Complete genetic linkage maps from an interspecific cross between *Fusarium circinatum* and *Fusarium subglutinans*

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Abstract

The *Gibberella fujikuroi* complex includes many plant pathogens of agricultural crops and trees, all of which have anamorphs assigned to the genus *Fusarium.* In this study, an interspecific hybrid cross between *Gibberella circinata* and *Gibberella subglutinans* was used to compile a genetic linkage map. A framework map was constructed using a total of 578 AFLP markers together with the mating type (*MAT-1* and *MAT-2*) genes and the histone (H3) gene. Twelve major linkage groups were identified ($n = 12$). Fifty percent of the markers showed significant deviation from the expected 1:1 transmission ratio in a haploid F_1 cross ($P < 0.05$). The transmission of the markers on the linkage map was biased towards alleles of the *G. subglutinans* parent, with an estimated 60% of the genome of F_1 individuals contributed by this parent. This map will serve as a powerful

tool to study the genetic architecture of interspecific differentiation and pathogenicity in the two parental genomes.

Article Outline

1. Introduction

Gibberella fujikuroi (Sawada) Wollenw. is the species complex associated with species that have anamorphs in *Fusarium* section *Liseola*. These include many important fungal pathogens of agricultural crops and trees. The *Fusarium* species associated with this complex include at least eleven different biological species (mating population A–K),

which are reproductively isolated ([Nirenberg and O'Donnell, 1998], [Samuels et al., 2001], [Zeller et al., 2003], [Phan et al., 2004] and [Lepoint et al., 2005]). Traditionally, the morphological species concept has been used to describe species in the *G. fujikuroi* complex, but differentiation between species following this approach has generally been unsatisfactory. One example is *Fusarium subglutinans sensu lato* within which three mating populations (B, E and H) have been identified, based on patterns of inter-isolate fertility. Recognition of these mating populations as distinct species is supported by multigene phylogenies ([Nirenberg and O'Donnell, 1998] and [O'Donnell et al., 1998]). *Fusarium subglutinans sensu stricto* is used for strains isolated from maize and belongs to mating population E ([Nirenberg and O'Donnell, 1998] and [O'Donnell et al., 1998]). *Fusarium circinatum* is the name applied to isolates from pine that cause pitch canker, which are associated with mating population H of the *G. fujikuori* complex ([Nirenberg and O'Donnell, 1998] and [Britz et al., 1999]). The biological species concept has been used extensively to characterise other species in the *G. fujikuroi* complex. However, this approach has limited value because the majority of species have no apparent sexual stage (Steenkamp et al., 2002).

Fusarium circinatum (mating population H), also known as the pitch canker fungus, is a pathogen of many pine species, and is especially damaging to *Pinus patula* and *Pinus radiata* ([McCain et al., 1987] and [Viljoen and Wingfield, 1994]). This fungus was first discovered in the United States in 1946 (Hepting and Roth, 1946) and has since spread to many other parts of the world including South Africa. In the latter country it causes root rot and damping off of *P. patula* and other susceptible pine species in seedling nurseries (Viljoen and Wingfield, 1994).

Fusarium subglutinans (mating population E) is a common pathogen of domesticated maize (*Zea mays* spp. *mays*). Desjardins et al. (2000) studied isolates of *Fusarium* from maize and the closely related wild teosinte (*Zea* spp.) in Mexico and Central America, in an attempt to characterize these isolates and determine an appropriate mating population for them. Strains identified based on morphology as *F. subglutinans* from maize and teosinte, were infertile when crossed with tester strains from mating populations B, E and H. However, one strain of *F. subglutinans* isolated from teosinte was moderately fertile in a cross with an isolate of *F. circinatum* (mating population H). Desjardins et al. (2000)

suggested that all *F. subglutinans* strains treated in this study represent a fourth distinct mating population associated with *F. subglutinans.* This was due to the almost complete infertility with the standard tester strains. Steenkamp et al. (2001) applied phylogenetic analyses and sexual compatibility tests to show that *F. subglutinans* isolates from teosinte belong to mating population E. They are phylogenetically more closely related to each other than to other mating populations within the *G. fujikuroi* species complex ([O'Donnell et al., 1998], [Steenkamp et al., 1999] and [Steenkamp et al., 2000]), indicating they share a greater ancestry. Hybridization of closely related fungal species, such as mating population E and H forming the basis of this study, has been documented in other fungi (Lind et al., 2005) as well as within the *Liseola* section of *Fusarium* ([Desjardins et al., 1997] and [Leslie et al., 2004b]).

Fusarium circinatum and *F. subglutinans* threaten forestry and maize production in South Africa. Both these industries are considered integral parts of South Africa's economy with maize being considered to be the staple diet of South Africans with approximately 25% of South Africa's total arable land use being planted to maize. Therefore, both commercial forestry and maize are of economic importance in South Africa and maintaining these sectors is of important to the economy of the country.

The cross between *F. circinatum* and *F. subglutinans* made by Desjardins et al. (2000) provided us with a unique opportunity to study genetic differentiation using genetic linkage mapping. Genetic linkage maps have also been used to study other fungi including *Fusarium verticillioides* ([Xu and Leslie, 1996] and [Jurgenson et al., 2002b]) and *Fusarium graminearum* ([Jurgenson et al., 2002a] and [Gale et al., 2005]). In general, AFLP markers are preferred when generating linkage maps. The objective of our study was to use AFLP markers (Vos et al., 1995) to generate a genetic linkage map for an interspecific cross between *Gibberella circinata* and *Gibberella subglutinans*. This map should provide a useful framework for further study of the architecture of the two parental genomes and elucidation of the genetic determinants of pathogenicity to pine.

2. Materials and methods

2.1. Fungal isolates

Isolates used for genetic linkage analysis were F_1 progeny from the cross between *G. circinata* (maternal parent; *MAT-1*) and *G. subglutinans* (paternal parent; *MAT-2*) from the study of Desjardins et al. (2000). The parents of this cross were isolates MRC7828 (*G. subglutinans*) and MRC7870 (*G. circinata*) (Table 1). Ninety-four F_1 isolates were randomly selected from 226 viable ascospore progeny obtained from 14 perithecia for use in this study.

Table 1.

Hosts, geographic origins and source of the F_1 parents used in this study

Isolate[a]	Host	Geographic origin	Source
MRC7828; Fst51[b]	*Zea mays* spp. *Mexicana*	Texcoco, Mexico	A.E. Desjardins
MRC7870; Fsp34[c]	*Pinus* spp.	California, USA	T.R. Gordon

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[b] *F. subglutinans* (mating population E).

[c] *F. circinatum* (mating population H).

2.2. DNA isolation

Isolates were grown on half strength PDA (potato dextrose agar; 20% potato dextrose agar and 5% agar) for 7 days at 25 °C in the dark. Mycelium was harvested and 300 µl extraction buffer (200 mM Tris [pH 8], 250 mM NaCl, 25 mM EDTA [pH 8], 0.5% w/v SDS) was added (Raeder and Broda, 1985). This mixture was homogenised at 4 m/s for 20 s using the Fastprep FP120 (QBIOgene, Farmingdale, NY, USA) system. Following homogenisation, the tissue was frozen in liquid nitrogen and then thawed in boiling water for 5 min. Phenol–chloroform (1:1) extractions were performed (10,600*g* for 5 min) until all cell debris had been removed. Thereafter, 0.1 volumes of 3 M sodium acetate (pH 8) and two volumes of cold absolute ethanol were added and the Eppendorf tubes were

inverted five times. After centrifugation at 10,600*g* for 5 min, 1 ml 70% ethanol was added to the supernatant and allowed to stand for 5 min at room temperature (Sambrook et al., 1989). The precipitated DNA was centrifuged for a further 5 min at 2700*g* and dried under vacuum. The DNA was resuspended in 500 μl low TE (10 mM Tris [pH 8], 0.1 mM EDTA).

2.3. AFLP analysis

AFLP analysis was performed essentially following the protocol of Vos et al. (1995). Restriction digestion of the genomic DNA was performed using *Eco*RI and *Mse*I (Zeller et al., 2000). These restriction fragments were ligated to the corresponding enzyme-specific oligonucleotide adapters (Vos et al., 1995). Preselective amplifications were performed with zero-base-addition *Eco*RI and *Mse*I adapter-specific primers using the following PCR conditions: 1 cycle of 30 s at 72 °C, 25 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C with an increase of 1 s per cycle and a final elongation step of 2 min at 72 °C. Final selective amplifications used *Eco*RI and *Mse*I primers (Table 2) with two-base-additions. The *Eco*RI primer was labelled with the infrared dyes, IRDye™ 700 or IRDye™ 800 (LI-COR, Lincoln, NE). PCR conditions were as follows: 13 cycles of 10 s at 94 °C, 30 s at 65 °C with a decrease of 0.7 °C per cycle and 1 min at 72 °C followed by 23 cycles of 10 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C with an increase of 1 s per cycle and a final elongation step of 1 min at 72 °C.

Table 2.

AFLP primer combinations used in this study

Primer combination	No. polymorphisms[a]	% Polymorphic bands/primer
M-AA + E-AA (700)	61	62.9
M-AA + E-CC (700)	44	56.4
M-AA + E-AC (700)	54	68.4
M-AA + E-TC (800)	55	70.7

Primer combination	No. polymorphisms[a]	% Polymorphic bands/primer
M-GA + E-CC (700)	32	48.5
M-GA + E-AC (700)	40	62.5
M-GA + E-TC (800)	42	56.0
M-AC + E-AA (700)	26	26.5
M-AG + E-AC (700)	32	53.3
M-AT + E-AC (700)	45	70.3
M-CA + E-TC (800)	63	66.3
M-AA + E-TT (800)	45	48.9
M-AG + E-AA (700)	39	42.4
Total	578	
Average	44.5 ± 11.3	56.4

In the first column, the primer combinations are given for the *Mse*I (M) selective nucleotides and the *Eco*RI (E) selective nucleotides. The value given in parenthesis refers to the IRDye™ (LI-COR, Lincoln, NE) used for fragment analysis.

[a] Only the markers used for framework map construction.

AFLP fragment analysis was performed on a model 4200 LI-COR® automated DNA sequencer as described by Myburg et al. (2001). Electrophoresis run parameters were set to the following: 1500 V, 35 mA, 35 W, 45 °C, motor speed 3 and signal filter 3. Electrophoresis prerun time was set to 30 min and the run time to 4 h.

Digital gel images obtained from the LI-COR system were analysed using the Saga[MX] AFLP® Analysis Software package (LI-COR) according to the manufacturers' instructions. Only markers that were polymorphic for the two hybrid parent strains were

scored with a '0' indicating absence, '1' indicating presence of bands and 'X' indicating missing data.

2.4. Additional marker analysis

PCR identification of the mating types of all the isolates was performed as described by Steenkamp et al. (2000). It was not possible to perform the multiplex PCR as described, but superior results were achieved when the *MAT-1* or *MAT-2* primer sets were used separately. An annealing temperature of 65 °C was used for the *MAT-2* PCR and 67 °C for *MAT-1*.

Because the F_1 isolates were hybrids of *F. circinatum* and *F. subglutinans*, PCR–RFLP analysis of the histone H3 gene was used to distinguish between parental alleles (Steenkamp et al., 1999). This PCR–RFLP technique successfully determined the parental origin of the histone H3 alleles segregating in the F_1 isolates. The results of all these amplifications were scored as a '0' for band absent and '1' for band present.

2.5. Framework linkage map construction

χ^2 analysis was performed on all markers to test for departure from the expected Mendelian segregation ratio (1:1) using a significance threshold of $\alpha = 0.05$. All markers, including those showing transmission ratio distortion, were included in framework map construction in order to optimise map coverage.

Based on the origin of each marker, the data were separated into two parental data sets. Linkage analysis was performed on the separate and joint data sets to obtain separate parental framework linkage maps and a F_1 framework linkage map. A maternal map of the *F. circinatum* parent and a paternal map of the *F. subglutinans* parent were generated, representing the two linkage phases of the F_1 map. The parental (linkage phase) maps were constructed separately to increase the confidence of marker ordering in the F_1 map. Markers having greater than 10% missing data were dropped from the data sets before framework map construction in MAPMAKER Macintosh V2.0 (Lander et al., 1987). Data were regarded as F_2 backcross configuration to accurately analyse segregation in the haploid F_1 genomes (Xu and Leslie, 1996). The Kosambi mapping function was used. The haploid chromosome number of *F. subglutinans* is known ($n = 12$) (Xu et al., 1995).

This information was used when distributing markers into linkage groups by evaluating the LOD linkage thresholds from 6 to 14, in incremental steps of 1.0, by using the 'Group' command (Myburg et al., 2003). The parental marker sets was separated into at least 12 linkage groups at LOD thresholds of 9 and 10.

To select framework markers, markers in each linkage group were subjected to the 'First Order' command of MAPMAKER to attain a starting order. Using the 'Drop Marker' command, internal markers that expanded the map by more than 11 cM were dropped. After a marker had been dropped, the 'First Order' step was repeated. Using the 'Ripple' function, the support of the remaining markers was evaluated. Markers that did not have a LOD interval support of at least 1.5 were removed from the map. The 'First Order' step was again repeated after each marker was dropped. Finally the terminal markers were evaluated with the 'TwoPoint/LOD Table' command. Terminal markers that showed stronger pairwise linkage to internal markers than to adjacent markers were dropped (Myburg et al., 2003).

In order to combine the parental maps and construct a single integrated map (referred to herein as the "F_1 map"), marker presence/absence data from the maternal data set was recoded to indicate that band absent ('0') represented *F. circinatum* markers. The two data sets (the recoded maternal data set and the paternal data set) were then combined into one data set and map construction was performed using the MAPMAKER program as described above. Markers were distributed into linkage groups using the 'Group' command by evaluating the LOD linkage thresholds from 6 to 14, in incremental steps of 1.0. The mapping set was separated into 12 major linkage groups at a LOD threshold of 9. Higher thresholds were used for the 'Drop Marker' command and 'Ripple' functions, with markers expanding the map length by 10 cM and not having a LOD interval support of at least 3.0 being dropped respectively.

Data from the F_1 map were subjected to the Graphical GenoTyping program (GGT) (Van Berloo, 1999). This was used to inspect the distribution of crossovers for each chromosome. This program was also used to determine if any nonrecombinant or duplicate progeny was found to show that this interspecific cross was the product of a heterothallic event.

2.6. Bin mapping of accessory markers

AFLP markers that did not meet framework marker criteria were mapped to the framework map as accessory markers using the bin mapping function of the MapPop V1.0 program. This program places accessory markers into map intervals contained in a previously constructed high confidence framework map (Vision et al., 2000). Only markers with $P > 0.95$ were placed into framework intervals. MapPop does not allow for the placement of accessory markers outside the terminal framework markers of each linkage group. Thus, AFLP markers not placed with MapPop were assessed for linkage to terminal markers using the 'Two Point/LOD' function of MAPMAKER (Myburg et al., 2003). Markers showing any linkage to the terminal markers at LOD 3.0 were placed as terminal accessory markers.

2.7. Estimated genome coverage and length

The total genome length (L) of the F_1 map was estimated using the Hulbert estimate (Hulbert et al., 1988) as modified in method 3 of Chakravarti et al. (1991), giving $L = n(n-1)d/k$. Here n is the total number of markers, d is the map distance which corresponds to the LOD threshold at which linkage was determined (Z) and k is the number of markers linked at the LOD threshold of Z or greater. The linkage threshold of LOD 9.0 (Z) was used to estimate genome length as the mapping set was separated into 12 linkage groups at this threshold.

Theoretical map coverage was calculated using the formula $c = 1 - e^{-2dn/L}$, where c is the proportion of the genome within d cM of a framework marker, n is the number of framework markers in the map and L is the estimated genome length (Lange and Boehnke, 1982).

3. Results

3.1. DNA isolation and AFLP analysis

DNA was successfully isolated from the 94 selected F_1 individuals as well as from the parents of the interspecific cross between *F. circinatum* and *F. subglutinans*. AFLP analyses were performed on these individuals (Table 2). Five hundred and seventy-eight polymorphic AFLP markers were identified with an average of 45 polymorphisms per

primer combination. In total, 56% of AFLP fragments were polymorphic. One marker was dropped for linkage analysis as it had more than 10% missing data. Missing data was defined as bands that could not be scored with confidence due to local gel irregularities, weak amplification, etc. This represented only 1% of the final data set.

Five hundred and eighty-two markers (578 AFLP markers and four other genetic markers) were generated. The parent-specific alleles of the *MAT* and H3 marker loci were considered as four different markers for mapping purposes. Of the 582 markers, 50% deviated significantly from the expected 1:1 ratio for a haploid F_1 cross ($\alpha = 0.05$, Table 3). One hundred and seventy-nine markers (31%) differed at the 1% level of significance and 97 (17%) at the 0.1% level of significance (results not shown). Only 12 of the markers that showed transmission ratio distortion at $\alpha = 0.05$ were skewed towards the *F. circinatum* parent with the remainder being skewed towards the *F. subglutinans* parent. The number of markers generated from each parent (296 from *F. subglutinans* and 286 from *F. circinatum*) did not differ significantly ($P = 0.68$).

Table 3.

Summary of markers in the framework linkage maps

	F. subglutinans (paternal)	*F. circinatum* (maternal)	F_1 hybrid (combined)
Markers			
Total no. of markers	296	286	582
No. of markers showing transmission ratio distortion[a]	128 (43.2%)	164 (57.3%)	292 (50.2%)
Markers included in framework map	104 (35.1%)	148 (51.7%)	252 (43.3%)
No. of markers in framework map showing disortion[a]	54 (51.9%)	85 (57.4%)	139 (55.2%)

	F. subglutinans (paternal)	*F. circinatum* (maternal)	F$_1$ hybrid (combined)
No. of accessory markers[b]	159 (53.7%)	112 (39.2%)	271 (46.6%)
No. of markers not mapped	33 (11.1%)	26 (9.1%)	59 (10.1%)
Framework maps[c]			
12 linkage groups each			
Average linkage group size (cM)	138.7	131.3	231.2
Average framework marker spacing (cM)[d]	18.1	11.6	11.6
Observed map length (cM)[e]	1664.4	1575.5	2774.4
Physical distance per unit of recombination [f] (kb/cM)	32.5	34.4	19.5
Estimation of genome length			
Hulbert estimate of genome length (cM)			2331.7
Framework map coverage[g]			
Map coverage ($c \times 100\%$) at $d = 20$ cM			98.7%

	F. subglutinans (paternal)	*F. circinatum* (maternal)	F$_1$ hybrid (combined)
Map coverage ($c \times 100\%$) at $d = 10$ cM			88.5%

[a] 5% level of significance (α) used to determine the departure of markers from the expected ratio of 1:1 of a haploid cross.

[b] AFLP markers that were not placed in the framework maps were mapped to the framework map using the bin mapping function of MapPop. Terminal markers were placed using the 'Two Point/LOD' function of MAPMAKER.

[c] Distances are in centiMorgan (cM) Kosambi.

[d] Calculated by dividing the summed length of all the linkage groups by the number of framework marker intervals (number of framework markers minus the number of linkage groups).

[e] Based on the classical estimate of recombination (r).

[f] **Xu et al. (1995)** estimated the genome size of *F. subglutinans* to be 54.1 Mbp. This estimate of genome size was used to calculate the physical distance per unit of recombination.

[g] The Hulbert estimate of genome length was used to estimate the framework map coverage.

3.2. Framework linkage map construction

All markers, including those showing transmission ratio distortion, were evaluated during framework map construction using the criteria described before. Using MAPMAKER, mapping sets were separated into at least 12 major linkage groups at LOD thresholds of 9 and 10. Subsequent analyses were performed on the linkage groups obtained at the LOD threshold of 9.0, as at this threshold more markers were incorporated into the 12 major linkage groups.

Twelve linkage groups emerged for the two parental framework maps as well as the F$_1$ map (Fig. 1). This corresponds to the haploid chromosome number reported for *F. subglutinans* (Xu et al., 1995). Only 252 markers (43%) met our criteria for framework

markers in the F_1 map (Table 3). Less stringent framework marker criteria were subsequently used for construction of the parental (linkage phase) framework maps to ensure that all markers placed in the F_1 map were also present in the parental maps. The 252 markers in the F_1 map corresponded to 104 markers in the *F. subglutinans* parental map and 148 markers in the *F. circinatum* parental map.
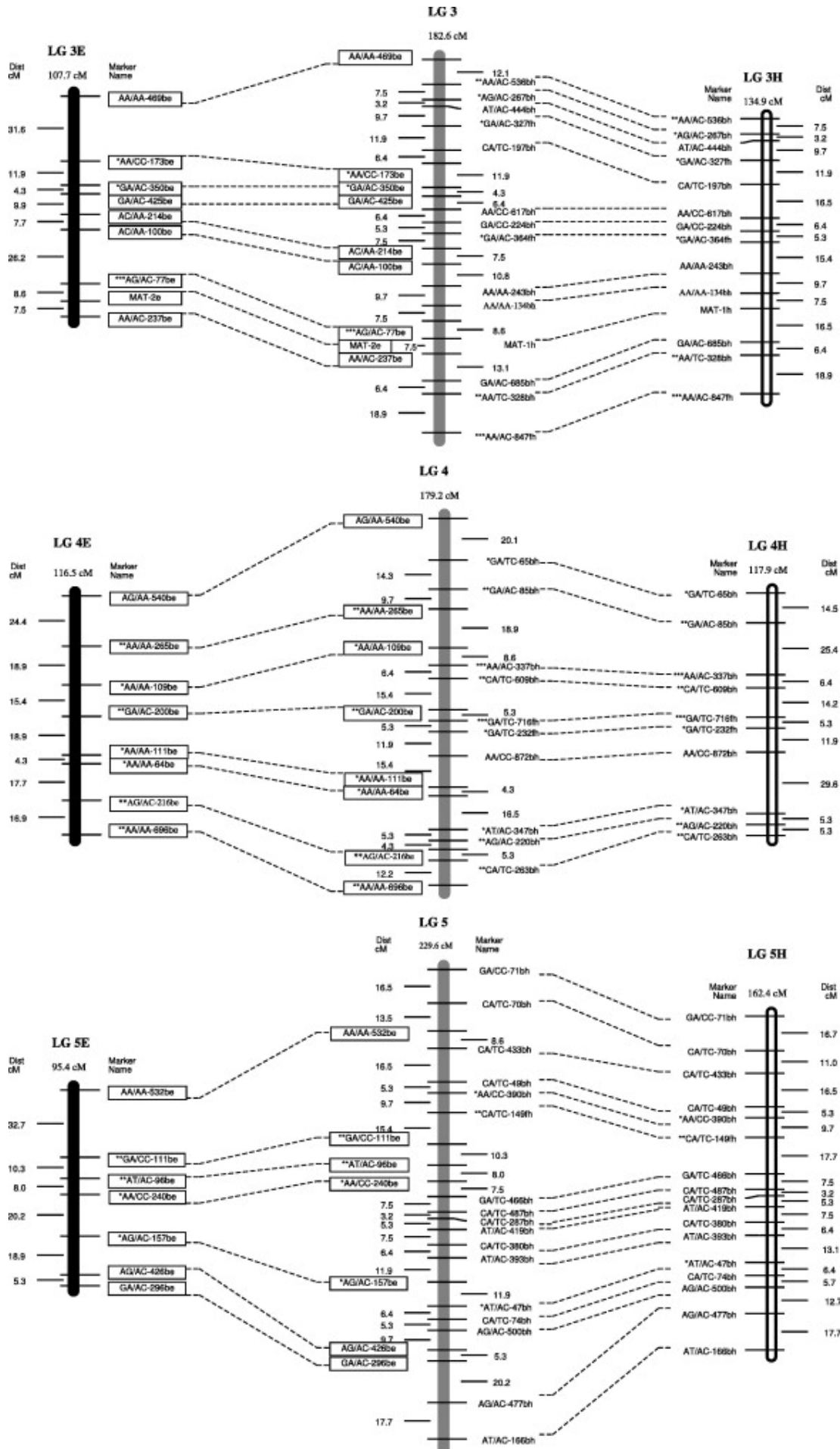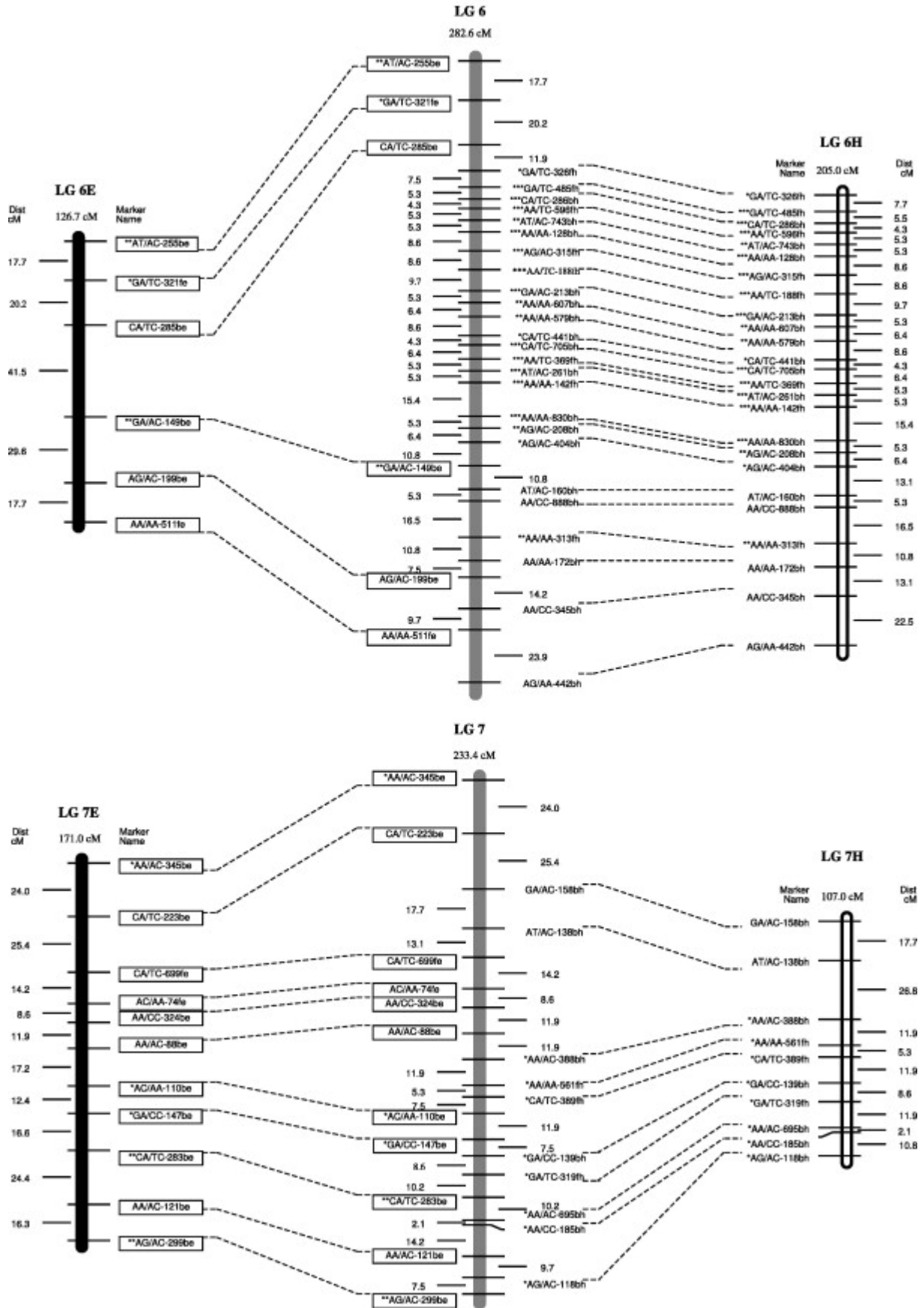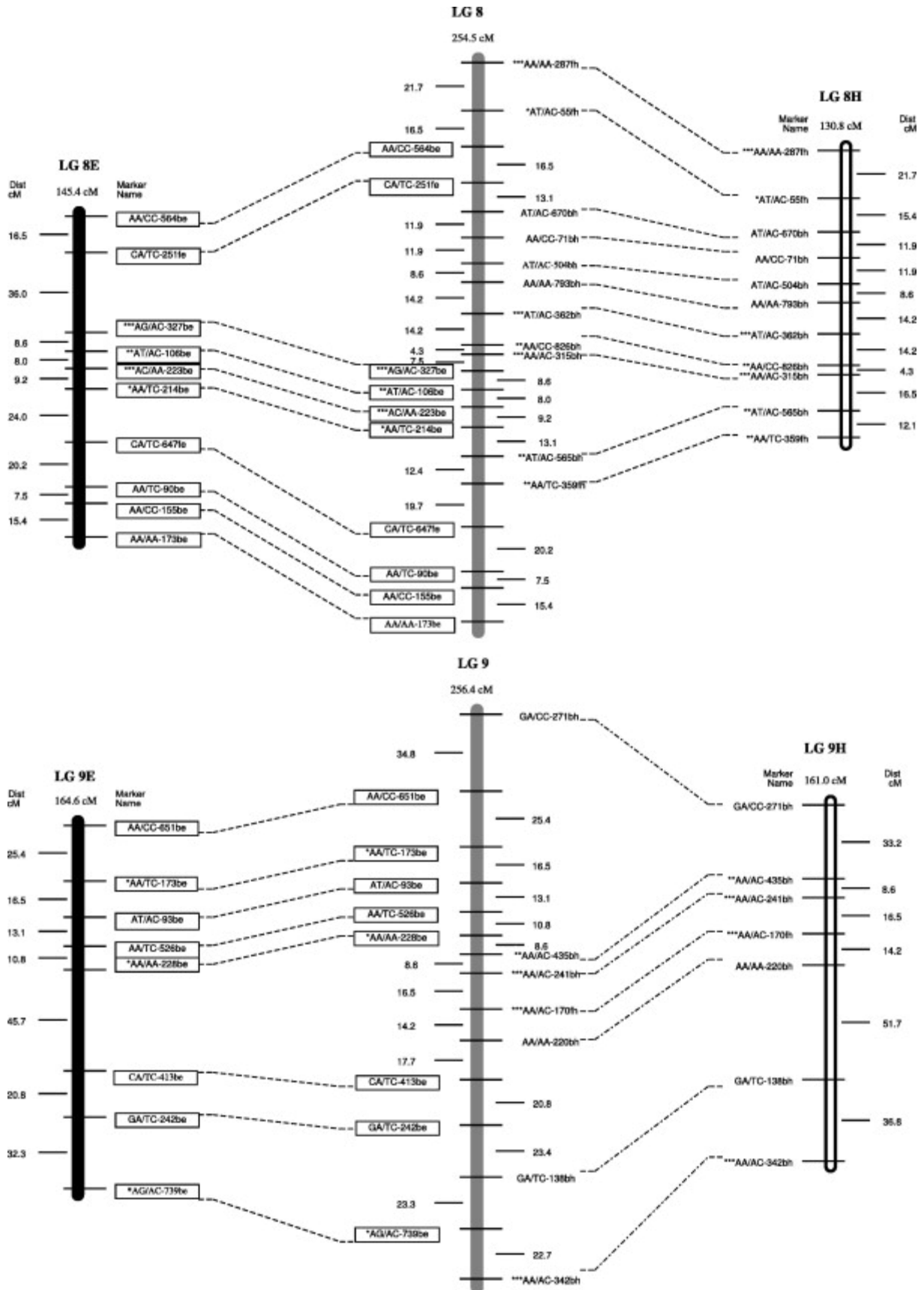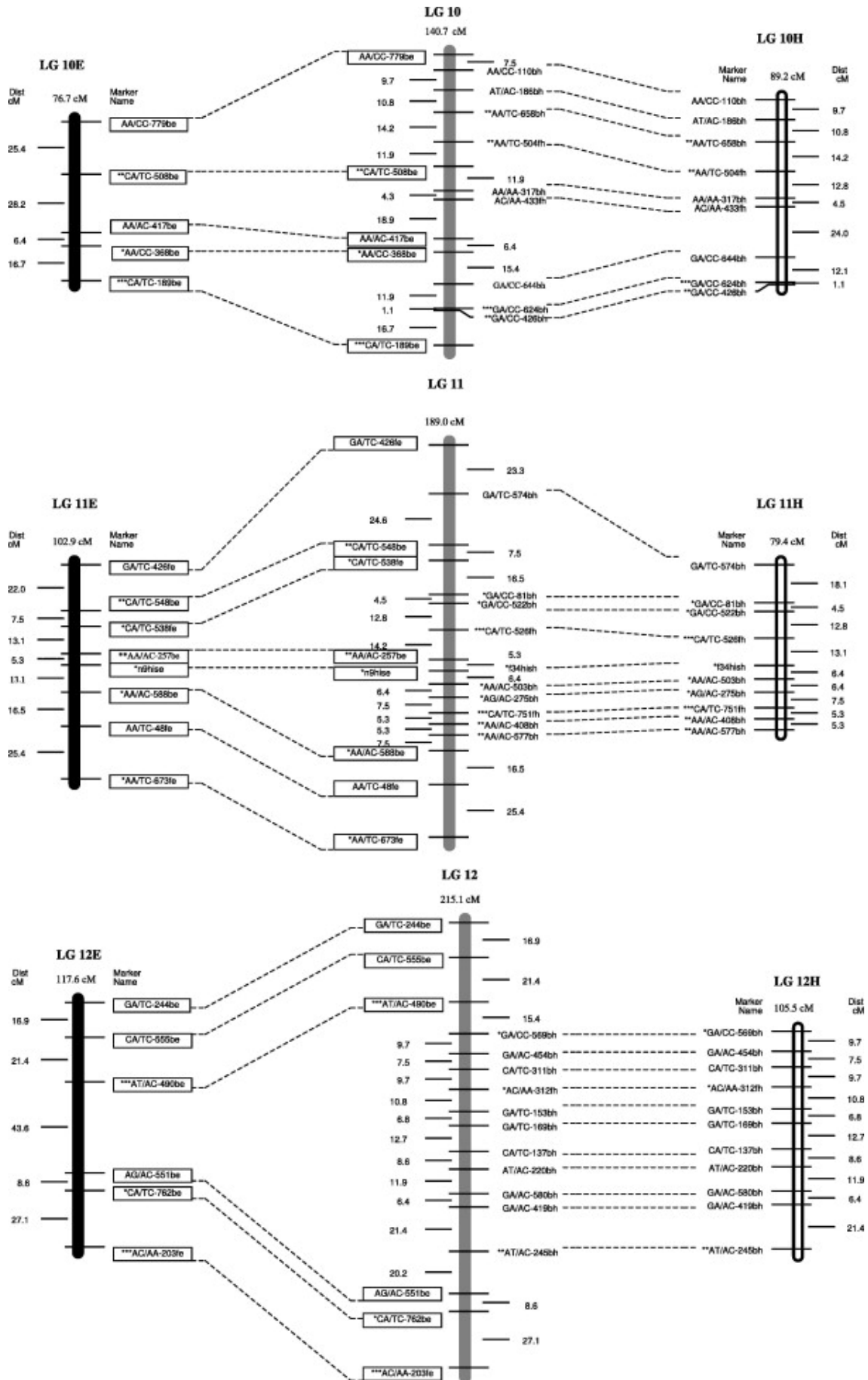
Fig. 1. Integrated framework maps of the cross between *F. subglutinans* and *F. circinatum*. Linkage group numbers followed by an 'E' indicate the *F. subglutinans* parental framework map and a 'H' the *F. circinatum* parental framework map. Bars shaded in black designate the *F. subglutinans* linkage map, those that are not shaded the *F. circinatum* linkage map and those that are shaded in grey the integrated F1 map of *F. subglutinans* and *F. circinatum*. Distances are given in centiMorgan (cM) Kosambi and the total map length of each linkage group is given at the top of each linkage group. Marker names consist of the *Mse*I selective nucleotides followed by the *Eco*RI selective nucleotides and the molecular size (bp), followed by a b (bright) or f (faint) indicating the quality of the fragment, and an 'e' and 'h' indicating markers originating from either *F. subglutinans* and *F. circinatum*, respectively. Marker names that are blocked originated from the *F. subglutinans* parent and unblocked from the *F. circinatum* parent. The dotted lines indicate those markers shared between maps. Markers exhibiting transmission ratio distortion are indicated with an asterisk ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < .001$).

Linkage groups in the F_1 map ranged in size from ~141 cM (Linkage Group 10) to ~ 358 cM (Linkage Group 1). The total observed length of the map was 2 774 cM and the average distance between markers was 12 cM. Significantly ($P = 0.041$) more markers from the *F. circinatum* parent were incorporated into the F_1 map than from the *F. subglutinans* parent (Table 3).

The *MAT* idiomorphs mapped to Linkage Group 3 (Fig. 1). Linkage Group 3, therefore, corresponds to chromosome 6 as previously reported for *F. verticillioides* (Xu and Leslie, 1996). The histone H3 gene mapped to linkage group 11 (Fig. 1).

Based on output from the Graphical GenoTyping (GGT) program, the estimated proportion of the genome of the F_1 progeny that was descended from *F. subglutinans* was 59.8% and from *F. circinatum* 39.7%, with 0.5% of the genome being unknown due to missing data that was scored as 'X'.

Using the GGT program, the number of progeny lacking any crossovers on each linkage group was determined (Table 4). Of the 12 linkage groups, Linkage Group 5 ($P < 0.05$) and 6 ($P < 0.001$) showed significant deviation from the expected 1:1 origin of markers. Both of these linkage groups had a substantial number of markers from *F. circinatum* (Table 4). This was also reflected in the fact that a significantly greater number of

markers were placed in the F_1 map from *F. circinatum*. F_1 progeny that received intact linkage groups tended to inherit these from the *F. subglutinans* parent, which was significant for Linkage Group 7 and 11 ($P < 0.05$), 1, 4, 8 and 12 ($P < 0.01$) and 10 ($P < 0.001$). No duplicate progeny was found, supporting the view that the interspecific cross forming the basis of this study was the product of a heterothallic event.

Table 4.

The number of F_1 individuals with parental types on each linkage group and the origin of framework markers in each linkage group

Linkage group	Intact parental linkage group[c]		Framework markers[f]	
	H[a]	**E**[b]	**H**[d]	**E**[e]
1	0	10**	12	16
2	6	13	12	10
3	9	19	14	9
4	4	17**	10	8
5	10	15	17*	7
6	6	11	25***	6
7	8	18*	10	11
8	4	19**	11	10
9	4	9	7	8
10	7	28***	9	5
11	10	24*	10	8
12	5	21**	11	6
Total	73	204***	148**	104

[a] Total number of intact linkage groups originating from *F. circinatum*.

[b] Total number of intact linkage groups originating from *F. subglutinans*.

[c] Significant deviation for progeny with an intact linkage group from each parent. Significant deviation is noted as follows: *5%, **1% and ***0.1%.

[d] Total number of framework markers originating from the *F. circinatum* parent.

[e] Total number of framework markers originating from the *F. subglutinans* parent.

[f] Significant deviation from the expected 1:1 marker frequency from each parent. Significant deviation is noted as follows: [*]5%, [**]1% and [***]0.1%.

3.3. Bin mapping of accessory markers

Using MapPop and MAPMAKER, 82% of the remaining markers were placed in the intervals between framework map markers as well as outside the terminal markers (Table 3). Approximately 10% of markers were not mapped to the framework maps. This is most likely due to scoring error or the markers being too distant from terminal markers ($\theta > 0.45$) to include them in the final map.

3.4. Estimated genome coverage and length

Estimation of genome length using the method of Hulbert ([Hulbert et al., 1988] and [Chakravarti et al., 1991]) showed that the Hulbert estimate was 16% lower than the observed map length for the F_1 map (Table 3). Using the Hulbert estimate of genome length, an estimated 99% of loci in the F_1 hybrid map were within 20 cM of a framework marker and an estimated 89% of loci were within 10 cM of a framework marker (Table 3).

4. Discussion

The F_1 progeny analysed in this study were the product of an interspecific cross between *F. circinatum* and *F. subglutinans*. Because these fungi are haploid, analysis of segregation patterns in F_1 progeny is similar to that of a backcross population in a diploid organism. No prior cloning or sequence data were required for the AFLP analyses and a large number of markers (average 45 markers produced per primer combination) were generated. Twelve linkage groups were found for the framework maps, which is consistent with the haploid chromosome number of *F. subglutinans* (Xu et al., 1995). The haploid chromosome number for *F. circinatum* is not known.

In this study, 582 polymorphic markers were generated and of these 252 were used to compile an F_1 framework linkage map. Two separate framework maps were also

generated for the parental strains of this interspecific cross in order to evaluate the stability of marker ordering and map distances in the parental (linkage phase) maps and the F_1 map. The ordering of markers in two different (mutually exclusive) sets allowed us to independently evaluate the possible local effects of specific marker combinations and their associated errors. As could be expected, the parental maps were generally shorter than the F_1 map due to lower map coverage. This is shown clearly in Linkage Group 1H, which has no markers originating from *F. circinatum* at the top end of the linkage group. The parental maps were also less inflated due to the presence of fewer markers. It has been shown previously that the addition of markers expands the length of linkage groups (Jurgenson et al., 2002b), which could be due to possible scoring error (Hackett and Broadfoot, 2003). A lower threshold was used in constructing the parental framework maps so that all markers present in the F_1 map could also be placed in the parental maps. When the same threshold was used for the parental and the F_1 maps, several markers could not be placed in the parental maps, especially in cases where map intervals became very large due to low map coverage.

The addition of the parental maps in this study has allowed us to compare the parental maps and the F_1 map. Even though a reasonable comparison could be drawn between them, significant differences do exist. Increasing the number of markers (as is the case with the F_1 map) provided better map coverage at linkage group terminals as well as increased statistical rigour to the framework linkage map.

Genetic maps have been published for other *Fusarium* species ([Xu and Leslie, 1996], [Jurgenson et al., 2002a], [Jurgenson et al., 2002b] and [Gale et al., 2005]). The genetic map of *F. verticillioides* had a total length of 1452 cM and a physical distance per unit of recombination of ~32 kb/cM (Xu and Leslie, 1996). Addition of AFLP markers to the existing RFLP map increased the map length to 2188 cM and the physical distance per unit of recombination decreased to ~21 kb/cM (Jurgenson et al., 2002b). The genetic map of *F. graminearum* had a map length of 1286 cM (Jurgenson et al., 2002a). However, phylogenetic evidence has subsequently shown that this map was based on an interspecific cross between *F. graminearum* and *Fusarium asiaticum* (O'Donnell et al., 2004). A genetic map of *F. graminearum* had a map length of 1234 cM (Gale et al., 2005). The F_1 map in this study had a map length of 2774 cM and the physical distance

per unit of recombination was ~20 kb/cM. Thus, the F_1 map produced in this study is consistent with previous published maps for *Fusarium* spp.

The observed map length for *F. subglutinans* in this study was only 5.6% larger than the *F. circinatum* map. This is despite the fact that there were 42.3% more framework markers included in the *F. circinatum* map. Conversely, approximately 60% of the F_1 genome was descended from the *F. subglutinans* parent. This is explained by the average spacing between framework markers being greater for *F. subglutinans* leading to a larger observed map length.

The two parental species, *F. circinatum* and *F. subglutinans,* shared 44% AFLP identity. Leslie et al. (2001) noted that, within the *Liseola* section of *Fusarium,* strains that share >65% band identity represent the same biological species. In contrast, those of different species usually share no more than 40% band identity, and often significantly less. Although the two parental strains used in this study represent discrete taxa, our results showed a higher level of band identity (44%) than isolates studied in other mapping studies using AFLP analysis for intraspecific crosses in *Fusarium* (Jurgenson et al., 2002a). Furthermore, in the map of an interspecific cross between *F. graminearum* and *F. asiaticum*, although not in the *Liseola* section of *Fusarium*, 50% band identity was observed between the two isolates used to construct the genetic map (Jurgenson et al., 2002a). In an interspecies cross between *G. fujikuroi* (mating population C) and *G. intermedia* (mating population D) band identity was approximately 50% ([Desjardins et al., 1997] and [Leslie et al., 2004b]). Although in separate mating populations, the authors hypothesized that these two species might be consolidated into a single species. Thus, genetic similarity as determined from the percentage band identity using AFLPs appears to be consistent with relationships inferred from phylogenetic analyses based on differences in DNA sequences. The two parental strains in this study are different species, but are more closely related than other members of the mating populations in the *Liseola* section of *Fusarium*, based on AFLP similarity.

Mendel's postulate of segregation dictates that during the formation of gametes, the paired unit factors segregate randomly so that each gamete receives one or the other with equal likelihood (Klug and Cummings, 1994). Zamir and Tadmor (1986) attributed transmission ratio distortion to linkage between markers and genetic factor(s) that affect

the fitness of gametes leading to unbalanced transmission of parental alleles to the next generation. In the present study, there was genome-wide selection for alleles of the *F. subglutinans* parent, with an estimated 59.8% of F_1 progeny genomes being received from the *F. subglutinans* parent. The F_1 progeny also showed a tendency to inherit intact parental linkage groups originating from the *F. subglutinans* parent (Table 4). Of the 292 markers that exhibited transmission ratio distortion, 96% were skewed towards the *F. subglutinans* parent. This interspecies cross therefore showed a clear bias towards the transmission of *F. subglutinans* alleles. We were not able to determine what proportion of the distorted loci exhibited epistatic interactions. However, 15% of the distorted markers exhibited a segregation ratio of approximately 3:1 ($P < 0.05$). In haploid organisms this is an indication of epistasis with two independent loci being involved in producing the phenotypic trait.

Marker loci exhibiting transmission ratio distortion suggest the presence of a distorting genetic factor in that region of the genome. However, it has also been shown that the greater the genetic divergence between the parental lines, the higher the levels of transmission ratio distortion ([Paterson et al., 1991] and [Grandillo and Tanksley, 1996]). In the present study, approximately 50% of the markers showed transmission ratio distortion. A more extreme case of transmission ratio distortion was reported in an interspecific cross between the domesticated tomato (*Lycopersicon esculentum*) and a wild relative (*Lycopersicon pennellii*), which showed 80% skewed segregation (DeVicente and Tanksley, 1993). Transmission ratio distortion ranging from 11 to 66% has also been reported in basidiomycetous fungi (*e.g.* [Larraya et al., 2000] and [Lind et al., 2005]) as well as in other ascomycetes (*e.g.* [Xu and Leslie, 1996], [Leslie et al., 2004b] and [Gale et al., 2005]). The authors of these studies have hypothesized that the distortion could be attributed to several factors such as bias in the collection of spores used for the mapping population ([Larraya et al., 2000] and [Lind et al., 2005]), error in scoring (Xu and Leslie, 1996), differential viability of certain ascospores (Xu and Leslie, 1996), or to structural rearrangements of chromosomes that may have caused distorted segregation patterns (Gale et al., 2005).

The *F. subglutinans* × *F. circinatum* cross showed a clear preferential inheritance of alleles as well as complete chromosomes from the *F. subglutinans* parent rather than

from the *F. circinatum* genome. This suggests a general fitness benefit for F_1 progeny that inherited *F. subglutinans* alleles. The reasons for this unidirectional distortion are not clear at present. It is possible that ascospores with *F. subglutinans* alleles were generally more viable on the specific crossing medium that was used, or that there were fewer negative interactions of *F. subglutinans* alleles with the hybrid genetic background. In this case, fitness selection would be due to the additive effects of individual genetic factors as mentioned previously. It is also possible that selection occurred in some cases against recombinant gametes because of co-evolved gene complexes that were broken up by recombination (Jurgenson et al., 2002a), but this would not explain the unidirectional bias observed.

Despite the use of the biological species concept in species delineation, interspecific crosses in *Fusarium* section *Liseola* have been reported previously. *Fusarium fujikuroi* (mating population C) and *Fusarium proliferatum* (mating population D) are defined as being different biological species, yet a few isolates have been shown to be sexually compatible ([Desjardins et al., 1997] and [Leslie et al., 2004b]), indicating the limitations of the biological species concept in the *Liseola* section of *Fusarium*. A naturally occurring hybrid has also been identified (Leslie et al., 2004a). One hypothesis to explain the presence of a naturally occurring hybrid is that of a hybrid swarm, possibly being geographically separated or occurring on a specific host. The standard tester strains are represented by distinct species, but a hybrid swarm might naturally exist between *F. fujikuroi* and *F. proliferatum* (Leslie et al., 2004b). The authors hypothesized that these two species might be consolidated into a single species. It may also be possible that the two species are in the final stages of speciation with some individuals in each species still being able to overcome crossing barriers. In the present study, isolates representing mating populations E and H of *G. fujikuroi* are phylogenetically closely related ([O'Donnell and Cigelnik, 1997] and [O'Donnell et al., 1998]), but are less similar to each other than mating populations C and D are to each other (Steenkamp et al., 2001). It is also important to highlight the fact that progeny used in this study were obtained from a laboratory cross rather from a natural cross and that only one isolate of *F. subglutinans* has been found to cross to one isolate of *F. circinatum*. The absence of host-specific

factors in the laboratory cross may have helped to overcome natural crossing barriers between these two species.

The framework map generated in this study will be used to identify QTLs for important quantitative traits such as pathogenicity in *F. circinatum*, which is an economically important pine tree pathogen. In a previous study (Friel et al., 2002), F_1 progeny of this same cross were found to be avirulent on pine trees. However, a backcross population involving a single F_1 individual crossed to the *F. circinatum* parental strain exhibited a wide range of virulence. We have constructed a similar backcross (unpublished results). This experimental population would be useful to identify QTLs associated with pathogenicity in the *F. circinatum* and *F. subglutinans* genomes and will aid us in gaining a better understanding of the genetic basis of *F. circinatum* virulence on *Pinus* species. It is reasonable to expect that there would be significant synteny between the genomes of *F. subglutinans* and *F. circinatum*, and that of *F. verticillioides*, which is completely sequenced and publicly available (http://www.broad.mit.edu). These resources should facilitate further investigation into the genetic determinants of pathogenicity of the pitch canker fungus.

The framework linkage map generated in this study will provide a means for studying genetic architecture of crossing barriers between *F. subglutinans* and *F. circinatum*. This will be possible with a more in depth analysis of the genome-wide pattern of segregation distortion observed in this mapping study. As mentioned, the genomic sequence of *F. verticillioides* is available and this genome is comparable to that of *F. subglutinans* in that there are also 12 chromosomes and the genome size is similar (Xu and Leslie, 1995). By sequencing selected AFLP framework markers generated in this study and finding a matching sequence in the *F. verticillioides* genome, we should be able to link the 12 linkage groups of this study to the *F. verticillioides* chromosomes. Putative hybrid fitness loci and pathogenesis-related QTLs could be fine-mapped and possibly identified by studying the corresponding genomic regions in the *F. verticillioides* genome.

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