Genomics of the Dutch elm disease pathosystem: are we there yet?

Louis Bernier, Mirella Aoun, Guillaume F Bouvet, André Comeau, Josée Dufour, Erika S Naruzawa, Martha Nigg, Karine V Plourde

During the last decades, the development of ever more powerful genetic, molecular and omic approaches has provided plant pathologists with a wide array of experimental tools for elucidating the intricacies of plant-pathogen interactions and proposing new control strategies. In the case of the Dutch elm disease (DED) pathosystem, these tools have been applied for advancing knowledge of the host (*Ulmus* spp.) and the causal agents (*Ophiostoma ulmi*, *O. novo-ulmi* and *O. himal-ulmi*). Genetic and molecular analyses have led to the identification, cloning and characterization of a few genes that contribute to parasitic fitness in the pathogens. Quantitative PCR and high-throughput methods, such as expressed sequence tag analysis, have been used for measuring gene expression and identifying subsets of elm genes that are differentially expressed in the presence of *O. novo-ulmi*. These analyses have also helped identify genes that were differentially expressed in DED fungi grown under defined experimental conditions. Until recently, however, functional analysis of the DED fungi was hampered by the lack of protocols for efficient gene knockout and by the unavailability of a full genome sequence. While the selective inactivation of *Ophiostoma* genes by insertional mutagenesis remains a challenge, an alternative approach based on RNA interference is now available for down-regulating the expression of targeted genes. In 2013, the genome sequences of *O. ulmi* and *O. novo-ulmi* were publicly released. The ongoing annotation of these genomes should spark a new wave of interest in the DED pathosystem, as it should lead to the formal identification of genes modulating parasitic fitness. A better understanding of DED, however, also requires that omic approaches are applied to the study of the other biotic components of this pathosystem.

Keywords: Dutch Elm Disease, *Ophiostoma* spp., Genetic and Molecular Analyses, Gene Expression, RNA Interference

Introduction

After nearly 100 years of documented Dutch elm disease (DED) presence, it seems only fitting to revisit this pathosystem from a genetic standpoint encompassing both traditional Mendelian genetics and the most recent developments in omic-based approaches. Ideally, this should be done in the context of integrative biology in which all biotic members of the pathosystem, as well as the intricate interactions among them, are considered. This review, however, will emphasize research on the DED pathogens. While this reflects the authors' expertise on fungi, it was also driven by the consideration that genetic investigations of other components of the pathosystem are lagging, as inferred from a survey of the peer reviewed literature published on DED. There are several reasons for such situation, including the fact that the pathogens are the component of the pathosystem that are the most easily analyzed genetically. Since their life cycle is short and can be completed under laboratory conditions, it is possible to cross sexually compatible individuals and obtain F_1 progeny within weeks. Highly inbred strains can be produced within one year. Mendelian genetic analysis of the pathogens is therefore easy and the heritability of any scorable phenotypic or genotypic trait can be assessed rapidly. This is clearly not the case for elms which, like other temperate tree species, have a long life cycle and take years before they reach sexual maturity. The small genomes of the DED fungi also facilitate genomic analyses. Molecular and omic approaches, however, represent technological advances that should allow rapid developments in the genetic analysis of elms and bark beetle vectors of the disease. In fact, this has already happened and some examples will be provided in this review.

Many pathosystems involve a single pathogen. In the case of DED, three fungal species are known to induce the disease. *Ophiostoma ulmi* caused the first DED pandemic, which spread throughout Europe since the early 1900's to the 1940's, when it started to decline (Brasier 1991). The closely related species *O. novo-ulmi* is responsible for the second, ongoing pandemic. Two subspecies (*novo-ulmi* and *americana*) are recognized within *O. novo-ulmi* (Brasier & Kirk 2001) which is more virulent (used here as a quantitative trait *sensu* Misaghi 1982) and generally fitter than *O. ulmi* (Brasier 1991). Over the years, *O. ulmi* and *O. novo-ulmi* were introduced into Europe, North America, central Asia, New Zealand, and Japan (Brasier 2001, Masuya et al. 2010). The third species, *O. himal-ulmi*, is endemic to the western part of the Himalayas where it is considered an endophyte of native elms (Brasier & Mehrotra 1995). Controlled inoculations of *O. himal-ulmi* to *U. procera* and *U.* x Commelin, however, have shown that it is pathogenic towards European species and hybrids (Brasier & Mehrotra 1995). This review will focus on *O. novo-ulmi*, which is the dominant pathogen in most areas where DED occurs.

Elm genomics

The genus *Ulmus* comprises over 40 known species that are indigenous to the northern hemisphere (Wiegrefe et al. 1994). There is, however, surprisingly little genomic information on elms compared to other broadleaved tree species found in the same parts of the world, such as poplars, beech or oaks (Kremer et al. 2012, Staszak & Paw-
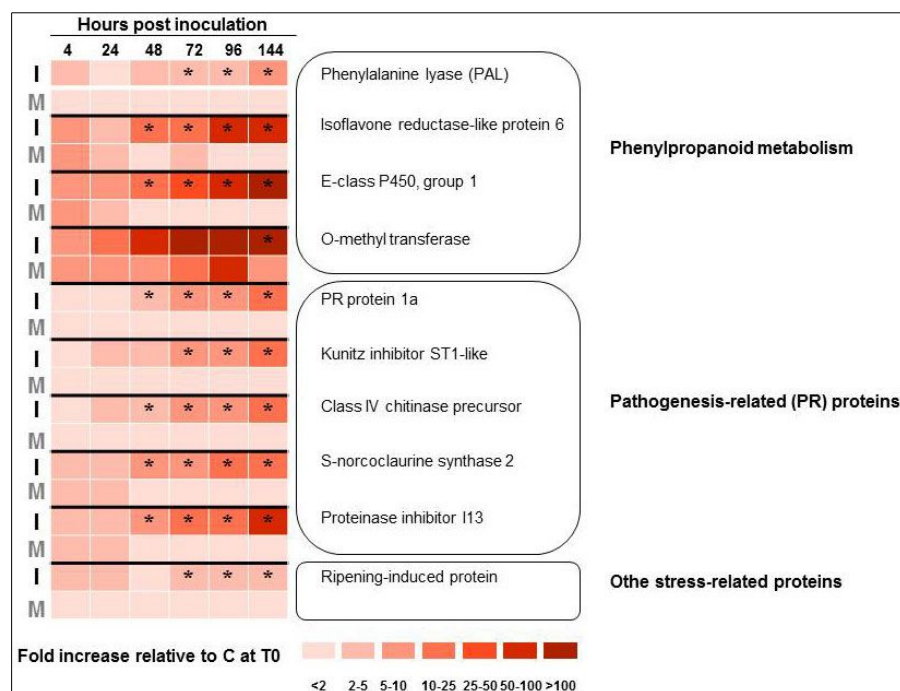
Fig. 1 - Transcript profiling of 10 *Ulmus americana* genes that are up-regulated upon infection of hard calli with *Ophiostoma novo-ulmi* subsp. *novo-ulmi*. Gene transcription profiles were monitored by quantitative reverse-transcriptase polymerase chain reaction, with 3 biological replicate and 2 technical replicates. Elm tissues were either mock-inoculated with sterile water (M) or inoculated with yeast-like cells of *O. novo-ulmi* H327 (I). Results are shown as fold increase in transcription level compared to healthy elm calli (C) harvested at the start of the experiment (T0). Data were analyzed by ANOVA and Tukey's test. (*): significant difference (*P* <0.05) between I and M treatments. Data from Aoun et al. (2009, 2010).

lowski 2012), even though elm breeding programs have been existing for over 80 years (Mittempergher & Santini 2004). Molecular and genomic studies of elms were mostly concerned with species identification (Coleman et al. 2000, Whiteley et al. 2003), population genetics and structure (Gil et al. 2004, Zalapa et al. 2008, Vakkari et al. 2009, Venturas et al. 2013), elm conservation (Goodall-Copestake et al. 2005) and introgression between invasive and native elm species (Zalapa et al. 2010, Brunet et al. 2013).

Information on the genetic determinants of elm response to biotic stresses such as DED remains fragmentary. Nasmith et al. (2008a, 2008b) studied *in vivo* the expression of three genes whose products are associated with plant defense, namely phenylalanine ammonia-lyase (PAL), chitinase (CHT) and polygalacturonase-inhibiting protein (PGIP). Based on Northern hybridization results, these authors reported that expression of PGIP and CHT was stronger in DED resistant *U. pumila* than in DED sensitive *U. americana*. Furthermore, expression was reported to be tissue-dependent: it was stronger in root tissues than in leaf midrib or inner bark, whereas only PAL expression was detectable in callus cultures (Nasmith et al. 2008a). The latter result corroborated previous observa-

tions of PAL activity in cell suspensions of *U. pumila* and *U. campestris* exposed to DED fungi (Corchete et al. 1993). Aoun et al. (2009) confirmed that PAL transcripts accumulated in *U. americana* hard calli inoculated with *O. novo-ulmi* and that this was correlated with the accumulation of suberin, phenols and lignin.

Rather than targeting specific genes, Aoun et al. (2010) used a large-scale cDNA sequencing approach to study the compatible interaction between *O. novo-ulmi* and *U. americana* hard calli. Following bioinformatics analyses of a host-pathogen interaction cDNA library enriched in up-regulated genes by suppression subtractive hybridization (SSH), 296 elm unique sequences were identified from 535 expressed sequence tags (ESTs). A time course analysis of gene expression was carried out on a subset of 18 elm transcripts by quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). Results from transcript profiling confirmed that several genes encoding pathogenesis-related (PR) proteins and enzymes belonging to the phenylpropanoid pathway were up-regulated (Fig. 1). None of the genes studied, however, showed a significant increase in transcription prior to 48 hours after inoculation, which prompted the

authors to suggest that the susceptibility of *U. americana* to DED might result partly from a delay in its response to infection (Aoun et al. 2010). More recently, Büchel et al. (2012) reported on the production and analysis of a much larger elm EST resource. These authors identified 361 196 *Ulmus minor* ESTs which clustered into 52 823 unique transcripts. While the study focused on the response of *U. minor* to egg laying by the elm leaf beetle (*Xanthogaleruca luteola*), the authors showed that up-regulated transcripts included several pathogen-related genes and genes involved in phytohormone (jasmonic acid) signaling.

Elm bark beetle genomics

Elm bark beetles known to vector DED fungi include species from the genera *Scolytus (S. kirchi, S. laevis, S. multistriatus, S. pygmaeus, S. schevyrewi, S. scolytus*) and *Hylurgopinus (H. rufipes)*. Unlike several other species of bark beetles which carry fungal associates in special organs called mycangia, elm bark beetles carry DED fungal spores on the surface of their body and in their gut (Webber 1990, 2004, Moser et al. 2010). They also carry phoretic mites which may contribute to increase the *O. ulmi* / *O. novo-ulmi* spore load of individual elm bark beetles (Moser et al. 2010). To our knowledge, very limited genomic information on any of the aforementioned species has been published in peer reviewed journals. The National Center for Biological Information (NCBI) database contains only five gene sequences from *S. scolytus*, which were analyzed within the context of a phylogenetic study of wood boring weevils (Jordal et al. 2011). The mitochondrial genome of *S. laevis* was also sequenced and annotated, again in the context of weevil phylogenetics (Haran et al. 2013). A growing body of information from genome-wide studies, however, is available for species of bark beetles associated with conifers. Thus, population genomics of the mountain pine beetle (*Dendroctonus ponderosae*) have been described in recent years (Mock et al. 2007, Gayathri Samarasekera et al. 2012), whereas Blomquist et al. (2010) reviewed the functional genomics of pheromone production in *Ips pini*. The transcriptome of *D. ponderosae* was recently analyzed by a combination of Sanger sequencing and Roche 454 pyrosequencing of 14 full-length enriched cDNA libraries (Keeling et al. 2012). Similar omic studies of elm bark beetles and their interactions with other components of the DED pathosystem are needed, especially in the light of the hypothesis that *O. novo-ulmi*, by up-regulating the production of semiochemicals in elms, manipulates host trees in order to enhance their apparency to foraging beetles (McLeod et al. 2005).

Genomics of Dutch elm disease fungi

The development of PCR-based approaches in the late 1980's stimulated the analysis of population structure in the DED fungi. Hoegger and co-workers used Random amplified polymorphic DNA (RAPD) markers to show that *O. ulmi* and the two subspecies of *O. novo-ulmi* were present in Switzerland (Hoegger et al. 1996). They further indicated that RAPDs were superior to restriction fragment length polymorphisms (RFLPs), used previously (Hintz et al. 1991, Jeng et al. 1991, Bates et al. 1993) for distinguishing between subspecies *americana* and *novo-ulmi* within *O. novo-ulmi*. Over the following years, additional population structure studies using PCR-derived markers were conducted in Italy (Santini et al. 2005), Spain (Solla et al. 2008), the Netherlands (Brasier & Kirk 2010) and Canada (Temple et al. 2006). From these and other studies, it is clear that European populations of DED fungi are more diverse than North American ones. *Ophiostoma ulmi* was still found in parts of Europe in the early 21st century (Solla et al. 2008), whereas this species has not been reported in North America since 1991 (Houston 1991). Moreover, hybrid isolates (between *O. ulmi* and *O. novo-ulmi,* between the two subspecies within *O. novo-ulmi*) are commonly reported in Europe (Hoegger et al. 1996, Brasier et al. 1998, Brasier 2001, Konrad et al. 2002, Santini et al. 2005, Brasier & Kirk 2010), but not in North America, where *O. novo-ulmi* subsp. *americana* seems to be the sole DED taxon and is characterized by large clonal populations (Temple et al. 2006). The population structure of the North American population of *O. novo-ulmi* is typical of that of recently-introduced pathogens experiencing a genetic bottleneck, and differs markedly from what has been reported in the closely-related species *O. piceae* (Gagné et al. 2001), as well as in the more distant Ophiostomatoid species *Grosmannia clavigera* (Tsui et al. 2012) which are genetically more diverse and appear to be indigenous to North America.

Molecular identification of genes controlling pathogenicity, virulence and other components of parasitic fitness in the DED fungi has proceeded slowly. So far, only three genes have been subjected to functional analyses. The first one, *Cu*, encodes the hydrophobin cerato-ulmin (CU) which was proposed to be a wilt toxin and thus a pathogenicity factor (Takai 1980). This hypothesis was weakened following the identification of naturally occurring non-CU producing mutants which were nevertheless pathogenic and virulent (Brasier et al. 1995). Furthermore, the *Cu* gene was cloned by Bowden et al. (1994) who subsequently produced knockout *cu*⁻ mutants that remained virulent (Bowden et al. 1996). Temple et al. (1997), based on the

analysis of an *O. ulmi* strain over-expressing CU and an *O. novo-ulmi cu*⁻ knockout mutant, proposed that CU contributed to parasitic fitness by improving yeast-like spore resistance to desiccation and attachment to the exoskeleton of elm bark beetles. Transcription of the *Cu* gene was reported to be 20-60% higher in mycelium than in yeast-like cells grown *in vitro* (Tadesse et al. 2003). The second gene, *Epg1*, encodes the lytic enzyme endopolygalacturonase, thought to be important for xylem colonization by DED fungi (Svaldi & Elgersma 1982). Temple et al. (2009) obtained *epg1*⁻ knockout mutants and reported these were not significantly less virulent than the wild-type strain. A third gene, *Col1*, was identified by Pereira et al. (2000) following random insertional mutagenesis of *O. novo-ulmi* with plasmid DNA (Royer et al. 1991). The authors noticed the occurrence of one hygromycin-resistant transformant with an unusually slow colony growth on nutrient agar. The *Col1* gene is orthologous to the *Saccharomyces cerevisiae* gene coding for U4/U6 splicing factor PRP24. Other genetic loci have been identified in *O. novo-ulmi*, including the *Pat1* locus thought to encode a pathogenicity factor (Et-Touil et al. 1999, 2005) and a locus associated with yeast/mycelial growth (Richards 1994), but have not been cloned. Sequences encoding adenylate cyclase (Binz et al. 1998) and chitin synthase (Hintz 1999), cloned from *O. novo-ulmi*, were not studied further.

The DED fungi are heterothallic species in which mating occurs between sexually-compatible individuals carrying different alleles (or idiomorphs) at the mating-type locus, designated *MAT1* according to standard genetic nomenclature (Debuchy & Turgeon 2006). The *MAT1-2* idiomorph was cloned by gene-walking (Paoletti et al. 2005), whereas a portion (*MAT1-1-3* gene) of the *MAT1-1* idiomorph was retrieved from an

EST library derived from *O. novo-ulmi* subsp. *novo-ulmi* strain H327 (Jacobi et al. 2010). The complete sequence of *MAT1-1* has since been obtained by gene-walking (Jacobi et al. unpublished) and confirmed by full genome sequencing of strain H327 (Forgetta et al. 2013). The structure of the *MAT1-1* and *MAT1-2* idiomorphs of DED fungi is similar to structures described in other heterothallic Sordariomycetes. The *MAT1-1* idiomorph contains three genes, namely *MAT1-1-1, MAT1-1-2* and *MAT1-1-3* encoding a α domain, a DNA-binding acidic amphipathic α-helix (HPG) domain, and a DNA-binding domain of the high mobility group (HMG), respectively. The *MAT1-2* idiomorph encodes a single protein, a HMG domain. Based on phenotypic characterization of DED fungi, Brasier originally suggested that early populations of *O. novo-ulmi* consisted uniquely of MAT1-2 individuals and that this species eventually acquired the *MAT1-1* idiomorph through interspecific hybridization with *O. ulmi* (Brasier 1988a). This hypothesis was later confirmed by genetic mapping of vegetative incompatibility (*Vic*) and *MAT1* loci in a large collection of DED fungi (Paoletti et al. 2006). Brasier also hypothesized that the *MAT1* locus acted as a key regulator of metabolism in the DED fungi (Brasier 1988b). While validation of this hypothesis awaits the development of efficient techniques for targeted gene/allele replacement, the acquisition of the *MAT1-1* idiomorph by *O. novo-ulmi* nevertheless illustrates the potential for rapid species evolution through interspecific hybridization. Another example of allele transfer was provided by the genetic and molecular analysis of a strain of *O. novo-ulmi* (AST27) with unusually low virulence (Et-Touil et al. 1999). Strain AST27 was shown to be an introgressant whose genome was enriched for *O. ulmi*-type RAPD sequences around the *Pat1* locus, suggesting that the

Tab. 1 - General features of the genomes of *Ophiostoma ulmi, O. novo-ulmi* and selected filamentous ascomycetes. (n/a): not applicable.

Species	Size (Mb)	Nb. Chromosomes	%GC	Nb. Genes
Fusarium oxysporum (Sordariomycetes)	61.5	15	48.5	17 735
Botrytis cinerea (Leotiomycetes)	39.5	n/a	43.5	16 448
Fusarium verticillioides (Sordariomycetes)	41.9	11	48.7	14 179
Magnaporthe grisea/M. oryzae (Sordariomycetes)	41	7	51.6	11 074
Neurospora crassa (Sordariomycetes)	41	7	48.2	9 734
Sclerotinia sclerotiorum (Leotiomycetes)	38.3	16	41.8	14 522
Aspergillus oryzae (Eurotiomycetes)	37.1	8	48.2	12 063
Fusarium graminearum (Sordariomycetes)	36.6	4	48.3	13 332
Chaetomium globosum (Sordariomycetes)	34.9	n/a	55.6	11 124
O. piceae (Sordariomycetes)	32.8	n/a	52.8	8 884
Neosartorya fischeri (Eurotiomycetes)	32.5	8	49.4	10 406
O. novo-ulmi (Sordariomycetes)	31.8	8	49.8	8 613
O. ulmi (Sordariomycetes)	31.5	n/a	50	8 639
Aspergillus nidulans/E. nidulans (Eurotiomycetes)	30.1	8	50.3	10 560
Grosmannia clavigera (Sordariomycetes)	29.8	7	53.4	8 301

latter originated from *O. ulmi*. The survival of such low-virulent introgressants in nature, however, appears unlikely unless they possess other attributes that may contribute positively to fitness.

Among eukaryotes, fungi are the organisms that are the most amenable to omic approaches because of their small genomes. Filamentous ascomycetes have genomes ranging from 25 to 160 Megabases (Mb) in size, with approximately 8000 to 18000 genes (Kelkar & Ochman 2012). In the case of the DED pathogens, original estimates of genome size varied from 30 to 40 Mb based on pulsed field gel electrophoresis data (Dewar & Bernier 1995, Dewar et al. 1997). In 2013, the genomic sequences of *O. novo-ulmi* subsp. *novo-ulmi* (Forgetta et al. 2013) and *O. ulmi* (Khoshraftar et al. 2013) were released within weeks of each other. The two genomes are very similar in size and gene number, with 31.8 Mb and 8640 nuclear genes predicted in *O. novo-ulmi* H327, compared to 31.5 Mb and 8639 predicted genes in *O. ulmi* W9 (Tab. 1). These statistics resemble those of ophiostomatoid associates of conifer bark beetles, such as *O. piceae* (Haridas et al. 2013) and *G. clavigera* (DiGuistini et al. 2009). When the genomes of these four ophiostomatoid fungi are compared to those of other plant pathogenic ascomycetes, however, one can see that the former are smaller and harbor fewer genes than most of the latter.

The sequencing of the genomes of *O. novo-ulmi* H327 and *O. ulmi* W9 represents a long-awaited accomplishment, but is only the beginning of a new phase of research on the DED fungi. The *O. novo-ulmi* H327 sequencing project (Forgetta et al. 2013) focused on reproducibility and quality control of the Roche 454 Titanium pyrosequencing technology, which resulted in obtaining a genome with high coverage (over 60X). This, plus the inclusion of additional paired-end sequencing, permitted the assembly of the nuclear genome into 8 scaffolds corresponding to chromosomes, and the full sequencing of the mitochondrial genome (66.4 Kb, 15 predicted genes). On the other hand, none of the 8640 nuclear genes predicted by automated gene-calling algorithms was annotated. Conversely, the genome of *O. ulmi* W9 (Khoshraftar et al. 2013) was not sequenced with the same depth and, as a consequence, could only be scaffolded and not completely assembled. The authors of this work, however, were able to provide an initial annotation of the *O. ulmi* W9 genome based on transcriptional data obtained by large-scale RNA sequencing (RNA-Seq).

The important work of *O. novo-ulmi* H327 annotation and comparison to other Sordariomycetes is currently underway in our group (Comeau et al. unpublished). Preliminary results show that approximately 76% of the proteins could be assigned functions using Gene Ontology analysis (Blast2GO). Additionally, 276 carbohydrate-active enzymes (CAZymes), 48 cytochrome P450s, and 763 proteins potentially involved in pathogen-host interaction, along with 8 clusters of fungal secondary metabolites, have been identified through searches in public databases. A relatively small portion of the genome is occupied by repeat sequences, however the mechanism of repeat-induced point mutation (RIP; see below) appears active in this genome.

A genome-to-genome alignment between *O. novo-ulmi* H327 against the appropriate scaffold from *O. ulmi* W9 showed a large region around the previously mentioned *MAT1* locus has very high identity between the two, consistent with the hypothesis of Paoletti et al. (2006) that the *MAT1-1* idiomorph was recently acquired by *O. novo-ulmi* from *O. ulmi*. Finally, on-going analyses of H327 peptidases, CAZymes and cytochrome P450s are showing various mechanisms and pathways whereby the fungus is able to detoxify and/or transform elm defenses (such as glycoproteins, phytoalexins and terpenes) in order to thrive in its interaction with its plant host and insect vector.

The genomes of *Ophiostoma* spp., like all eukaryotes, contain transposable elements (TEs) which can be divided into two main groups: Type I TEs include retrotransposons, whereas the Type II group is constituted by DNA transposons. Transposable elements can potentially generate novel phenotypes in their hosts. Three Type II TEs (*OPHIO1*, *OPHIO2* and *OPHIO3*) were originally described and characterized in *O. ulmi* and *O. novo-ulmi* (Bouvet et al. 2007, 2008). *OPHIO* transposons occur in low copy number (less than 10 copies) per genome, suggesting that they appeared recently in ophiostomatoid genomes. This hypothesis is supported by the observation that *OPHIO1* and *OPHIO2* are restricted to *O. ulmi* and *O. novo-ulmi* subsp. *novo-ulmi* (Bouvet et al. 2007) and can be induced to move from their genomic location by various biotic and abiotic stresses (Bouvet et al. 2008), which are two characteristics of a recent invasion. Based on the high variability of its Southern hybridization profiles, *OPHIO2* seems to have the highest level of mobility among *OPHIO* TEs. On the other hand, transposon *OPHIO3* displays less variation in its Southern hybridization profiles and was found to be heavily modified by RIP. The latter is a mechanism found in many fungi which allows them to inactivate repeated sequences prior to or during sexual recombination. RIP is a homology-dependent genome defense that introduces cytosine-to-thymine transition mutations in duplicated DNA sequences and is thought to control the proliferation of selfish repetitive DNA. Why only *OPHIO3*, and not *OPHIO1* and *OPHIO2*, was found to be RIPed in the DED fungal strains examined so far remains an open question. In addition, the extent to which TEs contribute to genomic and phenotypic plasticity of DED fungi remains unknown.

Surveys of natural populations of DED fungi have led to the identification of diseased individuals that display reduced growth rates, unstable "amoeboid" colony morphologies, low spore viability, decreased reproductive ability, and reduced virulence (Brasier 1983, Sutherland & Brasier 1995, Hintz et al. 2013). The lower fitness of such individuals is not the result of nuclear mutations. Rather, this particular phenotype has been associated with the presence of cytoplasmic elements originally known as d-factors (Brasier 1983) which have since been found to be double-stranded RNA viruses located in the mitochondria (mitoviruses). At least 15 different mitoviruses have been described in the DED fungi (Hintz et al. 2013), and genome sequences for nine of these are available in the NCBI database. How the mitoviruses found in *O. ulmi* and *O. novo-ulmi* act on their fungal host is unknown, but their dramatic effect on fungal physiology has prompted researchers to speculate on their use for biological control of DED (Hintz et al. 2013). One additional feature of the mitochondrial genome of DED fungi is the occurrence of LAGLIDADG homing endonuclease (*Heg*) genes within the *Rps3* gene encoding ribosomal protein S3 (Sethuraman et al. 2009). The biological significance of the presence of *Heg* genes in the mitochondrial genome of DED fungi, however, is not known.

Development of tools for genomic studies of the DED pathosystem

In order for the DED pathosystem to fully enter the omic era, tools and resources are required. Some of these tools are already available, whereas others need to be developed. In the case of the pathogens, omics are facilitated by the availability of simple procedures (Fig. 2) established decades ago for growing large numbers of colonies on solid media amended with deoxycholic acid (Bernier & Hubbes 1990a, 1990b), manipulating environmental parameters for obtaining either yeast or mycelial growth phases (Kulkarni & Nickerson 1981), and obtaining both asexual and sexual fruiting structures *in vitro* (Brasier 1981, Bernier & Hubbes 1990b). This has facilitated the construction of an EST expression library for the yeast growth phase of *O. novo-ulmi* (Hintz et al. 2011), as well as smaller EST datasets associated with the production of synnemata and perithecia (Jacobi et al. 2010). These are valuable resources for elucidating the molecular bases of biological functions in the DED fungi.

The mere association of ESTs with a given function, however, is not sufficient. Eventually, mutants are required for establishing the role of specific genes in these functions. As mentioned earlier in this review, genetic transformation of *O. novo-ulmi* was achieved in the early 1990s (Royer et al. 1991) and has been used both for random insertional mutagenesis (Pereira et al. 2000, Plourde et al. 2008) and inactivation of selected genes (Bowden et al. 1996, Temple et al. 2009). Problems, however, must be overcome before insertional mutants with the desired phenotype are recovered efficiently in the DED fungi. In the case of random insertional mutagenesis, it has proven difficult to clone mutated loci by recovering *Ophiostoma* DNA sequences flanking the plasmid insertion site due to large rearrangements (Plourde et al. 2008). On the other hand, targeted inactivation of specific genes may require a large screening effort: Temple et al. (2009) reported recovering one true *Δepg1* knockout mutants out of 50 transformants, whereas Bowden et al. (1996) had to screen 2500 transformants in order to recover one *Δcu* mutant. We recently recovered a mutant of *O. novo-ulmi* in which the gene *Mus52*, involved in non-homologous DNA repair (Ninomiya et al. 2004), has been inactivated (Naruzawa et al., unpublished). We are now using this *Δmus52* strain in further gene replacement experiments and assessing whether this improves the frequency at which knockout mutants are recovered. Meanwhile, protocols have been developed for gene knockdown in *O. novo-ulmi* by RNA interference (RNAi) technology (Carneiro et al. 2010). Furthermore, RNAi constructions including an endogenous promoter are now available for controlling the expression of the targeted gene by changing the source of carbon in the growth medium (Carneiro et al. 2013).

The EST expression libraries produced so far for the DED fungi are limited in scope. They are relatively small and only represent three physiological stages: the largest library, from yeast cells, contains 4386 sequences representing 2093 genes (Hintz et al. 2011), whereas the SSH-enriched libraries described by Jacobi et al. (2010) for synnemata and perithecia are even smaller, with tags for 181 and 128 genes, respectively. These libraries were constructed and analyzed at a time when ESTs were considered the most economical way to investigate the fungal transcriptome. This is no longer the case since newer technologies such as genome-wide RNA sequencing offer a much deeper coverage of the transcriptome for a fraction of the cost of standard EST analysis. This approach was used by Khoshraftar and colleagues to get a first glimpse of the transcriptome of *O. ulmi* W9 yeast cultures. Approximately 80% of the gene models identified *ab initio* were represented by at least one transcript in the
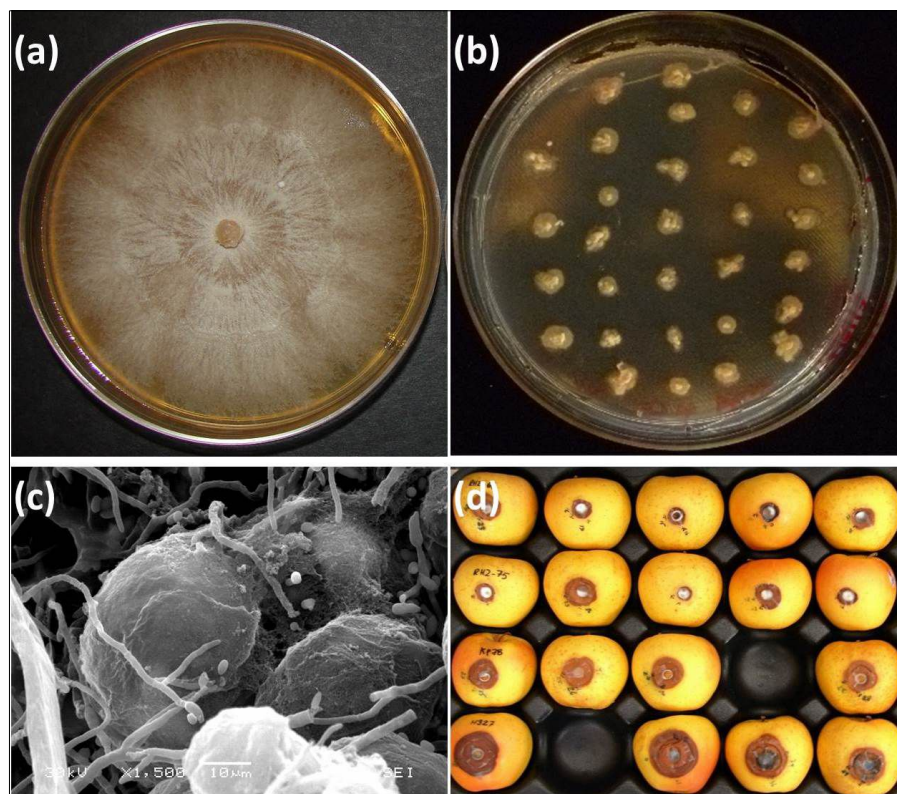

Fig. 2 - Approaches for screening high numbers of DED fungal strains *in vitro*. Extensive mycelial growth on standard solid media (a) precludes testing of several individuals on a single culture dish. Addition of deoxycholic acid (0.01%, v/v) induces a colonial growth pattern (b), without affecting cell germination, thus facilitating screening either directly or by replica plating. Callus tissues grown on solid media are suitable material for analyzing host-pathogen interactions (c). "Golden Delicious" apples (d) provide a rapid and efficient bioassay for around-the-year screening of mutant DED fungal strains derived from wild-type strain (arrow). Figure prepared from original data from Bernier & Hubbes (1990a), Aoun et al. (2010), and Plourde & Bernier (2014).

O. ulmi W9 RNA-Seq dataset (Khoshraftar et al. 2013), which represents a *ca* 3.5-fold improvement over previous EST data for *O. novo-ulmi* H327 (Hintz et al. 2011). The above-mentioned studies, however, were exploratory and did not include biological replicates. We are currently carrying out replicated RNA-Seq comparative analyses of the transcriptomes of yeast and mycelial cultures of *O. novo-ulmi* H327 in order to identify subsets of genes whose expression differs significantly between the two growth forms (Nigg et al. unpublished).

While no draft genome is available for any species within the genus *Ulmus*, resources are nevertheless available for elm omics and could help in the study of host-pathogen (or host-vector) interactions. The large EST dataset for the interaction between *U. minor* and the elm leaf beetle *X. luteola* (Büchel et al. 2012) is by far the most complete investigation into the elm transcriptome and likely contains information that can be used in the study of DED. The latter can be further facilitated by using elm cell cultures (Fig. 2), either in liquid medium (Corchete et al. 1993)

or on solid substrate (Pijut et al. 1990). While it may be argued that elm calli do not possess the characteristic arrangement of differentiated xylem in saplings and trees, they nevertheless express defense responses (such as accumulation of phenolics and production of suberin layers) typically encountered in trees (Aoun et al. 2009). Furthermore, they are more amenable than xylem tissue from twigs, branches or stem for transcriptomic analyses (Aoun et al. 2010). Putative defense-related genes identified in elm calli inoculated with the DED pathogens could be further characterized in saplings, through genetic transformation (Gartland et al. 2005, Newhouse et al. 2007). Although gene knockout experiments resulting in loss of function cannot be currently envisioned, gain of function through overexpression of selected genes following ectopic transformation is theoretically feasible.

Functional genomics of any pathosystem also requires that large-scale assays of mutant fungal strains or plant lines are carried out efficiently and in a cost-effective manner. In the case of DED, this rules out the

Tab. 2 - Some features of the Dutch elm disease pathosystem amenable to omic investigations.

Host	- Production of a draft genome for an *Ulmus* species
	- *In silico* identification of putative resistance genes by comparative genomics
	- Marker-assisted breeding of elms combining higher DED resistance and other desired traits
	- Production of DED resistant elms by targeted mutagenesis
Pathogen	- Production of a draft genome for *O. novo-ulmi* subsp. *americana* and *O. himal-ulmi*
	- *In silico* identification of putative pathogenicity and virulence genes by comparative genomics
	- Development of efficient procedures for producing knockout mutants
	- Functional analyses of other fitness traits, such as yeast-mycelium dimorphism, mating-type switching, and resistance to biotic/abiotic stresses
	- Determination of the fate and function of transposons and mycoviruses
Vector	- Production of a draft genome for *Hylurgopinus rufipes* and/or a *Scolytus* species
	- *In silico* identification of putative genes encoding elm and bark beetle pheromone receptors
	- Analysis of intraspecific and interspecific variation for targeted genes
Dutch elm disease interactome	- Transcriptomic analysis of elm bark beetle pheromone response genes in the presence of elm volatiles associated with healthy (feeding) and moribund (egg laying) elms
	- Transcriptomic analysis of elm response to young elm bark beetle adults feeding on phloem
	- Large-scale transcriptomic analysis of elm response to DED fungi
	- Gain/loss-of-function studies of targeted genes in host, pathogen and vector
	- Analysis of microbial diversity in elm bark beetle breeding galleries

inoculation of large sets of mutant *Ophiostoma* strains to elm saplings, because such inoculations are time- and material-consuming, must be conducted under confinement (especially when transformants of *Ophiostoma* and/or *Ulmus* are used), and require that inoculated elms be "receptive", *i.e.*, that they are inoculated when they are producing early wood. The use of elm tissue cultures alleviates some of these problems but remains technologically demanding. We recently adapted an apple based-assay (Fig. 2), designed originally for testing the virulence of the chestnut blight pathogen *Cryphonectria parasitica* (Fulbright 1984), for screening a library of insertional mutants of *O. novo-ulmi* H327 (Plourde & Bernier 2014). Parallel experiments in which mutants were inoculated to "Golden Delicious" apples and *Ulmus parvifolia* x *U. americana* hybrid saplings confirmed that the apple assay could be used to identify rapidly mutants with altered levels of virulence. This assay thus represents a useful tool for year-round screening of large numbers of *O. novo-ulmi* transformants. It could also likely be used for studying virulence in *O. himal-ulmi* (Bernier et al. unpublished).

Some aspects of DED worth visiting or revisiting

The interaction between *Ulmus* and pathogenic *Ophiostomas* has often been considered in a very reductionist way. For example, individual molecules such as cerato-ulmin (Takai 1980) and peptidorhamnomannans

(Nordin & Strobel 1981) were proposed as pathogenicity factors in *O. novo-ulmi*, whereas resistance was associated with the production of mansonone phytoalexins (Jeng et al. 1983). This oversimplified view of DED was in good part rooted in the type of experimental approaches that were available at the time. The omic revolution is rapidly changing the way we can address biological questions such as DED. The first analyses of the elm transcriptome have indeed shown that exposure to the pathogen (Aoun et al. 2010) or to eggs from leaf defoliators (Büchel et al. 2012) triggers a large array of genes coding for proteins involved in several different pathways. This is all the more obvious in the analysis carried out by Büchel et al. (2012) which showed that insects triggered responses that were also associated with pathogens. One can expect similar observations for the pathogen once *Ophiostoma* transcriptomes are systematically assessed from cultures harvested at different physiological states or exposed to various environmental conditions. For example, recent work on thermally controlled yeast-mycelium dimorphism in *Histoplasma capsulatum*, a soil-inhabiting pathogen of mammals, provides a genome-wide view of the complex transcriptional network linking pathogenicity and morphology in this species (Beyhan et al. 2013). Likewise, it seems reasonable to hypothesize that ongoing transcriptomic analyses of yeast-mycelium dimorphism in *O. novo-ulmi* will reveal significant shifts in the transcription of large subsets of genes. In the near fu-

ture, comparative genomic studies of elms with other tree species, and of the DED fungi with pathogenic species and with saprobes will likely help identify sets of genes that are common to most (if not all) plant-pathogen interactions, genes that may be specific to vascular wilts, and genes that are unique to the DED pathosystem. A partial list of features of the DED pathosystem that might be unravelled in future genomic investigations is presented in Tab. 2.

Concluding Remarks

Dutch elm disease is one of the most studied and documented fungal diseases of trees and has long been considered a textbook example of the devastating impact of exotic pathogens on plant populations. The last decades, however, have coincided with a dramatic decline in DED research, as only a few research groups are still actively studying this pathosystem. Ironically, this is happening at a time when powerful new analytical methods are available for gaining new insight into the biology of the protagonists. Meanwhile, omic approaches are being used for investigating other tree-pathogen interactions, such as chestnut blight (Barakat et al. 2009), *Melampsora* poplar leaf rust (Duplessis et al. 2011) or sudden oak death (Croucher et al. 2013). Pathogenomics of DED is clearly lagging behind the above systems, for reasons which are not biological in nature, as should be clear from the examples provided throughout this review. Rather, DED falls somewhere in the grey zone between the *Melampsora* poplar leaf rust "model pathosystem" and emerging diseases (such as ash dieback) which are now monopolizing attention and financial resources. Researchers interested in elms and their interactions with the environment should look at initiatives such as the Fagaceae Genomics Web (http://www.fagaceae.org) which has helped the chestnut blight research community secure funding for omic research, and has resulted in the creation of a large, public database for several tree species within the Fagaceae. Isn't it time for a cooperative, large-scale international research effort on DED, a disease with such historical and global impact?

References

Aoun M, Rioux D, Simard M, Bernier L (2009). Colonization and defense reactions in *Ulmus americana* callus cultures inoculated with *Ophiostoma novo-ulmi*. Phytopathology 99: 642-650. - doi: 10.1094/PHYTO-99-6-0642

Aoun M, Jacobi V, Boyle B, Bernier L (2010). Identification and monitoring of *Ulmus americana* transcripts during *in vitro* interactions with the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. Physiological and Molecular Plant Pathology 74: 254-266. - doi: 10.1016/j.pmpp.2010.04.003

Barakat A, DiLoreto DS, Zhang Y, Smith C, Baier K, Powell W, Wheeler N, Sederoff R, Carlson JE (2009). Comparison of transcriptome from cankers and healthy stems in American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*). BMC Plant Biology 9: 51. - doi: 10.1186/1471-2229-9-51

Bates MR, Buck KW, Brasier CM (1993). Molecular relationships between *Ophiostoma ulmi* and the NAN and EAN races of *O. novo-ulmi* determined by restriction fragment length polymorphisms of nuclear DNA. Mycological Research 97: 449-455. - doi: 10.1016/S0953-7562(09)80132-9

Bernier L, Hubbes M (1990a). Mutations in *Ophiostoma ulmi* induced by N-methyl-N'-nitro-N-nitrosoguanidine. Canadian Journal of Botany 68: 225-231. - doi: 10.1139/b90-031

Bernier L, Hubbes M (1990b). Meiotic analysis of induced mutations in *Ophiostoma ulmi*. Canadian Journal of Botany 68: 232-235. - doi: 10.1139/b90-032

Beyhan S, Gutierrez M, Voorhies M, Sil A (2013). A temperature-responsive network links cell shape and virulence traits in a primary fungal pathogen. PLoS Biology 11: e1001614. - doi: 10.1371/journal.pbio.1001614

Binz T, Jalali F, Horgen PA (1998). Isolation of adenylate cyclase gene-specific sequences from *Ophiostoma novo-ulmi*, *Candida albicans*, and *Agaricus bisporus* by PCR. Current Microbiology 37: 359-361. - doi: 10.1007/s002849900393

Blomquist GJ, Figueroa-Teran R, Aw M, Song M, Gorzalski A, Abbott NL, Chang E, Tittiger C (2010). Pheromone production in bark beetles. Insect Biochemistry and Molecular Biology 40: 699-712. - doi: 10.1016/j.ibmb.2010.07.013

Bouvet GF, Jacobi V, Bernier L (2007). Characterization of three DNA transposons in the Dutch elm disease fungi and evidence of repeat-induced point (RIP) mutations. Fungal Genetics and Biology 44: 430-443. - doi: 10.1016/j.fgb.2006.11.016

Bouvet GF, Jacobi V, Plourde KV, Bernier L (2008). Stress-induced mobility of OPHIO1 and OPHIO2, DNA transposons of the Dutch elm disease fungi. Fungal Genetics and Biology 45: 565-578. - doi: 10.1016/j.fgb.2007.12.007

Bowden CG, Hintz WE, Jeng RS, Hubbes M, Horgen PA (1994). Isolation and characterization of the cerato-ulmin gene of the Dutch elm disease pathogen, *Ophiostoma ulmi*. Current Genetics 25: 323-329. - doi: 10.1007/BF00351485

Bowden CG, Smalley E, Guries RP, Hubbes M, Temple B, Horgen PA (1996). Lack of association between cerato-ulmin production and virulence in *Ophiostoma novo-ulmi*. Molecular Plant-Microbe Interactions 9: 556-564. - doi: 10.1094/MPMI-9-0556

Brasier CM (1981). Laboratory investigation of *Ceratocystis ulmi*. In "Compendium of elm diseases" (Stipes RJ, Campana RJ eds). American Phytopathological Society, St. Paul, MN, USA, pp. 76-79.

Brasier CM (1983). A cytoplasmically transmitted disease of *Ceratocystis ulmi*. Nature 305 : 220-223. - doi: 10.1038/305220a0

Brasier CM (1988a). Rapid changes in genetic structure of epidemic populations of *Ophiostoma ulmi*. Nature 332: 538-541. - doi: 10.1038/332538a0

Brasier CM (1988b). *Ophiostoma ulmi*, cause of Dutch elm disease. In: "Advances in Plant Pathology, vol. 6" (Sidhu GS ed). Academic Press, New York, USA, pp. 207-223.

Brasier CM (1991). *Ophiostoma novo-ulmi* sp. *nov.*, causative agent of current Dutch elm disease pandemics. Mycopathologia 115: 151-161. - doi: 10.1007/BF00462219

Brasier CM (2001). Rapid evolution of introduced plant pathogens via interspecific hybridization. Bioscience 51: 123-33. - doi: 10.1641/0006-3568(2001)051[0123:REOIPP]2.0.CO;2

Brasier CM, Kirk SA (2001). Designation of the EAN and NAN races of *Ophiostoma novo-ulmi* as subspecies. Mycological Research 105: 547-554. - doi: 10.1017/S0953756201004087

Brasier CM, Kirk SA (2010). Rapid emergence of hybrids between the two subspecies of *Ophiostoma novo-ulmi* with a high level of pathogenic fitness. Plant Pathology 59: 186-199. - doi: 10.1111/j.1365-3059.2009.02157.x

Brasier CM, Kirk SA, Pipe ND, Buck KW (1998). Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. Mycological Research 102: 45-57. - doi: 10.1017/S0953756297004541

Brasier CM, Kirk SA, Tegli S (1995). Naturally occurring non ceratoulmin producing mutants of *Ophiostoma novo-ulmi* are pathogenic but lack aerial mycelium. Mycological Research 99: 436-440. - doi: 10.1016/S0953-7562(09)80641-2

Brasier CM, Mehrotra MD (1995). *Ophiostoma himal-ulmi* sp. *nov.*, a new species of Dutch elm disease fungus endemic to the Himalayas. Mycological Research 99: 205-215. - doi: 10.1016/S0953-7562(09)80887-3

Brunet J, Zalapa JE, Peccori F, Santini A (2013). Patterns of hybridization and introgression between the exotic Siberian elm, *Ulmus pumila*, and the native Field elm, *U. minor* in Italy. Biological Invasions 15: 2717-2730. - doi: 10.1007/s10530-013-0486-z

Büchel K, McDowell E, Nelson W, Descour A, Gershenzon J, Hilker M, Soderlund C, Gang DR, Fenning T, Meiners T (2012). An elm EST database for identifying leaf beetle egg-induced defense genes. BMC Genomics 13: 242. - doi: 10.1186/1471-2164-13-242

Carneiro JS, de la Bastide PY, Chabot M, Lerch L, Hintz WE (2010). Suppression of polygalacturonase gene expression in the phytopathogenic fungus *Ophiostoma novo-ulmi* by RNA interference. Fungal Genetics and Biology 47: 399-405. - doi: 10.1016/j.fgb.2010.02.005

Carneiro JS, de la Bastide PY, Hintz WE (2013). Regulated gene silencing in the fungal pathogen *Ophiostoma novo-ulmi*. Physiological and Molecular Plant Pathology 82: 28-34. - doi: 10.1016/j.pmpp.2013.01.001

Coleman M, Hollingsworth ML, Hollingsworth PM (2000). Application of RAPDs to the critical taxonomy of the English endemic elm *Ulmus plotii* Druce. Botanical Journal of the Linnean Society 133: 241-262. - doi: 10.1111/j.1095-8339.2000.tb01545.x

Corchete MP, Diez JJ, Valle T (1993). Phenylalanine ammonialyase activity in suspension cultures of *Ulmus pumila* and *U. campestris* treated with spores of *Ceratocystis ulmi*. Plant Cell Reports 13: 111-114. - doi: 10.1007/BF00235302

Croucher PJP, Mascheretti S, Garbelotto M (2013). Combining field epidemiological information and genetic data to comprehensively reconstruct the invasion history and the microevolution of the sudden oak death agent *Phytophthora ramorum* (Stramenopila: Oomycetes) in California. Biological Invasions 15: 2281-2297. - doi: 10.1007/s10530-013-0453-8

Debuchy R, Turgeon BG (2006). Mating-type structure, evolution, and function in Euascomycetes. In: "The Mycota I (2nd edn). Growth, Differentation and Sexuality" (Kües U, Fischer R eds). Springer-Verlag, Berlin & Heidelberg, Germany, pp. 293-323.

Dewar K, Bernier L (1995). Inheritance patterns of nonhomologous chromosomes in *Ophiostoma ulmi sensu lato*. Current Genetics 27: 541-549. - doi: 10.1007/BF00314445

Dewar K, Bousquet J, Dufour J, Bernier L (1997). A meiotically reproducible chromosome length polymorphism in the ascomycete fungus *Ophiostoma ulmi* (*sensu lato*). Molecular and General Genetics 255: 38-44. - doi: 10.1007/s004380050472

DiGuistini S, Liao NY, Platt D, Robertson G, Seidel M, Chan SK, Roderick Docking T, Birol I, Holt RA, Hirst M, Mardis E, Marra MA, Hamelin RC, Bohlmann J, Breuil C, Jones SJM (2009). *De novo* genome sequence assembly of a filamentous fungus using Sanger, 454 and Illumina sequence data. Genome Biology 10: R94. [online] URL: http://www.ncbi.nlm.nih.gov/pubmed/19747388

Duplessis S, Hacquard S, Delaruelle C, Tisserant E, Frey P, Martin F, Kohler A (2011). *Melampsora larici-populina* transcript profiling during germination and timecourse infection of poplar leaves reveals dynamic expression patterns associated with virulence and biotrophy. Molecular Plant-Microbe Interactions 24: 808-818. - doi: 10.1094/MPMI-01-11-0006

Et-Touil A, Brasier CM, Bernier L (1999). Localization of a pathogenicity gene in *Ophiostoma novo-ulmi* and evidence that it may be introgres-

sed from *O. ulmi*. Molecular Plant-Microbe Interactions 12: 6-15. - doi: 10.1094/MPMI.1999.12.1.6

Et-Touil A, Rioux D, Mathieu F, Bernier L (2005). External symptoms and histopathological changes following inoculation of genetically close strains of *Ophiostoma* to elms putatively resistant to Dutch elm disease. Canadian Journal of Botany 83: 656-667. - doi: 10.1139/b05-037

Forgetta V, Leveque G, Dias J, Grove D, Lyons Jr R, Genik S, Wright C, Singh S, Peterson N, Zianni M, Kieleczawa J, Hintz W, Jacobi V, Bernier L, Levesque R, Dewar K (2013). Comparison of multiple genome sequencing centers and analysis of the Dutch elm disease fungus genome Using the Roche/454 GS-FLX Titanium System. Journal of Biomolecular Techniques 24: 39-49. [online] URL: http://www.ncbi.nlm.nih.gov/pubmed/23542132

Fulbright DW (1984). Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology 74: 722-724. - doi: 10.1094/Phyto-74-722

Gagné P, Yang DQ, Hamelin RC, Bernier L (2001). Genetic variability of Canadian populations of the sapstain fungus *Ophiostoma piceae*. Phytopathology 91: 369-376. - doi: 10.1094/PHYTO.2001.91.4.369

Gartland KMA, McHugh AT, Crow RM, Garg A, Gartland JS (2005). Biotechnological progress in dealing with Dutch elm disease. In Vitro Cellular and Developmental Biology-Plant 41: 364-367. - doi: 10.1079/IVP2005646

Gayathri Samarasekera GDN, Bartell NV, Lindgren BS, Cooke, JEK, Davis CS, James PMA, Coltman DW, Mock KE, Murray BW (2012). Spatial genetic structure of the mountain pine beetle (*Dendroctonus ponderosae*) outbreak in western Canada: historical patterns and contemporary dispersal. Molecular Ecology 21: 2931-2948. - doi: 10.1111/j.1365-294X.2012.05587.x

Gil L, Fuentes-Utrilla P, Soto A, Cervera MT, Collada C (2004). English elm is a 2000-year-old Roman clone. Nature 431: 1053. - doi: 10.1038/4311053a

Goodall-Copestake WP, Hollingsworth ML, Hollingsworth PM, Jenkins GI, Collin E (2005). Molecular markers and *ex situ* conservation of the European elms (*Ulmus* spp.). Biological Conservation 122: 537-546. - doi: 10.1016/j.biocon.2004.09.011

Haran J, Timmermans MJTN, Vogler AP (2013). Mitogenome sequences stabilize the phylogenetics of weevils (Curculionoidea) and establish the monophyly of larval ectophagy. Molecular Phylogenetics and Evolution 67: 156-166. - doi: 10.1016/j.ympev.2012.12.022

Haridas S, Wang Y, Lim L, Massoumi Alamouti S, Jackman S, Docking R, Robertson G, Birol I, Bohlmann J, Breuil C (2013). The genome and transcriptome of the pine saprophyte *Ophiostoma piceae*, and a comparison with the bark beetle-associated pine pathogen *Grosmannia clavigera*. BMC Genomics 14: 373. - doi: 10.1186/1471-2164-14-373

Hintz WE (1999). Sequence analysis of the chitin

synthase A gene of the Dutch elm pathogen *Ophiostoma novo-ulmi* indicates a close association with the human pathogen *Sporothrix schenckii*. Gene 237: 215-221. - doi: 10.1016/S0378-1119(99)00291-7

Hintz WE, Jeng RS, Hubbes M, Horgen PA (1991). Identification of three populations of *Ophiostoma ulmi* (aggressive subgroup) by mitochondrial DNA restriction-site mapping and nuclear DNA fingerprinting. Experimental Mycology 15: 316-325. - doi: 10.1016/0147-5975(91)90035-C

Hintz W, Pinchback M, de la Bastide P, Burgess S, Jacobi V, Hamelin R, Breuil C, Bernier L (2011). Functional categorization of unique expressed sequence tags obtained from the yeast-like growth phase of the elm pathogen *Ophiostoma novo-ulmi*. BMC Genomics 12: 431. - doi: 10.1186/1471-2164-12-431

Hintz W, Carneiro JS, Kassatenko I, Varga A, James D (2013). Two novel mitoviruses from a Canadian isolate of the Dutch elm pathogen *Ophiostoma novo-ulmi* (93-1224). Virology Journal 10: 252. - doi: 10.1186/1743-422X-10-252

Hoegger PJ, Binz T, Heiniger U (1996). Detection of genetic variation between *Ophiostoma ulmi* and the NAN and EAN races of *O. novo-ulmi* in Switzerland using RAPD markers. European Journal of Forest Pathology 26: 57-68. - doi: 10.1111/j.1439-0329.1996.tb00710.x

Houston DR (1991). Changes in aggressive and nonaggressive subgroups of *Ophiostoma ulmi* within two populations of American elm in New England. Plant Disease 75: 720-722. - doi: 10.1094/PD-75-0720

Jacobi V, Dufour J, Bouvet GF, Aoun M, Bernier L (2010). Identification of transcripts upregulated in asexual and sexual fruiting bodies of the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. Canadian Journal of Microbiology 56: 697-705. - doi: 10.1139/W10-053

Jeng RS, Alfenas AC, Hubbes M, Dumas M (1983). Presence and accumulation of fungitoxic substances against *Ceratocystis ulmi* in *Ulmus americana*: possible relation to induced resistance. European Journal of Forest Pathology 13: 239-244. - doi: 10.1111/j.1439-0329.1983.tb00124.x

Jeng RS, Duchesne LC, Sabourin M, Hubbes M (1991). Mitochondrial DNA restriction fragment length polymorphisms of aggressive and non-aggressive isolates of *Ophiostoma ulmi*. Mycological Research 95: 537-542. - doi: 10.1016/S0953-7562(09)80065-8

Jordal BH, Sequeira AS, Cognato AI (2011). The age and phylogeny of wood boring weevils and the origin of subsociality. Molecular Phylogenetics and Evolution 59: 708-724. - doi: 10.1016/j.ympev.2011.03.016

Keeling CI, Henderson H, Li M, Yuen M, Clark EL, Fraser JD, Huber DPW, Liao NY, Docking TR, Birol I, Chan SK, Taylor GA, Palmquist D, Jones SJM, Bohlmann J (2012). Transcriptome and full-length cDNA resources for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins,

a major insect pest of pine forests. Insect Biochemistry and Molecular Biology 42: 525-536. - doi: 10.1016/j.ibmb.2012.03.010

Kelkar YD, Ochman H (2012). Causes and consequences of genome expansion in fungi. Genome Biology and Evolution 4: 13-23. - doi: 10.1093/gbe/evr124

Khoshraftar S, Hung S, Khan S, Gong Y, Tyagi V, Parkinson J, Sain M, Moses AM, Christendat D (2013). Sequencing and annotation of the *Ophiostoma ulmi* genome. BMC Genomics 14: 162. - doi: 10.1186/1471-2164-14-162

Konrad H, Kirisits T, Riegler M, Halmschlager E, Stauffer C (2002). Genetic evidence for natural hybridisation between the Dutch elm disease pathogens *Ophiostoma novo-ulmi* ssp. *novo-ulmi* and *Ophiostoma novo-ulmi* ssp. *americana*. Plant Pathology 51: 78-4. - doi: 10.1046/j.0032-0862.2001.00653.x

Kremer A, Abbott AG, Carlson JE, Manos PS, Plomion C, Sisco P, Staton ME, Ueno S, Vendramin GG. (2012). Genomics of Fagaceae. Tree Genetics and Genomes 8: 583-610. - doi: 10.1007/s11295-012-0498-3

Kulkarni RK, Nickerson KW (1981). Nutritional control of dimorphism in *Ceratocystis ulmi*. Experimental Mycology 5: 148-154. - doi: 10.1016/0147-5975(81)90015-3

Masuya H, Brasier C, Ichihara Y, Kubonoa T, Kanzaki N (2010). First report of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi* in Japan. Plant Pathology 59: 805. - doi: 10.1111/j.1365-3059.2009.02239.x

McLeod G, Gries R, von Reuß SH, Rahe JE, McIntosh R, König WA, Gries G (2005). The pathogen causing Dutch elm disease makes host trees attract insect vectors. Proceedings of the Royal Society B-Biological Sciences 272: 2499-2503 - doi: 10.1098/rspb.2005.3202

Misaghi IJ (1982). The role of pathogen produced cell-wall degrading enzymes in infection and damage. In: "Physiology and Biochemistry of Plant Pathogen Interactions" (Misaghi IJ ed). Plenum Press, New York, USA, pp. 17-34.

Mittempergher L, Santini A (2004). The history of elm breeding. Investigacion Agraria, Sistemas y Recursos Forestales 13: 161-177.

Mock KE, Bentz BJ, O'Neill EM, Chong P, Orwin J, Pfrender ME (2007). Landscape-scale genetic variation in a forest outbreak species, the mountain pine beetle (*Dendroctonus ponderosae*). Molecular Ecology 16: 553-568. - doi: 10.1111/j.1365-294X.2006.03158.x

Moser JC, Konrad H, Blomquist SR, Kirisits T (2010). Do mites phoretic on elm bark beetles contribute to the transmission of Dutch elm disease? Naturwissenschaften 97: 219-227. - doi: 10.1007/s00114-009-0630-x

Nasmith C, Jeng R, Hubbes M (2008a). Targeted gene analysis in *Ulmus americana* and *U. pumila* tissues. Forest Pathology 38: 90-103. - doi: 10.1111/j.1439-0329.2007.00523.x

Nasmith C, Jeng R, Hubbes M (2008b). A comparison of in vivo targeted gene expression during fungal colonization of DED-susceptible *Ulmus americana*. Forest Pathology 38: 104-112. -

doi: 10.1111/j.1439-0329.2007.00527.x

Newhouse AE, Schrodt F, Liang H, Maynard CA, Powell WA (2007). Transgenic American elm shows reduced Dutch elm disease symptoms and normal mycorrhizal colonization. Plant Cell Reports 26: 977-987. - doi: 10.1007/s00299-007-0313-z

Ninomiya Y, Suzuki K, Ishii C, Inoue H (2004). Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proceedings of the National Academy of Sciences USA 101: 12248-12253. - doi: 10.1073/pnas.0402780101

Nordin JH, Strobel GA (1981). Structural and immunochemical studies on the phytotoxic peptidorhamnomannan of *Ceratocystis ulmi*. Plant Physiology 67: 1208-1213. - doi: 10.1104/pp.67.6.1208

Paoletti M, Buck KW, Brasier CM (2005). Cloning and sequence analysis of the MAT-B (MAT-2) genes from the three Dutch elm disease pathogens, *Ophiostoma ulmi, O.novo-ulmi*, and *O. himal-ulmi*. Mycological Research 109: 983-991. - doi: 10.1017/S0953756205003308

Paoletti M, Buck KW, Brasier CM (2006). Selective acquisition of novel mating type and vegetative incompatibility genes via interspecies gene transfer in the globally invading eukaryote *Ophiostoma novo-ulmi*. Molecular Ecology 15: 249-262. - doi: 10.1111/j.1365-294X.2005.02728.x

Pereira V, Royer JC, Hintz WE, Field D, Bowden C, Kokurewicz K, Hubbes M, Horgen PA (2000). A gene associated with filamentous growth in *Ophiostoma novo-ulmi* has RNA-binding motifs and is similar to a yeast gene involved in mRNA splicing. Current Genetics 37: 94-103. - doi: 10.1007/s002940050015

Pijut PM, Lineberger RD, Domir SC, Ichida JM, Krause CR (1990). Ultrastructure of cells of *Ulmus americana* cultured *in vitro* and exposed to the culture filtrate of *Ceratocystis ulmi*. Phytopathology 80: 764-767. - doi: 10.1094/Phyto-80-764

Plourde KV, Bernier L (2014). Inoculation of apple (*Malus x domestica* "Golden Delicious") fruits provides a rapid virulence assay for the Dutch elm disease fungus *Ophiostoma novo-ulmi*. Plant Pathology. - doi: 10.1111/ppa.12177

Plourde KV, Jacobi V, Bernier L (2008). Use of insertional mutagenesis to tag putative parasitic fitness genes in the Dutch elm disease fungus *Ophiostoma novo-ulmi* subsp. *novo-ulmi*. Canadian Journal of Microbiology 54: 797-802. - doi: 10.1139/W08-068

Richards WC (1994). Nonsporulation in the Dutch elm disease fungus *Ophiostoma ulmi*: evidence for control by a single nuclear gene. Canadian

Journal of Botany 72: 461-467. - doi: 10.1139/b94-061

Royer JC, Dewar K, Hubbes M, Horgen PA (1991). Analysis of a high frequency transformation system for *Ophiostoma ulmi*, the causal agent of Dutch elm disease. Molecular and General Genetics 225: 168-176. - doi: 10.1007/BF00282655

Santini A, Montaghi A, Vendramin GG, Capretti P (2005). Analysis of the Italian Dutch elm disease fungal population. Journal of Phytopathology 153: 73-79. - doi: 10.1111/j.1439-0434.2004.00931.x

Sethuraman J, Majer A, Friedrich NC, Edgell DR, Hausner G (2009). Genes within genes: multiple LAGLIDADG homing endonucleases target the ribosomal protein S3 gene encoded within an rnl group I intron of *Ophiostoma* and related taxa. Molecular Biology and Evolution 26: 2299-2315. - doi: 10.1093/molbev/msp145

Solla A, Dacasa MC, Nasmith C, Hubbes M, Gil L (2008). Analysis of Spanish populations of *Ophiostoma ulmi* and *O. novo-ulmi* using phenotypic characteristics and RAPD markers. Plant Pathology 57: 33-44. - doi: 10.1111/j.1365-3059.2007.01692.x

Staszak AM, Pawlowski TA (2012). Forest tree research in post genomic era. Introduction to systems biology of broadleaves. Dendrobiology 68: 113-123.

Sutherland ML, Brasier CM (1995). Effect of d-factors on *in vitro* cerato-ulmin production by the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. Mycological Research 99: 1211-1217. - doi: 10.1016/S0953-7562(09)80281-5

Svaldi R, Elgersma DM (1982). Further studies on the activity of cell wall degrading enzymes of aggressive and non-aggressive isolates of *Ophiostoma ulmi*. European Journal of Forest Pathology 12: 29-36. - doi: 10.1111/j.1439-0329.1982.tb01369.x

Tadesse Y, Bernier L, Hintz WE, Horgen PA (2003). Real time RT-PCR quantification and Northern analysis of Cerato ulmin (CU) gene transcription in different strains of the phytopathogens *Ophiostoma ulmi* and *O. novo ulmi*. Molecular Genetics and Genomics 269: 789-796. - doi: 10.1007/s00438-003-0890-7

Takai S (1980). Relationship of the production of the toxin, cerato-ulmin, to synnemata formation, pathogenicity, mycelial habit, and growth of *Ceratocystis ulmi* isolates. Canadian Journal of Botany 58: 658-662. - doi: 10.1139/b80-083

Temple B, Horgen PA, Bernier L, Hintz WE (1997). Cerato-ulmin, a hydrophobin secreted by the causal agents of Dutch elm disease, is a parasitic fitness factor. Fungal Genetics and Biology 22: 39-53. - doi: 10.1006/fgbi.1997.0991

Temple B, Pines PA, Hintz WE (2006). A nine-year genetic survey of the causal agent of Dutch elm disease, *Ophiostoma novo-ulmi*, in Winnipeg, Canada. Mycological Research 110: 594-600. - doi: 10.1016/j.mycres.2006.01.005

Temple B, Bernier L, Hintz WE (2009). Characterization of the polygalacturonase gene of the Dutch elm disease pathogen *Ophiostoma novo-ulmi*. New Zealand Journal of Forestry Science 39: 29-37. [online] URL: http://www.cabdirect.org/abstracts/20103084436.html

Tsui CKM, Roe AD, El-Kassaby YA, Rice AV, Alamouti SM, Sperling FAH, Cooke JEK, Bohlmann J, Hamelin RC (2012). Population structure and migration pattern of a conifer pathogen, *Grosmannia clavigera*, as influenced by its symbiont, the mountain pine beetle. Molecular Ecology 21: 71-86. - doi: 10.1111/j.1365-294X.2011.05366.x

Vakkari P, Rusanen M, Kärkkäinen K (2009). High genetic differentiation in marginal populations of European white elm (*Ulmus laevis*). Silva Fennica 43: 185-196. - doi: 10.14214/sf.205

Venturas M, Fuentes-Utrilla P, Ennos R, Collada C, Gil L (2013). Human-induced changes on fine-scale genetic structure in *Ulmus laevis* Pallas wetland forests at its SW distribution limit. Plant Ecology 214: 317-327. - doi: 10.1007/s11258-013-0170-5

Webber JF (1990). Relative effectiveness of *Scolytus scolytus*, *S. multistriatus*, and *S. kirschi* as vectors of Dutch elm disease. European Journal of Forest Pathology 20: 184-192. - doi: 10.1111/j.1439-0329.1990.tb01129.x

Webber JF (2004). Experimental studies on factors influencing the transmission of Dutch elm disease. Investigacion Agraria, Sistemas y Recursos Forestales 13: 197-205.

Whiteley RE, Black-Samuelsson S, Jansson G (2003). Within and between population variation in adaptive traits in *Ulmus laevis* Pall., the European white elm. Forest Genetics 10: 313-323.

Wiegrefe SJ, Sytsma KJ, Guries RP (1994). Phylogeny of elms (*Ulmus*, Ulmaceae): molecular evidence for a sectional classification. Systematic Botany 19: 590-612. - doi: 10.2307/2419779

Zalapa JE, Brunet J, Guries RP (2008). Genetic diversity and relationships among Dutch elm disease tolerant *Ulmus pumila* L. accessions from China. Genome 51: 492-500. - doi: 10.1139/G08-034

Zalapa JE, Brunet J, Guries RP (2010). The extent of hybridization and its impact on the genetic diversity and population structure of an invasive tree, *Ulmus pumila* (Ulmaceae). Evolutionary Applications 3: 157-168. - doi: 10.1111/j.1752-4571.2009.00106.x