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Résumé de l'article

Les champignons pathogènes envahissants *Ophiostoma ulmi* et *O. novo-ulmi* ont causé deux pandémies successives de maladie hollandaise de l'orme depuis le début du 20^e siècle. On a mesuré la croissance et procédé à des analyses moléculaires chez l'isolat *O. novo-ulmi* AST27, modérément agressif et chez lequel le gène de pathogénie *Pat1-m* est introgressé; chez l'isolat *O. novo-ulmi* H327; chez les isolats *O. ulmi* Q412T et W9; ainsi que chez les descendants de croisements dirigés entre les isolats H327 et AST27. Les analyses génétiques indiquent que les loci *Pat1* et *Mat1* (type sexuel) sont situés sur des groupes de liaison différents qui correspondent respectivement aux chromosomes 1 et 2. Les différences moléculaires entre les génomes nucléaires des souches H327 et AST27 se retrouvent majoritairement dans le voisinage de *Pat1* et de *Mat1*. De plus, deux loci présumés de caractères quantitatifs, *Mgr1* et *Mgr2*, qui influencent la croissance mycélienne à 21 °C et à 28 °C (températures optimales pour *O. novo-ulmi* et *O. ulmi*) sont liés respectivement à *Mat1* et à *Pat1*.

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***Ophiostoma ulmi* DNA naturally introgressed into an isolate of *Ophiostoma novo-ulmi* is clustered around pathogenicity and mating type loci**

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The invasive fungal pathogens *Ophiostoma ulmi* and *O. novo-ulmi* have caused two successive pandemics of Dutch elm disease since the beginning of the 20th century. In nature, the highly aggressive *O. novo-ulmi* may hybridize with the less aggressive *O. ulmi*. Growth rate and molecular analyses were conducted on an unusual, moderately aggressive *O. novo-ulmi* isolate, AST27, carrying an introgressed pathogenicity gene, *Pat1-m*; on highly aggressive *O. novo-ulmi* isolate H327; on *O. ulmi* isolates Q412T and W9; and on progeny from laboratory crosses between H327 and AST27. Genetic analysis indicated that the *Pat1* and *Mat1* (mating type) loci were in different linkage groups corresponding to *O. novo-ulmi* H327 chromosomes 1 and 2, respectively. Most of the molecular differences between the nuclear genomes of H327 and AST27 occurred in the vicinity of *Pat1* and *Mat1*. In addition, two putative quantitative trait loci, *Mgr1* and *Mgr2*, which influence mycelial growth rate at 21°C and 28°C, the optima for *O. novo-ulmi* and *O. ulmi*, were linked to *Mat1* and *Pat1*, respectively.

Keywords: *Ophiostoma ulmi*, *Ophiostoma novo-ulmi*, Dutch elm disease, introgression.

[Détection d'ADN d'*Ophiostoma ulmi* introgressé naturellement dans les régions entourant les loci contrôlant la pathogénie et le type sexuel chez *O. novo-ulmi*]

Les champignons pathogènes envahissants *Ophiostoma ulmi* et *O. novo-ulmi* ont causé deux pandémies successives de maladie hollandaise de l'orme depuis le début du 20^e siècle. On a mesuré la croissance et procédé à des analyses moléculaires chez l'isolat *O. novo-ulmi* AST27, modérément agressif et chez lequel le gène de pathogénie *Pat1-m* est introgressé; chez l'isolat *O. novo-ulmi* H327; chez les isolats *O. ulmi* Q412T et W9; ainsi que chez les descendants de croisements dirigés entre les isolats H327 et AST27. Les analyses génétiques indiquent que les loci *Pat1* et *Mat1* (type sexuel) sont situés sur des groupes de liaison différents qui correspondent respectivement aux chromosomes 1 et 2. Les différences moléculaires entre les génomes nucléaires des souches H327 et AST27 se retrouvent majoritairement dans le voisinage de *Pat1* et de *Mat1*. De plus, deux loci présumés de caractères quantitatifs, *Mgr1* et *Mgr2*, qui influencent la croissance mycélienne à 21 °C et à 28 °C (températures optimales pour *O. novo-ulmi* et *O. ulmi*) sont liés respectivement à *Mat1* et à *Pat1*.

Mots clés : *Ophiostoma ulmi*, *Ophiostoma novo-ulmi*, maladie hollandaise de l'orme, introgression.

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INTRODUCTION

The invasive ascomycete fungus *Ophiostoma novo-ulmi* Brasier is a highly aggressive vascular wilt pathogen of elms and the principal agent of Dutch elm disease today. Over the past 50 years, it has progressively replaced the less aggressive *Ophiostoma ulmi* (Buisson) Nannf., which was responsible for the first pandemic of Dutch elm disease across Europe, central Asia and North America in the early 1900s (Brasier 1990). *Ophiostoma ulmi* and *O. novo-ulmi* differ in many behavioural and morphological properties. For example, they differ in their growth-temperature optima of ca 22°C and 28°C respectively and in their perithecial dimensions; *O. ulmi* secretes little or none of the cell surface hydrophobin cerato-ulmin while *O. novo-ulmi* secretes high levels; and the two species are characteristically non-overlapping in their levels of aggressiveness on elms of moderate resistance (Brasier 1991). They also differ extensively in their molecular architecture and are phylogenetically distinct and probably anciently divergent (Pipe *et al.* 1995). From their temperature relation and other properties, *O. ulmi* and *O. novo-ulmi* are believed to be adapted to tropical and temperate conditions in their respective centres of origin (Brasier and Mehrotra 1995).

Both species are outcrossing with two mating types, designated MAT-1 and MAT-2, controlled by alleles at a single *Mat* locus located on chromosome 2 of the *O. novo-ulmi* H327 genome (Comeau *et al.* 2015). They are also strongly but not totally reproductively isolated at the pre- and postzygotic levels (Brasier 1986b, 1991). Detailed analysis of natural populations in *O. ulmi*/*O. novo-ulmi* overlap zones has revealed the occurrence of rare and transient *O. ulmi* × *O. novo-ulmi* interspecific hybrids with distinct phenotypes and reduced growth and parasitic fitness (Brasier *et al.* 1995, 1998; Pipe *et al.* 2000). An unusual, only moderately pathogenic isolate of *O. novo-ulmi*, AST27 (Brasier 1986a, 1987), collected in Iran at a location where *O. novo-ulmi* had recently replaced *O. ulmi* (Brasier and Afsharpour 1979), was later shown to be an introgressant harbouring some *O. ulmi*-specific alleles (Bates *et al.* 1993; Et-Touil *et al.* 1999). These included *Pat1-m*, a putative *O. ulmi* pathogenicity allele conferring the moderately aggressive phenotype in AST27 (Et-Touil *et al.* 1999, 2005), and the *OPHIO2-int*. DNA transposon (Bouvet *et al.* 2008). Analysis of population samples collected in Portugal and Poland during the 1980s, when *O. novo-ulmi* was in the process of displacing *O. ulmi*, revealed other interspecific hybrids, including individuals with intermediate aggressiveness and a distinctive growth response to temperature (Brasier *et al.* 1998).

These hybrids and introgressants are known to have a major role in the continuing evolution of *O. novo-ulmi* (reviewed in Brasier *et al.* 2004). They also provide an opportunity to identify genes associated with fitness. Thus, previous genetic analysis of introgressant isolate AST27 allowed the identification of the *Pat1* pathogenicity locus (Et-Touil *et al.* 1999). Matching alleles for moderate (*Pat1-m*) and high (*Pat1-h*) aggressiveness were identified in AST27 and in normal and highly aggressive *O. novo-ulmi* isolate H327 respectively, whereas molecular studies with restriction fragment length polymorphisms (RFLPs; Bates *et al.* 1993) and random amplified polymorphic DNAs (RAPDs; Et-Touil *et al.* 1999) confirmed that a portion of the AST27 nuclear genome comprised *O. ulmi*-specific sequences. Some, but not all, of the latter were found to be linked to *Pat1* (Et-Touil *et al.* 1999).

In these previous studies, lower aggressiveness towards moderately resistant elms was the only phenotypic trait found to distinguish introgressant AST27 from other isolates of *O. novo-ulmi* (Brasier 1986a). However, the evidence for *O. ulmi*-like DNA sequences at various locations in the AST27 genome led us to investigate whether this introgressant isolate expressed other *O. ulmi*-like behavioural traits that might contribute to fitness. In the present study, mycelial growth rates at different temperatures, yeast phase development and cerato-ulmin production levels were quantified in progeny sets from controlled sexual crosses involving AST27 and in wild type isolates of *O. novo-ulmi* and *O. ulmi*. Molecular polymorphisms among the isolates were then investigated by polymerase chain reaction (PCR) using specific amplification of coding loci and random amplification of anonymous loci and possible associations between the various characters and isolates analyzed.

MATERIALS AND METHODS

Isolates and culture media

The following isolates, described by Et-Touil *et al.* (1999), were analyzed: highly aggressive *O. novo-ulmi* subsp. *novo-ulmi* isolate H327 (*Pat1-h*, MAT-1); moderately aggressive *O. novo-ulmi* subsp. *novo-ulmi* introgressant isolate AST27 (*Pat1-m*, MAT-2); 40 F₁ progeny from the cross H327 × AST27; and 50 progeny from the backcross H327 × A2P30 (*Pat1-m*, MAT-2). An additional set of 93 F₁ progeny was recovered from the cross H327 × AST27. *Ophiostoma novo-ulmi* subsp. *novo-ulmi* isolates AST20 (MAT-1) and CKT11 (MAT-2), *O. novo-ulmi* subsp. *americana* isolates FG245 (MAT-1), W2 (MAT-2), MH75 (MAT-2) and CESS16K (MAT-2), and *O. ulmi* isolates Q412T (MAT-1), R21 (MAT-1) and W9 (MAT-2) were also used in some tests. Isolates were routinely grown on solid complete medium (CM) with 2.0 g L⁻¹ ammonium sulfate as nitrogen source (Bernier and Hubbes 1990a). Genetic crosses were carried out on elm sapwood agar (Brasier 1981) supplemented with linoleic acid at 6 ml/L (Bernier and Hubbes 1990a, 1990b). The mating type of progeny strains was determined by crossing each individual with strains H327 and AST27. For long-term storage, aliquots of yeastlike cells grown in liquid CM with L-proline (1.15 g L⁻¹) as nitrogen source (Bernier and Hubbes 1990a) were mixed with glycerol (to a final concentration of 15%) and stored at -80°C (Bernier 1993). Modified 2% Oxoid (Nepean, ON, Canada) malt extract agar (MEA) was prepared as described in Brasier (1981).

Growth measurements

Mycelial growth rate on solid medium was measured according to Brasier (1981). Each isolate was inoculated centrally on two sets of MEA plates and incubated in the dark at 21°C and 28°C (Brasier *et al.* 1981). After 2 d (T₁) and 7 d (T₂), the diameter (D) of each colony was measured. The mean radial growth rate of each isolate was calculated from three replicates using the equation described by Brasier and Webber (1987): $D_2 - D_1 / 2(T_2 - T_1)$ = Radial growth rate per day.

Growth curves were established for yeastlike cell cultures in liquid CM using the method of Bernier and Hubbes (1990a). One-week-old cultures in liquid CM were filtered through eight layers of cheesecloth to remove clumps of cells and mycelia and centrifuged at 4100 × g for 5 min. The pellet was resuspended in sterile distilled water

at 1×10^6 cells mL⁻¹. A 500- μ L aliquot was added to 50 mL of medium in a 125-mL Erlenmeyer flask and incubated on a rotary shaker (120 rev min⁻¹) at 25°C in the dark. The cell concentration was quantified with a haemocytometer from three replicates and monitored over 8 d.

Cerato-ulmin measurement

Cerato-ulmin production levels in 1-wk-old culture filtrates of *O. novo-ulmi* H327, *O. ulmi* W9 and Q412T, introgressant AST27, F₁ progeny A2P5 (*Pat1-h*, MAT-2) and A2P30 (*Pat1-m*, MAT-2), and backcross progeny A3P48 (*Pat1-h*, MAT-2) and A3P11 (*Pat1-m*, MAT-1), grown in liquid shake culture at 25°C in CM, were determined by the turbidity method described by Takai and Richards (1978) and expressed by the cerato-ulmin production index (CPI). The CPI of each isolate was measured as 100 x the optical density at 400 nm from three replicates.

DNA extraction and amplification

Total genomic DNA was extracted from yeastlike cells grown in liquid CM, according to the procedure of Zolan and Pukkila (1986). DNA was amplified by the polymerase chain reaction (PCR) using RAPD primers (Operon Technologies, Alameda, CA, USA) as described by Et-Touil *et al.* (1999), as well as primers designed for the specific amplification of coding sequences (Dewar and Bernier 1995; Dusabenyagasani *et al.* 2000). Bulk segregant analysis (Michelson *et al.* 1991) was used to find RAPD markers linked to the mating-type locus. Two bulked DNA samples were prepared from 15 MAT-1 strains (A2P2, A2P3, A2P4, A2P10, A2P11, A2P16, A2P19, A2P20, A2P21, A2P22, A2P25, A2P26, A2P33, A2P35, A2P40) and 15 MAT-2 strains (A2P1, A2P5, A2P6, A2P7, A2P8, A2P9, A2P12, A2P13, A2P14, A2P15, A2P18, A2P23, A2P29, A2P31, A2P34) from the original set of 40 F₁ progeny from the H327 x AST27 cross (Et-Touil *et al.* 1999). The bulked DNA and parent DNA were screened for differences using 80 RAPD primers as described by Et-Touil *et al.* (1999). RAPD amplicons were electrophoresed in 1% agarose and 0.5% Synergel (Diversified Biotechnologies, Boston, MA, USA) gels in 0.09 M Tris-phosphate and 0.002 M EDTA, and visualized by ethidium bromide fluorescence. Gel electrophoresis, alone or in combination with RFLP or single strand conformation polymorphism (SSCP) analysis (Orita *et al.* 1989) was used to detect polymorphisms in PCR-amplified coding sequences located on six of the eight chromosomes (chr) found in *O. novo-ulmi* H327 (Comeau *et al.* 2015): 60S ribosomal protein L34-B and hypothetical eukaryotic translation initiation factor 6 (chr 1); β -tubulin, mitochondrial ATPase inhibitor, 60S ribosomal protein L35 and elongation factor 1- α (chr 2); 60S ribosomal protein L30, cytochrome C oxidase chain VIIc-like protein, histidyl-tRNA mitochondrial, cerato-ulmin and nuclear ribosomal rRNA (chr 3); O-glycosyl hydrolase (chr 4); probable eukaryotic translation initiation factor 2- β , BRCT-containing protein 1 and serine proteinase-like protein (chr 6); actin (chr 8); (Genbank accession numbers AF378547 to AF378553, and AF378555 to AF37867). In addition, Ins-OPHI2-AST27-L and R primers were used to amplify AST27-specific DNA transposon *OPHI2-int*. (Bouvet *et al.* 2008) in the H327 x AST27 F₁ progeny.

Statistical analysis of growth rate and linkage data

Mycelial growth rate data for the two sets of H327 x AST27 F₁ progeny, and the H327 x A2P30 backcross were subjected to ANOVA followed by a Duncan's multiple comparison test. The relationships between mycelial growth rate, pathogenicity, mating-type, and molecular markers were analyzed

by carrying out two-tailed Student's *t* tests. These statistical analyses were performed with SAS version 6.1 software package (SAS Institute Inc., Cary, NC, USA). Recombination among the physiological and molecular markers was studied in the first set of 40 H327 x AST27 F₁ progeny, using MapMaker Macintosh V2.0 Software (Lander *et al.* 1987). Linkage among markers segregating in Mendelian fashion was verified with a logarithm of the odds (LOD) score of 4.0 and Theta value of 0.3. Linkage distances, estimated using the Kosambi function, were first calculated by a two-point analysis, and then by multipoint analysis.

RESULTS

Forty H327 x AST27 F₁ progeny (F₁ set 1) and fifty H327 x A2P30 backcross progeny examined for pathogenicity by Et-Touil *et al.* (1999) during the identification of the *Pat1* locus were investigated for mating type, for other phenotypically observable traits, for segregation of putative quantitative trait loci (QTLs), and for additional associated molecular markers. A further 93 F₁ progeny from the cross H327 x AST27 (F₁ set 2) was also examined in many of the tests.

Mating types

The MAT-1 and MAT-2 mating types segregated in Mendelian fashion in H327 x AST27 F₁ set 1 (15:25; $\chi^2 = 2.500$) but not in set 2 (34:59; $\chi^2 = 6.720$, $P < 0.01$). The two mating types also segregated in Mendelian fashion (22:25; $\chi^2 = 0.191$) in the 47 isolates from the H327 x A2P30 backcross progeny for which mating reactions could be determined.

Mycelial growth rate at 21°C and 28°C

Growth rate tests of the two F₁ and one backcross progeny sets were conducted on MEA at 21°C and at 28°C, close to the optima for *O. novo-ulmi* and *O. ulmi*, respectively (Brasier *et al.* 1981). The data were then analyzed for their relationship to parental growth rates, to mating type and to pathogenicity (*Pat1-h* or *Pat1-m*). The pathogenicity data were available only for F₁ set 1 and for the backcross progeny (Et-Touil *et al.* 1999). The results are summarized in Tables 1 and 2 and Figs. 1 and 2.

At 21°C, *O. novo-ulmi* isolate H327 grew significantly faster (4.95 mm d⁻¹; $F = 7.70$, $P < 0.001$) than *O. novo-ulmi* introgressant AST27 (4.11 mm d⁻¹); and AST27 grew significantly faster than *O. ulmi* isolates Q412T and W9 (3.25 and 2.95 mm d⁻¹, respectively) (Fig. 1A). Both H327 and AST27 therefore show the *O. novo-ulmi* characteristic of a faster growth rate than *O. ulmi* at this temperature. Linear growth rates of both F₁ progeny sets were continuously distributed (Fig. 1A for set 1). For each set, the overall progeny mean did not differ significantly from the H327/AST27 midparent value. In set 1, the 21 *Pat1-h* and the 19 *Pat1-m* siblings exhibited comparable mean mycelial growth rates. The 15 MAT-1 individuals, however, grew significantly ($P < 0.01$) faster than their 25 MAT-2 siblings (Table 1). This behavior was also observed in the 93 F₁ progeny set 2.

At 28°C, *O. novo-ulmi* AST27 (3.05 mm d⁻¹) and *O. ulmi* Q412T (4.21 mm d⁻¹) grew significantly faster than *O. novo-ulmi* H327 (2.95 mm d⁻¹; $F = 6.72$, $P < 0.001$). Isolate AST27 also grew significantly faster than H327 at 28°C in two retests (4.98 vs 4.30 and 5.12 vs 4.17 mm d⁻¹, respectively) i.e. it exhibited a more *O. ulmi*-like property. In both F₁ progeny sets, the distribution of growth rates was again continuous (Fig. 1B for set 1). The overall means of the two

Table 1. Relationship between mycelial growth rate, mating type and pathogenicity in *O. novo-ulmi* H327 x AST27 progeny sets

PARAMETER	Mean growth rate (mm d ⁻¹)		
	H327 x AST27 F ₁		H327 x A2P30 backcross
	Set 1 (n = 40)	Set 2 (n = 93)	(n = 50)
21°C			
Overall progeny mean	4.47	3.54	3.94
Parent midpoint	4.53	3.75	3.86
<i>Pat1-h</i> progeny	4.36	nd	4.01
<i>Pat1-m</i> progeny	4.59	nd	3.86
MAT-1 progeny	4.85 **a	3.69 **	4.29 ***
MAT-2 progeny	4.24	3.41	3.61
28°C			
Overall progeny mean	2.80	3.89	3.49
Parent midpoint	3.00	3.42	3.52
<i>Pat1-h</i> progeny	2.62 ***	nd	3.48
<i>Pat1-m</i> progeny	3.00	nd	3.51
MAT-1 progeny	2.75	3.56	3.72 ***b
MAT-2 progeny	2.83	3.47	3.36

^a Vertical bar, significant difference between the two values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

^b In two retests, no significant differences in growth rate at 28°C were observed between MAT-1 and MAT-2 backcross progeny.

progeny sets were again similar to their respective H327 and AST27 midparent values. With set 1, however, the *Pat1-m* progeny grew at a significantly higher rate than *Pat1-h* progeny ($P < 0.001$), whereas the MAT-1 and MAT-2 progeny of both F₁ sets exhibited similar growth rates (Table 1).

The 50 H327 x A2P30 backcross progeny also exhibited continuous distributions for growth rates at 21°C and 28°C (Fig. 2). The 24 *Pat1-m* individuals and 26 *Pat1-h* individuals

displayed a similar growth rate at both 21°C and 28°C (Table 1). The 22 MAT-1 progeny grew significantly faster, on average, than the 25 MAT-2 progeny at 21°C ($P < 0.001$; Table 1).

Overall, therefore, these data suggested that genetic factors controlling growth rate were segregating among the H327 x AST27 progeny and that some of these factors might be associated with the *Mat1* or *Pat1* loci.

Table 2. Physiological characteristics of *O. novo-ulmi* (H327), introgressant (AST27), progeny from H327 x AST27 crosses (A2P5, A2P30), backcross progeny from H327 x A2P30 crosses (A3P11, A3P48), and *O. ulmi* (W9 and Q412T)

Character	Strains							
	H327 <i>O. novo-ulmi</i>	AST27 Introgressant	A2P5 F ₁	A2P30 F ₁	A3P48 Backcross	A3P11 Backcross	Q412T <i>O. ulmi</i>	W9 <i>O. ulmi</i>
<i>Pat1</i> allele	<i>Pat1-h</i>	<i>Pat1-m</i>	<i>Pat1-h</i>	<i>Pat1-m</i>	<i>Pat1-h</i>	<i>Pat1-m</i>	<i>Pat1-m</i> ^a	<i>Pat1-m</i> ^a
Mating type	MAT-1	MAT-2	MAT-2	MAT-2	MAT-2	MAT-1	MAT-1	MAT-2
Cerato-ulmin production index ^b	61 a	58 a	62 a	60 a	60 a	57 a	2 b	7 b
Growth rate at 21°C	4.95 a	4.11 c	3.95 c	4.36 b	3.93 c	4.88 a	3.25 d	2.95 e
Growth rate at 28°C	2.95 e	3.05 d	2.76 f	3.26 c	3.05 d	3.63 b	4.21 a	2.95 e

^a The *Pat1-m* allele is expected to occur in *O. ulmi* strains.

^b Within a line, means followed by a different letter differ significantly ($P < 0.05$) according to Duncan's multiple-range test following ANOVA.

Figure 1. Mycelial growth rate distribution at 21°C (A) and 28°C (B) of the 40 F₁ set 1 progeny from a cross between the highly aggressive *Ophiostoma novo-ulmi* H327 and the moderately aggressive *O. novo-ulmi* introgressant AST27. Isolates W9 and Q412T belong to *O. ulmi*. All measurements are means of three replicates. Vertical bars represent standard error. A continuous distribution at 21°C and 28°C was also observed for a second set of 93 F₁ progeny (data not shown).

Yeast growth and cerato-ulmin production

In liquid shake cultures, the yeast phases of *O. novo-ulmi* isolates H327 and AST27 grown in CM displayed nearly similar growth kinetics at 25°C. The only notable difference was observed after 96 h of incubation when cell concentrations of AST27 were lower than those recorded for H327 (Fig. 3). Both isolates grew more rapidly than *O. ulmi* Q412T and W9 after *ca* 80 h (Fig. 3). Similarly, H327 and AST27, two selected F₁ progeny isolates (A2P5 and A2P30) and two selected backcross isolates (A3P48 and A3P11) produced comparably high levels of cerato-ulmin, whereas isolates Q412T and W9 produced very small amounts of cerato-ulmin (Table 2) typical of *O. ulmi* (Scala et al. 1997; Takai 1980). Since yeast

growth kinetics and cerato-ulmin production were similar for parent isolates, these traits were not studied further in progeny isolates.

Molecular polymorphisms

Sixteen coding sequences which had been previously assigned to the six *O. novo-ulmi* chromosomes distinguishable by pulsed-field gel electrophoresis (Dewar and Bernier 1995; Dewar et al. 1997; Dusabenyagasani et al. unpubl.) were amplified in H327 and AST27 and subjected to RFLP or SSCP analysis to determine whether they corresponded to *O. ulmi* or *O. novo-ulmi* specific alleles. Results indicated that introgressant AST27 harboured no *O. ulmi*-specific alleles among

the 16 coding loci analyzed. Following sequencing and assembly of the *O. novo-ulmi* H327 nuclear genome into eight chromosomes (Forgetta *et al.* 2013) and its subsequent annotation (Comeau *et al.* 2015), it was found that two chromosomes (chr 5 and 7) were not represented in the set of 16 coding sequences. On the other hand, the *OPHIO2-int.* DNA transposon (Bouvet *et al.* 2008) segregated in the progeny (Table 1).

A comparison of RAPD patterns of H327 and AST27 yielded 26 additional reproducible polymorphisms. These included the 10 RAPD loci linked to *Pat1* reported previously (Et-Touil *et al.* 1999). These loci were assigned to chDNA II (Et-Touil *et al.* 1999) which corresponds to chromosome 1 of the *O. novo-ulmi* reference genome (Forgetta *et al.* 2013; Comeau *et al.* 2015). Seven RAPD loci were polymorphic between MAT-1 and MAT-2 bulked samples of F₁ progeny: OPA8₅₀₀, OPA11₁₉₀₀, OPA12₁₉₀₀, OPJ1₆₀₀, OPJ6₁₂₀₀, OPK12₅₀₀,

and OPK17₁₄₀₀. One of these markers, OPA12₁₉₀₀, was amplified in parent strain AST27 and in *O. ulmi* strains but not in parent strain H327 nor in other *O. novo-ulmi* strains tested. Recombination analysis of the first set of 40 F₁ progeny confirmed that the above markers were all linked to the *Mat1* locus, located on *O. novo-ulmi* H327 chromosome 2 (Comeau *et al.* 2015), and further indicated that an additional locus, OPJ1₆₈₀, was also linked to *Mat1*. These eight RAPD markers and the *Mat1* locus segregated independently from *Pat1* and the 11 molecular markers linked to it. Among the remaining eight RAPD polymorphisms, three loci (OPC2₆₃₅, OPC2₇₆₀ and OPK18₇₆₀) cosegregated only with each other and were assigned to linkage group III (*O. novo-ulmi* H327 chr 8). Locus OPC2₁₁₀₀ was tentatively assigned to chromosome 3 (Dusabenyagasani *et al.* unpubl), whereas OPC19₁₉₀₀, OPJ1₆₂₀, OPJ1₂₀₅₀ and OPK5₁₀₆₀ could not be assigned to any linkage group or chromosome (Fig. 4).

Figure 2. Mycelial growth rate distribution at 21°C (A) and 28°C (B) of 50 progeny from a backcross between the highly aggressive *Ophiostoma novo-ulmi* H327 and a moderately aggressive F₁ progeny (A2P30). All measurements are means of three replicates. Vertical bars represent standard error.

Figure 3. Yeastlike cell growth kinetics of *O. novo-ulmi* (H327), *O. novo-ulmi* introgressant (AST27) and *O. ulmi* (Q412T, W9) in liquid complete medium. Triplicate cultures were incubated on a rotary shaker (120 rev min⁻¹) at 25°C in the dark. Standard errors are shown only when they exceed the size of the symbol.

Figure 4. Linkage analysis of coding loci (*Pat1* and *Mat*), transposon *OPHIO2-int.* and 26 anonymous RAPD loci. The map is based on the analysis of 40 F₁ set 1 progeny from the cross between *Ophiostoma novo-ulmi* H327 (*Pat1-h*, MAT-1) x introgressant AST27 (*Pat1-m*, MAT-2). Map distances, estimated with the Kosambi function, were calculated by multipoint analysis with a LOD score of 4.0 and theta of 0.3, using MapMaker V2.0 for Macintosh.

Relationship between RAPD markers and growth rate

Mycelial growth rates of the 40 F₁ set 1 progeny data were compared to their allelic differences at the RAPD loci described above. Allelic differences at all eight RAPD loci linked to *Mat1* were associated with significant ($P < 0.01$) differences in mycelial growth rate at 21°C (Table 3). None

of the polymorphisms at the remaining RAPD loci were associated with the significant differences in mycelial growth rates observed at this temperature (Table 1). The *OPHIO2-int.* transposon and 10 RAPD polymorphisms linked to *Pat1* were associated with significant ($P < 0.01$) differences in mycelial growth rate at 28°C (Table 3). None of the other RAPD loci investigated showed a significant association with mycelial growth rate at 28°C.

Table 3. Segregation of parental *Pat1* or *Mat* alleles, molecular markers and mycelial growth rates in the set 1 F₁ progeny from the cross H327 x AST27

Inferred linkage group	Marker	H327 allele (<i>Pat1-h</i> or <i>Mat1-1</i>) α^a	AST27 allele (<i>Pat1-m</i> or <i>Mat1-2</i>) β	χ^2	Δ 21°C ($\alpha-\beta$) ^b	Δ 28°C ($\alpha-\beta$)
I	<i>Pat1</i>	21	19	0.100	-0.23	-0.38**
	OPA18 ₆₂₀	16	24	1.600	-0.25	-0.41**
	OPC16 ₁₈₉₀	19	21	0.100	-0.23	-0.38**
	OPC16 ₂₆₁₀	21	19	0.100	-0.23	-0.38**
	OPC18 ₉₄₀	15	25	2.500	+0.01	-0.33**
	OPC19 ₉₂₀	19	21	0.100	-0.18	-0.46**
	OPJ18 ₄₁₀	20	20	0.000	-0.22	-0.45**
	OPJ18 ₂₇₅₀	20	20	0.000	-0.22	-0.45**
	OPJ20 ₅₄₀	21	19	0.100	-0.23	-0.38**
	OPK3 ₁₀₅₀	21	19	0.100	-0.23	-0.38**
	OPK10 ₁₂₇₀	19	21	0.100	-0.17	-0.40**
	OPHIO2-int.	16	24	1.600	-0.14	-0.47**
II	<i>Mat</i>	15	25	2.500	+0.61**	-0.08
	OPA8 ₅₀₀	19	21	0.100	+0.45**	-0.01
	OPA11 ₁₉₀₀	17	23	0.900	+0.63**	-0.08
	OPA12 ₁₉₀₀	15	25	2.500	+0.61**	-0.08
	OPJ1 ₆₀₀	15	25	2.500	+0.61**	-0.08
	OPJ1 ₆₈₀	18	22	0.400	+0.43**	-0.03
	OPJ6 ₁₂₀₀	15	25	2.500	+0.61**	-0.08
	OPK12 ₅₀₀	15	25	2.500	+0.61**	-0.08
	OPK17 ₁₄₀₀	15	25	2.500	+0.61**	-0.08
III	OPC2 ₆₃₅	23	17	0.900	+0.14	-0.06
	OPC2 ₇₆₀	21	19	0.100	+0.18	-0.12
	OPK18 ₇₆₀	23	17	0.900	+0.16	-0.07
Unlinked	OPC2 ₁₁₀₀	25	15	2.500	-0.06	+0.01
	OPC19 ₁₉₀₀	18	22	0.400	+0.03	+0.15
	OPJ1 ₆₂₀	15	25	2.500	+0.26	-0.18
	OPJ1 ₂₀₅₀	14	26	3.600	-0.10	+0.17
	OPK5 ₁₀₆₀	20	20	0.000	+0.30	+0.13

^a α and β represent H327-type and AST27-type alleles, respectively.

^b For any given locus, values are the difference in mean mycelial growth rate (mm d⁻¹) between progeny with the H327 allele (α) or the AST27 allele (β). *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

In a previous study, the unusually low aggressiveness of AST27 relative to wild-type *O. novo-ulmi* isolates (Brasier 1986a) was shown to be due to a single nuclear allele, *Pat1-m*. Since this allele was also closely linked to two of the five *O. ulmi*-specific amplicons found in the AST27 genome, it was considered to be introgressed from *O. ulmi* (Et-Touil *et al.* 1999). In this study we have used a combination of physiological and molecular tests to identify other traits in AST27 and its derivatives that might be both genetically tractable and associated with interspecific hybridization.

One feature observed in AST27 was that it exhibited distinctive mycelial growth rates at 21°C and at 28°C, close to the optima for growth of *O. novo-ulmi* and *O. ulmi*, respectively (Brasier *et al.* 1981). When incubated at either temperature, isolate AST27 exhibited a growth rate that was intermediate between the rates of *O. novo-ulmi* H327 and *O. ulmi* Q412T. Two sets of H327 x AST27 F₁ progeny, and a backcross population showed a continuous distribution for growth rate at both 21°C and 28°C. This was in marked contrast to the heritability pattern of their pathogenicity phenotypes, which segregated 1:1 for highly vs moderate aggressiveness, consistent with monogenic control (Brasier 1987; Et-Touil *et al.* 1999). The observed differences in temperature-growth responses of H327 and AST27 at 21°C and 28°C were therefore most probably due to multiple genetic differences. The assumption that some of these loci were segregating independently from each other was supported by the fact that some of the fastest growing progeny isolates at 21°C were among the slowest growing at 28°C and vice versa (Figs. 1 and 2).

Co-analysis of additional markers with the F₁ set 1 progeny further supported the hypothesis that the growth rate differences between isolates H327 and AST27 were due to allelic differences at several loci. The MAT-1 progeny grew faster than their MAT-2 siblings at 21°C and the *Pat1-m* progeny grew faster than the *Pat1-h* progeny at 28°C. Similar results were obtained when linkage of molecular markers to *Pat1* or *Mat* was examined (Table 2). Linkage analysis of 29 genetic markers that included *Pat1*, *Mat1*, *OPHI02-int.* and 26 RAPD polymorphisms (Fig. 4) clearly showed that the *Pat1* and *Mat1* loci belonged to different linkage groups (I and II, respectively), as expected from markers located on different chromosomes (1 and 2, respectively, according to Comeau *et al.* 2015).

Among the F₁ and backcross progeny analyzed, however, some *Pat1-h* isolates grew significantly faster than both parents at 28°C. Likewise, some MAT-2 individuals were among the fastest growing isolates at 21°C. These findings suggest that the *Mat1* and *Pat1* loci themselves do not directly influence mycelial growth rates at 21°C and 28°C; but are rather linked to two genetic factors or putative quantitative trait loci (QTLs) controlling mycelial growth rate (*mgr*) that we propose to call *Mgr1* and *Mgr2*, respectively. The precise localization of these putative QTLs is unclear owing to the low number of informative genetic polymorphisms found so far between H327 and AST27 and the small sets of progeny analyzed. Nevertheless, it is evident from the RAPD and growth rate data that both putative *mgr* QTLs are located in regions of the AST27 nuclear genome that contain many *O. ulmi*-specific sequences. It therefore appears likely that they are derived from *O. ulmi* and that introgression of *O. ulmi* DNA is responsible for the distinctive growth phenotype of AST27 in addition to its reduced aggressiveness (Et-Touil *et al.* 1999). We found no evidence

for *mgr* QTLs elsewhere in the *Ophiostoma* genome. This is not surprising since we analyzed progeny derived from only two field isolates (H327 and AST27) and sampled a very small portion of the ca. 32-megabase nuclear genome of the Dutch elm disease fungi (Forgetta *et al.* 2013; Khoshraftar *et al.* 2013). Nevertheless, our data indicate that a region of chromosome 8 in introgressant AST27 also contains *O. ulmi*-specific RAPD markers. We therefore expect neighbouring genes to be polymorphic between isolates H327 and AST27.

The significant difference in mycelial growth rate between *Pat1-m* and *Pat1-h* F₁ progeny at 28°C was not observed in the H327 x A2P30 backcross population. This apparent discrepancy may be explained by the genotype of backcross parent isolate A2P30, which not only carried the *Pat1-m* and *Mat1-2* alleles from AST27 but also H327-type alleles for four of the 26 RAPD loci analyzed, viz. OPJ1₆₈₀, OPC2₁₁₀₀, OPC19₁₉₀₀ and OPK5₁₀₆₀. H327-type coding genes linked to any of these markers may have interacted with the *Mgr1* locus.

The clustering of *O. ulmi* DNA around the *Mat1* and *Pat1* loci may have adaptive significance. Little is known about the genome reorganization in interspecific hybrids among filamentous fungi, but it is likely that natural selection will have a strong influence on the process (Brasier 1995). It has been shown that as *O. novo-ulmi* has replaced *O. ulmi* across the Northern Hemisphere it has acquired the *Mat1-1* allele and vegetative compatibility (*Vic*) alleles from *O. ulmi* at a high frequency (Paoletti *et al.* 2006). We have also found that most *O. ulmi* DNA detected in the *O. novo-ulmi* isolates examined flanked the introgressed *Mat1* and *Vic* loci. Brasier *et al.* (2004) proposed that the most likely mode of introgression of the *Mat1* and *Vic* loci was via sexual hybridization of *O. novo-ulmi* with *O. ulmi* followed by sequential backcrossing of resulting F₁s to *O. novo-ulmi*. *O. ulmi* x *O. novo-ulmi* F₁ progeny tend to be highly unfit and transient in nature, most being of very low pathogenicity. Backcross genotypes still carrying “unuseful” *O. ulmi* genes, i.e. alleles conferring low fitness on *O. novo-ulmi*, are also probably transient, i.e. eliminated by natural selection. The end result is likely to be selective acquisition and fixation by *O. novo-ulmi* of “useful” *O. ulmi* alleles (in this case, alleles at the *Mat* and *Vic* loci that confer resistance to fungal viruses) and a clustering of residual neutral *O. ulmi* DNA around these loci.

Isolate AST27 exhibits higher pathogenic fitness than most *O. ulmi* x *O. novo-ulmi* F₁ progeny and is also more aggressive to healthy elms than is *O. ulmi*. It was originally isolated from the xylem of a symptomatic elm sapling and was therefore successful in the vascular wilt phase of the pathogen. AST27 shares most of its other properties with *O. novo-ulmi*. It may therefore be an intermediate or late backcross product in which some phenotypically important *O. ulmi* genes, including *O. ulmi* genes for lower aggressiveness (*Pat1-m*) and faster growth rate at 28°C (*Mgr2*), are still present. In areas with populations of moderately to highly resistant elms, it is likely that AST27 would soon have been eliminated through competition with fitter *O. novo-ulmi* genotypes. However, in areas where susceptible elms are frequent, an AST27-type introgressant could overcome host resistance (Et-Touil *et al.* 2005) and be disseminated efficiently by elm bark beetles given its ability to secrete high amounts of cerato-ulmin (Temple *et al.* 1997). Genetic analysis of progeny from laboratory crosses suggests that such introgressants might contribute additional useful traits to *O. novo-ulmi* through sexual hybridization. For instance, H327 x AST27 F₁ isolate A2P33 and backcross isolates A3P26 and A3P33 grew well at both 21°C and 28°C (Figs. 1 and 2) and were highly aggressive (Et-Touil *et al.* 1999). Large scale

genomic analyses of *O. novo-ulmi* populations in historical *O. ulmi/O. novo-ulmi* overlap zones are needed to determine whether, apart from *O. ulmi* *Vic* and *Mat1* loci, *O. ulmi* loci governing some other adaptive traits have been fixed or simply eliminated by intense post-epidemic selection.

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