Chromosome composition of an F_2 *Triticum aestivum* x *T. turgidum* spp. *durum* cross analysed by DArT markers and MCFISH[1]

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Running title: **DArT and MCFISH analyses of pentaploid wheat progeny**

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Abstract. This study has employed multicolour fluorescence *in situ* hybridisation and Diversity Array Technology markers to determine the segregation of parental A, B and D genome material into the progeny of a cross between a hexaploid bread wheat (*Triticum aestivum* L. var. 2-49) and a tetraploid durum wheat (*T. turgidum* L. spp. *durum* (Desf.) var. Bellaroi). In the F_2 progeny from a 2-49/Bellaroi cross, 82 out of 83 F_2 plants investigated with DArT analysis had some D genome material, principally as entire chromosomes, while 40 plants had at least one complete copy of all seven D genome chromosomes. Twelve plants containing partial D chromosomes were identified. MCFISH analysis of 26 additional F_2 plants of the same cross showed that all 26 plants contained varying amounts of D genome material of which three carried single A-D translocations. In addition two telocentric D genome chromosomes were detected. The D genome content of each line and the breakpoint positions of the three A-D translocations were confirmed with DArT marker analysis. Overall results indicate a random recombination of A and B genome loci from the hexaploid female parent and the tetraploid male parent in this F_2 population and a significant retention of the maternal D genome material. This study illustrates that the combined application of the MCFISH and DArT techniques provides a powerful approach for the analysis of crosses between cereal genotypes of different ploidy.

Keywords: DArT, durum wheat, MCFISH, hexaploid x durum crosses

Introduction

Tetraploid durum wheat (*Triticum turgidum* L spp. *durum* (Desf.)), with the chromosome constitution AABB (2n=4x=28), is grown in several cereal growing regions of Australia to supply both domestic and overseas manufacturers of pasta and related food products. Hexaploid bread wheats (*T. aestivum* L.), with the chromosome constitution AABBDD (2n=6x=42), provide an excellent resource for the genetic enhancement of durum, due to the shared AABB tetraploid chromosome compliment and the potential for transfer of desirable D genome loci into durum. For example, the gluten quality of durum wheat has been significantly improved by the introgression of D genome segments (Ceoloni *et al.* 1996; Pogna and Mazza 1996).

Although there have been some recent studies on hexaploid by tetraploid crosses (Gilbert 2000; Wang *et al.* 2005; Lanning *et al.* 2008), the pioneering cytological research into the fate of chromosomes in *T. aestivum* x *T. turgidum* spp. *durum* crosses was conducted in the 1920's (Sax 1922; Kihara 1925; Thompson and Hollinghead 1927). These discoveries were detailed in a comprehensive review by Kihara (1982), which described his observations that the pentaploid F_1 plants contained 35 chromosomes consisting of 14 bivalents and seven univalents. In successive generations, plants divided into an 'increasing group', comprised of families which returned to the hexaploid state over several generations and a 'declining group', which lost all D genome chromosomes, resulting in a tetraploid state. The main feature of the increasing group was that the number of bivalents plus the number of univalents was 21 at the F_2 generation. While this pioneering work, using traditional cytological methods, revealed much about the fate of the unpaired D genome chromosomes in subsequent generations, they could not easily determine the genomic identity of the chromosomes, nor routinely identify intergenomic translocations between the A, B and D genome chromosomes. Modern cytogenetic and molecular techniques, such as Multicolour fluorescence *in situ* hybridization (MCFISH) and Diversity Arrays Technology (DArT) markers have now given us the tools to observe these details. Furthermore, marker technologies can reveal in the progeny the segregation of genetic sequences inherited from each parent in the interspecific cross.

We are currently engaged in a program which seeks to introgress, from hexaploid wheat into durum, partial resistance against the cereal disease crown rot, caused principally by

the fungus *Fusarium pseudograminearum* (Aoki and O'Donnell 1999). This approach has been adopted due to the general susceptibility of durum germplasm to crown rot, despite wide screening of genotypes in search of resistance. In order to achieve efficient resistance introgression, it is necessary to understand the cytogenetics of hexaploid x tetraploid crosses.

The objectives of this study were to determine whether there is a random segregation of the parental A and B genomes in the F_2 progeny of a hexaploid x tetraploid cross; to observe the quantity of D genome material retained in F_2 progeny of the cross; and to measure the frequency of D genome introgression into either the A or B genome.

Materials and methods

Plant materials

The hexaploid wheat line 2-49 (AUS 29532 - Australian Winter Cereal Collection Accession number) was used as the maternal parent and crossed with the durum wheat line Bellaroi (AUS 30781) at NSW Industry and Investment, Tamworth Agricultural Institute, Tamworth, Australia. The line 2-49 (Gluyas Early/Gala) is recognised as a source of partial resistance to crown rot (Collard *et al.* 2005). Bellaroi is an elite Australian durum variety with excellent semolina colour and dough strength. Plants from the F_2 seed of the 2-49/Bellaroi cross were grown in the glasshouse either at the University of Southern Queensland, Toowoomba or at the University of Sydney, Plant Breeding Institute, Cobbitty.

DArT analysis

DNA was extracted from leaf samples of 83 F_2 seedlings and the parents using a DNeasy Plant Mini kit (Qiagen Pty Ltd, Victoria, Australia). The protocol provided by the supplier was used. The DNA samples were sent to Triticarte Pty Ltd, ACT, Australia (http://www.diversityarrays.com) for DArT analysis (Wenzl *et al.* 2004). DNA was hybridised to a wheat array, a genomic representation of a mixture of mainly hexaploid wheat cultivars and a small number of durum cultivars. For each marker a P value (based on ANOVA) was established, which represents an estimate of marker

quality. Only markers with a P value greater than 77 were considered, as this is the recommended benchmark value (Akbari *et al.* 2006).

DArT markers were grouped into linkage groups using Map Manger QTX (Manly *et al*. 2001). The best order of the markers within a linkage group was determined using the program Record (Van Os *et al.* 2005). A χ^2 goodness of fit test was conducted on the linked DArT marker data.

MCFISH

Seed germinations, root-tip pre-treatments, squash preparations, slide pre-treatments and denaturations were performed according to Zhang *et al.* (2001). Root-tips were collected separately from each seedling and chromosome numbers were counted in root-tip squashes stained with Acetocarmine before the slide was analysed with MCFISH.

BAC 676D4 and 9M13 DNA was isolated using a Qiagen Plasmid Midi Kit (Qiagen, Australia). The BACs 676D4 and 9M13 contain A and D genome specific dispersed repetitive sequences, respectively (Zhang *et al.* 2004). One microgram each of 676D4 and 9M13 was labelled with Tetramethyl-Rhodamine-5-dUTP (Roche Applied Science, Australia) using nick translation and biotin-14-dATP (BioNick Labelling System, Invitrogen Life Technologies, Australia), respectively. The protocol provided by the supplier was used. The hybridisation and post-hybridisation washes were conducted as described in Zhang *et al.* (2004). The biotin-labeled probe was detected with fluorescein-avidin DN (Vector Laboratories, Burlingame, CA). Chromosome preparations were analysed with an epifluorescence Zeiss Axio Imager microscope. Images were captured with a Retiga EXi CCD (charge-coupled device) camera (QImaging, Surry, BC, Canada) operated with Image-Pro Plus 6.2 software (Media Cybernetics Inc., Bethesda, MD) and processed with Photoshop v8.0 software (Adobe Systems, San Jose, CA).

Results

DArT analysis of 83 F_2 plants of the 2-49/Bellaroi cross

Four-hundred and fifty-eight polymorphic DArT markers hybridised onto the DNA of the 83 2-49/Bellaroi F_2 plants. Of the analysed DArT markers, 88% gave a positive

signal on the maternal parent reflecting the predominance of hexaploid DNA sources used to construct the array. More than twice as many markers mapped to the B genome (148) compared to the A genome (64). Two-hundred and forty-six markers mapped to the D genome. None of the F_2 plants had a solely maternal genotype, indicating that self-pollination did not occur during crossing (hybridization). The χ^2 -test indicated that there was no significant deviation from the expected Mendelian segregation ratio of 3:1 (signal : no signal) across the A and B genomes, given the dominant character of DArT markers. Segregation distortion ($P< 0.01$) was only observed in the case of ten closely linked markers on chromosome 5B which favoured inheritance of the durum alleles. The distribution of D genome material based on the DArT markers is illustrated in Table 1. Only one plant (line 55) had lost all seven D genome chromosomes. Forty plants (48%) contained at least one copy of all seven D genome chromosomes. Thirty-nine different chromosome combinations were observed with most combinations present only once. A large majority of the D chromosomes were inherited in their entirety, however, 12 (presumably single copy) partial chromosomes were detected (Table 2). These would include both translocated D chromosome segments and telocentric chromosomes. Partial chromosomes in lines also containing an entire homologous copy were not detected. The loss of pairs of complete D chromosomes appeared to be random with respect to which of the seven chromosomes were missing (Table 2).

MCFISH and DArT analysis of 26 F_2 plants of 2-49/Bellaroi cross

A further 26 F_2 plants from the 2-49/Bellaroi population were analysed with MCFISH. The plants contained varying amounts of D genome material, with chromosome numbers varying from 29 to 42 (Table 3). Three plants retained 42 chromosomes (lines 5, 20 and 25), while the most common chromosome number was 31, observed in five of the plants. Translocations (T) between A and D chromosomes were observed in lines 13, 22, and 24 (Table 3; Fig. 1). Whole-arm translocations were observed in lines 13 and 22, whereas in plant 24 a small D genome segment translocated to the long arm of an A genome chromosome. Telocentric (t) chromosomes were present in lines 7 and 23 (Table 3).

The DNA of the 26 F_2 plants used for MCFISH was extracted and sent to Triticarte Pty Ltd for DArT analysis. Four-hundred and fifty-seven DArT markers hybridised with the DNA. Good agreement was observed between the DArT markers and the MCFISH results with regard to retention of the D genome material (Table 3). Generation of these two data sets allows the number of single copy D chromosomes present to be determined. Ten of the 12 lines which had more than seven D genome chromosomes had at least one copy of each D genome chromosome indicated by the DArT markers present. One line had 11 D genome chromosomes with no copy of chromosome 4D (line 12; Table 3) and line 13 only had a fragment of 6D. The DArT results suggest that chromosomes 6D, 3D, and 1D were involved in the A-D translocations observed by MCFISH in lines 13, 22, and 24, respectively (Table 3). The A genome chromosome involved in these translocations could not be determined. The telocentric chromosome in line 7 (Table 3) could not be identified from its DArT fingerprint, while the source of the telocentric chromosome fragment detected in line 23 could only be narrowed down to 2D, 4D, or 7D, since all three chromosomes had at least one entire copy present.

Discussion

Hexaploid x durum crosses are being increasingly considered as a means of transferring desired genes in either direction (Wang *et al.* 2005; Lanning *et al.* 2008). The first barrier is the formation of an F_1 kernel that grows into a viable plant. Further obstacles include the zero or relatively low yield of fertile F_1 seed which results from some crosses, the challenge of establishing stable recurrent ploidy of choice (28 or 42) in subsequent generations and the potential loss of blocks of desirable alleles from the recurrent parent genome. In the successful hexaploid x durum cross examined in this study, polymorphic DArT alleles from the hexaploid and tetraploid parents segregated randomly among the progeny across almost all loci in the A and B genomes. Interestingly, twice as many polymorphic DArT markers mapped to the B genome chromosomes compared to the A genome chromosomes, consistent with previous observations of higher levels of polymorphism in the B genome of modern wheats (Boeuf *et al.* 2003). Clearly this also applies to comparisons between hexaploid bread and tetraploid durum wheats, reflecting both their common ancestry and subsequent limited evolutionary divergence. The segregation distortion observed for a group of

closely linked markers on chromosome 5B is consistent with studies in hexaploid wheats which have also reported distortion on 5B (Kumar *et al.* 2007).

With regard to the D genome, we observed a very significant retention of this material in the F_2 progeny of the cross. Of the 83 F_2 lines analysed with the dominant DArT markers, 48% retained at least one apparently entire copy of each D genome chromosome. Where both copies of a particular D genome chromosome had been lost, there was no evidence that loss or retention of particular chromosomes (or groups of chromosomes) was favoured. In a previous study, 24 (56%) out of 43 F_2 lines from a Sumai 3 (hexaploid) x DT486 (tetraploid) wheat cross analysed with microsatellites had at least one copy of all seven D genome chromosomes (Gilbert *et al.* 2000). While our MCFISH studies indicated that 14 (54%) out of 26 lines retained 35 or more chromosomes, Wang *et al.* (2005) found that 80% of 55 F_2 plants of a As195 (durum) by Chuannong (hexaploid) cross were in this class.

The retention of some D genome material in all but one of the total of 109 progeny examined indicates successful transfer of many unpaired D genome chromosomes through meiosis in selfed F_1 plants. Based on the comparison of the DArT and MCFISH results for 26 lines, D genome chromosomes were predominantly inherited as complete chromosomes. However, single telocentric chromosomes and translocation events were observed in five of these lines (19%). In the larger sub-population analysed only by DArT, partial chromosomes were observed with a slightly lower frequency (14%). It is not possible, based on DArT analysis alone, to determine whether these are translocations or telocentric chromosomes. Furthermore, in the absence of MCFISH analysis, some incomplete chromosomes will go undetected due to the presence of the entire homologous D genome chromosome. For similar reasons, A or B genome chromosomes involved in translocations with D genome material cannot be identified using DArT markers.

All three translocations of D genome material characterised by MCFISH were confirmed by DArT marker analysis, indicating that the homologous D genome chromosome was absent.

While this particular population shows minimal segregation distortion of parental alleles, further F_2 populations derived from pentaploid F_1s of other hexaploid x durum crosses are currently being examined to establish whether this is a general rule.

Subsequent generations of this and other crosses will be screened to determine whether different crosses retain different levels of D genome material and to observe the rate at which D genome material is lost at each meiosis event.
MCFISH is a labour intensive cytogenetic technique, requiring a high level of operator expertise, which does not lend itself to high throughput screening of population lines. Nevertheless, its judicious use together with high throughput marker-based techniques such as DArT screening has begun to reveal much that will be of practical application to the development of hexaploid x durum derived materials in breeding programs.

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Table 1. D genome material in 83 F_2 plants of the 2-49 x Bellaroi cross analysed with DArT markers. Each column represents one of the 83 F_2 plants analysed. Each row represents a different marker and different D genome chromosomes (Chrom) are indicated in the first column. Alleles from 2-49 are indicated in grey, Bellaroi null alleles in white and missing values in black

Table 2. Distribution of complete and partial D genome chromosomes in 83 F_2 plants

Table 3. D genome material present in 26 F_2 plants of 2-49 x Bellaroi cross, analysed by both DArT markers and MCFISH. Each column represents one of the 26 F_2 plants analysed. In the DArT results each row represents a different marker and different D genome chromosomes (Chrom) are indicated. Alleles from 2-49 are indicated in grey, Bellaroi null alleles in white and missing values in black. Under the MCFISH results the chromosome counts for the A, B and D genomes are shown. Translocations (T) and telocentric (t) chromosomes are also indicated.

Fig. 1. MCFISH analysis of mitotic metaphase cells of F_2 root tips showing a) a hexaploid cell and b) The A-D translocation in line 13. The arrow points to the translocation breakpoint at the centromere. A genome chromosomes are red, B genome chromosomes brown, and D genome chromosomes green. Scale bars equal 10 μm.

Table 1

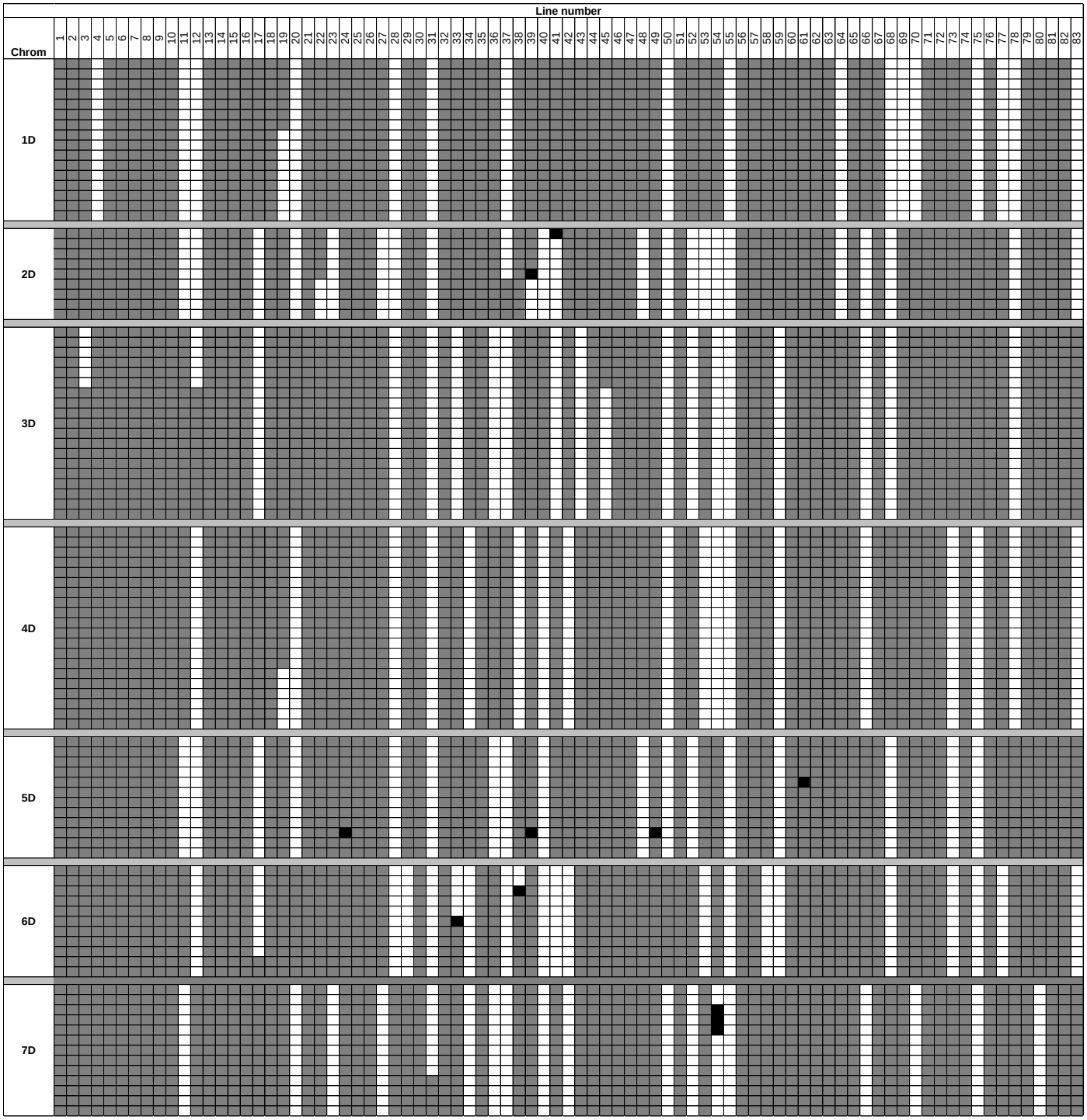
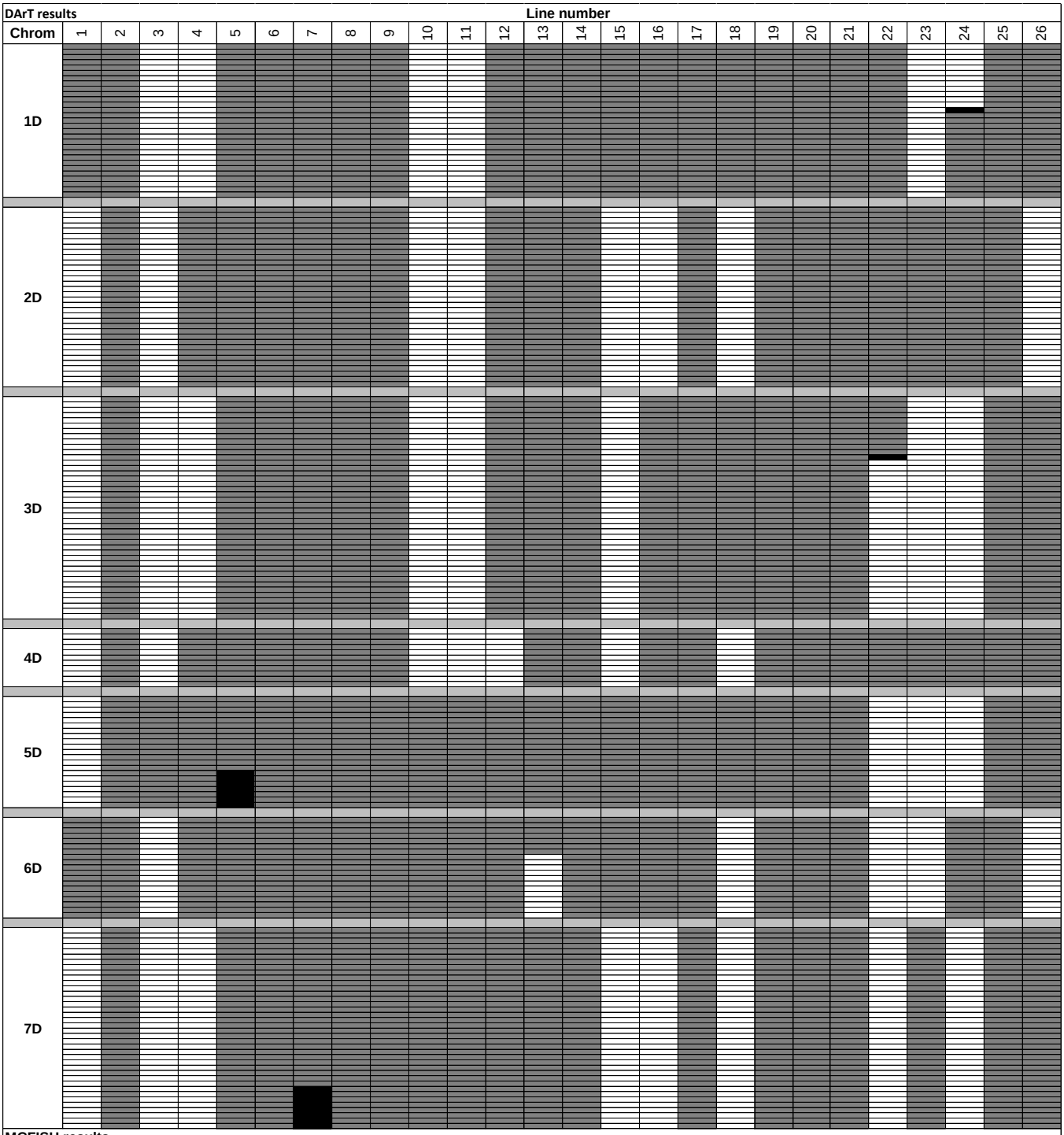

Line number
Chrom
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83
1D
2D
3D
4D
5D
6D
7D

Table 2

D chromosome	1D	2D	3D	4D	5D	6D	7D
At least one complete	65	59	64	64	66	62	65
Unpaired partial	1	3	3	1	0	3	1
Null	17	21	16	18	17	18	17

Table 3



MCFISH results																											
A	14	14	14	14	14	14	14	14	14	14	14	14	13+T	14	14	14	14	14	14	14	14	13+T	14	14+T	14	14
B	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
D	2	12	1	4	14	7	12+t	11	11	3	3	11	9+T	9	4	5	10	3	12	14	7	3+T	4+t	3+T	14	5
Total	30	40	29	32	42	35	40+t	39	39	31	31	39	37	37	32	33	38	31	40	42	35	31	32+t	31	42	33

Fig.1