Dutch elm disease in Austria

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Abstract

Dutch elm disease (DED) has first been recorded in Austria in 1928 and subsequently caused severe damage to elms in this country. In the 1990s studies on the incidence and geographical distribution of *Ophiostoma ulmi* and the subspecies of *O. novo-ulmi* in Austria have been initiated. Additionally, efforts were made to identify hybrids between these subspecies. In this paper we present a synthesis of this research and review the history and impact of DED in Austria. By the 1990s only *O. novo-ulmi* was present in Austria and *O. ulmi* had already disappeared. Based on its unique colony morphology and its female sterility, one isolate is suspected of being a hybrid or introgressant between *O. ulmi* and *O. novo-ulmi*. Both subspecies of *O. novo-ulmi* were detected in Austria, with ssp. *novo-ulmi* occurring more frequently than ssp. *americana*, and their ranges overlap, which suggests that they hybridize in this part of Europe. Examination of seven isolates of *O. novo-ulmi* for their fertility response as donor to authenticated isolates of *O. novo-ulmi* and by PCR-RFLP of the cerato-ulmin (*cu*) gene and colony type (*col1*) gene have unequivocally shown that genetic recombination occurs between ssp. *novo-ulmi* and ssp. *americana* and that Austria forms part of a hybrid zone between *O. novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana*.

Key words: *Ulmus, Ophiostoma ulmi, Ophiostoma novo-ulmi,* hybridization.

Resumen

La grafiosis de los olmos en Austria

La grafiosis del olmo se detectó por primera vez en Austria en 1928. Posteriormente causó graves daños a los olmos de este país. En la década de 1990 se iniciaron en Austria los estudios sobre la incidencia y la distribución geográfica de *Ophiostoma ulmi* y de las subespecies de *O. novo-ulmi*. Igualmente se ha realizado esfuerzos para identificar híbridos entre ambas especies. En este artículo presentamos una síntesis de estas investigaciones y una revisión de la historia y el impacto de la grafiosis en Austria. Hacia 1990 únicamente *O. novo-ulmi* estaba presente en Austria, habiendo ya desaparecido *O. ulmi*. Se sospecha, teniendo en cuenta la presencia de una morfología colonial única y de esterilidad femenina, que uno de los aislamientos es un híbrido o el resultado de una introgresión entre *O. ulmi* y *O. novo-ulmi*. Ambas subespecies de *O. novo-ulmi* se han detectado en Austria, siendo más frecuente la ssp. *novo-ulmi* que la ssp. *americana*, y existiendo una superposición en su área de distribución, lo que sugiere que se pueden hibridar en esta parte de Europa. El estudio de siete aislamientos de *O. novo-ulmi* en cuanto a su fertilidad como donantes frente a aislamientos conocidos, y en cuanto a su análisis mediante PCR-RFLP de los genes cerato-ulmina (*cu*) y tipo de colonia (*col1*) ha mostrado inequívocamente que existe una recombinación genética entre las subespecies *novo-ulmi* y *americana* y que Austria forma parte de una zona de hibridación entre *O. novo-ulmi* ssp. *novo-ulmi* y *O. novo-ulmi* ssp. *americana*.

Palabras clave: *Ulmus, Ophiostoma ulmi, Ophiostoma novo-ulmi*, hibridación.

Introduction

Dutch elm disease (DED) is among the best known tree diseases world-wide. It is a warning example for the fatal consequences of the accidental introduction of pathogens into areas outside of their natural range where they come in contact with highly susceptible host trees. This destructive vascular wilt disease of elm (*Ulmus* spp.) is effectively transmitted by elm bark beetles. The disease is caused by three biological forms within the ascomycete genus *Ophiostoma*, namely *Ophiostoma ulmi* and the two subspecies of *Ophiostoma novo-ulmi* (ssp. *novo-ulmi* and ssp. *americana*) (Brasier, 1991; Brasier and Kirk, 2001).

Dutch elm disease occurred in two pandemics that have been caused by *O. ulmi* and *O. novo-ulmi*, respectively (Brasier, 1990, 2000). The first one was triggered

by *O. ulmi* and started around 1910 in north-western Europe (Gibbs, 1978; Brasier, 1990). Within a few years DED had spread in Europe and North America and given rise to substantial losses to the various elm species in both continents (Gibbs, 1978; Brasier, 1990). In the 1970s another, much more aggressive DED pathogen was discovered (Gibbs and Brasier, 1973). This fungus was subsequently recognized as a new species and described as *O. novo-ulmi* (Brasier, 1991). *Ophiostoma ulmi* and *O. novo-ulmi* display a strong, though not total reproductive barrier (Brasier, 1977; Kile and Brasier, 1990; Brasier, 1991) and they differ in many of their biological, physiological, pathological and molecular characteristics (Brasier, 1991).

Ophiostoma novo-ulmi is separated into two subspecies, ssp. *novo-ulmi* and ssp. *americana* (Brasier and Kirk, 2001), which have formerly been known as Eurasian (EAN) and North American (NAN) races, respectively (Brasier, 1979). They were originally defined on the basis of a partial reproductive barrier that operates between them (Brasier, 1979, 1981). In addition, the two subspecies exhibit differences in their perithecial dimensions, colony morphologies, growth rates as well as pathogenicity to elm (Brasier, 1991; Brasier and Kirk, 2001), and can be separated by a number of genetic markers (e. g. Bates *et al.*, 1993a, 1993b, Hoegger *et al.*, 1996; Pipe *et al.*, 2000; Harrington *et al.*, 2001). Although *O. novo-ulmi* has been discovered in the 1970s, it is thought to have appeared earlier, possibly after two independent introductions in the 1940s. While ssp. *novo-ulmi* is suspected to have been introduced into eastern Europe, ssp. *americana* probably appeared about the same time in central North America and was subsequently also introduced from North America into Europe (Brasier, 1990). As a result of these introductions the ranges of the two subspecies presently overlap in several parts of Europe (Brasier and Kirk, 2001; Brasier, 2000a).

After the appearance of *O. novo-ulmi*, remarkable interactions between the three DED pathogens occurred that are thought to have been of great significance for the epidemiology of DED (Brasier, 2000a, 2001). The most obvious phenomenon was the gradual displacement of *O. ulmi* by the more virulent and more competitive *O. novo-ulmi* (Gibbs *et al.*, 1979; Brasier, 2000a). While *O. ulmi* is presently threatened to disappear completely as a separate species, recent evidence has shown that *O. ulmi* forms hybrids with and introgresses into *O. novo-ulmi* in nature (Brasier *et al.*, 1998, 2002; Et-Touil *et al.*, 1999). In addition, in those parts of Europe where the ranges of the subspecies are presently overlapping free hybridization between *O. novo-ulmi* spp. *novo-ulmi* and ssp. *americana* is suspected to occur (Brasier, 2000a, 2001). As a consequence, subspecies hybrids may become the dominant forms of *O. novo-ulmi* in Europe. Both the gene flow from *O. ulmi* into *O. novo-ulmi* and the hybridization between the subspecies of *O. novo-ulmi* have resulted in the rapid, still ongoing evolution of *O. novo-ulmi* (Brasier, 2000a). Likewise, both hybridization events are suspected to have been advantageous for *O. novo-ulmi*, because they improved the fitness of its emerging population (Brasier, 2000b, 2001).

As in most European countries, DED has caused severe damage to elms in Austria (Mayer and Reimoser, 1978; Kirisits and Halmschlager, 1997a, 1997b). Despite the devastating impact of the disease, virtually no research on the DED pathogens has been conducted in Austria until recently. Also, there is very little published information on the history and impact of DED in this country. In the 1990s, studies have been initiated to investigate the incidence and geographical distribution of *O. ulmi* and the subspecies of *O. novo-ulmi* in Austria (Kirisits *et al.*, 2001; Konrad, 2002). In addition, efforts were made to identify hybrids between the subspecies of *O. novo-ulmi* (Konrad *et al.*, 2002; Konrad, 2002). This paper presents a synthesis of these recent studies with an attempt to review the history and impact of the disease in this central European country.

Importance of elm in Austria

As in many other parts of Europe three species of elm, field elm (*Ulmus minor*), wych elm (*Ulmus glabra*) and European white elm (*Ulmus laevis*) are native to Austria (Mayer, 1984; Röhrig, 1996; Adler *et al.*, 1994; Collin, 2000). Wych elm, which occurs in parts of the Alps and their foothills as well as at mountainous or hilly areas outside of the Alps, differs considerably in its ecology from the other two species (Mayer, 1984; Röhrig, 1996). It is distributed up to 1400 m a.s.l. altitude and occurs on nutrient-rich and moist sites with high air humidity, particularly in steep canyons, where it often forms part of rare and endangered forest communities (Mayer, 1971, 1974, 1984; Brennsteiner, 1984; Kargl, 1992; Franz, 1994; Fischer, 1997). Field elm and European white elm are mainly distributed in the lowlands, especially in riparian fo-

rests along big rivers in eastern Austria (Danube, Morawa), where they grow together with other mesophilic hardwoods (Jelem, 1974, 1975; Mayer, 1974, 1984). Apart from riparian ecosystems *U. minor* and sometimes also *U. laevis* occur in oak-hornbeam forests and other hardwood forest communities in flat and hilly areas up to 600 m a.s.l. altitude (Mayer, 1974; Röhrig, 1996).

For economical and ecological reasons elms were appreciated as forest trees. Both *U. glabra* and *U. minor* produce valuable timber which is preferentially used for furniture (Mayer, 1984; Röhrig, 1996). The timber of *U. laevis* is less valuable, since it has a lower portion of heartwood (Röhrig, 1996). The overall economic importance of elms in Austria´s forestry has been low, but in the riparian forests of the Danube east of Vienna and along the Morawa, field elm was one of the most common tree species and thus of considerable economical importance for the owners of these forests (Jelem, 1974, 1975; Damm, 1997). Apart from the forest, elms were also valuable shade trees in cities, able to withstand the various abiotic stresses in the urban environment. In some parts of Austria they have also been planted as solitary trees, being a characteristic component of the landscape. In addition, field elm and wych elm have been used in shelterbelts in order to stabilize steep slopes and to protect agricultural areas from erosion. Due to its nutrient-rich foliage and bark, wych elm was one of the preferred tree species for lopping in order to obtain twigs and leaves as forage for cattle and sheep. All the functions of elm mentioned above mainly refer to the time preceding the DED outbreaks, since today most mature trees have vanished and elms are presently truly missed from an economical, ecological and nature conservation point of view.

History and impact of Dutch elm disease in Austria

Of the three elm species native to Austria *U. glabra* and *U. laevis* are known to be highly susceptible to DED, whereas *U. minor* is moderately susceptible (Brasier, 1977; Heybroek, 1981). The incidence of the disease is not only linked to the susceptibility of the trees to *O. ulmi* and *O. novo-ulmi*, but is also strongly influenced by their attractiveness to maturation feeding by the bark beetle vectors. Indeed, field elm is much more attractive to infestation by *Scolytus* spp.

than wych elm and European white elm (Sacchetti *et al.*, 1990; Webber, 2000).

Field elm has been decimated, especially in the riparian forest ecosystems, but mature wych elm trees can still be found, particularly in remote valleys and canyons. In most situations, healthy, diseased and dead wych elm trees occur together, indicating that the disease is still progressing and that the remaining healthy trees are highly threatened of also becoming infected. Concerning *U. laevis*, the elm species least attractive to beetle feeding (Saccetti *et al.*, 1990; Webber, 2000), relatively large numbers of mature trees still exist (Günzl, 1999). For example, numerous trees with diameters up to 1.2 m and heights reaching 35 m occur in the «Wiener Prater» (Vienna) (Kirisits and Halmschlager, 1997a, 1997b), a former riparian forest of the Danube (Fig. 1), and are also common in the riparian forests along the Danube east of Vienna (na-

Figure 1. Mature European white elm *(Ulmus laevis)* tree, a hitherto escape of Dutch elm disease («Wiener Prater», Vienna, 1997).

tional park «Donauauen») and the Morawa (Günzl, 1999). *Ulmus minor* was originally much more abundant than *U. laevis* in these forests (Jelem, 1974, 1975), but as a result of DED this situation has reversed (Günzl, 1999). The observations from Austria that European white elm is least affected by DED are consistent with reports from other parts of Europe (e. g. Collin, 2000; Mackenthun, 2000).

As a consequence of DED mature and large elm trees have become rare in Austria, and the elms have almost completely lost their importance as forest and shade trees as well as commercially important timber species. Even more important than these direct economical losses, is the devastating impact of the disease on natural forest ecosystems that have changed in their composition and structure after the decimation of elm (Jelem, 1974, 1975; Mayer and Reimoser, 1978; Ramskogler, 1990; Damm, 1997). Although elms are still present —sometimes rather abundantly— as sprouts, saplings and small- to medium-sized trees, they eventually will become attractive to the *Scolytus* beetles, leading to new cycles of the disease.

The history of DED in Austria and other parts of central Europe is surrounded by much uncertainty and speculation, since very few detailed observations on the development of the disease have been published. Although the author of the first record of the disease in Austria is unknown, Gibbs (1978, 1981) reported that it has been present in Austria since 1928. In 1931, the disease was detected in Vienna where it gave rise to the destruction of elms that were then common in avenues of Austria´s capital (E. Donaubauer, personal communication). These reports refer with certainty to the first epidemic caused by *O. ulmi*. It appears that elms that had been predisposed by drought or other agents as well as shade and ornamental trees, were initially mostly affected, whereas during its early years in Austria and other parts of central Europe DED seemed to have had relatively little impact on vigorous elm trees growing in the forests (Maschning, 1974; Mayer and Reimoser, 1978; Mayer, 1984; E. Donaubauer, personal communication).

This situation changed by the mid-1950s when the incidence and severity of DED increased dramatically in the riparian forests along the Danube and Morawa in eastern Austria, where *U. minor* and *U. laevis* occurred very abundantly (E. Donaubauer, personal communication). In the management plan from 1949 to 1958 of a forest enterprise (Stiftung Fürst Liechtenstein Guts- und Forstbetrieb Wilfersdorf) that ma-

naged lowland forests with a high proportion of elm trees along the Morawa near Hohenau in the most eastern part of Austria, Dutch elm disease was already mentioned as being severe by 1949 (Damm, 1997). In the light of this report, it is suspected that the outbreak of DED in the riparian ecosystems in eastern Austria occurred at even an earlier time. In the late 1950s and 1960s the disease spread quickly in these eastern parts of the country as well as westwards through the lowland forests of the Danube and by 1970 the majority of mature field elm trees in these ecosystems had been decimated (Damm, 1997; E. Donaubauer, personal communication). In the forests along the Morawa the proportions of elms decreased from 20-25% before the arrival of DED to 1% today (Günzl, 1999). Likewise, along the Danube east of Vienna, elm had in the 1950s a proportion of 5-10% which had also decreased to about 1% by 1982 (Günzl, 1999).

In the distribution range of wych elm it took much longer than in the riparian forests before severe damage occurred. The first reports of intensive outbreaks of DED on this species in Austria are documented from the mid-1970s. Mayer and Reimoser (1978) reported about the death of virtually all mature wych elm trees in the forest reservation Dobra (Waldviertel, Lower Austria) between 1974 and 1977. In the mid-1970s an outbreak of DED is also documented in the Salzach valley around Lend (district Pinzgau, province Salzburg) (E. Donaubauer, personal communication). After a time lag of a few years the epidemic of DED also spread in west-east direction through the upper Mur valley (Fig. 2) in the provinces Salzburg and Styria (Donaubauer, personal communication). It is very li-

Figure 2. An example of wide-spread mortality of wych elm *(Ulmus glabra)* due to Dutch elm disease in the upper Mur valley (St. Ruprecht ob Murau, Styria).

kely that all these reports coincide with the appearance of *O. novo-ulmi*. In neighbouring Bavaria and Baden-Württemberg the second epidemic of DED and its causative agent, *O. novo-ulmi* were first recorded in 1973 (Maschning, 1974; Gibbs, 1978), around the same time when severe damage on wych elm was registered in Austria. In the late 1970s and during the 1980s and 1990s DED continued to spread in the distribution range of wych elm in the Alps and caused catastrophic damage to the population of this elm species as well.

The widespread occurrence of the disease in Austria is also paralleled by the geographical distribution of isolates of the DED pathogens collected during a recent survey (Fig. 3; Kirisits *et al.*, 2001). There is evidence that isolated trees or groups of wych elms, especially in remote areas and at higher elevations, may still be healthy, as exemplified by a

Figure 3. A) Known geographical distribution of *Ophiostoma novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana* in Austria based on a sample of 92 isolates collected between 1997 and 1999. Designation of isolates to the two subspecies was done by a fertility test in the laboratory and by PCR-RFLP of the *cerato-ulmin* gene. One dot may represent more than one isolate. B) Geographical distribution of known hybrid isolates between *Ophiostoma novo-ulmi* ssp *novo-ulmi* and *O. novo-ulmi* ssp. *americana*, collected between 1997 and 1999. Hybrids were identified on the basis of their fertility response and PCR-RFLP of the *cerato-ulmin* gene and the *colony type* gene. One dot may represent more than one isolate.

small, isolated stand in southern Carinthia, in which DED has so far not been recorded (Franz, 1994; Kirisits *et al.*, 2001) and also by other stands in the Alps (e. g. Brennsteiner, 1984; Kargl, 1992). The absence of the disease on wych elm in some alpine stands may be due to unfavourable conditions for the insect vectors of the disease. These escapes are nevertheless at high risk of becoming devastated within a few years, if DED comes to reach these stands. This emphasises the need for an ex-situ conservation of elm genotypes from such forests before they become destroyed (Collin, 2000).

Although foresters and the general public have testified much concern about DED, virtually no attempts have been made to manage the disease in Austria, neither in the forest nor in the cities (Donaubauer, personal communication). In the forests, the death of elm was accepted as unavoidable and in most forest enterprises only removal of dead trees was undertaken. Elm was dropped from the list of economically important timber species and it was recommended not to plant and promote elm any longer in the forest (e. g. Damm, 1997). This situation slightly changed in the late 1980s and 1990s, when rare and endangered tree species, including elm, generally received more attention. It was then again recommended to plant and promote elm trees as a forest and landscape tree, despite the danger of infection by DED (Müller, 1992, 1997).

Since 1994, measures for conservation of the native elm species have been carried out in Austria (Müller and Frank, 1994; Müller, 1996, 1997). This program includes registration of remaining mature elm trees, harvest of seeds from selected trees, propagation of trees from seeds and by softwood cuttings, as well as establishment of plantations (Müller, 1996, 1997). Three kinds of field plots, containing half-siblings from selected mother trees were established, namely plantations for seed production, family plots (both managed by the Federal Office and Research Centre for Forests, Vienna), and plantations managed by forest enterprises in Lower and Upper Austria (Müller, 1996, 1997). The family plots have been managed as coppice stands with cuttings performed at short intervals in order to keep the trees small to avoid infestation by elm bark beetles. From trees of the family plots, cuttings for vegetative propagation have also been obtained. In addition to these ex-situ measures, in-situ preservation of wych elm is achieved in forest reserves where populations shall be maintained by natural regeneration (Müller, 1997).

Occurrence of *O. ulmi* and of the subspecies of *O. novo-ulmi* in Austria

Prior to the 1990s almost nothing was known about the DED pathogens in Austria. The only previous report was that of Brasier (1979) who identified two isolates from Tyrol as *O. novo-ulmi* ssp. *americana*. The lack of knowledge about the incidence and geographical distribution of *O. ulmi* and of the subspecies of *O. novo-ulmi* in Austria prompted our recent surveys of these pathogens, which were conducted in 1993 and 1994 and from 1997 till 1999 (Kirisits *et al.*, 2001). In 1993 and 1994, strains were mainly obtained from Vienna and the surrounding areas in Lower Austria, whereas in the subsequent surveys most isolates originated from the provinces Lower Austria, Burgenland, Styria, Upper Austria and Tyrol, but a few strains were also obtained from the most southern (province Carinthia) and most western parts (province Vorarlberg) of the country (Table 1, Fig. 3).

Identification of 151 isolates based on growth studies at 20°C and 33°C and assessments of colony morphology (Brasier, 1981; Fig. 4) clearly indicated that *O. novo-ulmi* is the only DED pathogen presently occurring in Austria, with but one exception (Kirisits *et al.*, 2001). This isolate has a unique colony morphology, atypical of either *O. ulmi* or *O. novo-ulmi* (Fig. 5). The absence of *O. ulmi* in the isolates collected in the 1990s (Kirisits *et al.*, 2001) may follow the pattern of replacement by *O. novo-ulmi* as observed in North America and Europe (e. g. Gibbs *et al.*, 1979; Hoegger *et al.*, 1996; Brasier, 2000a).
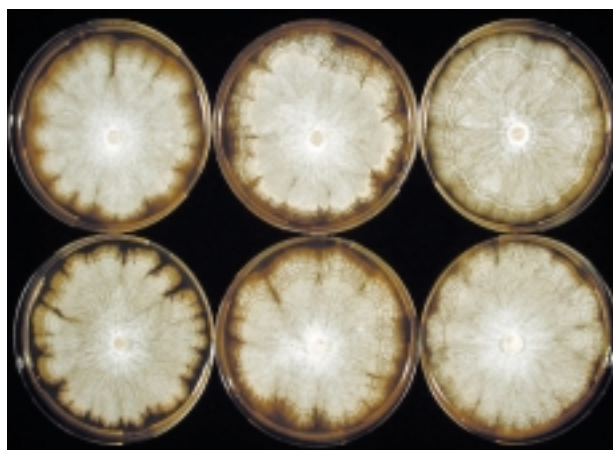

Figure 4. Various colony morphologies of *O. novo-ulmi* isolates from Austria. The isolates were grown on Oxoid malt extract agar at 20°C in the dark for 7 days and subsequently exposed to diffuse daylight at room temperature for 10-14 days.

A set of 99 isolates of *O. novo-ulmi* from various parts of Austria was selected for the mating type tests and for subspecies identification. Twenty of the 99 isolates belonged to mating type A, and 79 to mating type B (Table 1), figures which are consistent with the values reported in the literature (Brasier, 1988). Isolates of mating type B are usually dominant in early epidemic and late epidemic populations of *O. novo-ulmi* in Europe (Brasier, 1988, 2000b), since they are slightly more virulent, have a faster growth rate and are thus more competitive than those of mating type A (Brasier and Gibbs, 1975).

Results of the fertility tests showed that 66 isolates belonged to ssp. *novo-ulmi* and 33 to ssp. *americana*

Table 1. Isolates in each of the nine Austrian provinces and in whole Austria that were identified as *Ophiostoma novo-ulmi* ssp. *novo-ulmi* and *O. novo-ulmi* ssp. *americana* in the fertility tests and as A- and B- mating type in the mating type tests. The seven hybrids between ssp. *novo-ulmi* and ssp. *americana* (Table 2) are also included

Province	N	*O. novo-ulmi* ssp. *novo-ulmi*				*O. novo-ulmi* ssp. *americana*			
		n	%*	A	B	n	%*	A	B
Burgenland	17	12	70.6	3	9	5	29.4	1	4
Carinthia	3	1	33.3	0	1	2	66.7	1	1
Lower Austria	21	12	57.1	1	11	9	42.9	0	9
Salzburg	1	0	0.0	0	0	1	100.0	0	1
Styria	27	23	85.2	6	17	4	14.8	1	3
Tyrol	11	6	54.5	2	4	5	45.5	0	5
Upper Austria	12	7	58.3	2	5	5	41.7	1	4
Vorarlberg	4	4	100.0	0	4	0	0.0	0	0
Vienna	3	1	33.3	1	0	2	66.7	1	1
Austria (total)	99	66	66.7	15	51	33	33.3	5	29

* Percentages refer to the portion of isolates in each province or in whole Austria belonging to ssp. *novo-ulmi* and ssp. *americana*, respectively.
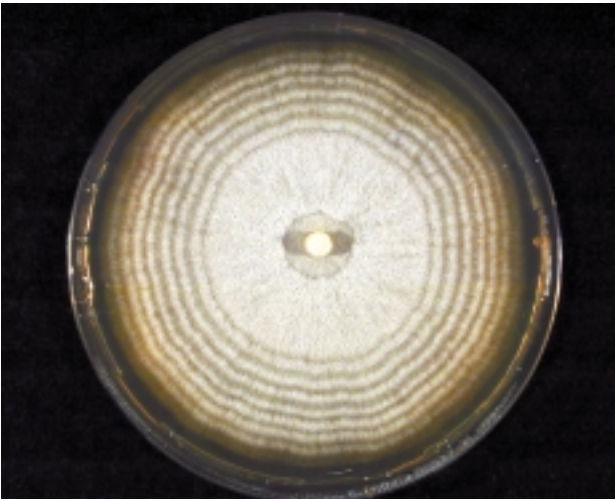
Figure 5. Colony morphology of isolate L/16, neither resembling that of *Ophiostoma ulmi* nor that of *Ophiostoma novo-ulmi*. Note the regular concentric pattern of growth, the strong diurnal zonation and the abundant production of undifferentiated aerial mycelium. Conditions for growth as described in Fig. 4. From Kirisits et al. (2001), Forstwissenschaftliches Centralblatt 120, 231-241. Reproduced with permission from Blackwell Publishing Ltd.

(Table 1), with one isolate of the latter (AT146) showing an intermediate fertility response, characterized by the formation of few fertile, but numerous partly developed perithecia in its crosses as donor with a tester isolate of *O. novo-ulmi* ssp. *novo-ulmi* as recipient (Konrad *et al.*, 2002). This unusual result was considered as indication of the possible hybrid origin of isolate AT146. This strain was subsequently included in studies on hybridization between the subspecies of *O. novo-ulmi* in Austria (see below) (Konrad *et al.*, 2002).

The two subspecies of *O. novo-ulmi* occur intermingled throughout the country, often in close proximity of one to another (Table 1; Fig. 3A). Hence, ssp. *americana* was not restricted to the western part of the country and ssp. *novo-ulmi* to the eastern part, as it might have been inferred from previous surveys conducted in Europe (Brasier, 1979, 2000a, 2001; Brasier and Kirk, 2001). Indeed, in Vorarlberg, the most western province, only ssp. *novo-ulmi* was detected. Comparatively, a recently conducted study in Switzerland, indicated that ssp. *americana* predominates therein, whereas ssp. *novo-ulmi* was generally rare (Hoegger *et al.*, 1996). Records of *O. novo-ulmi* ssp. *americana* from the eastern part of Austria represent the most eastern occurrence of this taxon in central Europe and they are also among of the most eastern reports for whole Europe (Brasier and Kirk, 2001; Brasier, 2000a).

Hybridization between the subspecies of *O. novo-ulmi* in Austria

Although it has been proposed that hybrids between *O. novo-ulmi* ssp. *novo-ulmi* and ssp. *americana* commonly arise in parts of Europe where both subspecies occur (Brasier, 2000a, 2001; Brasier and Kirk, 2001), identification of these hybrids is presently very difficult and only a few have so far been detected in natural populations of *O. novo-ulmi*. Prior to our investigations there have only been two studies that presented clear experimental evidence for identification of ssp. *novo-ulmi* x ssp. *americana* hybrid isolates. Based on isozyme analyses, Jeng *et al.* (1988) suggested a French isolate to be a subspecies hybrid and Hoegger *et al.* (1996) proposed that a Swiss isolate giving conflicting results in fertility tests and RAPD analyses is a subspecies hybrid.

Due to the overlap in the distribution of the subspecies of *O. novo-ulm* in Austria, it was suspected that they commonly hybridize in this part of Europe. To verify this possibility, methods of DNA sequencing and PCR-RFLP of nuclear genes that are polymorphic and consistently distinguish the subspecies of *O. novo-ulmi* were followed (Konrad *et al.*, 2002). The cerato-ulmin gene *cu* (Bowden *et al.*, 1994; Jeng *et al.*, 1996; Pipe *et al.*, 1997) and the colony type gene *col1* (Pereira *et al.*, 2000) were selected for the studies.

In a first step the 99 isolates which had previously been designated to ssp. *novo-ulmi* or ssp. *americana* on the basis of their fertility response (Table 1, see above) were examined by PCR-RFLP of the *cu* gene. In 93 isolates, the fertility test and RFLP of *cu* gave identical results. However, in six isolates, the fertility response and *cu* RFLP profile (Fig. 6A) differed (Table 2), indicating that they likely corresponded to *O. novo-ulmi* ssp. *novo-ulmi* x ssp. *americana* hybrids (Konrad *et al.*, 2002). In order to find further evidence for hybridization between the subspecies of *O. novo-ulmi*, these six isolates (AT21, AT73, AT83, AT125, AT145 and AT149) and one with an unusual fertility response (AT146, see above) were investigated by RFLP of the *col1* gene (Table 2, Fig. 6B). In two of the Austrian isolates (AT73 and AT146) recombination between the two subspecies of *O. novo-ulmi* in the *cu* and *col1* RFLP profiles occurred (Table 2, Fig. 6), providing unequivocal molecular evidence that these isolates were hybrids between these subspecies (Konrad *et al.*, 2002). The other five Austrian isolates are also subspecies hybrids, since they gave conflicting results in
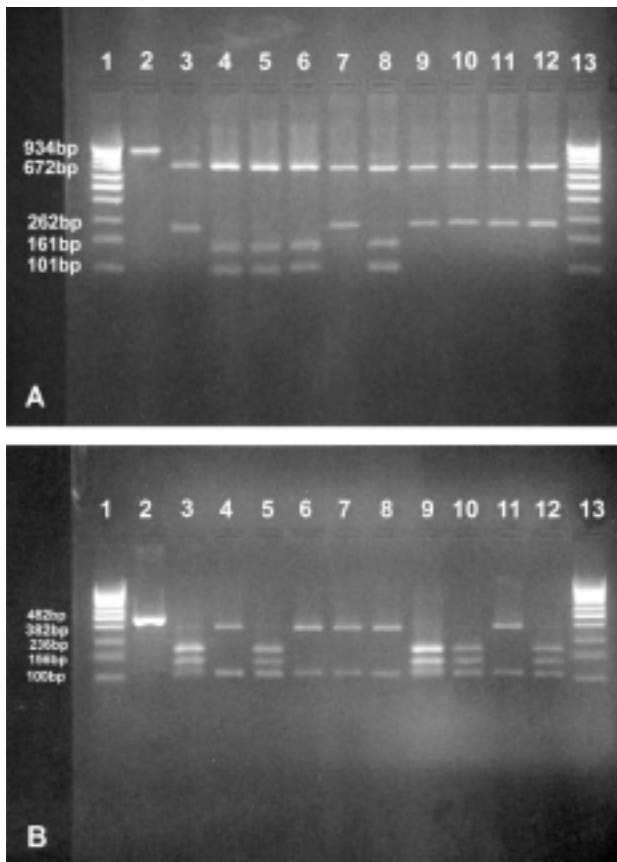
Figure 6. A) RFLP banding patterns of cu from *Ophiostoma novo-ulmi* digested with *Hph*I after separation on a 2% agarose gel. **B**) RFLP banding patterns of *col1* from *O. novo-ulmi* digested with *Bfa*I after separation on a 2% agarose gel. Lanes 1 and 13: 100 bp DNA ladder. Lane 2: uncut product of isolate AT 73; lane 3: ssp. *americana* isolate PG402; lane 4: ssp. *novo-ulmi* isolate CA4. Lanes 5-12, suggested ssp. *novo-ulmi* x ssp. *americana* hybrid isolates: P150, AT21, AT73, AT83, AT125, AT145, AT146, AT149. From Konrad *et al.* (2002), Plant Pathology 51, 78-84. Reproduced with permission from Blackwell Publishing Ltd.

the fertility response on the one hand and the *cu/col1* RFLP analyses (Fig. 6) on the other hand (Table 2). The seven hybrid isolates were detected in various parts of Austria, including eastern (province Burgenland; AT145, AT146, AT149), western (province Tyrol; AT73), southern (province Carinthia; AT21, AT125) and north-central (province Upper Austria; AT83) parts of the country (Fig. 3B).

Clear molecular evidence for hybridization between ssp. *novo-ulmi* and ssp. *americana* was also provided for a Polish strain of *O. novo-ulmi* (P150) that had originally been included as a tester strain for ssp. *novo-ulmi* (Konrad *et al.*, 2002; Table 2; Fig. 6). This result was surprising, since no isolate belonging to O. *novo-ulmi*

ssp. *americana* has so far been detected in Poland (Brasier, 2000a, 2001; Brasier and Kirk, 2001). The examples of the hybrid isolates from Austria and Poland show that the fertility test for distinguishing the two subspecies is no longer sufficient for definite designation of isolates of *O. novo-ulmi*, since ssp. *novo-ulmi* x ssp. *americana* hybrids can show fertility responses characteristic of those of either subspecies (Table 2).

Similar to the gene flow between *O. ulmi* and *O. novo-ulmi* (Brasier *et al.*, 1998), hybridization between the subspecies of *O. novo-ulmi* could increase the genetic diversity and fitness of the population of the DED pathogens in Europe (Brasier, 2000a, 2000b, 2001), and in particular their different vegetative compatibility (vc) types (Brasier, 2000b; Konrad *et al.*, 2002). Populations of *O. novo-ulmi* with many different vc types are a greater deterrent to the spread of deleterious mycoviroses (d-factors) than populations containing lower numbers of vc types (Brasier, 2000b). Preliminary studies on the pathogenicity of ssp. *novo-ulmi* x ssp. *americana* hybrids have shown that these might be as virulent as ssp. *americana* (Brasier, 1986). If the hybrids are as fit and virulent as their parents, this could lead to the expansion of the hybrid zone, and to the predominance of a new hybrid form of the pathogen in Europe (Brasier, 2000a, 2001; Konrad *et al.*, 2002).

A putative hybrid or introgressant between *O. ulmi* and *O. novo-ulmi* from Austria

As already mentioned, strain «L/16» (culture collection of IFFF-BOKU), isolated in 1994 from a twig sample of a diseased field elm tree at the locality «Laaerwald (Vienna)» could not be identified as either *O. ulmi* or *O. novo-ulmi* on the basis of its colony morphology (Kirisits *et al.*, 2001). Under controlled laboratory conditions (Brasier, 1981) this isolate was characterized by a regular concentric pattern of growth, strong diurnal zonation and abundant production of undifferentiated aerial mycelium (Fig. 5). Its unusual colony morphology resembled that of certain *O. ulmi* x *O. novo-ulmi* laboratory generated hybrids (Fig. 9F in Brasier, 1993; Brasier, personal communication). This suggested that isolate L/16 might represent one of the rare, naturally occurring hybrids or an unusual introgressant between *O. ulmi* and *O. novo-ulmi* (Kirisits *et al.*, 2001). In order to prove this possibility, the isolate was examined for its growth rates at 20°C

Table 2. Data concerning *O. novo-ulmi* ssp. *novo-ulmi* x ssp. *americana* hybrid isolates from Austria and Poland as well as results of mating type and fertility tests and PCR-RFLP of the *cerato-ulmin* (cu) gene and the *colony type* (col1) gene. Modified from Konrad *et al.* (2002)

Isolate*	Locality of isolation	Year of isolation	Sampled by**	Mating type	Fertility test	*Cu*	*Col 1*
P150	Szechin, PL	1980	CMB	A	ssp. *novo-ulmi*	ssp. *novo-ulmi*	ssp. *americana*
AT21	Freistritz, Carinthia	1997	SK & TK	A	ssp. *americana*	ssp. *novo-ulmi*	ssp. *novo-ulmi*
AT73	Landeck, Tyrol	1997	SK & TK	A	ssp. *novo-ulmi*	ssp. *americana*	ssp. *novo-ulmi*
AT83	Grünburg, Upper Austria	1997	SK & TK	B	ssp. *americana*	ssp. *novo-ulmi*	ssp. *novo-ulmi*
AT125	Wolfsberg, Carinthia	1998	SK & TK	B	ssp. *novo-ulmi*	ssp. *americana*	ssp. *americana*
AT145	Güssing, Burgenland	1999	HK	B	ssp. *novo-ulmi*	ssp. *americana*	ssp. *americana*
AT146	Güssing, Burgenland	1999	HK	B	ssp. *americana*	ssp. *americana*	ssp. *novo-ulmi*
AT149	Güssing, Burgenland	1999	HK	B	ssp. *novo-ulmi*	ssp. *americana*	ssp. *americana*

* All isolates are maintained in the Culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU), Vienna, Austria. ** C. M. Brasier (CMB); S. Krumböck (SK); T. Kirisits (TK); H. Konrad (HK).

and 33°C (Brasier, 1981), mating type (Brasier, 1981), fertility response as donor (male) and recipient (female) to authenticated isolates of the DED pathogens (Brasier, 1981; Brasier *et al.*, 1998), PCR-RFLP pattern of the *cerato-ulmin* gene and for the DNA sequences of the *colony type* gene (Konrad *et al.*, 2002) (Table 3).

The growth rate of isolate L/16 at 20°C was at the extreme upper limit of *O. ulmi* and at the lower limit of *O. novo-ulmi* (Table 3; Brasier, 1981). In the mating studies it proved to be sterile as recipient in crosses with *O. ulmi* and both subspecies of *O. novo-ulmi* as donor. When it was paired as donor with both subspecies of *O. novo-ulmi* as recipients, isolate L/16 behaved like *O. novo-ulmi* ssp. *americana*. The RFLP profile of the *cerato-ulmin* gene and the DNA sequences of the *colony type* gene were also identical to those of *O. novo-ulmi* ssp. *americana*.

In addition to its unique colony morphology, the female sterility of this *Ophiostoma* isolate hint to the possibilty that it could have arisen from hybridization between *O. ulmi* and *O. novo-ulmi*. Unusual colony morphologies and female sterility are common in laboratory generated and naturally occurring *O. ulmi* x *O. novo-ulmi* hybrids (Kile and Brasier, 1990; Brasier *et al.*, 1998). We believe that strain L/16 could be a key isolate for studying natural hybridization between *O. ulmi* and *O. novo-ulmi*, since it was isolated when *O. ulmi* had already been replaced by *O. novo-ulmi* (Kirisits *et al.*, 2001). The isolate may therefore represent a genotype with considerable fitness, which has enabled it to compete successfully with *O. novo-ulmi*. This postulate requires thorough investigation and examination of this isolate is continuing in order to prove its possible interspecific origin.

Conclusions and future plans

Our recent studies have greatly improved the knowledge on the DED pathogens in Austria and also provided new insights for a better understanding of the di-

Table 3. Morphological, physiological, biological and molecular characteristics of the putative *O. ulmi* x *O. novo-ulmi* hybrid isolate «L/16»

Colony morphology	Unique
Growth rate at 20°C	3.08-3.30 mm/day
Growth rate at 33°C	0.1 mm/day
Mating type	B
Fertility response as recipient to *O. ulmi* and the subspecies of *O. novo-ulmi*	Sterile
Fertility response as donor to *O. novo-ulmi* ssp. *novo-ulmi* and ssp. *americana*	Like *O. novo-ulmi* ssp. *americana*
PCR-RFLP profile of the *cerato-ulmin* gene	Identical to *O. novo-ulmi* ssp. *americana*
DNA sequence analyses of the *colony type* gene	Identical to *O. novo-ulmi* ssp. *americana*

sease in the whole of Europe. Austria forms part of a hybrid zone between *O. novo-ulmi* ssp. *novo-ulmi* and ssp. *americana*. This has been first suspected because of the overlap of the subspecies in Austria and has subsequently been proven through the unequivocal identification of ssp. *novo-ulmi* x ssp. *americana* hybrids in several parts of the country. Most recently, not only seven (Konrad *et al.*, 2002), but 28 out of 99 isolates from 18 localities in Austria were identified as hybrids between ssp. *novo-ulmi* and spp. *americana*, based on fertility response and RFLP of the *cerato-ulmin* gene and *colony-type* gene (H. Konrad, C. Stauffer and T. Kirisits, unpublished data). This strongly suggests that hybrids of the subspecies of *O. novo-ulmi* may soon become predominate in Austria, as it has previously been suggested by Brasier (2000a, 2001) for various parts of Europe.

Examination of isolates of *O. novo-ulmi* for their RFLP patterns of the *cerato-ulmin* gene and *colony type* gene, combined with fertility tests, offers the possibility for rapid, reliable and repeatable identification of ssp. *novo-ulmi* x ssp. *americana* hybrids. However, a portion of hybrids will remain undetected even by using this approach. Despite this disadvantage, populations of *O. novo-ulmi* can be screened by this method in order to determine the relative abundance of hybrids in various parts of Europe. Thus, it would allow determining and mapping putative hybrid zones on the European continent. It is certainly desirable to improve the present method for detection of ssp. *novo-ulmi* x *americana* hybrids. It will thus be necessary to include other polymorphic nuclear or mitochondrial genes or anonymous markers such as AFLP and RAPD, to obtain highly accurate information on the presence of subspecies hybrids in natural populations of *O. novo-ulmi*. Furthermore, the development of single sequence repeat (SSR) microsatellite markers (Zhou *et al.*, 2002) for the DED pathogens may provide a powerful tool to study hybridization between *O. ulmi* and *O. novo-ulmi* as well as between the subspecies of *O. novo-ulmi*.

Relatively little is presently known about the major characteristics of naturally occurring ssp. *novo-ulmi* x ssp. *americana* hybrids. Hence, as part of our future work we intend to examine and compare subspecies hybrids showing various patterns of recombination. These investigations will include morphology (perithecial dimensions), fertility response as recipient to tester strains of *O. novo-ulmi*, growth rate at various temperatures, tests on vegetative compatibility, ability to colonize elm bark as well as pathogenicity to elm.

The ongoing hybridization between ssp. *novo-ulmi* and ssp. *americana* is of significant ecological and epidemiological importance for the development of DED in Europe. A complete understanding of the disease requires careful examination of the unusual hybridization events between the DED pathogens. Unambiguous identification and characterization of ssp. *novo-ulmi* x ssp. *americana* hybrids will be key issues in future research on Dutch elm disease.

Acknowledgements

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