European Journal of Plant Pathology (2006) 114: 17–31
DOI 10.1007/s10658-005-3168-6

Genetic linkage maps and genomic organization in *Leptosphaeria maculans*

Marie-Line Kuhn[1], Lilian Gout[1,2], Barbara J. Howlett[3], Delphine Melayah[1,4], Michel Meyer[1], Marie-Hélène Balesdent[1] and Thierry Rouxel[1,*]
[1]*INRA-PMDV, Route de St Cyr, 78026 , Versailles Cedex, France;* [2]*Protection des Plantes, INA-PG, F-78850, Thiverval-Grignon, France;* [3]*School of Botany, The University of Melbourne, 3010, Victoria, Australia;* [4]*Laboratoire d'Ecologie Microbienne, Université Claude Bernard Lyon 1, 69622, Villeurbane cedex, France; *Author for correspondence (Phone: +33-130833229; Fax: +33-130833195; E-mail: rouxel@versailles.inra.fr)*

Accepted 6 September 2005

Key words: chromosome length polymorphism, genetic map, genome structure, physical map, repetitive DNA, retrotransposons

Abstract

Leptosphaeria maculans is a haploid outcrossing ascomycete with a genome size of about 34 Megabases (Mb) which is predicted to have between 10,000 and 12,000 genes. The chromosomes of *L. maculans* are of a size range (0.7–3.5 Mb) and number (15–16) that can be readily resolved by pulsed field gel electrophoresis. Chromosome length polymorphisms are generated by meiosis giving rise to size differences as high as 57%, in the case of the ribosomal DNA-harbouring chromosome whose size varies between 1.8 and 4.2 Mb. Genetic maps are characterised by linkage groups comprising an accumulation of markers based on retrotransposon sequences. This, along with sequencing of pericentromeric regions and stretches of ORF-rich regions, suggest that the genome of *L. maculans* consists of a mosaic of GC-equilibrated coding regions with no or few transposons, and of stretches of highly degenerated and truncated retrotransposons, encompassing very few genes. Chromosome length polymorphisms are linked with the loss of dispensable regions. We suggest that the degree of length polymorphism for a particular chromosome correlates to the amount of dispensable retrotransposons, and that some gene-rich chromosomes may be less prone to undergo chromosome length polymorphisms than other chromosomes.

Abbreviations: AFLP – amplified fragment length polymorphism; BAC – bacterial artificial chromosome; CHEF – contour-clamped homogeneous electric field; CLP – chromosome length polymorphism; ISSR – inter-simple sequence repeat; LG – linkage group; LTR – long terminal repeats; RAPD – random amplified polymorphic DNA; Rep-PCR – repetitive element-based PCR; RIP – repeat induced point mutation; TAFE – transverse alternating field electrophoresis

Introduction

Leptosphaeria maculans is an ascomycete fungus, which has historically been classified as a Loculoascomycete, a class comprising over 6000 species (Silva-Hanlin and Hanlin, 1999). However, taxonomic revisions now assign *L. maculans* to the largest order of the Dothideomycetes class (only partly corresponding to the Loculoascomycete class), the Pleosporales (Liew et al., 2000). Pleosporales also encompass other important plant pathogens such as the closely related *Phaeosphaeria (Stagonospora) nodorum* (formerly known as *Leptosphaeria nodorum)*, and other genera such as *Cochliobolus*, *Pleospora*, *Alternaria*, *Venturia* and *Pyrenophora* (Berbee, 2001).

Due to the incidence of phoma stem canker worldwide (Fitt et al., 2005) and the experimental

tractability of *L. maculans*, major efforts in understanding the epidemiology and the population genetics of the fungus, and its interaction with the host have been recently undertaken (Balesdent et al., 2002; Delourme et al., 2004; Howlett, 2004; Aubertot et al., 2005; Delourme et al., 2005; Fitt et al., 2005). In addition *L. maculans* exemplifies Dothideomycete infection strategies and has characteristics in its life traits, including pathogenicity, that render it an excellent model to address numerous issues related to pathogen evolution, pathogenicity and host specificity. The importance of sexual recombination in the wild also renders *L. maculans* an appropriate model for studying molecular evolution of genes submitted to selection pressure (avirulence genes, fungicide resistance genes) (Rouxel and Balesdent, 2005).

Numerous tools have been developed that will allow the *L. maculans* scientific community to enter the 'genomic era' through the use of a gene-by-gene approach. These include efficient transformation techniques, large collections of segregating populations (including tetrads), genetic maps, Bacterial Artificial Chromosomes (BACs) and cosmid libraries, Expressed Sequence Tag libraries, including subtractive libraries of the plant pathogen interaction, and collections of strains tagged with selectable markers that can mutate genes (for a review see Rouxel and Balesdent, 2005). However, to date, like other Dothideomycetes, the genome structure of *L. maculans* is poorly characterised. The genome size of the fungus is about 34 Megabases (Mb) and this is predicted to contain between 10,000 and 12,000 genes (Cozijnsen et al., 2000). The chromosomes of *L. maculans* are of a size range (0.7–3.5 Mb) and number (15–16) that can be readily resolved by pulsed field gel electrophoresis (Howlett et al., 2001).

This paper presents our current knowledge on the genome of *L. maculans* focusing on structural features that can be deduced from (i) genetic maps (ii) physical maps built by hybridisation of probes to electrokaryotypes, and (iii) sequence data on clusters of coding regions or repeats. Our data strongly suggest that, at least for some chromosomes, the genome *of L. maculans* encompasses mosaics of large coding regions and large dispensable zones, which may be responsible for chromosome length polymorphisms generated following meiosis.

Materials and methods

Fungal isolates and in vitro crosses

Three crosses were used to build genetic maps at INRA, Versailles. Cross #11 between two French field isolates (a.2 and H5) heterozygous at the avirulence *AvrLm1* locus, and cross #23 between two isolates (v11.3.6, a progeny of the a.2 × H5 *in vitro* cross, and 21.3.1, a progeny of a cross between a German field isolate and a French lab. isolate) heterozygous at the *AvrLm1* and *AvrLm4* loci, were described by Attard et al. (2002). Cross #30 between v23.1.2 (a progeny of the v11.3.6 × 21.3.1 cross displaying the *AvrLm7* allele) and the New Zealand field isolate Nzt 4 (virulent for this locus) was described by Balesdent et al. (2002). Maps 11, 23 and 30 were constructed from 98, 42 and 46 F_1 progeny, respectively. Isolates were cultured and maintained as previously described (Ansan-Melayah et al., 1995; Attard et al., 2002).

Markers used to generate the genetic maps

Except for biological (mating type) and phytopathological (host-specificity, i.e., avirulence) loci, markers were mostly anonymous and PCR-based (Table 1). These encompass RAPD (Ansan-Melayah et al., 1995), ISSR, e.g., markers designed to amplify between microsatellites (West et al., 2002), AFLP, and Rep-PCR markers (Jedryczka et al., 1999), i.e., mostly markers generated by using the sequence polymorphism of the Long Terminal Repeat (LTR) retrotransposon *Pholy* (Attard et al., 2005) to amplify between tandem truncated copies of *Pholy* (Jedryczka et al., 1999). Protocols for generation, separation and visualization of RAPD, ISSR and Rep-PCR are described in Ansan-Melayah et al. (1995), West et al. (2002), and Jedryczka et al. (1999), respectively.

AFLP analysis was performed according to Vos et al. (1995) with slight modifications and amplified products run on acrylamide gels. Genomic DNA (approx. 500 ng) from parents and F_1 progeny was digested with 5 U of *Mse*I (Invitrogen) (30 min digestion at 37 °C), followed by a digestion with 10 U of *Eco*RI (Invitrogen) (overnight digestion at 37 °C). Adaptors were designed and ligated to the restriction fragments according

Table 1. Characteristics of three genetic maps of *Leptosphaeria maculans* developed at INRA, Versailles

Cross number	11	23	30
Total number of markers in the map, including	443	177	266
RAPD	102	6	4
ISSR	48	6	29
Rep-PCR	35	0	1
Mini- and microsatellites	3	0	2
AFLP	235	163	229
Others	20	2	1
Major linkage groups			
More than 20 cM	41 (3–13 markers)	7 (4–9 markers)	7 (4–9 markers)
More than 6 markers	20	4	7
Doublets	17	19	27
Unlinked markers	76 (17.1%)	60 (33.9%)	54 (20.3%)
Total number of linkage groups	78	35	61
Map coverage (cM)	2129	430	867
Average physical/genetic distance (kb/cM) [a]	16	79	39

[a]As estimated on the basis of a 34 Mb genome size (Cozijnsen et al., 2000).

to Vos et al. (1995). A 3 μl part of the mix was first amplified with a +1-*Mse*I primer and a +1-*Eco*RI primer, i.e., primers with one additional selective nucleotide, followed by a second round of selective amplification using *Eco*RI + 1 and *Mse*I + 2 primer combination. In accordance with findings of Cozjinsen et al. (2000) this combination gave rise to more resolvable bands per lane than the other combinations and consequently more polymorphic bands between parents. Amplification took place in a 20 μl volume with 1 μM of each primer, 0.1 μM of each dNTP, 2 mM $MgCl_2$, 2 μl 10× Taq buffer and 1.25 U Taq Polymerase (Applied Biosystems). Amplification took place in a PE-9600 Thermal Cycler (Applied Biosystems). Cycling conditions were as follows: first round of amplification: cycle 1–20; 1 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C, with 0.01 ramp. Selective amplification: cycle 1; 2 min at 94 °C, 30 s at 65 °C, 2 min at 72 °C. Cycle 2–9; 1 s at 94 °C, 30 s at 64 °C with a progressive drop of 1 °C annealing temperature in each cycle, 2 min at 72 °C. Cycle 10–32; 1 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C. Two microlitres of the amplification products were separated in a CastAway Precast 5.5% Long Ranger acrylamide gel (Stratagene) and electrophoresed in a CastAway Sequencing Device (Stratagene) according to the manufacturer's recommendations. After electrophoresis the gel was stained with silver nitrate as described by Chalhoub et al. (1997).

Genetic map construction

Polymorphic bands were recorded as present/absent in the progeny and monogenic segregation was checked with a Chi-squared test ($P = 0.05$). Monogenic markers were submitted to multipoint linkage analysis using the Mapmaker/Exp 3.0 software (Lander et al., 1987) set for F_2 backcross segregating population, with log of the likelihood ratio (LOD) value of 3.0, a maximum distance of 20 cM. Linkage groups were drawn with the Mappit version 1.3 software (L. Gianfranschi and B. Koller, Swiss Federal Institute of Technology, http://www.pa. ipw.agrl.ethz.ch/).

Cloning and characterization of markers

AFLP bands were isolated from the acrylamide gels as described by Chalhoub et al. (1997) and fragments ligated into vector pGEM-T Easy (Promega). Cloning and sequencing was as described previously (Attard et al., 2001). Minisatellites were obtained as described by Eckert et al. (2005). Chromosomal DNA was prepared according to Plummer and Howlett (1995) and resolved by contour-clamped homogeneous electric fields (CHEF) electrophoresis using the conditions described by Morales et al. (1993). Chromosomal DNA was vacuum blotted on nylon N^+ membranes and hybridized as described previously (Leclair et al., 1996).

Results and discussion

Genetic linkage maps of L.maculans

Genetic linkage maps have been developed for *L. maculans* by Pongam et al. (1998), Cozijnsen et al. 2000) and by the INRA-PMDV group (this study). The more detailed one is presented in Figure 1. The map developed by Pongam et al. (1998) comprised 56 AFLP markers on 67 progeny from a cross between the Australian isolate PHW1223 and the French isolate PHW1245 (IBCN74) that segregated for an avirulence gene that the authors named *alm1*. This gene probably corresponds to *AvrLm4* (Delourme et al., 2004). Nine linkage groups and five pairs of markers were assigned with a total genome size of 340 cM and an average distance between loci of 6.1 cM. Cozijnsen et al. (2000) constructed a genetic map comprising 155 AFLP markers, three RAPD markers, the mating

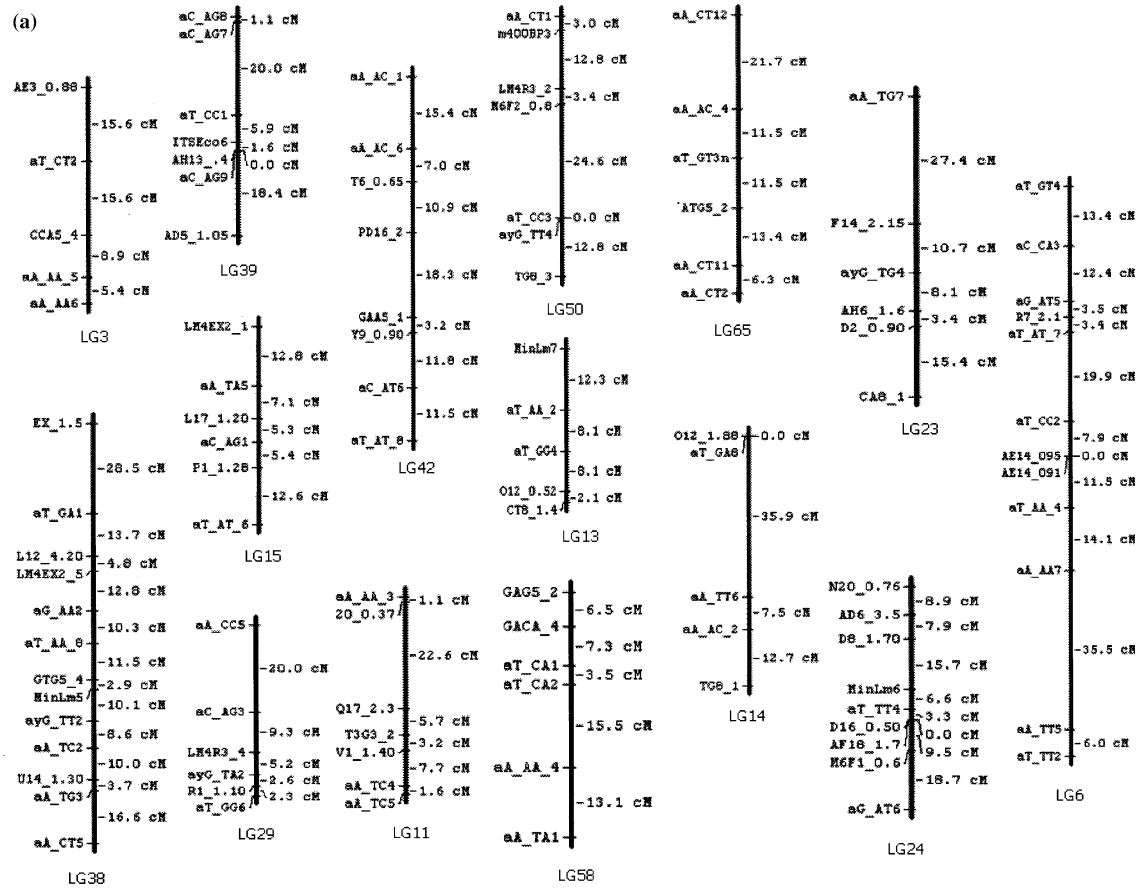

Figure 1. The linkage map of *Leptosphaeria maculans* cross #11. (a) shows the major linkage groups (LG) (5 markers or more, and more than 30 cM) (b) shows LG harbouring 5 markers or more, with accumulation of markers over a small genetic distance. The map was derived by analysis with Mapmaker/Exp 3.0 software set for F_2 backcross segregating population (LOD = 3.0, maximum distance = 20 cM). The marker names are shown to the left of the LG and the distance between markers (cM) is indicated on the right of the LG. AFLP markers are indicated with an 'a' followed by the additional 3' nucleotides of the primer combination (e.g., A_TA) and the number of the polymorphic band on the AFLP gel. ISSR markers are indicated as the name of the repeat used as primer (e.g., TG8 or GAG5) followed by the number of the polymorphic band on the agarose gel. RAPD markers are indicated as the name of the primer (as per Operon Technology; e.g., L12, U14 or AH13) followed by the size (kb) of the polymorphic band. Rep-PCR markers are indicated in the FxRy form (some are LmxEXy or LmxRy) followed by the size (kb) of the marker band. MAT denotes the mating type locus, AvrLmx corresponds to avirulence loci, ITSEco6 to the rDNA locus, and MinLmx to minisatellite loci.

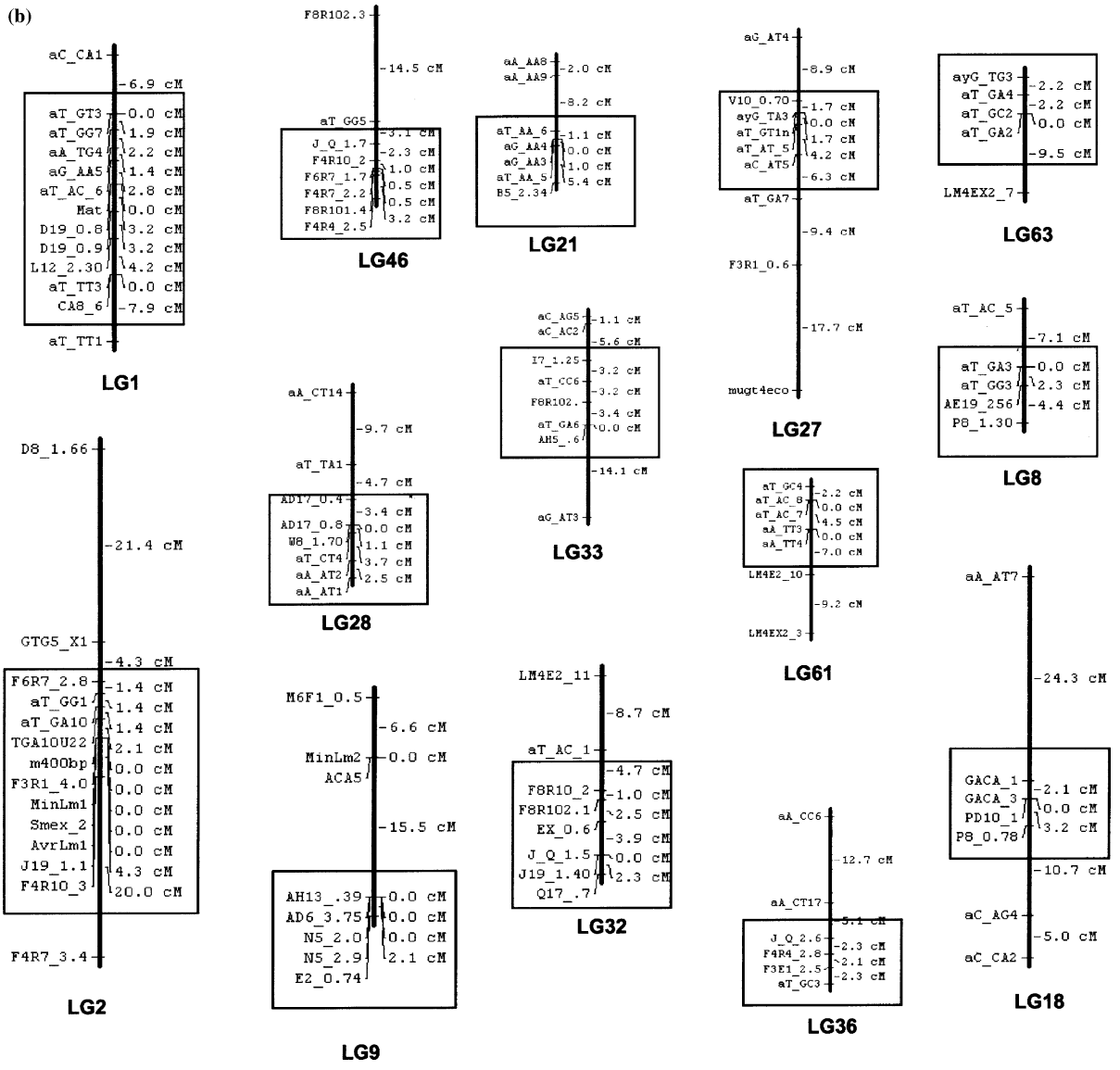

Figure 1. (Continued).

type locus, and a *B. juncea* host specificity locus. The cross was between Australian isolates C13 and M1 (IBCN17 and 18, respectively) (Figure 2) and the mapping was performed on 58 F_1 progeny. Twenty-one linkage groups, five pairs, and 18 unlinked markers were assigned, and the coverage was 1520 cM.

The *L. maculans* INRA maps comprised between 177 and 443 markers, mainly including anonymous PCR-based markers (Table 1). The most detailed map (from cross #11) was obtained using a F_1 segregating population of 98 progeny. This map had 15 major groups (comprising 5 markers or more, and covering more than 30 cM) (Figure 1a). Its overall coverage (2129 cM), and physical to genetic ratio is comparable to the most detailed linkage maps currently available for other fungal species (Table 2). However, 14 additional groups that could be considered as major groups in terms of number of markers, were characterised by an accumulation of markers in a very small genetic distance (Figure 1b). This was also the case for the less detailed genetic maps developed from crosses #23 and #30, with each map showing seven linkage

groups again with several markers in a small genetic distance (data not shown). The mating-type locus, *MAT*, and the avirulence *AvrLm1* locus mapped to these linkage groups (Figure 1b).

Our data indicate that the other two avirulence loci *AvrLm4* and *AvrLm7* mapped in crosses #23 and #30 are also in a region with many markers in a small distance (Figure 3). Such a location of avirulence/virulence loci in linkage groups with many markers in a small genetic distance, and/or clustering of avirulence loci so that they form a separated linkage group, seems to be a usual feature of fungal genetic maps, as noted for *Blumeria graminis* (Pedersen et al., 2002) and for the two Dothideomycetes, *Mycosphaerella graminicola* (Kema et al., 2002) and *Cochliobolus sativus* (Zhong et al., 2002). In addition, for our *L. maculans* maps, there was a difference between these two types of linkage groups in terms of the major markers that defined them. Groups that had many markers over a small genetic distance were rich in Rep-PCR markers, i.e. markers based on the *Pholy* retrotransposon (23 Rep-PCR markers out of 113), whereas these markers were almost absent from the major linkage groups displayed on Figure 1a (5 markers out of 108). The opposite situation was observed for ISSR markers (10.2% in the major groups compared to 4.4% in groups showing accumulation of markers) (Figure 1). This difference was not observed for AFLP

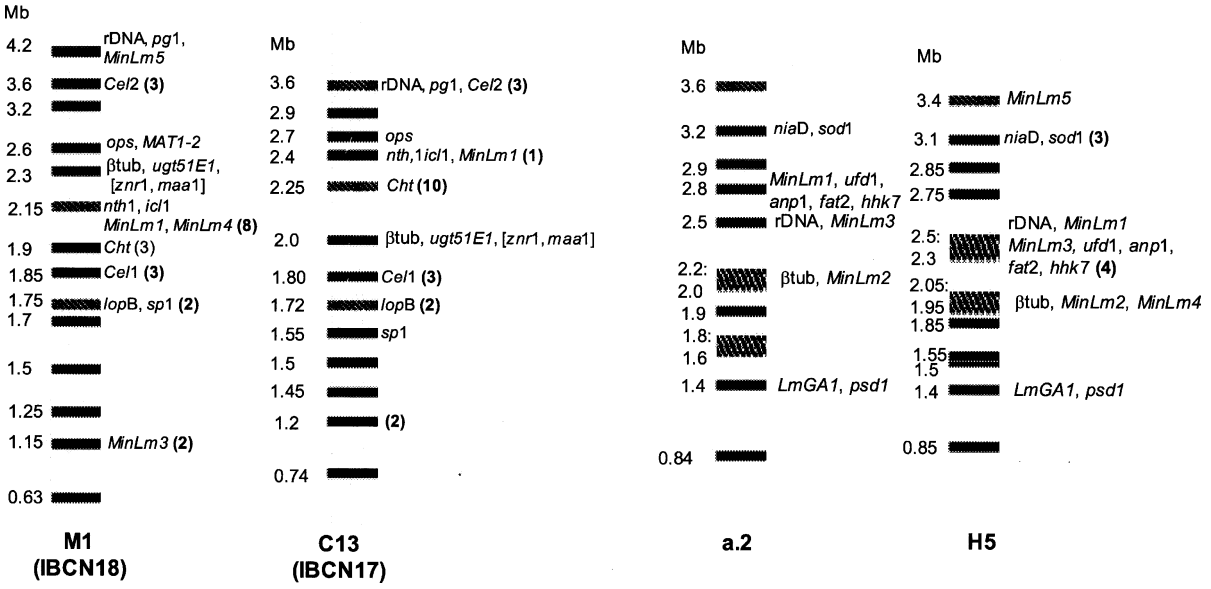

Figure 2. Schematic diagram of chromosomes of isolates M1, C13, a.2 and H5 showing estimated sizes (Mb) and location of probes corresponding to identified genes or minisatellites of *L. maculans*. The pattern is a synthetic representation based on separations done on gels run under several conditions to resolve the whole karyotype. In addition, the number of cloned markers originating from the genetic maps and allocated to a given chromosome is indicated in bold in parentheses. Bands that stain brightly with ethidium bromide (indicated by diagonal striping) probably comprise more than one chromosome. The smallest chromosome (sized 0.63–0.85 Mb) is thought to be a dispensable 'B' chromosome (Leclair et al., 1996), with no single-copy sequence detected to date. Probes used for hybridisation are: *MinLm1-5*, minisatellite loci (Eckert et al., 2005); *LmGA1*, G protein alpha subunit, physically linked to *psd1*, phosphatidyl serine decarboxylase; *sod1*, superoxide dismutase 1; *ufd1*, ubiquitin fusion degradation protein 1; *anp1*, similar to ANP1 protein; *fat2*, fatty acid transporter 2; *hhk7*, histidine kinase 7 (this study); βtub, β-tubulin (Cozijnsen et al., 2000; this study); rDNA, ribosomal DNA (Howlett et al., 1997; this study); *niaD*, nitrate reductase, physically linked to *niiA*, nitrite reductase (Williams et al., 1994, 1995; this study); *pg1;* polygalacturonase 1; *Cel1*, cellulase 1; *Cel2*, cellulase 2 (Sexton et al., 2000)*; ops*, opsin (Idnurm and Howlett, 2001); *MAT1–2*, mating-type locus, physically linked to *GAP*, GTPase activating protein, and *DNA-L*, a DNA-Lyase homologue (Cozijnsen and Howlett, 2003); *icl1*, isocitrate lyase physically linked to *prs5*, ribose phosphate pyrophosphokinase (Idnurm and Howlett, 2002); *znr1* and *maa1*, physically linked to multidrug facilitator 1 (*mfs1*), elongation factor (*ef1b*), heat shock protein (*hsp78*), DNA mismatch repair protein (*msh5*) (Idnurm et al., 2003a); *nth1*, neutral trehalase 1, physically linked to *ade3*, C1 tetrahydrofolate synthase; *ugt51E1*, UDP-glucose:sterol glucosyltransferase, physically linked to MAP kinase (Idnurm et al., 2003b); *Cht*, cyanide hydratase (Sexton and Howlett, 2000); *lop-B*, loss of pathogenicity B (Idnurm and Howlett, 2003); *sp1*, secreted protein 1, physically linked to *chp1*, conserved hypothetical protein (Wilson et al., 2002). Minisatellites *MinLm1*, *MinLm2*, and *MinLm5* map to LG2, LG9 and LG38 of the cross #11 genetic map, respectively (see Figure 1).

Table 2. General features of the genetic linkage maps constructed for fungal species using anonymous molecular marker[a]

Fungal species	Progeny[b]	Markers[b]	Type of Markers[c]	Chromosomes	Major LG	Accumulation of markers in LGs	Physical map[d]	CLP[e]	Map coverage (cM)	Physical/genetic Ratio (kb/cM)	References[f]
Basidiomycetes											
Cryptococcus neoformans var *neoformans*	100	181	AFLP/RAPD	13	14	Limited	Yes	Yes	1917	13.6	(1)
Coprinus cinereus	94	289	RFLP/SSR	14	20	Yes	Yes	Yes	1536	13.2	(2)
Pleurotus ostreatus	40	256	RAPD/RFLP	13	13	Yes	Yes	Yes	1346	27.9	(3)
Ascomycetes: Sordariomycetes	80	189	RAPD/RFLP	11	11	No	Yes	Yes	1001	35.1	(4)
Magnaporthe grisea	61	254	RFLP/Rep/SSR	7	7	Yes	Yes	?	900	44.4	(5,6)
Gibberella zeae	99	1052	AFLP	4	9	Yes	No	?	1300	30.7	(7,8)
Gibberella moniliformis	121	636	AFLP/RFLP	12	12	Yes	Yes	?	2188	21	(9)
Ascomycetes: Leotiomycetes											
Blumeria graminis	81	359	AFLP/RFLP/SNP/Rep	7 or 8	34	Yes	No	?	2114	*c*. 19	(10)
Ascomycetes: Dothideomycetes											
Mycosphaerella graminicola	61	282	AFLP/RAPD	17–18	23	Yes	Limited	Yes	1216	28	(11)
Cochliobolus heterostrophus	91	120	RFLP	15–16	26	No	Yes	Yes	1505	23	(12)
Cochliobolus sativus	104	134	AFLP/RFLP	15	27	Yes	Yes	Yes	1329	25	(13)

[a]'Founding' fungal species whose extremely saturated maps encompass hundreds of genes initially identified by phenotypes are not included in this Table. These include *Saccharomyces cerevisiae*, *Neurospora crassa* and, to a lesser extent *Aspergillus nidulans*.
Information and links to the genetic maps of these species are at http://www.broad.mit.edu/annotation/fungi/and http://www.yeastgenome.org/
[b]Number of progeny and markers used to construct the genetic map.
[c]Major types of markers used to generate the map.
[d]Hybridization of markers to electrokaryotypes and assignment of LG to given chromosomes.
[e]Existence of large Chromosome Length Polymorphisms (CLPs) in the fungal species.
[f]References, (1) Forche et al. (2000); (2) Marra et al. (2004); (3) Muraguchi et al. (2003); (4) Larraya et al. (2000); (5) Nitta et al. (1997); (6) http://www.broad.mit.edu/annotation/fungi/magnaporthe/; (7) Jurgenson et al. (2002a); (8) description of another *G. zeae* map is at http://www.broad.mit.edu/annotation/fungi/fusarium; (9) Jurgenson et al. (2002b); (10) Pedersen et al. (2002); (11) Kema et al. (2002), (12) Tzeng et al. (1992); (13) Zhong et al. (2002).

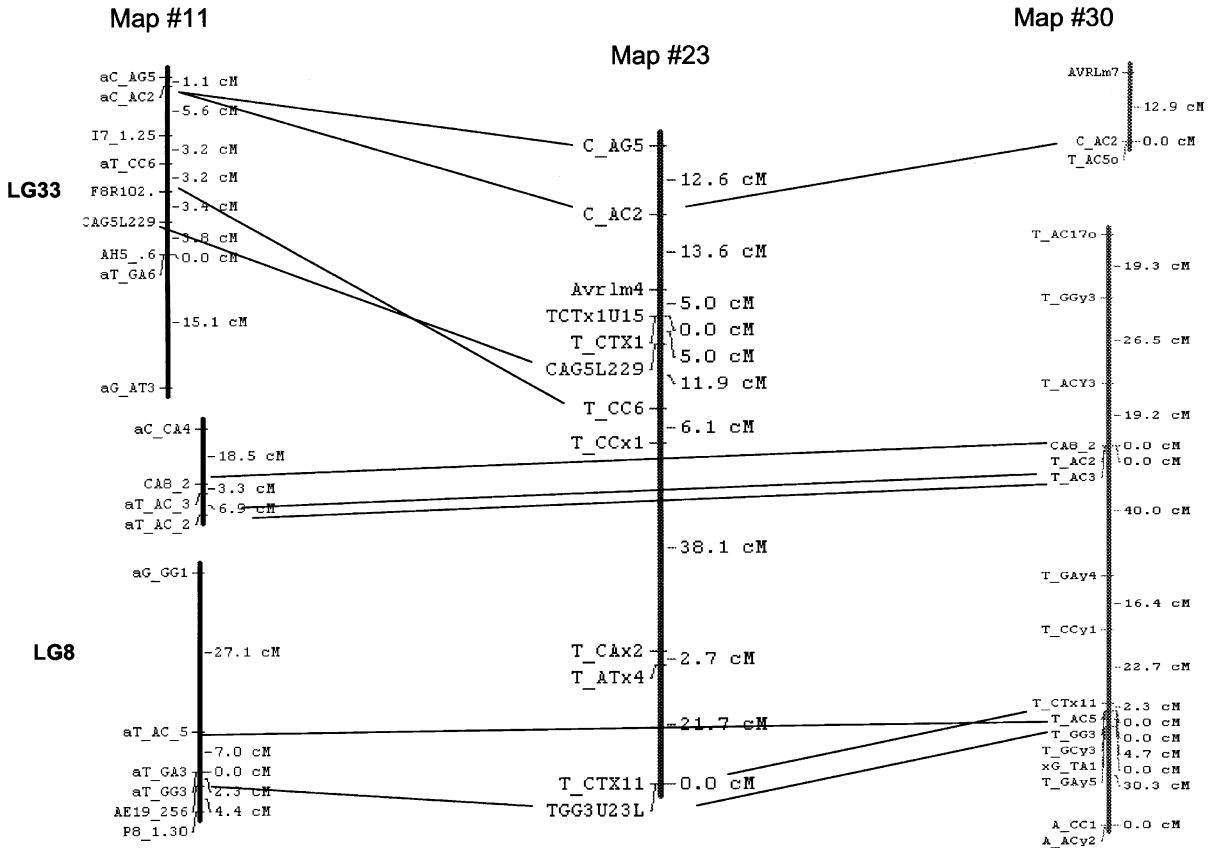

Figure 3. An example of co-linearization between the three *L. maculans* maps developed at INRA. The maps were constructed with Mapmaker/Exp 3.0 software set for F_2 backcross segregating population (LOD = 3.0, maximum distance = 30 cM). Common AFLP markers between the three maps are indicated with a plain line. Marker name nomenclature and distances are as in Figure 1, except that names of AFLP markers do not begin with a lowercase 'a' in maps #23 and #30.

markers (53.7% in the major groups compared to 50% in groups showing accumulation of markers) or for RAPD markers (24.1% in the major groups compared to 23% in groups showing accumulation of markers) (Figure 1). Again, accumulation of specific types of markers, particularly anonymous or retrotransposon-based markers, is commonly observed in fungal genetic maps (Table 2). For example, retrotransposons such as those of the MAGGY Gypsy-type are clustered at specific locations in the *Magnaporthe grisea* integrated genetic map as are other types of repetitive DNA (Nitta et al., 1997). However, markers based on repeats are still uncommon in fungal genetic maps and clustering of markers is often attributed to the use of AFLP markers whereas RAPD, and mainly RFLP markers or microsatellites are less prone to such clustering (e.g. Kema et al., 2002; Jurgenson et al., 2002a, 2002b; Zhong et al., 2002). As emphasized by Jurgenson et al. (2002a), the most likely explanation for clustering of AFLP markers is the map saturation that can be attained with such markers. Accordingly, the relatively low number of AFLP markers used here in the *L. maculans* genetic map could explain the lack of specific clustering of these markers. Other explanations for clustering of such markers suggested by Jurgenson et al. (2002a) include non-random distribution of AT-rich regions in the genome, which would contain a higher number of *Eco*RI and *Mse*I restriction sites, and/or recombination suppression in some genomic regions.

AFLP markers common between the three INRA maps were investigated and cloned in order to co-linearize the three maps. Forty-three markers out of 98 tested (43.8%) common between map #11 and map #23 were obtained; 64 common markers out of 187 tested (34.2%) were obtained

between map #11 and map #30; and 16 markers out of 80 tested (20.0%) were common between map #23 and map #30. This latter low number of common markers between map #23 and map #30 led to only eight markers being common to the three maps. Using standard MapMaker settings (LOD 3.0, max distance 20 cM), the co-linearization strategy was unable to combine linkage groups. When maximum distance was increased to 30 cM, several previously unlinked groups were combined for some maps (Figure 3). This extensive mapping showed that, even though the markers were scattered throughout the genome, increasing the number of markers usually increased the size of given linkage groups without reducing their number. Accumulation of markers in some linkage groups further suggested that Rep-PCR, ISSR, and to a lesser extent that AFLP or RAPD markers, could specifically target some regions of the genome whereas other genomic regions are extremely poor targets for these markers, therefore leading to gaps in the maps, and to many more linkage groups than chromosomes. This can also be illustrated by similar findings in the linkage map of the Dothideomycete *C. sativus*, with chromosomes encompassing up to three linkage groups, one of which is characterized by accumulation of markers in a small genetic distance (Zhong et al., 2002). In this respect, the recent development of new types of markers, i.e., locus-specific minisatellites, were able to target parts of the genome not covered by other markers, as exemplified by the *MinLm6* minisatellite which allowed two previously unlinked minor LGs to be joined (LG24, Figure 1a).

Cloning of markers and their assignment to physical maps

Cozjinsen et al. (2000) cloned three polymorphic RAPD bands and 18 polymorphic AFLP bands of their map to provide anchoring markers for chromosomes. These AFLP and RAPD markers were hybridised to Southern blots of chromosomal DNA of parent isolates, but only the three RAPD markers and eight of the AFLP markers were chromosome-specific. The remaining 10 AFLP markers bound to all chromosomes. Eight of these were sequenced; two (GenBank AF293766 and AF293768) corresponded to portions of the *Copia* LTR retrotransposon *Pholy* (previously named LMR1; Cozijnsen et al., 2000) whereas the six other markers did not have any match in the database until recently. Here, we have shown that three of these (6 unknown AFLP markers) (GenBank AF293767, AF293769 and AF293770) correspond to various parts of the recently identified *Gypsy*-like LTR retrotransposon *Polly*, and another one (GenBank AF293771) correspond to part of the recently identified *Gypsy*-like LTR retrotransposon *Rolly* (Gout, 2005). Single-copy minisatellites identified in *L. maculans* by Eckert et al. (2005) were also assigned to chromosomes. A schematic representation of the chromosomes of the two parents of the Cozijnsen et al. (2000) map (of isolates M1 and C13) and of map #11 of the present study (of isolates a.2 and H5) is shown in Figure 2, where the letters and numbers next to each band refer to chromosome-specific markers, corresponding to known genes or to minisatellites. The number of anonymous RAPD or AFLP markers per chromosome is also indicated. Most chromosomes have several markers. In contrast, as exemplified by the most extensive physical map available, that of isolate M1 (Figure 2), six (or seven, if including the minichromosome) chromosomes do not possess any marker from the genetic maps. However, as mentioned above, many anonymous RAPD and AFLP markers tested bound to all chromosomes.

Chromosome length polymorphisms

Large variation in chromosome sizes is a feature of many fungal electrokaryotypes (Table 2). Chromosome Length Polymorphisms (CLPs) were observed between natural isolates of *L. maculans* firstly using TAFE (transverse alternating field electrophoresis) and then CHEF (contour-clamped homogeneous electric fields) separation of chromosomal DNA (Taylor et al., 1991; Morales et al., 1993). Morales et al. (1993) also hybridised chromosomal DNA with conserved heterologous sequences, such as β-tubulin, and thus suggested that homologous chromosomes may have very different sizes. As for other Dothideomycetes species (Tzeng et al., 1992; Zhong et al., 2002; Kema et al., 2002), major chromosome length polymorphisms were shown to be generated by meiosis (Plummer and Howlett, 1993, 1995) with significant karyotype variation linked with the assortment of parental homologues of different

sizes. However, in some cases, novel-sized homologues were observed that varied in size from those of the parents (Plummer and Howlett, 1995; Leclair et al., 1996). Zolan (1995) suggested that size changes in chromosomes represented gain or loss of non-essential sequences. In *L. maculans*, large CLPs generated by meiosis were described particularly for the chromosome harbouring ribosomal DNA (rDNA) sequences (Howlett et al., 1997). Ribosomal DNA probes hybridised to chromosomal DNA ranging between 2.5 and 4.2 Mb (Figure 2), and rDNA was shown to be borne by a 1.8 Mb chromosome in the Canadian isolate Leroy (Morales et al., 1993). However, significant size polymorphism was also observed for the smallest chromosome (Leclair et al., 1996), and in the present study for other putative homologues, such as the chromosome harbouring *MinLm1*, which ranged in size from 2.15 to 2.8 Mb or the chromosome harbouring *MinLm3* which ranged in size from 1.15 to 2.5 Mb (Figure 2).

In contrast, other chromosomes only show a smaller degree of CLP, as illustrated here by the chromosome harbouring β-tubulin which ranged between 1.95 Mb and 2.3 Mb, and was previously described to range between 1.6 and 2.0 Mb in a series of Canadian isolates (Morales et al., 1993). This chromosome is characterised by the numerous genes that it is currently thought to contain and by the lack of genetic markers from the maps that could be hybridised to it (Figure 2). Leclair et al. (1996) suggested that a dispensable 'B' chromosome was present and that this was composed of repetitive sequences. Until now, no gene-coding sequences have been identified on this chromosome. Here, neither ORF-based nor repeat-based single-copy probes (e.g., minisatellites) hybridised to the minichromosome (Figure 2). Finally, mitochondrial DNA, as diffuse bands sized 100 to 150 kb, and linear plasmids (sized 9 and 10 kb) can also be resolved (Howlett, 1997).

L. maculans *genome structure as a mosaic of gene-rich and repeat-rich regions*

As yet, few regions of the genome of *L. maculans* have been sequenced and only about 300 gene sequences and Expressed Sequenced Tags (ESTs) are available on GenBank and COGEME databases (http://www.cogeme.man.ac.uk/). A 184 kb contig sequence has been obtained through the INRA-PMDV-Genoscope collaboration (Attard, 2001; Attard et al., 2002; Attard et al., 2005). Sequence data have also been obtained and analysed extensively for three gene-rich regions: 10 kb encompassing the mating type locus in two isolates (Cozijnsen and Howlett, 2003); the sirodesmin cluster region (68 kb) (Gardner et al., 2004); a 38 kb region with genes possibly involved in secondary metabolism (Idnurm et al., 2003a). This research, along with studies on particular genes or randomly mutated genes (Idnurm and Howlett, 2002; Idnurm and Howlett, 2003; Idnurm et al., 2003b; Meyer et al., 2004) has resulted in additional annotated genes published on international databases e.g. opsin, isocitrate lyase, cyanide hydratase, neutral trehalase, secreted proteins sp1 and sp2, G_{α} subunit (Idnurm and Howlett, 2001; Sexton and Howlett, 2000; Idnurm and Howlett, 2002; Wilson et al., 2002; Idnurm et al., 2003b; Wilson and Howlett, 2005; M. Meyer, this study) (Figure 2).

The occurrence of repeats in the *L. maculans* genome has been investigated via hybridisation of High-Density spotted BAC libraries with randomly labelled whole genomic DNA. This approach showed that 20% of the BAC clones contained high amounts of repeated DNA (Attard et al., 2005). The 184 kb contig sequence appears to be a pericentromeric region and contains the inactive Long Terminal Repeat (LTR)-retrotransposon '*Pholy*', also present in 17.1% of the genomic BAC clones (Attard et al., 2005). Comparative analysis of the two largest regions currently sequenced showed a marked difference between these two genomic regions. The sirodesmin gene cluster had 18 genes within a 55 kb region flanked by genes involved in other aspects of metabolism of *L. maculans*, very few non-coding sequences, and short intergenic regions. The sirodesmin gene cluster appears to be 'protected' from repetitive elements, although *Pholy* fragments flanked one end. In contrast to the sirodesmin gene cluster, the 184 kb region was AT-rich with many diverse and repeated elements, and only 11 open reading frames clustered within 32 kb. This mosaic of transposons was characterised by the occurrence of numerous truncated copies, including solo-LTRs, and large-scale degeneracy of the retrotransposons by Repeat Induced Point mutation (RIP) phenomenon, a mechanism by which repeated sequences are inactivated following

meiosis. This process was recently shown to be active in *L. maculans* whereby copies of an introduced selectable marker (hygromycin resistance gene) were inactivated (Idnurm and Howlett, 2003). In addition to *Pholy* (*Copia*-type), at least two other *Gypsy*-type degenerate and truncated LTR-retrotransposons were clustered within this region, amounting to up to 70% of the sequence (Figure 4). Finally, preliminary data from the on-going sequencing of 1.3 Mb of the 2.8 Mb-chromosome of isolate JN3 suggest that up to 80% of the sequence consisted of large arrays of degenerate and composite repetitive elements (Gout and Ross, unpublished data; Gout, 2005).

Concluding remarks

Genetic linkage maps are still the primary tool for genome knowledge in a series of plant and animal models. For plants they also are a basic tool for positional cloning and breeding for agronomically desirable traits, including quantitative traits, eventually leading to marker-assisted selection. Due to their small genome size and laboratory tractability, fungal models such as the ascomycete yeast *Saccharomyces cerevisiae*, and the filamentous ascomycete *Neurospora crassa*, have been pioneers in the process of building genetic maps (Arnold, 1997; see also http://www.yeastgenome.org/; http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/markers.html). This has led to maps containing hundreds of gene-based markers at a time when anonymous markers just began to emerge to be used for other models. In this respect, the development of genetic linkage maps has been very slow for other fungal species, being very often slowed down by the impossibility of achieving fertile crosses in laboratory conditions (or the lack of a sexual stage), the difficulty to visualize fungal chromosomes and the lack of genetic markers. By the beginning of the 1990s, both the development of high-throughput anonymous molecular markers and the assignment of linkage groups to chromosomes separated by pulsed field gel electrophoresis were instrumental for genetic mapping in fungi (Table 2). Genetic maps associated with physical assignment to chromosomes were developed to analyse genome structure, and to target and clone genes of interest such as avirulence/pathogenicity genes of pathogenic fungi, or quality genes for edible mushrooms, and nowadays, to prepare the numerous genome initiatives underway for numerous fungal species.

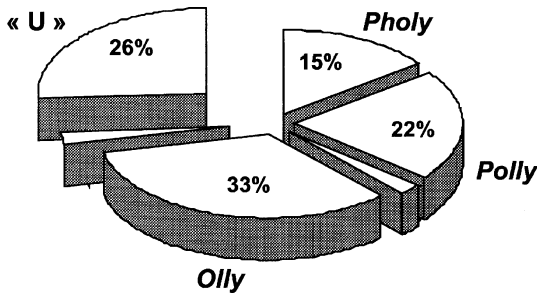

Figure 4. Sequence analysis of a 184 kb *L. maculans* genomic region (GenBank accessions AL732383, AL732384 and AL732385) expressed as the number of basepair corresponding to one given class of repeated element or 'unique' sequences (Gout, 2005). The region comprises numerous copies of more or less truncated copies of the *Copia* retrotransposon *Pholy*, and of the two recently identified *Gypsy* retrotransposons *Olly* and *Polly*. 'U' denotes non-repeated sequences either corresponding to ORFs or to other repeats of which only one copy is present in this region. Other repeats are present and correspond to a minor proportion of the sequence (minor unlabelled segments).

In the present study, the moderately dense genetic map presented along with preliminary assignment to chromosomes suggest that the genome of *L. maculans* consists of a mosaic of GC-equilibrated coding regions with no or few transposons, and of stretches of degenerate and truncated retrotransposons, encompassing very few ORFs. Here, we confirm that each chromosome contains stretches of degenerate retrotransposons, such as *Pholy*. We also suggest that, at least for some chromosomes, degenerate retrotransposons are clustered together, and often constitute a very large part of the chromosome whereas ORF-rich regions seem to be 'protected' from retrotransposon invasion. Apart from partial sequence data, this is substantiated by the observation that 20% of BAC clones are rich in repeats whereas other BAC clones did not hybridise to repeats, and by the fact that the genetic maps have linkage groups with clustered rep-PCR markers that are absent from larger linkage groups. In addition, the two minisatellites which resulted from sequencing of the BAC-end, chosen so that the corresponding BAC did not hybridize to repeated elements, *MinLm5* and *MinLm6* (Eckert

et al., 2005), were genetically mapped here to major LG, poor in rep-PCR markers. It remains to be seen whether large clustered arrays of repeats are a peculiarity of the *L. maculans* genome since, regardless of potential gaps in the genomic sequences, such arrays of repeats are generally not observed in the currently fully sequenced genomes of model fungi such as *S. cerivisiae* or *N. crassa*, or phytopathogenic fungi such as *M. grisea* or *Fusarium graminearum* (see www.broad.mit.edu/annotation/fungi/). The present study, along with previously published work also suggests large chromosomal length polymorphisms are linked with the loss of dispensable retrotransposon-rich regions. The dispersed nature of this repetitive DNA may play a role in generating chromosomal length polymorphisms (Plummer and Howlett, 1993, 1995) and homologues of varying sizes may be due to the presence of different numbers of repetitive sequences spaced between coding sequences, arising from unequal pairing of chromosomes. During meiosis these sequences would act as sites for reciprocal recombination, leading to production of chromosomes of novel sizes in the progeny (Plummer and Howlett, 1995). This was recently demonstrated by Gout (2005) who showed that up to 600 kb (i.e. 25% of the corresponding chromosome) consisted of mosaics of retrotransposons and other repeats, and that these regions were absent from homologues when a range of isolates was compared. This has led to the hypothesis that chromosomes may have a minimal functional size where they mainly consist of coding regions (Gout, 2005). The present study is consistent with this hypothesis and suggests that some chromosomes may be less prone to large CLP than others, suggesting they reach a minimal functional size, and are rich in ORFs. In addition, this postulate can probably be expanded to other fungal models such as *C. sativus*, where significant CLPs are noticed for the chromosome harbouring the VHv1 virulence locus (size ranging between 2.2 and 2.8 Mb) whereas other chromosomes may show only limited size polymorphism (Zhong et al., 2002).

In most fungal genome initiatives that are currently underway, a high priority is given to the construction of integrated genome maps encompassing (i) high-resolution genetic maps, (ii) physical maps, (iii) complete genomic sequence and (iv) annotation with special reference to the genes (Xu et al., 2003). Such an integrated genetic map, initiated for *L. maculans* here, is a prerequisite to understand genome structure, function and evolution of fungal genomes. The economic importance of the *L. maculans-B. napus* interaction, the properties of this interaction as a model host-pathogen system, and the need to better characterise the genome structure of the fungus has led to the establishment of the *L. maculans* genome sequence initiative. This whole-genome sequencing project is led by INRA-PMDV (T. Rouxel and M.H. Balesdent) and The University of Melbourne (B. Howlett) and will be done by the French sequencing agency Genoscope (www.cns.fr) in 2006. The objective of the project is to attain a 12-x coverage of the *L. maculans* genome, using isolate JN3 (v23.1.3) (Attard et al., 2002). The sequencing strategy will include the three following steps: (1) shot gun sequencing of chromosomal DNA of isolate v23.1.3, (2) sequencing of both ends of 12,500 BAC clones, in order to facilitate the assembly of small contigs generated by shot-gun sequences, (3) sequencing of cDNA libraries (25,000 reads), in order to facilitate and validate the automatic annotation of the sequences (Rouxel and Balesdent, 2005). Only such a large-scale sequencing project will allow us to generate the ultimate integrated maps for *L. maculans*, and enable us to validate the hypotheses on genome structure discussed in this paper based on current genetic and physical map information.

Acknowledgements

This work was partly supported by EU contracts FAIR3CT96–1669 and QLK5-CT-2002–01813 'SECURE' and also by the Australian Grains Research and Development Corporation and the Australian Research Council. Large-scale sequencing was performed in collaboration with the French sequencing agency, Genoscope (www.cns.fr). We are greatly indebted to L. Cattolico and J. Weissenbach (Genoscope, Evry, France) for the high-quality data. Thanks are due to the numerous students, post-docs and scientists who contributed to generate the INRA genetic and physical maps: F. Blaise, L. de Roquemaurel, M. Eckert, J.P. Narcy, S. Laroche, F. Parlange, S. Ross.

References

Ansan-Melayah D, Balesdent MH, Buée M and Rouxel T (1995) Genetic characterization of *AvrLm1*, the first avirulence gene of *Leptosphaeria maculans*. Phytopathology 85: 1525–1529

Arnold J (1997) Editorial. Fungal Genetics and Biology 21: 254–257

Attard A (2001) Le gène d'avirulence *AvrLm1* chez l'ascomycète phytopathogène *Leptosphaeria maculans*: Marquage moléculaire et caractérisation de son environnement génomique. Ph.D. Thesis, Institut National Agronomique, Paris, France

Attard A, Gourgues M, Gout L, Schmit J, Roux J, Narcy JP, Balesdent MH and Rouxel T (2001) Molecular characterisation and polymorphism of *MinLm1*, a minisatellite from the phytopathogenic ascomycete *Leptosphaeria maculans*. Current Genetics 40: 54–64

Attard A, Gout L, Gourgues M, Kuhn ML, Schmit J, Laroche S, Ansan-Melayah D, Billault A, Cattolico L, Balesdent MH and Rouxel T (2002) Analysis of molecular markers genetically linked to the *Leptosphaeria maculans* avirulence gene *AvrLm1* in field populations indicates a highly conserved event leading to virulence on *Rlm1* genotypes. Molecular Plant-Microbe Interactions 15: 672–682

Attard A, Gout L, Ross S, Parlange F, Cattolico L, Balesdent MH and Rouxel T (2005) Truncated and RIP-degenerated copies of the LTR retrotransposon *Pholy* are clustered in a pericentromeric region of the *Leptosphaeria maculans* genome. Fungal Genetics and Biology 42: 30–41

Aubertot JN, West J, Bousset-Vaslin L, Salam MU, Barbetti MJ and Diggle AJ (2006) Improved resistance management for durable disease control: a case study of phoma stem canker of oilseed rape (*Brassica napus*). European Journal of Plant Pathology, 114: 91–106

Balesdent MH, Attard A, Kuhn ML and Rouxel T (2002) New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. Phytopathology 92: 1122–1133

Berbee ML (2001) The phylogeny of plant and animal pathogens in the Ascomycota. Physiological and Molecular Plant Pathology 59: 165–187

Chalhoub BA, Thibault S, Laucou V, Rameau C, Hofte H and Cousin R (1997) Silver staining and recovery of AFLP™ amplification products on large denaturing polyacrylamide gels. BioTechniques 22: 216–220

Cozijnsen AJ and Howlett BJ (2003) Characterisation of the mating-type locus of the plant pathogenic ascomycete *Leptosphaeria maculans*. Current Genetics 43: 351–357

Cozijnsen AJ, Popa KM, Purwantara A, Rolls BD and Howlett BJ (2000) Genome analysis of the plant pathogenic ascomycete *Leptosphaeria maculansi*; mapping mating type and host specificity loci. Molecular Plant Pathology 1: 293–302

Delourme R, Chèvre A-M, Brun H, Rouxel T, Balesdent M-H, Dias JS, Salisbury PA and Rimmer RS (2006) Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape. European Journal of Plant Pathology, 114: 41–52

Delourme R, Pilet-Nayel ML, Archipiano M, Horvais R, Tanguy X, Rouxel T, Brun H, Renard M and Balesdent MH (2004) A cluster of major specific resistance genes to *Leptosphaeria maculans* in *Brassica napus*. Phytopathology 94: 578–583

Eckert M, Gout L, Rouxel T, Blaise F, Jedryczka M, Fitt BDL and Balesdent MH (2005) Identification and characterisation of polymorphic minisatellites in the phytopathogenic ascomycete *Leptosphaeria maculans*. Current Genetics 47: 37–48

Fitt BDL, Brun H, Barbetti MJ and Rimmer SR (2006) Worldwide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). European Journal of Plant Patholoy, 114: 3–15

Forche A, Xu JP, Vilgalys R and Mitchell TG (2000) Development and characterization of a genetic linkage map of *Cryptococcus neoformans* var. *neoformans* using amplified fragment length polymorphisms and other markers. Fungal Genetics and Biology 31: 189–203

Gardiner DM, Cozijnsen AJ, Wilson LM, Pedras MSC and Howlett BJ (2004) The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. Molecular Microbiology 53: 1307–1318

Gout L (2005) Clonage et évolution du gène d'avirulence *AvrLm1* chez l'ascomycète phytopathogène *Leptosphaeria maculans*. Ph.D. thesis, Institut National Agronomique, Paris-Grignon, Paris, France

Howlett BJ (1997) Genome analysis of the fungal plant pathogen *Leptosphaeria maculans* using pulsed field gel electrophoresis. Electrophoresis 18: 1544–1547

Howlett BJ (2004) Current knowledge of the interaction between *Brassica napus* and *Leptosphaeria maculans*. Canadian Journal of Plant Pathology 26: 245–252

Howlett BJ, Idnurm A and Pedras MSC (2001) *Leptosphaeria maculans*, the causal agent of blackleg disease of Brassicas. Fungal Genetics and Biology 33: 1–14

Howlett BJ, Rolls BD and Cozijnsen AJ (1997) Organisation of ribosomal DNA in the ascomycete *Leptosphaeria maculans*. Microbiological Research 152: 261–267

Idnurm A and Howlett BJ (2001) Characterization of an opsin gene from the ascomycete *Leptosphaeria maculans*. Genome 44: 167–171

Idnurm A and Howlett BJ (2002) Isocitrate lyase is essential for pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). Eukaryotic Cell 1: 719–724

Idnurm A and Howlett BJ (2003) Analysis of loss of pathogenicity mutants reveals that repeat-induced point mutations can occur in the Dothideomycete *Leptosphaeria maculans*. Fungal Genetics and Biology 39: 31–37

Idnurm A, Taylor JL, Pedras MSC and Howlett BJ (2003a) Small scale functional genomics of the blackleg fungus, *Leptosphaeria maculans*: analysis of a 38 kb region. Australasian Plant Pathology 32: 511–519

Idnurm A, Warnecke DC, Heinz E and Howlett BJ (2003b) Characterisation of neutral trehalase and UDP-glucose: sterol glucosyltransferase genes from the plant pathogenic fungus *Leptosphaeria maculans*. Physiological and Molecular Plant Pathology 62: 305–313

Jedryczka M, Rouxel T and Balesdent MH (1999) Rep-PCR based genomic fingerprinting of isolates of *Leptosphaeria maculans* from Poland. European Journal of Plant Pathology 105: 813–823

Jurgenson JE, Bowden RL, Zeller KA, Leslie JF, Alexander NJ and Plattner RD (2002a) A genetic map of *Gibberella zeae* (*Fusarium graminearum*). Genetics 160: 1451–1460

Jurgenson JE, Zeller KA and Leslie JF (2002b) Expanded genetic map of *Gibberella moniliformis* (*Fusarium verticillioides*). Applied and Environmental Microbiology 68: 1972–1979

Kema GHJ, Goodwin SB, Hamza S, Verstappen ECP, Cavaletto JR, Van der Lee TAJ, de Weerdt M, Bonants PJM and Waalwijk C (2002) A combined amplified fragment length polymorphism and randomly amplified polymorphism DNA genetic linkage map of *Mycosphaerella graminicola*, the septoria tritici leaf blotch pathogen of wheat. Genetics 161: 1497–1505

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE and Newburg L (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181

Larraya LM, Perez G, Ritter E, Pisabarro AG and Ramirez L (2000) Genetic linkage map of the edible basidiomycete *Pleurotus ostreatus*. Applied and Environmental Microbiology 66: 5290–5300

Leclair S, Ansan-Melayah D, Rouxel T and Balesdent MH (1996) Meiotic behaviour of the minichromosome in the phytopathogenic ascomycete *Leptosphaeria maculans*. Current Genetics 30: 541–548

Liew ECY, Aptroot A and Hyde KD (2000) Phylogenetic significance of the pseudoparaphyses in Loculoascomycete taxonomy. Molecular Phylogenetics and Evolution 16: 392–402

Marra RE, Huang JC, Fung E, Nielsen K, Heitman J, Vilgalys R and Mitchell TG (2004) A genetic linkage map of *Cryptococcus neoformans* variety neoformans serotype D (*Filobasidiella neoformans*). Genetics 167: 619–631

Meyer M, Blaise F, Remy E, Zhou L, Tourneur C, Narcy JP, Rouxel T and Balesdent MH (2004) *Agrobacterium tumefaciens*-mediated transformation of *Leptosphaeria maculans*. IOBC Bulletin 27: 179–189

Morales VM, Séguin Swartz G and Taylor JL (1993) Chromosome size polymorphism in *Leptosphaeria maculans*. Phytopathology 83: 503–509

Muraguchi H, Ito Y, Kamada T and Yanagi SO (2003) A linkage map of the basidiomycete *Coprinus cinereus* based on random amplified polymorphic DNAs and restriction fragment length polymorphisms. Fungal Genetics and Biology 40: 93–102

Nitta N, Farman ML and Leong SA (1997) Genome organization of *Magnaporthe grisea*: Integration of genetic maps, clustering of transposable elements and identification of genome duplications and rearrangements. Theoretical and Applied Genetics 95: 20–32

Pedersen C, Rasmussen SW and Giese H (2002) A genetic map of *Blumeria graminis* based on functional genes, avirulence genes, and molecular markers. Fungal Genetics and Biology 35: 235–246

Plummer KM and Howlett BJ (1993) Major Chromosomal Length Polymorphisms are evident after meiosis in the phytopathogenic fungus *Leptosphaeria maculans*. Current Genetics 24: 107–113

Plummer KM and Howlett BJ (1995) Inheritance of Chromosomal Length Polymorphisms in the ascomycete *Leptosphaeria maculans*. Molecular and General Genetics 247: 416–422

Pongam P, Osborn TC and Williams PH (1998) Genetic analysis and identification of Amplified Fragment Length Polymorphism markers linked to the *alm1* avirulence gene of *Leptosphaeria maculans*. Phytopathology 88: 1068–1072

Rouxel T and Balesdent MH (2005) The stem canker (blackleg) fungus, *Leptosphaeria maculans*, enters the genomic era. Molecular Plant Pathology 6: 225–241

Sexton AC and Howlett BJ (2000) Characterisation of a cyanide hydratase gene in the phytopathogenic fungus *Leptosphaeria maculans*. Molecular and General Genetics 263: 463–470

Sexton AC, Paulsen M, Woestemeyer J and Howlett BJ (2000) Cloning, characterization and chromosomal location of three genes encoding host-cell-wall-degrading enzymes in *Leptosphaeria maculans*, a fungal pathogen of *Brassica* spp. Gene 248: 89–97

Silva-Hanlin DMW and Hanlin RT (1999) Small subunit ribosomal RNA gene phylogeny of several loculoascomycetes and its taxonomic implications. Mycological Research 103: 153–160

Taylor JL, Borgmann IE and Séguin Swartz G (1991) Electrophoretic karyotyping of *Leptosphaeria maculans* differentiates highly virulent from weakly virulent isolates. Current Genetics 19: 273–277

Tzeng TH, Lyngholm LK, Ford CF and Bronson CR (1992) A Restriction Fragment Length Polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. Genetics 130: 81–96

Vos P, Hogers R, Bleeker M, Reijans M, van der Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407–4414

West JS, Balesdent MH, Rouxel T, Narcy JP, Huang YJ, Roux J, Steed JM, Fitt BDL and Schmit J (2002) Colonization of winter oilseed rape tissues by A/Tox^{+} and B/Tox^{0} *Leptosphaeria maculans* (phoma stem canker) in France and England. Plant Pathology 51: 311–321

Williams RSB, Davis MA and Howlett BJ (1994) Nitrate reductase of the ascomycetous fungus, *Leptosphaeria maculans* – gene sequence and chromosomal location. Molecular and General Genetics 244: 1–8

Williams RSB, Davis MA and Howlett BJ (1995) The nitrate and nitrite reductase-encoding genes of *Leptosphaeria maculans* are closely linked and transcribed in the same direction. Gene 158: 153–154

Wilson LM and Howlett BJ (2005) *Leptosphaeria maculans*, a fungal pathogen of *Brassica napus*, secretes a subtilisin-like serine protease. European Journal of Plant Pathology 112: 112–232

Wilson LM, Idnurm A and Howlett BJ (2002) Characterization of a gene (*sp1*) encoding a secreted protein from *Leptosphaeria maculans*, the blackleg pathogen of *Brassica napus*. Molecular Plant Pathology 3: 487–493

Xu Z, Lance B, Vargas C, Arpinar B, Bhandarkar S, Kraemer E, Kochut KJ, Miller JA, Wagner JR, Weise MJ, Wunderlich JK, Stringer J, Smulian G, Cushion MT and Arnold J (2003) Mapping by sequencing the Pneumocystis genome

using the Ordering DNA Sequences V3 tool. Genetics 163: 1299–1313

Zhong SB, Steffenson BJ, Martinez JP and Ciuffetti LM (2002) A molecular genetic map and electrophoretic karyotype of the plant pathogenic fungus *Cochliobolus sativus*. Molecular Plant Microbe Interactions 15: 481–492

Zolan ME (1995) Chromosome-Length Polymorphism in fungi. Microbiological Review 59: 686–698