for primary roots) and 30 h at 22 °C (for secondary roots). Roots were pre-treated in 0.025% colchicine for 1 h 10 min to shorten the chromosomes, fixed in 45% acetic acid for 8 min, washed at least five-times in distilled water and stored in 70% ethanol at –20 °C. The meristematic root tips were cut off and digested using an enzyme mixture, according to Fukui and Kakeda (1990), consisting of 2.5% pectolyase Y-23 and 2.5% cellulase 'Onokuza R-10' diluted in 75 mM of KCl and 7.5 mM of EDTA (pH 4.0) at room temperature. After 2 h of maceration, the root tips were briefly washed in 45% acetic acid and squashed on cover slips (32 × 12 mm) which were removed after freezing with liquid nitrogen. Air-dried preparations were used immediately or stored in 96% ethanol at –20 °C.

Chromosome microdissection

Microdissections were performed essentially as described by Macas et al. (1993). Using a mechanically driven sliding micromanipulator (Eppendorf, 5171) combined with an inverted phase contrast microscope (Axiovert 100, Zeiss), chromosomes or prophase nuclei were isolated at 630-fold magnification and transferred into a collection drop of 1 µl of TE buffer within an Eppendorf tube to avoid evaporation.

DOP-PCR

DNA was amplified with the degenerate oligonucleotide primer (DOP, Telenius et al. 1992) in a PCR-system 9600 thermocycler (Perkin Elmer). The droplet with collected chromosomes or nuclei was added to 50 µl of PCR-mix containing 0.7 µM of degenerated primer (5′-CCG ACT CGA GNN NNN NAT GTG G-3′, 6-Mw), 0.2 mM of each dNTP, 2.5 mM of $MgCl_2$, 1 × *Taq* DNA polymerase buffer (Boehringer Mannheim) and 2.5 units of *Taq* polymerase (Boehringer Mannheim). Five cycles were run at 94 °C for 1 min, 30 °C for 1.5 min, and 72 °C for 3 min with a transition time of 3 min to 72 °C. This was followed by 35 cycles at 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min with an auto-extension step of 14 s/cycle, and a final extension at 72 °C for 7 min. Negative controls without DNA were processed identically.

STS primers and PCR with chromosome-specific DNA

The 16 RFLP probes listed in Table 1 were selected based on data derived from linkage maps of diploid oat species (O'Donoughue et al. 1992; Rayapati et al. 1994; Van Deynze et al. 1995, 1998) according to the following criteria: (1) originated from cultivated *A. sativa*; (2) located within different linkage groups of diploid oat maps; (3) characterized by good hybridization signals in Southern analysis; and (4) present as single-copy or in low copy number. To convert the oat cDNA markers (CDO) into STS markers, primers were designed using the DNA 3′ end sequences available from the database GrainGenes. For the internal transcribed spacer (ITS) region of both the 18S-5.8S-26S and the 5S rDNA, the primers were synthesized according to sequence data reported by Hsiao et al. (1995) and Cox et al. (1992), respectively.

PCR was performed using either chromosome-specific DNA amplified by DOP-PCR or directly by the DNA of microdissected chromosomes/nuclei as templates. The PCR-mix (final volume 25 µl) contained 2 µl of DOP-PCR-amplified DNA (or the collection drop with microdissected chromosomes/nuclei), 1.5 units of *Taq* polymerase (MBI Fermentas), 0.2 mM of each dNTP, 0.2 µM of each primer, and 2 mM of $MgCl_2$. PCR conditions were optimized for each specific primer pair. Amplified DNA fragments were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. Fragment sizes were estimated using a DNA length marker VI (Boehringer Mannheim) or a pUC marker (MBI Fermentas).

Photo documentation

For figures, the images were digitalized, stored on a PhotoCD and processed using the Adobe Photoshop. Only those functions that apply equally to all pixels in the image were used.

Results and discussion

Microdissection of individual chromosomes

The precise identification of microdissected chromosomes is an indispensable prerequisite for the reliability of results obtained in this study. The GTG-banding technique, routine in human chromosome identification for microdissection (Lüdecke et al. 1989; Weimer et al. 1999), is of little use in plants (Anderson et al. 1982). The HCl treatment required for diagnostic C-banding excludes its use, owing to DNA damage, for PCR amplification from microdissected chromosomes. Consequently, only chromosomes with well-discernible morphology in mitotic metaphases can bypass these drawbacks. In *A. strigosa*, only three chromosome pairs are readily identifiable in unstained metaphase plates: chromosome 2 with a long satellite in the short arm, chromosome 3 with a short satellite in the short arm and chromosome 7, the only subtelocentric pair of the complement (Rajhathy and Thomas, 1974).

Optimal spreading of *A. strigosa* metaphase plates by squashing requires fixation in acetic acid at 45%. To minimize the acid-depurination of DNA, this treatment was reduced to 8 min. Only metaphases with unequivocally identifiable and spatially well-separated chromosomes were used for microdissection (see Fig. 1). Fifteen or 30 chromosomes of each selected pair were collected for DOP-PCR or direct PCR with primer sets for STS markers, respectively. In contrast to the technique applied to barley, collection drops consisting of TE buffer were used instead of the pick-up buffer specified by Künzel et al. (2000).

As exemplified by this study, inability to discriminate all of the individual chromosomes in unstained mitotic metaphases of native karyotypes often limits the applicability of plant microdissection cytogenetics. This limitation may be overcome by generating lines with defined chromosomal translocations or aneuploid stocks. Such materials have been used successfully in barley (Korzun and Künzel 1996; Künzel et al. 2000) and *A. sativa* (Chen and Armstrong 1995) to individualize microdissection chromosomes not otherwise discernible.

Molecular check of microdissected chromosomes

FISH data show that the clones pTa71 (18S-5.8S-26S rDNA) and pTa794 (5S rDNA) hybridize to the two satellited chromosome pairs, 2 and 3, of *A. strigosa*. The

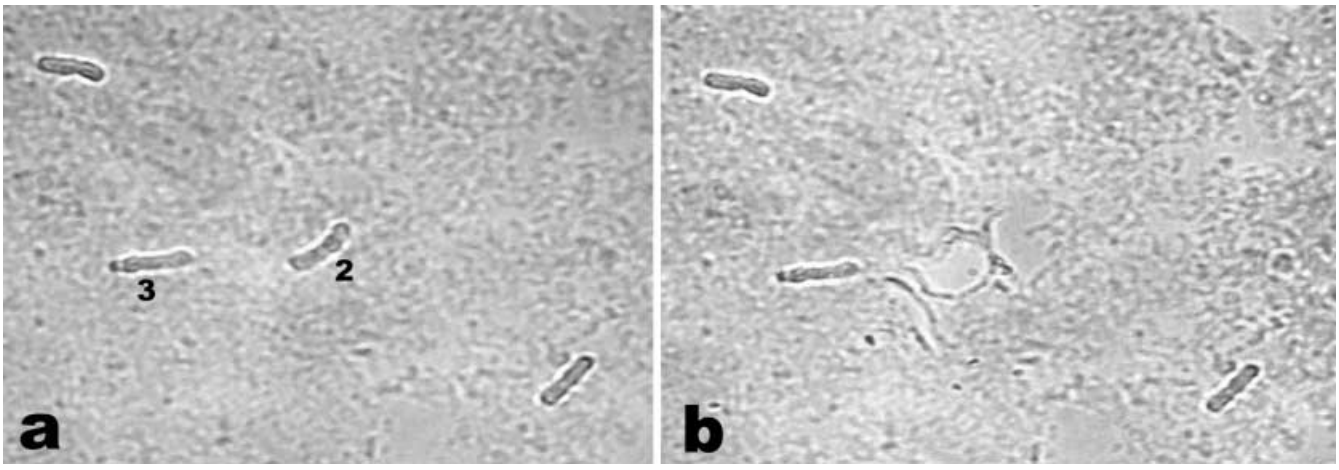

Fig. 1a, b Part of an unstained metaphase spread of *A. strigosa* showing four chromosomes. **a** The satellited chromosomes 2 and 3 are indicated by their *numbers*, **b** The same metaphase after dissection of chromosome 2

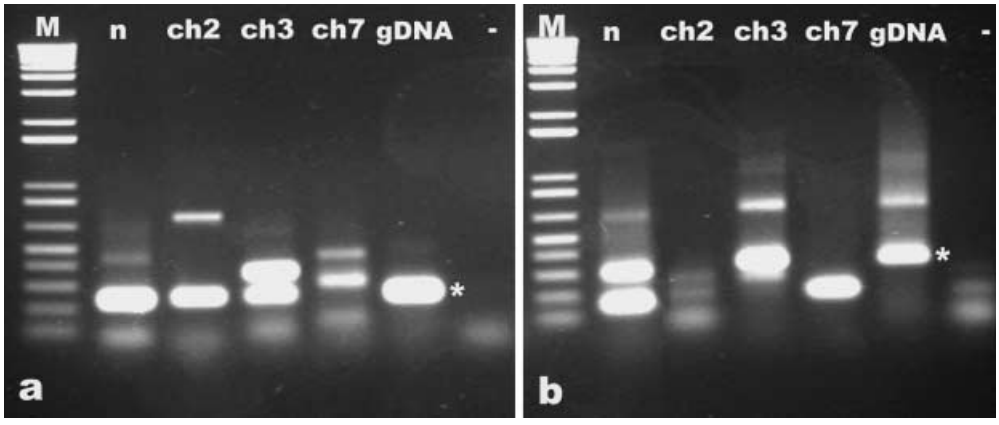

Fig. 2a, b PCR products obtained with sequence-specific primer sets for the 18S-5.8S-26S (**a**) and 5S rDNA (**b**) probes. PCR was performed with template DNA (lane gDNA) and no DNA (*lane* -) from *A. strigosa* as a control, with DOP-PCR products of micro-dissected nuclei (*lane n*), chromosome 2 (*lane ch2*), chromosome 3 (*lane ch3*) and chromosome 7 (*lane7*). Lane M corresponds to the molecular-weight marker 1 kb + (Gibco) In **a** and **b**, *asterisks* indicate the diagnostic bands

first clone physically maps to the nucleolus-organizing regions (NORs) of both chromosomes at their secondary constrictions, while the second clone detects one site each within the short and the long arm of chromosome 3 (Linares et al. 1996). Based on this knowledge the purity and identity of each collection of microdissected chromosomes was tested by PCR.

The DNAs of separate collections of chromosomes 2, 3, 7 and prophase nuclei were subjected to DOP-PCR. The DOP-PCR products were subsequently amplified separately by PCR using primers specific for one of the internal transcribed spacer regions of the 18S-5.8S-26S rDNA (ITS1) or of the 5S rDNA (see Table 1). As shown in Fig 2, the NOR-specific rDNA was present in chromosomes 2 and 3, and absent in chromosome 7, while the 5S rDNA was present only in chromosome 3. These results support the FISH data and indicate the correct selection of microdissected chromosomes.

PCR results for STS markers with the DNA of microdissected chromosomes

Because the primers were designed from RFLP probes derived from *A. sativa*, at first their functionality was tested with genomic DNA of *A. strigosa*, either conventionally isolated from etiolated leaves or DOP-PCR-amplified from micro-isolated prophase nuclei. Of the 16 primer pairs used, 13 yielded a single PCR product of the expected size (see examples in Fig. 3). The primers for CDO1479, CDO507 and CDO938 gave several bands in addition to the STS-specific bands of 310-bp, 104-bp and 650-bp length, respectively, given in Table 1.

To avoid the labour-intensive repeated dissection of individual chromosomes for testing each of the 16 STS markers, at first, DOP-PCR products obtained from the DNA of microdissected chromosomes 2, 3 and 7, or prophase nuclei, were used as a template for PCR with the respective primer sets. However, these PCR variants often amplified a mixture of fragments of different lengths, causing problems in recognizing the STS-specific bands. Therefore, PCR based on the DNA of micro-dissected chromosomes was used directly to definitively confirm the presence or absence of STS markers.

As summarized in Table 1, markers relating to linkage groups A, B and D (CDO98, CDO385 and CDO89, respectively) were not amplified from the DNA of any of the microdissected chromosomes. Both of the tested markers of linkage group C (CDO460 and CDO1479)

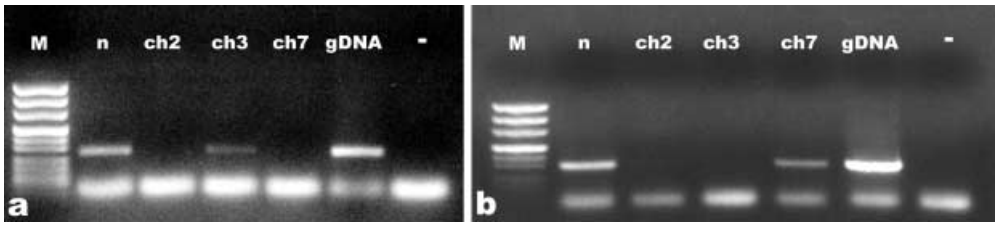

Fig. 3a, b PCR products obtained with sequence-specific primer sets for RFLP probes CDO57 (**a**) and CDO64 (**b**). PCR was performed with template DNA (*lane gDNA*) and no DNA (*lane* -) from *A. strigosa* as a control, with microdissected nuclei (*lane n*), chromosome 2 (*lane ch2*), chromosome 3 (*lane ch3*) and chromosome 7 (*lane ch7*). *Lane M* corresponds to molecular-weight marker pUC8 (MBI Fermentas)

were present in the DNA of chromosome 2 and absent in the DNA of chromosomes 3 and 7. The four markers of linkage group E (CDO57, CDO241A, CDO241B and CDO457) were all amplified exclusively with the DNA of chromosome 3. In contrast to chromosomes 2 and 3, the DNA of chromosome 7 contained markers of three different linkage groups. These markers were CDO680, which mapped to group F of the *A. strigosa* × *Avena wiestii* map (Rayapati et al. 1994) but to group B of the *A. atlantica* × *A. hirtula* map (O'Donoughue et al. 1992), CDO64, CDO749 and CDO938 mapping to group F in the *A. atlantica* × *A. hirtula* map (O'Donoughue et al. 1992), and CDO497 and CDO507 mapping to group G in the *A. atlantica* × *A. hirtula* map (O'Donoughue et al. 1992). However, marker CDO105, also belonging to group G in the *A. atlantica* × *A. hirtula* map, was missing in chromosome 7.

Assignment of linkage groups to microdissected chromosomes

Considering the PCR results altogether, there were no experimental contradictions against the conclusion that the *A. strigosa* chromosomes 2 and 3 correspond to the oat linkage groups C and E, respectively.

With chromosome 7 the situation is complicated by the fact that markers of three different linkage groups were found to be associated. An examination of the map positions of the respective markers suggests the following interpretation. Because CDO64, CDO749 and CDO938, all assigned to chromosome 7, genetically map within the central part of group F (Van Deynze et al. 1995), it is reasonable to assume that the main body of *A. strigosa* chromosome 7 corresponds to linkage group F of the *A. atlantica* × *A. hirtula*-derived map. The presence of CDO680 in chromosome 7 substantiates the suggestion of Portyanko et al. (2001) to consider linkage groups F and B as one group. Of the three markers from linkage group G, the two markers CDO507 and CDO497, both present in chromosome 7, are located on the opposite end of the linkage map as compared to the missing marker CDO105. This suggests that chromosome 7 is involved in an unidentified *A. strigosa*-specific chromosomal translocation relative to the genomes of *A. atlantica* and *A. hirtula*.

The conclusions drawn for chromosome 7 are supported by comparing the map positions of loci detected by the barley cDNA marker BCD1829. This probe is one of the few RFLP markers which the *A. atlantica* × *A. hirtula* map (O'Donoughue et al. 1992) and the *A. strigosa* × *A. wiestii* map (Rayapati et al. 1994) have in common. In the *A. atlantica* × *A. hirtula* map, BCD1829 detects a locus on each of the linkage groups B, E and G, of which the locus on group G maps at a subterminal position 8 cM proximal to CDO497. In the *A. strigosa* × *A. wiestii* map, the same probe detects only one locus at a subterminal position on group F. Another common marker is CDO1255, which is located on group G in both maps but at different positions (more central in *A. atlantica* × *A. hirtula* as compared to subterminal in *A. strigosa* × *A. wiestii*). These examples show that markers distally located on the *A. atlantica* × *A. hirtula*-derived group G are found at terminal positions on the *A. strigosa* × *A. wiestii*-derived linkage group F. Recent results of Kremer et al. (2001) indicated a weak linkage (LOD < 2.0) of CDO497 and CDO1255 to one end of group G.

The experimental findings for chromosome 7 can be caused theoretically also by locus duplication. Even though duplicated loci within different oat linkage groups have been reported (Rayapati et al. 1994), there is no evidence for extended duplications in diploid oats (O'Donoughue et al. 1992). Additionally, the RFLP probes used as a source of STS markers in this study do not detect loci simultaneously present in linkage groups F and G of the two diploid oat maps. On the other hand, translocations between chromosomes belonging to different genomes of hexaploid oats have been well documented by FISH (Chen and Armstrong 1994; Jellen et al. 1994; Fominaya et al. 1995; Leggett and Markhand 1995; Linares et al. 1996, 2000).

Although data obtained from RFLP-derived STS markers bear the risk that the PCR products may not necessarily be derived from the target locus (Blake et al. 1996; Erpelding et al. 1996s), the results presented seem to be uninfluenced by such mismatching between RFLP probes and their respective STS primers.

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