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Molecular analysis of evolutionary changes in populations of *Ophiostma novo-ulmi*

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Abstract

The spread of *Ophiostoma novo-ulmi* across Europe, North America and central Asia, resulted in the current, highly destructive Dutch elm disease (DED) pandemic, replacing *O. ulmi*, responsible for the first DED pandemic in the early 1900s. This process has resulted in a series of remarkable evolutionary and adaptive developments. Studies of *O. novo-ulmi* populations in the 1980s, especially in Spain and Portugal, showed the following: 1) that *O. novo-ulmi* initially spread across Europe as a series of genetic clones; 2) that deleterious RNA viruses were transmitted within the *O. novo-ulmi* clones; 3) that natural hybrids between *O. novo-ulmi* subspecies *americana* and subsp. *novo-ulmi*, emerged widely across Europe; 4) that there has also been a widespread emergence, across Europe, of natural hybrids between *O. novo-ulmi* subspecies *americana* and also subsp. *novo-ulmi*. The factors driving these changes have been examined by molecular analysis. Results show: 1) that the rapid change from clonality to genetic variability involved the acquisition of 'useful' mating type, vegetative compatibility type and other genes by *O. novo-ulmi* from *O. ulmi* via lateral (or interspecies) gene transfer; whereas 'unuseful' *O. ulmi* genes were discarded; 2) that the RNA viruses occurring in the *O. novo-ulmi* populations probably originated from *O. ulmi*; and 3) and that where *O. novo-ulmi* subsp. *americana* and subsp. *novo-ulmi* co-exist, natural hybrids are occurring very freely; in some areas most *O. novo-ulmi* isolates are already complex subspp. *americana* x *novo-ulmi* hybrids. These phenomena features are unique, and have considerable implications for the invasion history, successful spread and future behaviour of *O. novo-ulmi*.

Key words: Dutch elm disease, clones, hybridisation, viruses.

Resumen

Análisis molecular de los cambios evolutivos en poblaciones de *Ophiostoma novo-ulmi*

La expansión de *Ophiostoma novo-ulmi* en Europa, Norteamérica y Asia central provocó la actual pandemia de grafiosis, altamente destructiva, y reemplazó a *O. ulmi*, responsable de la primera pandemia de grafiosis a principios del siglo XX. Este proceso ha provocado una serie de destacables desarrollos evolutivos y adaptativos. Los estudios realizados en la década de 1980 en poblaciones de *O. novo-ulmi*, especialmente en España y Portugal, mostraron lo siguiente: 1) que inicialmente *O. novo-ulmi* se expandió a través de Europa como una serie de clones genéticos; 2) que virus deletéreos de RNA se trsmitieron dentro de los clones de *O. novo-ulmi*; 3) que híbridos naturales entre las subespecies *americana* y *novo-ulmi* de *O. novo-ulmi* aparecieron en muchas zonas de Europa; 4) que en toda Europa está surgiendo un gran número de híbridos naturales entre las subespecies *americana* y *novo-ulmi* de *O. novo-ulmi*. Los factores conducentes a estos cambios han sido examinados mediante análisis molecular. Los resultados son: 1) que el rápido paso desde una situación de clonalidad a otra con gran variabilidad genética supuso la aparición de formas «utiles» para el apareamiento, formas con compatibilidad vegetativa, y otros genes de *O. novo-ulmi* por transferencia lateral (o interespecífica) a partir de *O. ulmi*; mientras que se descartó la presencia de genes «inútiles» de *O. ulmi*; 2) que los virus ARN presentes en las poblaciones de *O. novo-ulmi* se originaron probablemente a partir de *O. ulmi*; y 3) que en los lugares donde *O. novo-ulmi* subsp. *americana* y subsp. *novo-ulmi* coexisten, los híbridos naturales se generan libremente; en algunas áreas la mayor parte de los *O. novo-ulmi* aislados son en realidad complejos híbridos subsp. *americana* x subsp. *novo-ulmi*. Estas características son únicas, y tiene considerable implicaciones para la historia invasora, la exitosa dispersión y el futuro comportamiento de *O. novo-ulmi*.

Palabras clave: grafiosis, clones, hibridación, virus.

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Introduction

Two destructive pandemics of Dutch elm disease (DED) have occurred across Europe and North America over the past 100 years, each caused by a different species of *Ophiostoma*. The first pandemic was caused by the more weakly aggressive *O. ulmi* (Buisman) Nannfeldt and the second by the highly aggressive *O. novo-ulmi* Brasier, which itself occurs as two distinct subspecies, subsp. *novo-ulmi* and subsp. *americana*. The history of spread of *O. ulmi* and *O. novo-ulmi* is summarised in Fig. 1. The biological characteristics of these two pathogens and their role in the two pandemics have been summarised in more detail elsewhere (e.g. Brasier, 1984, 1986a, 1990, 1991, 2000a; Brasier and Kirk, 2001).

O. novo-ulmi has rapidly replaced *O. ulmi* at a relative incidence of about 10% per annum at each location. The replacement process has resulted in some of the most remarkable and rapid evolutionary developments ever observed in fungal populations. Studies on *O. novo-ulmi* populations in the 1980s, especially in Spain and Portugal, showed the following. (1) *O. novo-ulmi* initially spread across Europe as a series of genetic clones. (2) Deleterious fungal viruses then spread within the *O. novo-ulmi* clones. (3) These clones then rapidly —and inexplicably— became highly genetically variable. (4) There has been widespread emergence, across Europe, of natural hybrids between *O. novo-ulmi* subsp. *americana* and subsp. *novo-ulmi*. The above processes were summarised at the First International Elm Conference in 1998 (Brasier, 2000a, b). Other details of these developments are given elsewhere (e.g. Brasier, 1988, 1991; Brasier and Buck, 2002; Brasier and Kirk, 2000; Buck *et al.*, 2002). Recently, a number of the factors driving these changes have been investigated or corroborated by molecular analyses. The results of these analyses will now be presented and their implications discussed.

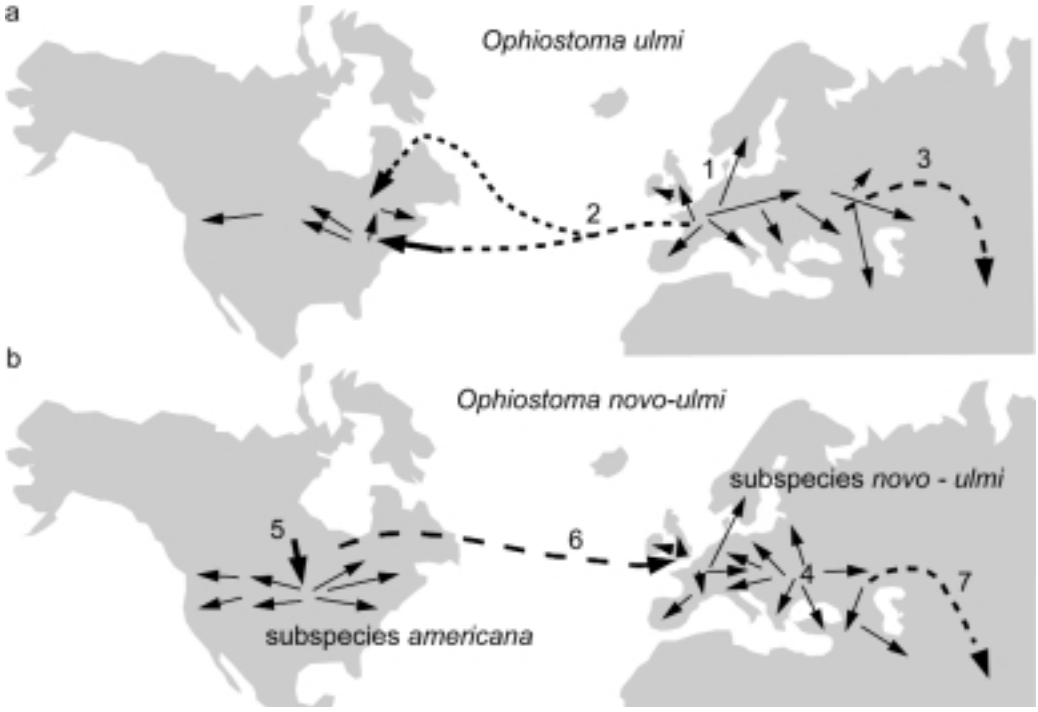

Figure 1. Intercontinental spread of *Ophiostoma ulmi* and *O. novo-ulmi* in the first and second pandemics of Dutch elm disease. Solid arrows, natural migrations from probable sites of introduction. Dashed arrows, subsequent spread via additional importation events. (a) Spread of *O. ulmi*. 1: Its appearance in Northwest Europe around 1910. 2: Introduction to North America in the 1920s. 3: Introduction from Krasnodar to Tashkent, late 1930s. (b) Spread of the two subspecies of *O. novo-ulmi*. 4, 5: Original centres of appearance of subsp. *novo-ulmi* and *americana* in the Romania-Moldova and southern Great Lakes regions, respectively. 6: Introduction of subsp. americana from Toronto area to Britain, ca. 1960. 7: Introduction of subsp. *novo-ulmi* to Tashkent area, 1970s. Adapted from Brasier (1990). Introductions reflect movement of infested timber.

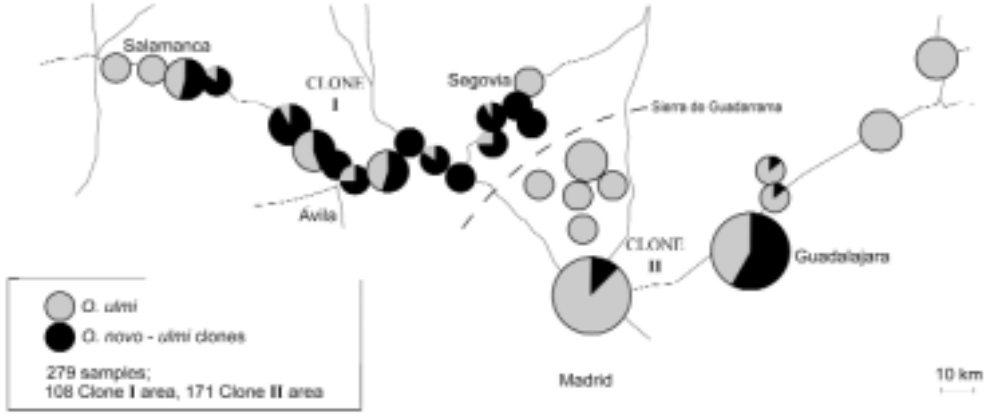

Figure 2. Presence of two epidemic front clones of *Ophiostoma novo-ulmi* in samples collected in the Madrid area of central Spain in 1984. Note that, at the time, *O. ulmi* (light hatching) was still present throughout the area, sometimes comprising 100% of a sample. *O. novo-ulmi* clone I occurred in the Salamanca-Ávila-Segovia area, north of the Sierra de Guadarrama. Clone II occurred in the Madrid-Guadalajara area. Clone I was subsequently called the Penaranda *vic* clone and Clone II the Guadalajara *vic* clone (see Brasier 1987, 1988, Brasier and Kirk, 1990).

Rapid increase in genetic diversity of *O. novo-ulmi* clones

O. novo-ulmi has spread across much of North America, Europe and southwest and central Asia as a series of vegetative compatibility type and mating type clones. The vegetative compatibility (vc) system of *O. novo-ulmi* is a multi-locus, multi-allelic genetic system analogous to tissue incompatibility (*vic*) systems in animals. The system can generate large numbers of different vc types. It limits the spread through populations of parasitic deleterious agents such as viruses, which in fungi are transmitted by cell fusions. It operates by confining viable hyphal fusions to genetically identical *vic* genotypes. Fusions between hyphae of different *vic* genotypes result in cell death and greatly restricted virus transmission. Both *O. ulmi* and *O. novo-ulmi* are also obligatorily sexually outcrossing, with two mating types, A and B, determined by different alleles at the *mat* locus (Brasier 1984).

The occurrence of *O. novo-ulmi* as *vic* and *mat* B clones at epidemic fronts was revealed by both conventional genetic analyses of populations and also by the unusual morphological uniformity of the clones themselves. Fig. 2 shows the presence of two such clones of *O. novo-ulmi* in the Madrid area of central Spain in 1984, a few years after the arrival of *O. novo-ulmi* in the area. It also shows that, at the time the *O. novo-ulmi* clones were beginning to spread, a high proportion of the local DED pathogen population still comprised *O. ulmi* (see Brasier 1987).

An unexpected and rapid change in *O. novo-ulmi* populations, from clonality to genetic heterogeneity, then occurred at epidemic fronts. This was likewise demonstrated by conventional genetic and morphological analysis. Fig. 3 shows the occurrence of a single

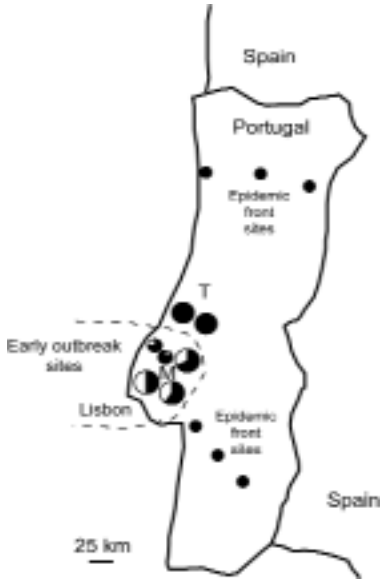

Figure 3. Structure of the *Ophiostoma novo-ulmi* population in Portugal in 1985, showing the presence of a single dominant vic clone, of B compatibility type at new epidemic front sites (black); and the emergence of a heterogeneous component (of multiple new *vic* types and the A mating type) alongside the residual clone in the older outbreak areas around Lisbon (white). *O. novo-ulmi* probably first arrived in the Lisbon areas the late 1970s. Within Tomar (T) and Mafra (M) sites, the transition from clonality to heterogeneity was studied in detail (see Brasier, 1988).

O. novo-ulmi vic clone of B mating type associated with the advance of fresh epidemic fronts in northern and southern Portugal in 1985, and the emergence, at older epidemic areas close to Lisbon, of a genetically highly heterogeneous subpopulation of *O. novo-ulmi* alongside the residual clone. As in other sites across Europe, this emerging heterogeneous component of the population comprised large numbers of mainly unique *vic* types. Its emergence was accompanied by the appearance of the previously absent A mating-type; by a change from morphological uniformity of colony types to morphological diversity (Fig. 4); and by a temporary reduction in aggressiveness among isolates of the heterogeneous component (Brasier, 1988). Confirmation that the epidemic front *vic* clones were indeed «true» genetic clones came from RFLP markers (Bates, 1990) and from RAPD Analysis (Brasier and Kirk, 2000). Thus a uniformity of RFLP or RAPD profiles was observed within the different *vic* clones, both in Europe and in North America. One North American *vic* clone was also present across much of Europe and in New Zealand, where it again exhibited an identical RAPD profile.

In Europe, the change from clonality to heterogeneity was very rapid, taking only ca. 5-6 years (Fig. 5). Such a rapid increase in genetic diversity was considered highly unusual. Among the hypotheses proposed to account for it were: hypervariability at the *vic* loci; insertion of mobile genetic elements; and acquisition of *vic* and *mat* genes from *O. ulmi* via interspecific gene transfer. Three lines of evidence tended to support the possibility of interspecific gene transfer. First, an opportunity for genetic exchange was presented by the close physical proximity of *O. novo-ulmi* and *O. ulmi* in elm bark during the former's replacement of *O. ulmi*. Second, *O. ulmi* and *O. novo-ulmi* are known to be strongly, but not totally reproductively isolated at the pre- and post- zygotic levels: perithecia, or sexual fruiting bodies, are produced at a low frequency between the two species, but the resulting F_1 progeny are highly unfit; see e.g. Kile and Brasier, 1990; Brasier and Mehrotra, 1995. Third, rare but transient *O. ulmi* x *O. novo-ulmi* hybrids have been found in two populations in Europe (Brasier *et al.*, 1998).

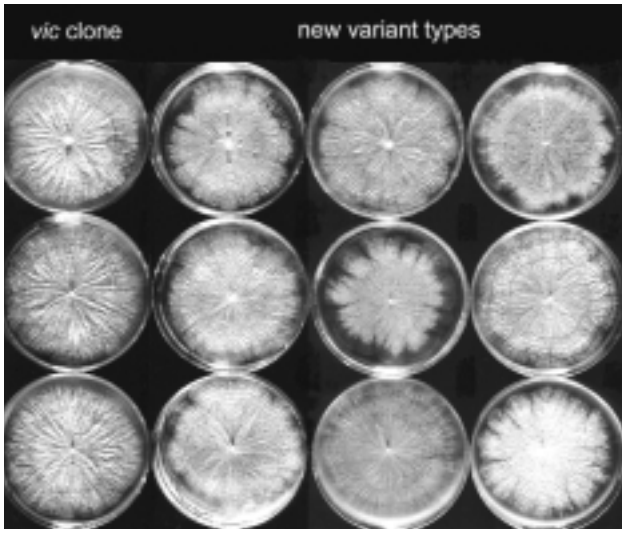

Figure 4. Colony phenotypes of *Ophiostoma novo-ulmi* isolates sampled during the transition from clonality to heterogeneity. Left column, 3 representative colonies of the frontal *vic* clone at Tomar, Portugal. Note the colony uniformity. Other columns, colonies of the novel *vic* types appearing in the Tomar population, showing that the change to vic heterogeneity was accompanied by a marked increase in colony variability. C. M. Brasier and S. A. Kirk, previously unpublished.

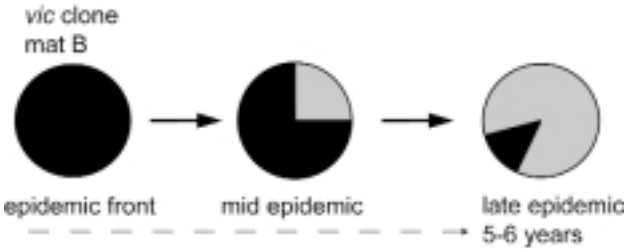

Figure 5. Rapid rate of change from *vic* clonality (black) to *vic* heterogeneity (grey) in local *O. novo-ulmi* populations in Europe (ca. 5-6 years). The appearance of the novel *vic* types was also accompanied by the appearance of the A mating-type, a temporary reduction in aggressiveness, and a marked increase in colony variation.

Recently, the interspecific gene transfer hypothesis has been examined by molecular analysis. More specifically, the origins of the novel *vic* and *mat* A genes among isolates of the emerging heterogeneous component at epidemic fronts have been investigated. To this end, a combination of conventional and molecular genetic analysis was used. Crosses were conducted between A-mating type isolates of novel *vic* type and an isolate of the local *vic* B-type clone. Bulked-segregant analysis of *vic* loci and AFLP markers among the F_1 progeny was carried out to determine whether the DNA linked to a novel *vic* locus was *O. novo-ulmi* or *O. ulmi* DNA. Most AFLP markers (c. 67%) around these *vic* loci where shown to be *O. ulmi* markers, indicating that the novel *vic* genes were most probably derived from *O. ulmi* (M. Paoletti, K. W. Buck and C. M. Brasier, unpublished).

To investigate the origin of the «new» A-mating types, the flanking regions of the *O. ulmi* and *O. novo-ulmi mat* genes have been cloned and sequenced. This has shown that the flanking sequences of the *O. ulmi* and *O. novo-ulmi mat* loci are distinct. Based on these differences, primers were developed that would distinguish the *O. ulmi* from the *O. novo-ulmi* sequence type. Use of these primers to screen for *mat* gene types demonstrated that emerging epidemic front isolates of A mating-type carried *O. ulmi mat* A genes, whereas most B mating-type isolates, including the frontal *vic* clones, carried 'true' *O. novo-ulmi mat*-B genes (M. Paoletti, K. W. Buck and C. M. Brasier, unpublished).

This study shows therefore that the rapid change from clonality to genetic heterogeneity in epidemic front *O. novo-ulmi* populations has involved the acquisition of both new *vic* loci and the *mat* A locus from *O. ulmi*. Surveys of *mat* genes among larger numbers of *O. ulmi* isolates from across Europe and North America have also been carried out using the PCR protocol. These results showed that some *O. novo-ulmi* isolates carrying the «true» *O. novo-ulmi mat* A gene also existed, but were almost entirely confined to the Moscow – Volgograd – Black Sea area (M. Paoletti, K. W. Buck and C. M. Brasier, unpublished), close to where *O. novo-ulmi* subspecies *novo-ulmi* is believed to have first appeared following its introduction into Europe (Brasier, 1990; Fig.1). Probably, therefore, *O. novo-ulmi* was originally introduced into this region with both of its «true» mating types, A and B, but the A-types were quickly lost as the organism migrated, resulting in the prevalence of the B-type clones. Apparently, as *O. novo-ulmi* has spread, the «missing» A mating-type has continually been newly acquired from *O. ulmi*. *O. ulmi* itself has been rendered virtually extinct during the process.

Origin of deleterious viruses spreading in the *O. novo-ulmi* clones

Studies on European populations of *O. novo-ulmi* have also shown that deleterious, cytoplasmic dsRNA-based fungal viruses spread widely within the epidemic front *O. novo-ulmi vic* clones. These viruses can greatly reduce the growth vigour of the fungus (Fig. 6) and the viability of its asexual spores (conidia), and thus its ability to infect healthy elms. The viruses, therefore, have the potential to break the cycle of Dutch elm disease (see Brasier, 2000b; Buck and Brasier, 2001).

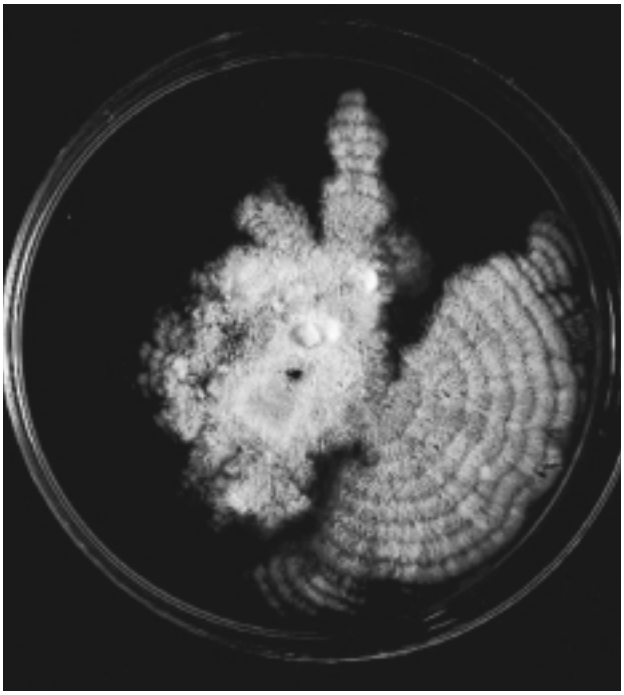

Figure 6. A virus (d-infected) culture of *Ophiostoma novo-ulmi* showing the typical irregular and unstable («amoeboid») growth pattern associated with deleterious virus infection.

The fact that epidemic front populations of *O. novo-ulmi* were clonal in terms of their *vic* genes would enable viable hyphal fusions between adjacent colonies of the pathogen in elm bark. This in turn would allow ready transmission of viruses between colonies. If, however, adjacent colonies were of different *vic* types, most attempted hyphal fusions would not be viable, restricting the dissemination of viruses. Indeed it was shown that the frequency of deleterious virus infection in the clones increased rapidly as a local epidemic developed, up to 90% of isolates becoming virus-infected in the samples examined (based on the frequency of severely diseased or «amoeboid» isolates among the samples) (Figs. 6 and 7; Brasier 1988). However, as the heterogeneous *vic* component became predominant in the population, i.e. as many new vc-types appeared, the frequency of virus infected isolates declined. In the post–epidemic period, when almost every *O. novo-ulmi* isolate in Europe is of a different *vic* type, the frequency of deleterious virus infection tends to be very low (< 0.5%; C. M. Brasier and S. A. Kirk, unpublished).

A related question is the origin of the viruses that became so abundant in the *O. novo-ulmi vic* clones. In view of the circumstantial evidence suggesting that the

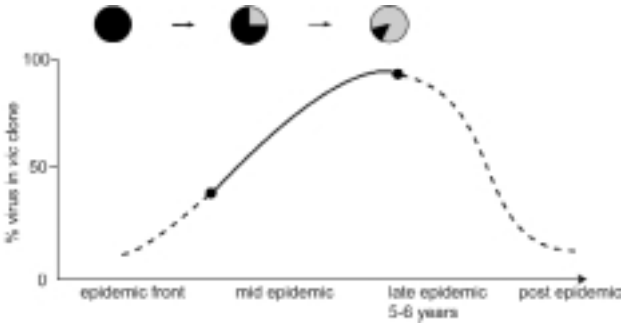

Figure 7. Rapid increase in frequency of virus-infected isolates of *Ophiostoma novo-ulmi* during the change from *vic* clonality to *vic* heterogeneity at epidemic fronts. Based on observations at multiple sites across Europe (composite figure; C. M. Brasier and S. A. Kirk, previously unpublished; see also Brasier, 1988; Brasier and Kirk, 1990).

novel *vic* and *mat* genes might be acquired from *O. ulmi*, it was postulated that the viruses, also, might have been acquired from *O. ulmi* - rather than being «native» *O. novo-ulmi* viruses. This possibility was tested by cloning and sequencing regions of the viral RNA that encode the viral polymerase (RdRp) genes. Isolates of *O. ulmi* and *O. novo-ulmi* obtained from within the same pieces of scolytid colonised elm bark (i.e. during the pathogens' saprotrophic phase) at an epidemic front site at Tomar, Portugal (Fig. 3), were investigated, together with isolates obtained from other geographic locations. From the RdRp RNA sequences, the amino-acid sequence of the polymerase peptide can be inferred. Studies were concentrated on a particular species of virus, known as *Ophiostoma* mitovirus 5 (OMV5), which is found in both *O. ulmi* and *O. novo-ulmi* (L. J. Crawford, K. W. Buck and C. M. Brasier, unpublished).

Two important deductions could be made from the data. First, the OMV5 RdRp sequences clustered on a geographical basis rather than according to the fungal species (i.e. as *O. ulmi* or *O. novo-ulmi*). Second, *O. ulmi* has been present in Portugal since the 1920's, whereas *O. novo-ulmi* reached this country only in the late 1970s (probably from North America via other parts of Europe). Yet the similarities between the OMV5 RdRp sequences of *O. ulmi* and *O. novo-ulmi* isolates from the Tomar site (ca. 96.8-100% identity) were greater than between these isolates and those from other countries (ca. 91.6-93.2%). This evidence was consistent with horizontal transmission of OMV5 between *O. ulmi* and *O. novo-ulmi*. Particularly persuasive was the close homology between the RdRp OMV5 sequences of two Tomar isolates *O. ulmi* elb and *O. novo-ulmi* e1a. These isolates were obtained from the same small (4 mm^2) segment of elm bark i.e. physical contact between the mycelia of ela and elb had almost certainly occurred. Other circumstantial evidence suggests that the direction of virus transmission is from *O. ulmi* to *O. novo-ulmi* rather than *vice versa* (cf. Buck *et al.* 2002). To summarise, it is most probable that the deleterious viruses spreading in the *O. novo-ulmi* clones were acquired from *O. ulmi*.

Link between incidence of deleterious viruses and increasing *vic* diversity

Other evidence suggests that the effect of the deleterious viruses on the survival of *O. novo-ulmi* provided the selection pressure that favoured the acquisition of the novel *mat* A and *vic* genes. In Europe, the incidence of deleterious viruses in the *vic* clones at epidemic fronts fell markedly following the change to a heterogeneous population structure. Whereas in North America, where virus incidence in the frontal *vic* clones has remained almost undetectably low, only a partial and locally sporadic change from clonality to multiple *vic* genotypes is occurring. In New Zealand, where both the viruses and *O. ulmi* are absent, the single introduced *O. novo-ulmi vic mat*-B clone (the same *vic* clone as that at Tomar in Portugal) has remained genetically stable and unchanged (Brasier, 2000b). That introgression of the *O. ulmi mat*-B locus into *O. novo-ulmi* has been rare, both in Europe and North America, probably reflects the lack of a selective advantage for such introgression to the originally clonal *mat*-B *O. novo-ulmi* populations.

Transfer of viruses from *O. ulmi* to *O. novo-ulmi* is most likely to occur during the early stages of an epidemic. At this time mycelia of an *O. novo-ulmi vic* clone colonizing elm bark will tend to be surrounded by mycelia of *O. ulmi*. In both Europe and North America, most *O. ulmi* isolates are virus infected (L. Sutherland, A. G. Mitchell and C. M. Brasier, unpublished). *O. novo-ulmi* colonies are therefore likely to become infected with *O. ulmi* viruses. Since adjacent mycelia of an emerging *O. novo-ulmi* clone in bark will be of an identical *vic* type, transfer of any newly acquired *O. ulmi* viruses to other *O. novo-ulmi* mycelia would occur very freely, resulting in the observed rapid virus build up in the *O. novo-ulmi* clones (Fig. 7).

However, *O. novo-ulmi* isolates that had acquired novel *vic* genes from *O. ulmi* via gene transfer i.e. isolates of a novel *vic* type, would be better able to resist virus transmission and so would have a marked fitness advantage over the increasingly virus-infected clone. Also, through its parallel acquisition of the *mat* A locus, *O. novo-ulmi* would also acquire the ability to sexually reproduce. This would result, in turn, in recombination of the novel *vic* genes, and so to yet more novel vc-types in the population. Furthermore, since ascospores (the sexual spores) tend to be virus-free, the relative fitness of the sexual recombinants over the clone would be yet further enhanced.

Other evidence indicates that the acquisition and fixation of the *vic* and *mat* genes by *O. novo-ulmi* is selective. First, most introgressed *O. ulmi* DNA observed in *O. novo-ulmi* appears to be linked to the *vic* loci. Second, other *O. ulmi* DNA/loci, such as genes governing pathogenicity and cerato-ulmin production, are probably acquired by *O. novo-ulmi* during the gene transfer process (e.g. Et Touil, *et al.*, 1999; Pipe, *et al.*, 2000). However, they are not detected in post-epidemic *O. novo-ulmi* populations. This is probably because they have a strong negative effect on fitness, and in consequence are quickly eliminated by natural selection.

To summarise it appears: 1) that the rapid genetic change that occurs in epidemic front *O. novo-ulmi* populations results from the acquisition of novel *vic*, *mat* A and other genes from *O. ulmi*; 2) that the process is driven by the spread within the *vic* clones of deleterious viruses, most probably also acquired from *O. ulmi*; and 3) that the fixation of the *vic* and *mat* genes is selective, the other genes being lost.

Mode of transfer genes and viruses between *O. ulmi* and *O. novo-ulmi*

The precise mode of transfer of the genes and viruses between *O. ulmi* and *O. novo-ulmi* is unknown, but may be surmised. In the case of the nuclear *vic*, *mat*-A and other nuclear genes the transient *O. ulmi* x *O. novo-ulmi* sexual hybrids shown to occur in nature may be involved. Although generally unfit, the hybrids may act as a genetic bridge, the «useful» *vic* and *mat* A loci being integrated into and fixed within the *O. novo-ulmi* genome via sequential backcrosses; with natural selection eliminating the less fit (e.g. less virus resistant or less pathogenic) intercross or backcross products. Regarding the latter, as has already been mentioned above, the appearance of the new *vic* types at epidemic fronts was accompanied by a temporary reduction in aggressiveness and a marked increase in colony variation (Fig. 4; Brasier, 1988). This increase in phenotypic diversity may well reflect the transfer to *O. novo-ulmi* of additional *O. ulmi* genes. The end result of this process of gene transfer and selection is probably an array of *O. novo-ulmi* genotypes that carry the novel *vic* and *mat* A loci but are otherwise relatively isogenic with the original *O. novo-ulmi* clone.

It is known that the viruses are not normally transferred into the sexual ascospores of *O. novo-ulmi*. Therefore, virus transfer is more likely to occur via rare somatic fusions between adjacent *O. ulmi* and *O. novo-ulmi* mycelia in elm bark than via a sexual route. The existence of *O. novo-ulmi* isolates with *O. ulmi*-like or recombinant mitochondrial DNA profiles (Pipe *et al.*, 1995) provides indirect evidence that somatic fusions occur in nature between *O. ulmi* and *O. novo-ulmi*.

Unrestricted hybridisation between the two subspecies of *O. novo-ulmi*

The two subspecies of *O. novo-ulmi*, subsp. *americana* and subsp. *novo-ulmi* (previously the NAN and EAN races) are now overlapping in many parts of Europe (Fig. 8). They differ in a number of phenotypic properties, such as colony development, occurrence of «*up-mut*» colony mode, perithecial morphology and average growth rate and aggressiveness (Brasier and Kirk, 2001). Since these subspecies are only weakly reproductively isolated at the pre-zygotic level, it has long been predicted that hybrids will occur between them (Brasier, 1986b, 1995, 2000, 2001; Brasier and Kirk, 2001; Brasier and Buck, 2002).

The first inter-subspecies hybrids were detected in an *O. novo-ulmi* population in Limburg Provence, Netherlands, where the two subspecies have overlapped since the late 1970s. Detailed sequential sampling was carried out during the 1980s. Initially, the hybrids were identified on their combination of *vic* type; their fertility reaction against a sexually compatible subsp. *novo-ulmi* tester isolate (a weak pre-zygotic isolating mechanism is operated by subsp. *novo-ulmi* against subsp. *americana*, resulting in greatly reduced perithecial frequency; see Brasier 1979); and on the occurrence of the *up-mut* colony dimorphism (see Bra-

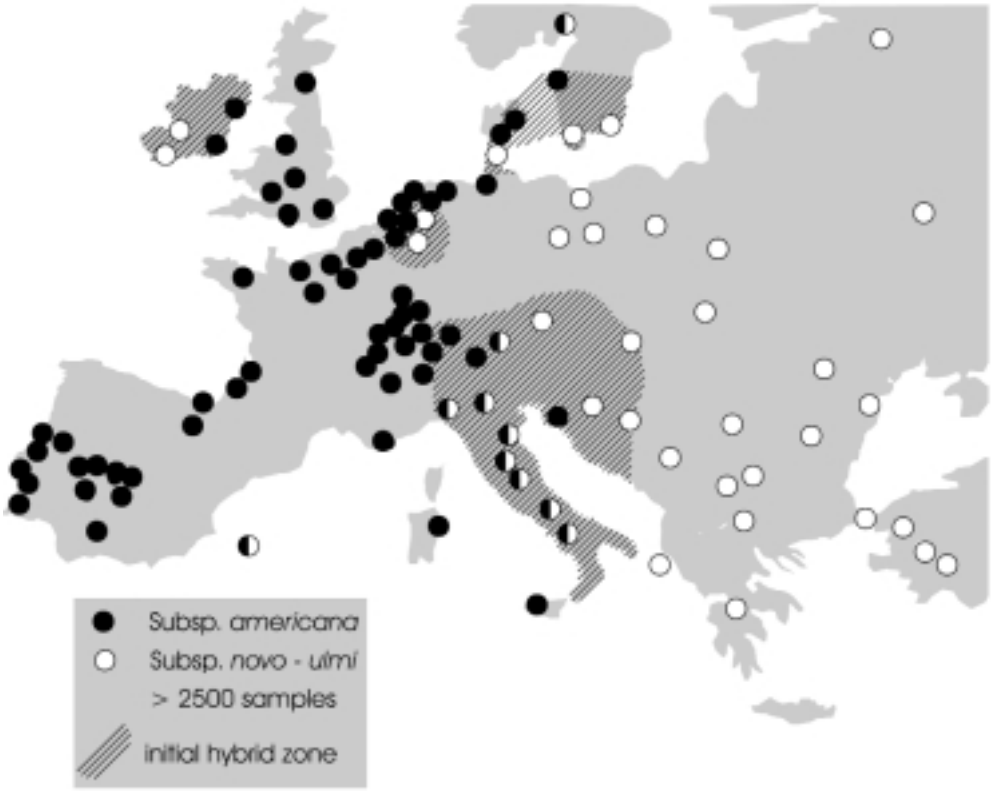

Figure 8. Known distribution of *Ophiostoma novo-ulmi* subsp. *novo-ulmi* (white circles) and subsp. *americana* (black circles) across Europe in 1990. (Representative sample points only, based on > 2,500 samples). Locations where subsp. *novo-ulmi* and *americana* overlapped included Ireland, Netherlands, Scandinavia, Germany and Italy. Hatched areas, predicted zones of emergence of subsp. *novo-ulmi* and x subsp. *americana* hybrids. Based on Brasier 1986b, 2000; Brasier and Kirk (2001).

sier 1986a). In 1980, for example, 1 of 23 isolates sampled (4.3%) had the characteristics of a hybrid. By 1983 the equivalent figure was 55 isolates out of 107 (51.4%) (Brasier, 1986b). Subsequently, hybrids between the two subspecies were detected on a similar basis among samples from Italy, Ireland, and Hungary.

To corroborate the evidence for hybridisation and to analyse the extent of recombination, a RAPD analysis has been used. Initially, 14 subsp. *americana* and 14 subsp. *novo-ulmi* isolates from a wide range of geographic locations across Europe and North America were screened for polymorphisms against a large number of oligonucleotide primers. Eight primers that clearly discriminated the two subspecies (producing either a unique major band product or resulting in band presence versus band absence) were selected (Fig. 9). The same primers were then used to analyse a sample of *O. novo-ulmi* isolates collected in the Netherlands, in 1983. The data for a representative subset of these isolates is also shown in Fig. 9. As can be seen, a majority of the isolates are recombinant for the markers used. A similar result was obtained with a sample of *O. novo-ulmi* isolates from Orvieto, Italy, collected in 1989 and from smaller samples of isolates from Ireland and Hungary (C.M. Braser and S.A. Kirk, unpublished).

It is clear therefore that a large proportion of the *O. novo-ulmi* genotypes present in the potential hybrid zones illustrated in Fig. 8 can now be expected to be recombinants. This is further supported by a recent study by Konrad *et al.* (2002), who have used PCR detection of molecular polymorphisms in the *cu* (ceratoulmin) and *col 1* (colony type) genes to analyse 6 Austrian isolates. These results showed that two of the Austrian isolates, plus a Polish control isolate, were subsp. *novo-ulmi* x subsp. *americana* hybrids. It remains to be seen whether natural selection will result in the emergence of a particular phenotype from among these hybrids (see Brasier 1986b, 1995, 2001) or whether a wide range of phenotypes, analagous to a hybrid swarm, will persist in the hybrid areas.

The finding by Konrad *et al.* (2002) that a control isolate of *O. novo-ulmi,* collected in Poland in 1980

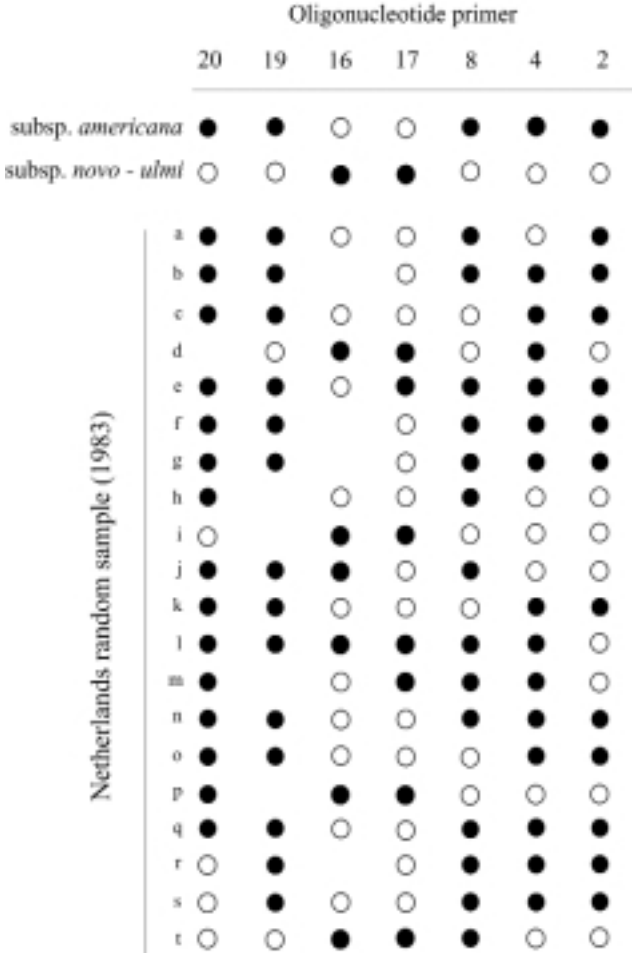

Figure 9. RAPD banding patterns generated among *O. novo-ulmi* isolates with 7 selected oligonucleotide primers. First two rows, typical RAPD banding patterns of *O. novo-ulmi* subsp. *americana* and subsp. *novo-ulmi* respectively. Rows a-t, recombinant RAPD patterns among a random sample of isolates collected in a developing subsp. *americana* /subsp. *novo-ulmi* overlap zone in the Netherlands in 1983.

(Brasier and Kirk, 2000), was a hybrid is of additional interest. In 1980, north-western areas of Poland were on the fringes of what might be considered the potential subsp. *americana* x subsp. *novo-ulmi* hybrid zone (Fig. 8). However, all 150 isolates collected across Poland in 1980 were of a typical subsp. *novo-ulmi* phenotype (Brasier and Kirk, 2000) and it was not anticipated that hybrids might already be present in the population. To investigate this phenomenon further, 20 isolates representative of the Polish sample were analysed with the above RAPD primers. While the majority of isolates gave a subsp. *novo-ulmi* type profile, three of them gave a recombinant profile, exhibiting a combination of subsp. *americana* and subsp. *novo-ulmi* polymorphisms. One of these three isolates is the Polish isolate examined by Konrad *et al.* (2002). All three came from a region near the Baltic Ports/German border. It appears, therefore, that some introgression of subsp. *americana* DNA had already occurred in this region by 1980 (C.M. Brasier and S.A. Kirk, unpublished).

By coincidence, the herbarium type material for *O. novo-ulmi* subsp. *novo-ulmi* comes from Poland (see Brasier and Kirk 2000). The two isolates recorded as the «type» material have since been screened for the *cu* and *col 1* gene polymorphisms (H. Konrad, personal communication) and with the above RAPD primers. No subsp. *americana* DNA has been detected. However, it is clear that in the future many common, and therefore representative, *O. novo-ulmi* isolates can be expected to exhibit «novel» DNA from another species, subspecies or population; for example an introgressed mating type gene from *O. ulmi* or, as in this case, DNA introgressed from another *O. novo-ulmi* subspecies.

Concluding comments: wider genetic risk issues arising

It is again evident from this work that molecular methods provide powerful tools for confirming or consolidating population characteristics in fungal pathogens. For people interested in the preservation of elms, what is particularly pertinent is that these molecular studies provide clear evidence that the invasive DED pathogens are continuing to evolve through interspecific gene transfer. The latter is a relatively new and indeed somewhat concerning issue in fungal population biology (Brasier, 2001).

Tree breeders attempting to develop trees resistant to aggressive invasive pathogens, such as *O. novo-ulmi*, need to be aware of this issue. A «favourable» gene transferred from one pathogen species to another could well be a pathogenicity or host specificity gene. If there were large-scale deployment of highly disease resistant hybrid elms, the increased selection pressure on the pathogen might favour interspecific acquisition of genes for increased aggressiveness. Those interested in using fungal viruses as potential biological control agents against invasive pathogens such as *O. novo-ulmi* also need to take the issue into consideration. Both the *O. ulmi* to *O. novo-ulmi* gene transfer process discussed here, and the continuing hybridisa-

tion between subsp. *americana* and subsp. *novo-ulmi*, are likely to result in increased heterogeneity for *vic* genes in the *O. novo-ulmi* population. Since *vic* genes enhance virus resistance in the pathogen, increased *vic* gene heterogeneity will reduce the chances of success in deploying fungal viruses as biocontrol agents unless the vc system itself can be bypassed e.g. by insertion of viruses into the unclear DNA of the pathogen (see Buck and Brasier, 2001).

Fungal taxonomists also need to be aware of the interspecific gene transfer issue. It means that a taxon defined as «species X» today (or equally, one defined as «species X» 50 years ago) might be very different, both in genotype and phenotype, tomorrow. This is again well illustrated by *O. novo-ulmi*. The most common genotypes of this species across Europe and North America now carry *O. ulmi vic* and *mat* genes, almost by definition. Furthermore, in terms of the current subsp. *americana x* subsp. *novo-ulmi* hybridization process, *O. novo-ulmi* is virtually reinventing itself as a species.

From an international plant health perspective, both the present work and other recent studies show that gene transfer between invasive and resident pathogens can lead to evolution of entirely new organisms and new diseases (cf. Brasier 1995, 2000, 2001). The newly identified *Phytophthora* complex now attacking *Alnus* trees across Europe is a swarm of interspecific hybrids. Probably neither of the two «parental» *Phytophthora* species involved can attack alder. The hybrid products appear therefore to have acquired a new host specificity during the hybridisation process (Brasier *et al.*, 1999). The process itself may well have occurred in a horticultural nursery in Europe (Brasier and Jung, 2003). Del Sorbo *et al*. (2000) have shown experimentally that if the *O. novo-ulmi* cerato-ulmin toxin gene is artificially inserted into the genome of the saprotrophic oak inhabitant *O. quercus* (previously *O. piceae*), it results in *O. quercus* causing typical vascular wilt symptoms in elm. Under the explosive conditions of a DED epidemic, physical contact between *O. novo-ulmi* and *O. quercus* i.e. the opportunity for genetic exchange between them, is more than a purely theoretical possibility (Brasier, 1990). In 1986, at Rubena, northern Spain a colony of *O. piceae* was obtained from a sample of diseased elm bark entirely surrounded by mycelia of *O. novo-ulmi* and *O. ulmi* (*O. piceae* isolate H926; J. F. Webber and C. M. Brasier, unpublished). Other evidence indicates that *O. quercus* may have been present on European elms prior to the arrival of DED in the early 1900s (Brasier, 1990).

In terms of their evolutionary opportunities and their evolutionary potential, therefore, invasive pathogens must be considered to be fluid, dynamic and unpredictable.

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