The Identification of *Ophiostoma novo-ulmi* subsp. *americana* from Portland Elms

by

Benjamin K. Au

A PROJECT

Submitted to

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AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____

Melodie Putnam

Dutch elm disease (DED) is a disease of elm trees caused by three species of Ascomycota fungi: *Ophiostoma ulmi*, *Ophiostoma novo-ulmi*, and *Ophiostoma himal-ulmi*. There are also two subspecies of *O. novo-ulmi*: subsp. *americana* and subsp. *novo-ulmi*. The pathogen is spread by bark beetles, which inhabit and traverse different elms. *O. novo-ulmi* is noted to be more aggressive than *O. ulmi*, and thus many areas in which *O. ulmi* had been dominant are being replaced by *O. novo-ulmi*.

Epidemiology of DED has been studied in areas including Spain, New Zealand, and Austria. Studies of the disease in the United States are not as prevalent. This study attempts to identify to subspecies, 14 fungal strains isolated from diseased elms growing in Portland, Oregon. Goals include determination of the relative abundance of *O. novo-ulmi* and *O. ulmi*. Most elm surveys categorize diseased elms as having signs of DED, but do not specify the causal species or subspecies. Another goal is to develop methods that can be used to differentiate between the species and subspecies of *Ophiostoma*, based on growth rate and polymerase chain reaction (PCR). A final goal of this study is to devise a protocol for Oregon State University's Plant Clinic to type *Ophiostoma* by a method other than morphology.

Typing of isolates was done using the mtsr primers (Hafez and Hausner 2011), which target gene sequences specific to *O. ulmi* and *O. novo-ulmi*. Subspecies differentiation of *O. novo-ulmi* was done using the CU primers (Konrad et al. 2002), which target the specific gene sequences needed to differentiate between subsp. *americana* and subsp. *novo-ulmi*. A growth rate experiment was also conducted using different optimal growth temperatures for each species.

Results suggest that *O. novo-ulmi* is more abundant than *O. ulmi* around Portland, Oregon, and that subsp. *americana* is more abundant than subsp. *novo-ulmi*. Growth rates did not appear as useful as the PCR screen to differentiate between *O. ulmi* and *O. novo-ulmi*, and cannot differentiate the *O. novo-ulmi* subspecies. PCR is a more reliable method to differentiate between *O. ulmi* and *O. novo-ulmi*, as well as the two subspecies of *O. novo-ulmi*. These findings could be used to further the knowledge of the DED pandemic that is currently occurring.

Keywords: Dutch elm disease, pandemic, *O. novo-ulmi*, *O. ulmi*, mtsr, CU.

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Benjamin K. Au, Author

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Literature Review and Background

I. The killer: Dutch elm disease

Dutch elm disease (DED) causes a fatal wilt of elms, and is caused by *Ophiostoma*, a fungal pathogen exotic to the US. The disease was first described in Holland in 1919 (Webber 2009), but could have possibly been noted earlier in the decade by other researchers. DED began to spread to different parts of the world with the transport of infected trees for furniture or firewood use (Webber 2009). A Dutch scientist, M.B. Schwarz, is credited for identifying the causal agent (Webber 2009), and was among several scientists of that country who conducted surveys of DED between 1919 and 1934.

DED spread eastward through Europe in the 1920s. Austria cites the first appearance of the disease in 1928 (Kirisits and Konrad 2004). It had spread to Turkey as well as Ukraine by the 1930s, and only a few years later reached Russia in 1939 (Brasier 2000). An appraisal in 1990 suggests that most elms in China are infected (Brasier 1990). The disease has crossed oceans with the movement of infected wood, and it was found in New Zealand elms by 1989 (Gadgil et al. 2000). Australia is yet to discover the disease, although the bark beetle vector was first recorded in Melbourne in 1974 (Lefoe et al. 2001). There have been no reports of DED in South America or Africa yet (Six et al. 2005), although this is likely because these areas are outside the natural habitable range of elms and bark beetles.

DED appeared in North America around the 1920s. Logs infested with beetles carrying the pathogen which had been imported from the Netherlands to the US were the likely source (Gibbs 1978). European bark beetles, which act as a disease vector, were brought to North America through trade routes years before the causal fungi were introduced.

The disease spread to eastern Canada in the 1940s, although it has not been established if

this was due to the same introduction event from the United States, or by a second introduction from overseas during World War II (Brasier and Buck 2002). By 1945, it was noted to have spread through northern Quebec (D'Arcy 2005). The disease reached Manitoba by 1975 (Forestry Branch 2013). To date, there have been no reports of DED as far west as British Columbia (Ministry of Agriculture 2012), although the first report of the disease in neighboring Alberta occurred in 1998 (Agriculture and Rural Development 2010).

After DED arrived in the eastern United States in the 1920s, it began to advance westward across North America. C.J. Buisman, another Dutch scientist, first observed DED in Ohio in the 1930s, and the disease was found in Kentucky around the 1940s (D'Arcy 2005). However, information on the spread of DED in the United States became less prevalent as time passed. Newspapers reported DED to be in Michigan by 1950, Illinois by 1960, and in Minnesota by 1970 (Byers 2006, New York Times 1989).

In the Pacific Northwest, infected trees were noted to be in Boise, Idaho as early as 1968, and spread to Washington in 1974 (City of Seattle 2002). However, like the rest of the United States, awareness of the pandemic dropped as time passed. Local *Oregonian* news reports provide a record of when DED appears in each community. Several cases appear in Oregon around 1974, in the cities of Ontario, La Grande, and Union. Portland, Oregon reported a single diseased tree in 1976. Other Oregon cities that reported the presence of DED include Hillsboro in 1987, Salem in 1988, Corvallis in 1995, Heppner in 1999, and Medford in 2006 (Pscheidt and Ocamb 2014). The Portland City Council passed an ordinance concerning DED in 1987, showing that the disease was already a problem after the disease spread was first detected in Eugene, Oregon (Parks & Recreation, 2014).

The disease was found in California in 1973 (Byers 2006), although this is a few years

before DED was regularly reported.

II. The causal agents of DED

Dutch elm disease (DED) is caused by fungi of the genus *Ophiostoma: Ophiostoma ulmi, Ophiostoma novo-ulmi,* and *Ophiostoma himal-ulmi*. *Ophtiostoma novo-ulmi* consists of two subspecies, subsp. *americana* and subsp. *novo-ulmi* (Brasier and Buck 2002).

O. himal-ulmi, which is endemic to the Himalayas (Brasier and Buck 2002), was thought to have originated as a hybrid of *O. ulmi* and *O. novo-ulmi*, but new evidence suggests that this is not the case. Current evidence indicates that *O. himal-ulmi* evolved from a common ancestor of *O. novo-ulmi* and *O. ulmi*, due to reproductive isolation. It shares some characteristics with *O. novo-ulmi*, such as high rates of cerato-ulmin production—a hydrophobin toxic to elms—and aggressiveness toward *Ulmus laevis* and *Ulmus americana* (Brasier and Mehrotra 1995).

Ophiostoma novo-ulmi was previously described as consisting of two different races (Brasier 1990). The North American race (NAN) was used to label the species that was prevalent in North America. The Eurasian race (EAN) was used to label the species predominately in Europe and in parts of Russia and China. However, the NAN race of *O. novo-ulmi* has since been reclassified as *O. novo-ulmi* subsp. *americana*, while the EAN race of *O. novo-ulmi* is now recognized as *O. novo-ulmi* subsp. *novo-ulmi* (Brasier and Buck 2002).

Two pandemics of DED have occurred since its identification in the early 20th century. The first pandemic was caused by *O. ulmi* and lasted from the 1920s to the 1940s, and originated in Northwest Europe (Brasier and Buck 2002). DED spread to Europe, Russia, and Southwest Asia, as well as to the United States and the United Kingdom due to infected timber imports (Brasier and Buck 2002). The second, current, pandemic is thought to be caused by *O.*

novo-ulmi and has predominated since the 1940s (Brasier and Buck 2002). It is thought that subsp. *americana* originated in the southern Great Lakes area of the United States, while subsp. *novo-ulmi* originated in Romania (Brasier and Buck 2002). Areas afflicted with the second pandemic
include most of Europe and Asia, as well as Canada and the United States (Brasier and Buck 2002).

There is evidence to suggest that *O. novo-ulmi* and *O. ulmi* are in a competitive interaction during this second pandemic, whereas before they were temporally isolated. *O. novo-ulmi* is more aggressive, and is thought to have displaced O. *ulmi* in regions where it occurred (Brasier and Buck 2002). The rapid spread of *O. novo-ulmi* in areas predominated by *O. ulmi* suggests consequences such as the potential for genetic exchange between the *Ophiostoma* species, which could lead to accelerated evolution. Elms planted in the United States have little resistance to this exotic pathogen (Brasier and Buck 2002).

III. Differentiation of *Ophiostoma ulmi, O. novo-ulmi,* and subspecies

The two species of *Ophiostoma* which cause DED in the US vary morphologically, and also have different optimal growth conditions which are useful for differentiating isolates. When grown on solid nutrient media, *O. ulmi* is able to grow at 30°C, and exhibits a slight undulating margin of growth. It is more yeast-like in appearance, and grows close to the surface of the medium. In contrast, *O. novo-ulmi* is unable to grow at 30°C, but grows faster than *O. ulmi* at 20°C. At 20°C, *O. novo-ulmi* has distinct rings and radial lines emanating from the colony center (Brasier and Buck 2002).

Another means of distinguishing the species is amplification of the mt-rns intron by

polymerase chain reaction (PCR) (Hafez and Hausner 2011). This intron (a non-coding region of a eukaryotic gene) is a part of the mitochondrial small subunit ribosomal RNA gene present in several fungal taxa, including the species of *Ophiostoma* that cause DED (Hafez and Hausner 2011). Secondary structure models of the mt-rns RNA show that there is a 12 nucleotide difference in the RNA and encoding DNA between *O. ulmi* and *O. novo-ulmi* ssp. *americana* (Hafez and Hausner 2011). The DNA encoding the mt-rns intron is amplified using the mtsr primer. The two species of fungi then can be differentiated by the size of the resulting amplicon (PCR product).

The mtsr primers are not useful for distinguishing subspecies within *O. novo-ulmi;* hence a second gene region is necessary to differentiate the subspecies. A second set of primers that may be used target the cerato-ulmin (CU) gene (Konrad and Kirisits 2002). Cerato-ulmin is a hydrophobin protein secreted at high levels in *O. novo-ulmi,* but at lower levels in *O. ulmi* (Konrad and Kirisits 2002). Amplification of the CU gene forms a product of the same size for both subsp. *americana* and subsp. *novo-ulmi.* However, there is a transversion in the CU gene of subsp. *americana* relative to subsp. *novo-ulmi* (Konrad and Kirisits 2002). *Hph*I is a restriction endonuclease that cuts within the CU gene. Because of the transversion, *Hph*I digest of the PCR product results in different sized fragments, depending on whether the sequence was from subsp. *americana* or subsp. *novo-ulmi*.

IV. Uses and value of elm trees

The native range of the American elm, *Ulmus americana*, covers the entire eastern half of the United States, as well as some parts of southeastern Canada. Due to factors such as human cultivation and planting, *U. americana* is present throughout the continental United States, as

well as the southern latitudes of Canada from British Columbia to Newfoundland (Bey 2005). The number of American elms lost to DED in North America alone is estimated to be in the hundreds of millions (Brasier and Buck 2002). For comparison, it is estimated that 28 million European elms in Britain have died from DED since the introduction of *O. novo-ulmi* from Toronto in the 1960s (Brasier and Buck 2002).

There are species of elm trees that are resistant to DED. Such species include the Siberian and Chinese elms, as well as their hybrids. However, elms that are resistant to DED are less desirable as street trees because they tend to be more susceptible to damaging abiotic factors. For example, the Siberian elm, *Ulmus pumila*, has a reputation for having brittle wood. Its major limbs have a tendency to split, especially in stormy weather (Gilman and Watson 1994). The Chinese elm, *Ulmus parvifolia*, is less tolerant of harsh environments, and grows poorly on rock or wet soil conditions (Niemiera 2012).

In terms of human uses, elms are highly valued as street trees due to their high arching branches, and historically have constituted an important component of the urban forest, particularly in the Midwest, where they were extensively planted. Street trees add an immense contribution to property values. In Portland, Oregon, street trees were found to increase property values by an average of 7000 USD, depending on the number of trees in front of the house and the crown area of the tree within 100 feet of the house. In the same study, researchers concluded that the annual benefit of all street trees in Portland is estimated to be 45 million USD annually when compounded, but only costs around 4.6 million USD for annual maintenance (USDA 2008).

Elm wood has also served other purposes as a materials product, and has been used in the production of cardboard boxes, baskets, crates, barrels, and caskets (Painter 2014). According to

Purdue University, the three most important species of elm used for lumber are *Ulmus americana, Ulmus thomasii,* and *Ulmus ruba* (Cassens 2007). The trees were used for furniture back in the 1900s, although this has become less prevalent due to the development of stronger materials for furniture (Painter 2014). Elm wood has also been used for firewood (Painter 2014).

The tree plays a large role in the greater ecosystem. Elms have been widely used as shelterbelts or windbreaks, particularly on the plains where winds can erode soils (Bey 2005). The extensive root system of the trees adds to the structural integrity of the soil, and also alleviates the risk of wind damage to crops.

Many organisms rely on the elm tree as a food source, habitat, or symbiotic partner. Examples of such include beavers, squirrels, honey bees, and various species of birds. Elm trees also provide homes for mycorrhizal fungi, and lichens. In one joint study by Plantlife International and English Nature, over 200 species of lichen were identified on British elms. Elm trees are an important part in urban and natural ecosystems; the continuing loss of elms due to DED has had a profound impact on these systems.

V. Disease cycle of DED

Members of the genus *Ophiostoma* are able to reproduce both sexually and asexually. The asexual stage consists of two synanamorphs, which are two different spore-producing stages (anamorphs) that share the same sexual state. One anamorph is *Graphium,* and the other is *Sporothrix.* The *Graphium*-type spores are produced in a sticky mass at the top of a specialized structure called a coremium. Coremia consist of hyphae that aggregate into dark, consolidated stalks known as synnemata. At the top of each synnema is a flared head of hyaline hyphae on which the spores are produced. The spores adhere together in mucilaginous, globose droplets.

The *Graphium* spores are typically produced in dead or dying DED-infected elms (Agrios 2005).

The *Sporothrix* stage produces dry spores that tend to form when the elm is first infected, after the fungus has reached the large xylem vessels. The spores are formed by yeast-like budding, and are then transported throughout the plant with the flow of water in the xylem. This spreads the fungus to limbs distant from the original infection site and between trees via root grafts (Webber 2009).

In addition to asexual spore production, *Ophiostoma* species can also undergo genetic recombination via sexual reproduction. Members of *Ophiostoma* are heterothallic, meaning that the fungus is self-sterile—mating types must be sexually compatible in order to successfully reproduce (Agrios 2005). When cells of different mating types come together, sexual fruiting bodies known as perithecia are formed (Agrios 2005). Perithecia are spherical fruiting structures with an opening at the end of a long stalk, or neck. Asci, sac-like structures in which the sexual spores are formed, line the interior of the perithecium. Ascospores are produced within the asci, which eventually break down to release the spores. When mature, the spores are pushed up the perithecial neck through osmotic pressure, where they accumulate in mucilaginous droplets at the opening. *Ophiostoma* undergoes sexual reproduction less frequently than asexual reproduction due to the prevalence of a single mating type within a large infected area (Agrios 2005, D'Arcy 2005).

VI. The beetle vector

Dutch elm disease is primarily spread by bark beetles, which overwinter in dead or dying elms, under the bark. They emerge in the spring, carrying with them the sticky spores of the *Graphium* stage of the fungus.

The beetles feed on healthy elm twigs, introducing the fungal spores into feeding wounds. As the beetles mature, they seek out dead and dying trees in which to breed. The females bore underneath the bark to lay their eggs. The larvae, as they hatch, eat the wood beneath the bark, forming tiny tunnels known as galleries as they go. The beetles feed during summer, and overwinter under the bark. Meanwhile, the *Graphium* stage grows in the diseased wood, producing synnemata which protrude from the galleries, their spore-laden surfaces projecting into the air. The beetle offspring continue to bore underneath the bark as they mature. After the beetles mature, they migrate from the galleries to the surface and collect fungal spores that adhere to their backs and legs. The spores are then transported as the beetles move to healthy elms on which to feed (Savonen 2004).

There are three species of bark beetle that are known to vector DED. The European bark beetle, also known as *Scolytus multistriatus*, is thought to be the species that spread DED throughout Europe during the first pandemic. *S. multistriatus* is known to be an invasive species, and now has a habitat range from the United States, Canada, and to Europe (Davis 2011). The banded elm bark beetle, also known as *Scolytus schevyrewi*, has a habitable range including the United States, northern China, and central Asia (Davis 2011). The American Elm Bark Beetle, *Hylurgopinus rufipes*, is less prominent as a disease vector worldwide. This is because (as its common name implies) the species range is currently limited to the central and eastern United States (Davis 2011).

VII. Treatments and the importance of early detection

There are two avenues to treating a tree with DED. One avenue is to target the beetle vector. Pruning symptomatic branches, proper removal of diseased elms, and quick identification

of DED reduces the amount of breeding sites available to the beetles. Trees that have been removed should be debarked before the mature beetles emerge in springtime. Beetles may also be targeted with insecticides, but this is not a preferred treatment due to the non-specificity of insecticides and difficulty in achieving thorough coverage (USDA 2008).

The second avenue is to target the fungus itself. Fungicides can be injected into the tree to prevent infection from *Ophiostoma*, but the treatment is costly over time. Fungicide treatments must be repeated every one to three seasons. As a result, the United States Department of Agriculture only recommends this for historically important or high-value trees. Another method of control is to disrupt root grafts between trees. *Ophiostoma* can spread through the roots of trees once it reaches the tree's vascular system. The breaking of root grafts, followed by the removal of the infected tree, can reduce the risk of infection. Trees that have been infected via root grafts cannot be successfully treated with fungicides or pruning (USDA 2008).

While there are solutions to inhibit the spread of the fungus or the spread of the beetles, the reality is that trees that are found to be diseased are rarely treated successfully. Treatment of a plant with DED is more likely to be effective if the disease is quickly detected. If DED is detected in the early stages, there is a chance the tree can be saved by pruning, fungicides, or both. Pruning is most effective if the newly infected tree has less than five percent of its crown affected, and is the most successful treatment when combined with fungicide. However, pruning is only viable if a tree is identified as having DED as early as possible (USDA 2008).

It is important to know which species of *Ophiostoma* is present in infected trees. *O. novo-ulmi* is highly aggressive, much more so than *O. ulmi*. *O. novo-ulmi* has the ability to reproduce quickly, spread to new trees rapidly, and kills trees within a shorter time frame than *O. ulmi* (Brasier and Buck 2002). This possibility increases the urgency for prompt detection and

treatment of elms that become afflicted with DED, as pruning has a higher probability of being successful the sooner DED is identified.

Introduction to the Experiment

This project is part of a larger study looking at the species composition of *Ophiostoma* recovered from diseased elms, and the degree of genetic diversity present within the fungi. The purpose here was to address the following hypotheses:

- *O. ulmi* is not represented in diseased elms submitted to the OSU Plant Clinic from urban settings in Portland, Oregon.

- The population of *O. novo-ulmi* present in Portland, Oregon, consists solely of subspecies *americana*; subspecies *novo-ulmi* (abundant in Europe) and *O. himal-ulmi* are not expected to be present in Oregon.

Materials and Methods

Sample preparation and DNA extraction

Samples of diseased elm branches that were submitted to the OSU Plant Clinic, largely from trees on public land, were used for this study. Tissue from symptomatic branches was plated onto ¼ strength potato dextrose agar amended with 100 ppm streptomycin sulfate (¼ SPDA plates). The medium was made according to these specifications: 10.0 grams of potato

dextrose agar plus 7.0 grams of agar per liter of de-ionized water. This mix was autoclaved for 20 minutes at 103.42 kpa and 121°C. After the medium cooled to 50°C, streptomycin was added (0.100 mg/L), mixed, poured into petri dishes and used after all surface moisture had evaporated. Plates with tissue pieces were incubated at 20°C for a week.

Putative *Ophiostoma* cultures were identified when *Graphium* and *Sporothrix* spore stages were observed growing from tissues. Spores were collected using a dissecting scope and a scalpel, and placed into a tube of 3 mL sterile, de-ionized water. The tube was then vortexed gently for 10 seconds and 100µL of the suspension was then spread out on another ¼ SPDA plate using sterile technique. The spores were allowed to germinate, and a single spore was transferred to a fresh ¼ SPDA plate. Cultures derived from these single spores were used in DNA extraction.

Spore suspensions were prepared by placing 5 mm plugs from the margin of 7-day old cultures into 10 mL of PD broth per isolate (PD broth consists of 20.0 grams of dehydrated potato dextrose medium in 1 liter of de-ionized water, and then autoclaved). The inoculated tubes were shaken at 200 RPM (Lab-Line 3250 Orbit Shaker) at room temperature for 7 days, at which time the broth was cloudy. Spore production was confirmed by examining a drop of broth culture at 400x magnification.

The broth tubes were centrifuged at 3000 RPM for 10 minutes using the Allegra X22-R centrifuge (Beckman Coulter, Inc.), and the supernatant was decanted. A portion of the remaining pellet, 0.20g, was put into lysing buffer and disrupted (Lysing Matrix, MP Biomedicals). DNA was extracted using the MP Biomedical Fast Spin Extraction Kit according to the manufacturer's directions.

The quantity of the DNA was then determined using a fluorometer (Qubit), as well as a

spectrophotometer (NanoDrop).

PCR Experiment for mtsr primers

The PCR mix for the mtsr primers consisted of 17.75µl of sterile water, 2.5µl of 10X reaction buffer (Invitrogen), 0.5µl of 50mM $MgCl_2$, and 1.0µl each of 10µm mtsr-1, 10µm mtsr-2 (Table 1), and 2.5mM dNTPs. 0.25µl of Taq (5U/µl, Invitrogen) was added, followed by 1.0µl of template DNA. The PCR conditions were as follows: 93 °C for 3 minutes, followed by a cycle of 93°C for 1 minute, 56.2°C for 1 minute 30 seconds, and 72°C for 4 minutes, repeated 25 times, followed by a final extension time of 10 minutes at 72°C.

After amplification, 25µl of product was added to an additional 25µl reaction mix prepared as above, except the template was replaced with 1µl sterile molecular grade water. The mix was subjected to the same PCR conditions a second time. This further amplified the target sequence.

Amplicons were separated on a 1% agrose gel made with 1x TBE (Tris/Borate/Ethylenediaminetetraacetic acid) buffer, at 160 volts for 35 minutes. The TBE buffer was made at 10X concentration, by mixing and autoclaving 1 liter of de-ionized water, 58g of boric acid, 108g of Tris base, and 80mL of 0.25M EDTA. The gel was then stained in an ethidium bromide bath at 0.5 mg/ml concentration for 30 minutes before being imaged under a UV light.

Samples that exhibited a 3kb product were identified as *Ophiostoma ulmi*. Samples that exhibited a 1.2kb product were identified as *Ophiostoma novo-ulmi*.

Table 1. mtsr forward and reverse primer sequences.

mtsr-1	5’-AGT GGT GTA CAG GTG AG-3'
mtsr-2	5’-CGA GTG GTT AGT ACC AAT CC-3’

PCR Experiment for CU primers

The samples that had a likely chance of being *O. novo-ulmi*, based on PCR with the mtsr primers, were then subjected to PCR using the CU primers (Table 2) to determine the subspecies. The PCR mix was done as follows with the Invitrogen Taq system: 16.75µl of sterile water, 2.5µl of 10X reaction buffer, 0.5µl of 50mM $MgCl_2$, 2.0µl of 2.5mM dNTPs, 1.0µl of CU1, 1.0µl of CU2, and 0.25µl of Taq at 5U/µl. The PCR program was: 94°C for 3 minutes, followed by a cycle of 94°C for 15 seconds, 68°C for 1 minute, and 72°C for 2 minutes. The cycle was repeated 40 times, with a final extension of 72°C for 5 minutes. The target sequence is expected to be 934bp. The PCR product for each sample was then digested using endonuclease *HphI* (New England Biolabs) (Table 3). To each PCR product (the entire 25µl reaction), 5.0µl of enzyme buffer was added, along with 1.0µl of *Hph*I at 5U/µl. 19.0µl of sterile water was added to bring the total reaction volume to 50.0µl. The PCR products were then incubated at 37°C for 60 minutes. The restriction enzyme cuts the 934bp amplicon to differentiate between the subspecies.

DNA amplicons were then separated on a 1.5% electrophoresis gel made with 1x TBE buffer, at 160 volts, for 35 minutes. The gel was then stained in an ethidium bromide bath at 0.5mg/ml concentration for 30 minutes before being imaged under a UV light.

Samples that exhibited products at 672bp and 262bp are identified as *Ophiostoma novo-ulmi*, subsp. *americana*. Samples that exhibited products at 672bp, 161bp and 101bp were identified as *Ophiostoma novo-ulmi* subsp. *novo-ulmi*.

Table 2. CU forward and reverse primer sequences.

CU1	5’-GGG CAG CTT ACC AGA GTG AAC-3’
CU2	5’-GCG TTA TGA TGT AGC GGT GGC-3’

Table 3. *Hph*I target sequence.

*Hph*I	5’-GGTGA (N_8) [cut]...3’ 3’-CCACT (N_7) [cut]...5’

Growth Experiment

For each single spore isolate, two 5mm diameter plugs from actively growing cultures were each transferred to one plate each of ¼ SPDA. One plate was placed in a 20°C incubator and the other plate was placed in a 30°C incubator (two plates for each isolate). Growth was recorded every 2 or 3 days for 14 days. The furthest growth was recorded on two axes. Internal temperatures of the chambers were monitored at the same time as measurements were made. This experiment was repeated three times for a total of three replicates and results were subjected to regression analysis.

Results

Table 4 lists the sample numbers as well as their identity before experimentation began. Figures 1 and 2 are gel images of the assays done with the mtsr primers. In Figure 1 and Figure 2, sample C1186 was the *O. ulmi* positive control in lane 8, and sample C1187 was the *O. novo-ulmi* control in lane 9. Lane 10 contained the no-template control.

Figures 3 and 4 are the gel images of the assays done with the CU primers. In Figure 3

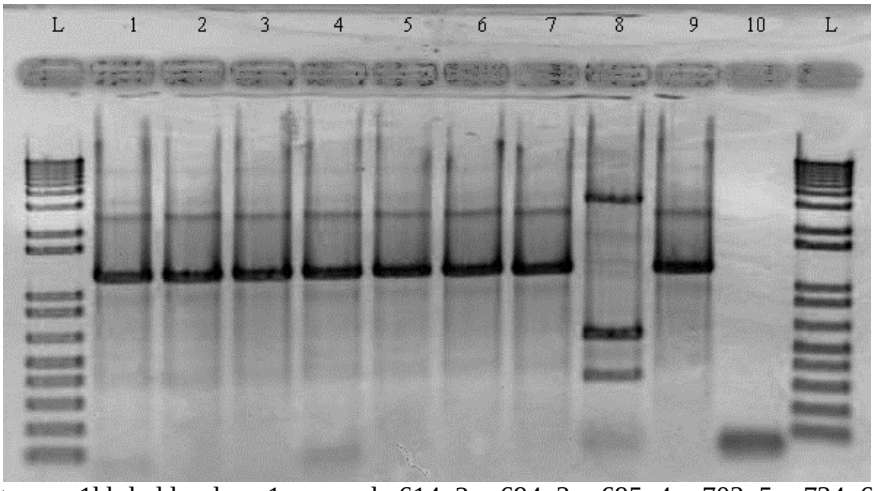
and Figure 4, sample C1184 was the *O. novo-ulmi* subsp. *americana* control in lane 8, and sample C1185 was the *O. novo-ulmi* subsp. *novo-ulmi* control in lane 9. Lane 10 contained the no-template control.

Figure 5 and Figure 6 show typical results of the growth experiment, using representative samples to demonstrate the patterns observed. Figure 5 is a scatterplot of *O. ulmi*, *O. novo-ulmi*, and three samples at 20°C, and Figure 6 is a scatterplot of *O. ulmi*, *O. novo-ulmi*, and three samples at 30°C.

Table 4. Isolates used in experiments.

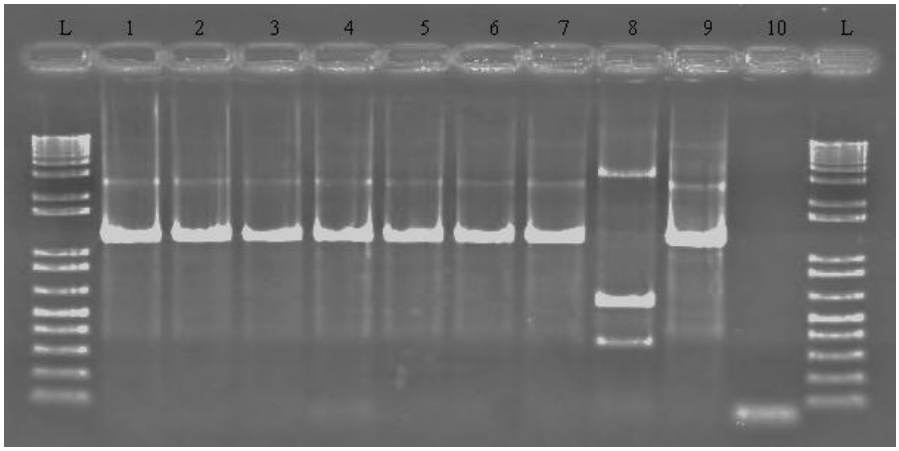
Sample Number	Identity
614	Unknown
694	Unknown
695	Unknown
703	Unknown
734	Unknown
735	Unknown
745	Unknown
804	Unknown
820	Unknown
896	Unknown
920	Unknown
1105	Unknown
1118	Unknown
1173	Unknown
C1182	*O. ulmi*
C1184	*O. novo-ulmi* subsp. *americana*
C1185	*O. novo-ulmi* subsp. *novo-ulmi*
C1186	*O. ulmi*
C1187	*O. novo-ulmi* subsp. *americana*

Figure 1. An inverse image of the agarose gel of samples 614 through 745 using mtsr primers.



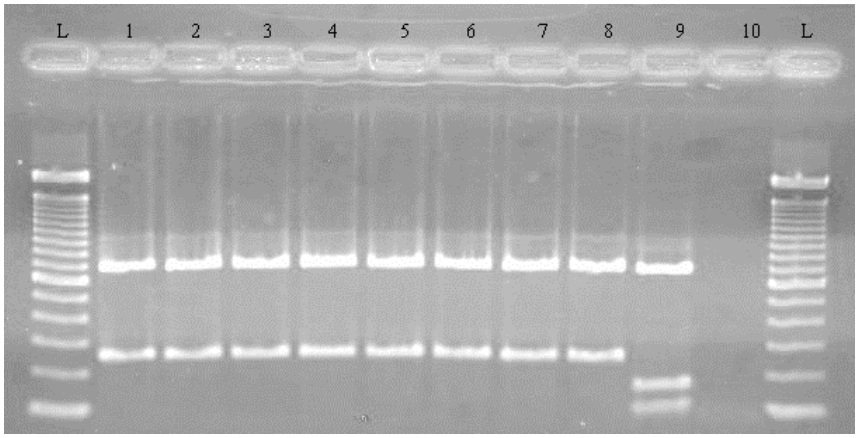
L = Invitrogen 1kb ladder, lane 1 = sample 614, 2 = 694, 3 = 695 ,4 = 703, 5 = 734, 6 = 735, 7 = 745, 8 = C1186, *Ophiostoma ulmi* control, 9 = C1187, *Ophiostoma novo-ulmi* control, 10 = Water, no-template control.

Figure 2. Agarose gel of samples 804 through 1173 using mtsr primers.



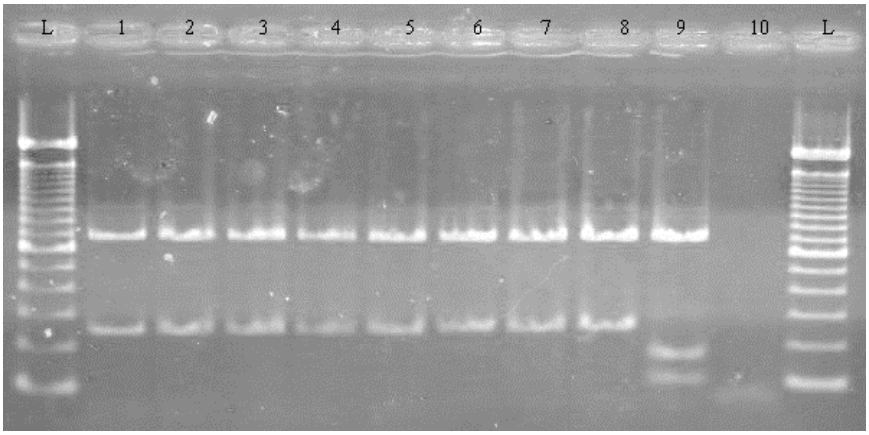
L = Invitrogen 1kb ladder, lane 1 = sample 804, 2 = 820, 3 = 896, 4 = 920, 5 = 1105, 6 = 1118, 7 = 1173, 8 = C1186, *Ophiostoma ulmi* control, 9 = C1187 *Ophiostoma novo-ulmi* control, 10 = Water, no-template control.

Figure 3. Agrose gel of samples 614 through 745 using CU primers.



L = Invitrogen 100bp ladder, lane 1 = sample 614, 2 = 694, 3 = 695, 4 = 703, 5 = 734, 6 = 735, 7 = 745, 8 =, C1184, *Ophiostoma novo-ulmi ssp. americana,* control, 9 = C1185, *Ophiostoma novo-ulmi ssp. novo-ulmi* control, 10 = Water, no-template control.

Figure 4. Agrose gel of samples 804 through 1173 with CU primers.



L = Invitrogen 100bp ladder, lane 1 = sample 804, 2 = 820, 3 = 896, 4 = 920, 5 = 1105, 6 = 1118, 7 = 1173, 8 = C1184, *Ophiostoma novo-ulmi ssp. americana,* control, 9 = C1185, *Ophiostoma novo-ulmi ssp. novo-ulmi,* control, 10 = Water, no-template control.

Figure 5. Growth of C1182 (*Ophiostoma ulmi*), C1187 (*O. novo-ulmi)*, and Portland elm isolates 614, 804, and 1173 at 20°C (Replicate 2)

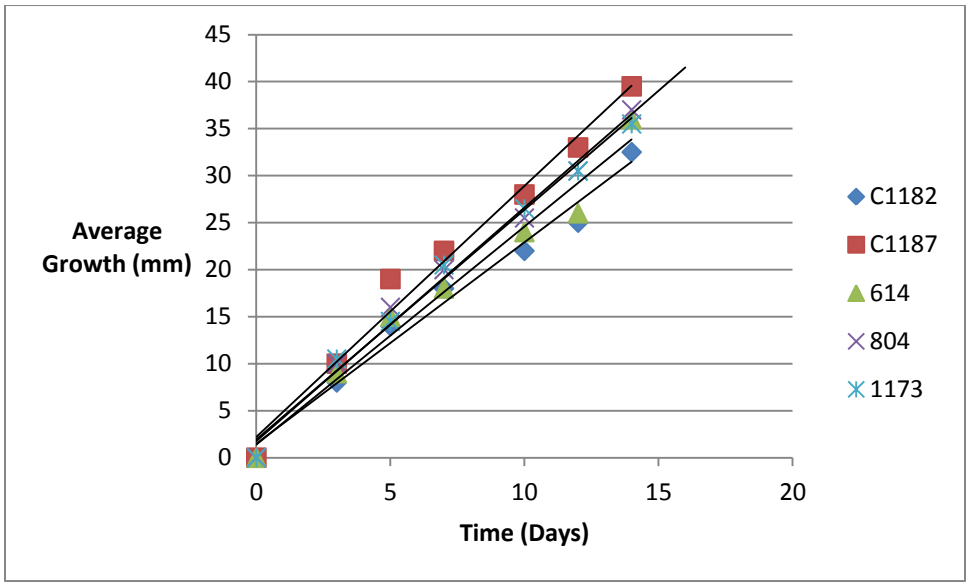
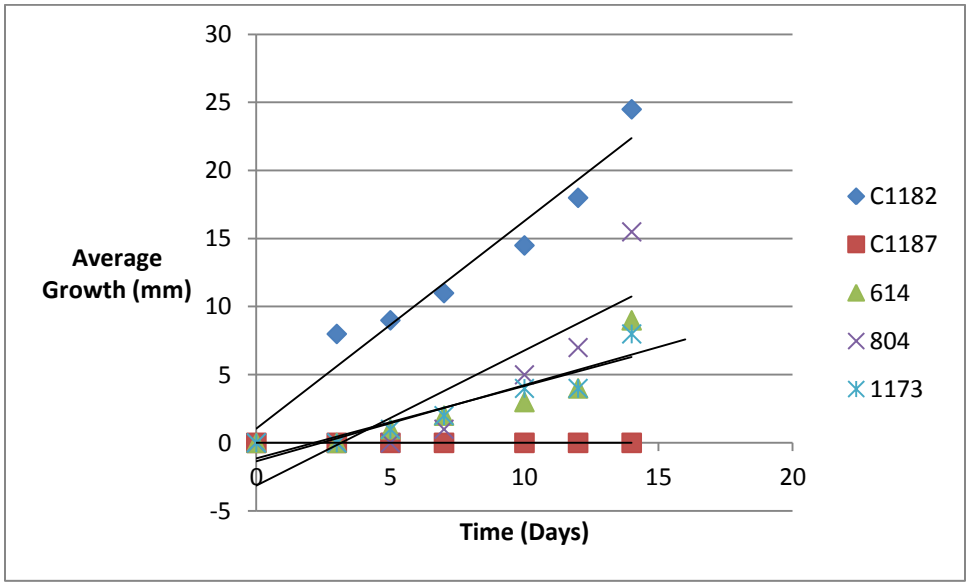


Figure 6. Growth of C1182 (*Ophiostoma ulmi*), C1187 (*O. novo-ulmi)*, and Portland elm isolates C1182, C1187, 614, 804, and 1173 at 30°C (Replicate 2)



Discussion

Analysis of mtsr primers for differentiating between O. ulmi and O.novo-ulmi

The mtsr primers were able to differentiate between *O. ulmi* and *O. novo-ulmi* with stark clarity. The expected PCR product of *O. ulmi* is 3kb, while the expected PCR product of *O. novo-ulmi* is 1.2kb. All 14 isolates from Portland elms in the year 2012 tested positive for *O. novo-ulmi*; there were no isolates that tested positive for *O. ulmi* (Figure 1 and Figure 2). The amplicons of all the samples in lanes 1 through 7 for both Figure 1 and Figure 2 were the same size as the *O. novo-ulmi* positive in lane 9; thus, these samples are likely *O. novo-ulmi*.

These results support existing evidence for the hypothesis that *O. novo-ulmi* has moved into areas that may have once been inhabited by *O. ulmi*. However, since the first detection of DED in the Pacific Northwest was as late as the 1970s compared to *O. novo-ulmi*'s explosive outbreak in the 1940s, it is possible that *O. ulmi* never arrived in the Pacific Northwest before the second pandemic. Historical records are unclear since the epidemiology of *O. ulmi* in comparison to *O. novo-ulmi* was not well documented.

While the use of the mtsr primers may seem like a reliable method for differentiation between *O. ulmi* and *O. novo-ulmi*, due to the large difference in amplicon size, the protocol requires a very specific annealing temperature. Thus, gradient runs (with varying annealing temperatures) are recommended before extensive use of these primers for *Ophiostoma* species differentiation, in order to account for variation in thermocyclers and polymerases. Another drawback is that this particular protocol required two repeats of the same thermocycler program to achieve results mentioned in peer-reviewed literature—this means that the total time required for the PCR protocol is a minimum of seven hours; this estimate excludes the time required for

sample preparation, DNA extraction, and gel electrophoresis. There is an alternative method for differentiating the *Ophiosomta* species by first determining whether one has *O. novo-ulmi* subsp. *americana* or *O. novo-ulmi* subsp. *novo-ulmi*. *O. ulmi* does not form any PCR products with the CU primers. This suggests that it may be more time efficient to test an elm sample with the CU primers first, as *O. novo-ulmi* is apparently more abundant than *O. ulmi*. If any sample appears negative, the mtsr primers would be used as the second test to determine if the sample were *O. ulmi*.

It may also be wise to test the DNA extractions for their concentration and purity before attempting PCR, in order to ensure sufficient material for amplification. The DNA concentrations for the elm samples used in this experiment varied from 20 ng/µl to 130 ng/µl.

Analysis of CU primers for differentiating between O. ulmi and O. novo-ulmi

The CU primers were helpful in differentiating between *O. novo-ulmi* subsp. *americana* and *O. novo-ulmi* subsp. *novo-ulmi* in combination with the restriction enzyme *Hph*I. Before the introduction of the restriction enzyme into the PCR product, the resulting amplicon of *O. novo-ulmi*, regardless of subspecies, would appear at 934bp. After use of the restriction enzyme, samples that were *O. novo-ulmi* subsp. *americana* exhibited bands at 672bp and 262bp. However, samples that were *O. novo-ulmi* subsp. *novo-ulmi* produced three bands: at 672bp, 161bp, and 101 bp.

The results in Figure 3 and Figure 4 show that *O. novo-ulmi* subsp. *americana* was the only subspecies detected in isolates obtained from sick elms in Portland, Oregon during 2012. The amplicons of all the samples in lanes 1 through 7 for both Figure 3 and Figure 4 are identical to the *O. novo-ulmi* subsp. *americana* positive in lane 8; thus, these samples are likely *O. novo-*

ulmi subsp. *americana.*

Analysis of the growth experiment

Analysis of the data from the growth experiment (Appendix A) was done with the statistical program MiniTab. It has been reported that *O. novo-ulmi* grows faster than *O. ulmi* at 20°C, while *O. ulmi* grows faster than *O. novo-ulmi* at 30°C. The goal of the growth experiment was to see if this difference in average growth rate at 20°C and 30°C is statistically significant between *O. ulmi* and *O. novo-ulmi*, such that it could be used to differentiate between the species. The positive controls were analyzed first to examine if either treatment would be useful for differentiating between the species, before applying the same analysis to the samples.

The measurements of colony diameter (taken at right angles to each other) were averaged, and a linear regression analysis was done for each of the samples. The slopes of the regression lines, which signify growth rates, were averaged across the three replicates. An analysis of variance was performed to determine if growth rates between the two species were significantly different at two temperatures.

There was no significant difference ($p = 0.48$) between the average growth rates of *O. novo-ulmi* (2.59 mm/day) and *O. ulmi* (2.57 mm/day) at 20°C, suggesting this criterion is not a useful one for differentiating the species. The discrepancy between these results and what has been reported in the literature may be due to a small sample size or difference due to length of time the controls have been in culture (which is unknown). Figure 5 demonstrates this, where the linear regression for C1182 (*O. ulmi*) and C1187 (*O. novo-ulmi*) are similar to each other and with three sample isolates.

In contrast, there was a greater difference in growth rates at 30°C, with *O. ulmi* averaging

1.3 mm/day whereas the average growth rate for *O. novo-ulmi* was only 0.18 mm/day, a difference which is statistically meaningful ($p < 0.005$). Figure 6 shows the large difference in growth rates for C1182 and C1187. Thus, growth at 30°C may be useful for determining if an isolate is *O. novo-ulmi* or *O. ulmi*.

However, analyses of the 14 samples growing at 30°C are inconclusive. Variance tests indicate there is a high degree of variability between the growth rates of the test isolates and the known isolates ($p > 0.05$) so that, based on growth rates alone, the 14 samples cannot be identified as *O. novo-ulmi* or *O. ulmi*. Figure 6 shows that the three representative samples have regression lines that fall in between those of C1182 and C1187; this pattern illustrates the inconclusive results due to the variability.

The amount of variance in average growth rates was large enough so that samples could not be identified under statistical guidelines; however, the morphology of the test isolates at 20°C was more similar to the *O. novo-ulmi* controls than the *O. ulmi* controls. Characteristics that matched between the test isolates and the *O. novo-ulmi* controls included concentric, circular rings around the center of the single-spore isolate, and with radial lines emanating from the center. In contrast, the *O. ulmi* controls did not exhibit any noticeable rings, and was more yeast-like in appearance.

Limitations of the Experiment

A crucial limitation of the growth experiment is the lack of a sufficiently large sample size. While there were enough replicates to assert a difference in average growth rates within the controls, the values obtained were likely to have a high amount of variance due to a small sample size. There were not enough samples to apply the central limit theorem which states that a

sufficiently large sample size will follow a normal distribution, leading to more accurate data; a larger sample size would make the variation in growth rates more normally distributed, thus providing a more reliable estimate of what might be the true average growth rate for *O. novo-ulmi* and *O. ulmi*. This can also be applied to the samples to increase confidence in the statistical analysis.

Testing of Portland elm samples from the years 2010, 2011, and 2013 are not presented in this study due to time restrictions. Preliminary analysis of samples using the CU primers from these years indicates that all isolates test positive for *O. novo-ulmi* subsp. *americana*, but additional tests are required to confirm these results.

Conclusions

Molecular analysis using PCR is likely the more reliable way to identify the species and sub-species of *Ophiostoma* that cause DED. Growth rate experiments are much more time consuming to collect and analyze data than it is to use PCR. One key flaw in the growth experiment is that while established literature mentions the difference in optimal growth temperatures for *O. ulmi* and *O. novo-ulmi*, there is no literature to suggest that there is a difference in optimal growth temperatures between the *O. novo-ulmi* subspecies. Thus, the growth experiment cannot differentiate between the subspecies while the CU primers can.

The current focus on preserving elm trees in the Pacific Northwest is centered on planting elm species that are resistant to DED, and efficient detection of the disease so that a tree may be treated quickly. Knowing that there is a high probability that *O.novo-ulmi* subsp. *americana* is the causal agent of DED in the Pacific Northwest is important in documenting the epidemiology of the second pandemic, and for the development of targeted treatments.

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Appendix A: Tables for Growth Experiment

Replicate 1, Day 0

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	0	0	0	0
694	0	0	0	0
695	0	0	0	0
703	0	0	0	0
734	0	0	0	0
735	0	0	0	0
745	0	0	0	0
804	0	0	0	0
820	0	0	0	0
896	0	0	0	0
920	0	0	0	0
1105	0	0	0	0
1118	0	0	0	0
1173	0	0	0	0
C1182 (*O. ulmi.)*	0	0	0	0
C1184 (*O. novo-ulmi.)*	0	0	0	0
C1185 (*O. novo-ulmi.)*	0	0	0	0
C1186 (*O. ulmi.)*	0	0	0	0
C1187 (*O. novo-ulmi.)*	0	0	0	0

Replicate 1, Day 3

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	9	9	0	0
694	11	10	1	1
695	10	9	1	1
703	10	9	1	0
734	12	12	2	2
735	10	12	2	1
745	9	10	0	0
804	10	10	0	0
820	11	11	2	0
896	9	9	0	0
920	11	13	1	0
1105	13	12	3	3
1118	12	11	1	1
1173	11	10	0	0
C1182 (*O. ulmi.)*	9	9	9	9
C1184 (*O. novo-ulmi.)*	22	19	0	0
C1185 (*O. novo-ulmi.)*	19	15	0	0
C1186 (*O. ulmi.)*	9	9	7	7
C1187 (*O. novo-ulmi.)*	10	9	0	0

Replicate 1, Day 5

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	15	14	0	0
694	15	16	1	1
695	12	13	1	1
703	14	14	2	1
734	16	16	2	4
735	15	14	4	4
745	15	15	0	0
804	16	16	0	0
820	15	15	5	1
896	14	14	0	0
920	15	15	1	1
1105	17	14	5	5
1118	14	15	1	1
1173	15	15	1	1
C1182 (*O. ulmi.)*	12	14	9	9
C1184 (*O. novo-ulmi.)*	28	24	0	0
C1185 (*O. novo-ulmi.)*	28	20	0	0
C1186 (*O. ulmi.)*	18	18	8	8
C1187 (*O. novo-ulmi.)*	19	17	0	0

Replicate 1, Day 7

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	19	18	2	2
694	20	18	2	2
695	18	17	1	2
703	19	19	2	1
734	20	20	7	4
735	22	20	10	10
745	20	19	0	0
804	21	20	1	1
820	21	21	7	2
896	25	18	0	0
920	21	21	3	3
1105	22	21	5	5
1118	20	20	2	2
1173	20	20	2	2
C1182 (*O. ulmi.)*	17	18	11	11
C1184 (*O. novo-ulmi.)*	33	34	0	0
C1185 (*O. novo-ulmi.)*	32	25	0	0
C1186 (*O. ulmi.)*	22	23	9	9
C1187 (*O. novo-ulmi.)*	25	22	0	0

Replicate 1, Day 10

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	24	24	7	3
694	28	24	4	4
695	23	23	4	4
703	24	24	4	4
734	26	27	10	10
735	26	26	11	10
745	26	25	2	2
804	25	28	6	5
820	27	27	3	7
896	35	35	0	0
920	26	26	5	4
1105	27	27	5	5
1118	28	27	4	4
1173	27	25	4	4
C1182 (*O. ulmi.)*	23	22	14	14
C1184 (*O. novo-ulmi.)*	38	36	0	0
C1185 (*O. novo-ulmi.)*	37	29	0	0
C1186 (*O. ulmi.)*	30	29	9	10
C1187 (*O. novo-ulmi.)*	28	28	0	0

Replicate 1, Day 12

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	26	26	4	10
694	29	29	11	10
695	27	27	5	5
703	29	29	8	8
734	31	32	14	15
735	30	30	12	15
745	31	29	3	3
804	30	30	10	10
820	31	30	5	10
896	38	40	0	1
920	29	28	7	7
1105	29	27	7	7
1118	30	29	7	6
1173	31	30	4	4
C1182 (*O. ulmi.)*	24	25	20	20
C1184 (*O. novo-ulmi.)*	43	40	1	1
C1185 (*O. novo-ulmi.)*	39	31	0	0
C1186 (*O. ulmi.)*	30	35	0	0
C1187 (*O. novo-ulmi.)*	33	33	16	15

Replicate 1, Day 14

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	36	40	13	9
694	40	40	15	16
695	35	35	10	9
703	35	35	12	11
734	37	36	20	20
735	38	40	22	20
745	36	36	11	11
804	35	37	17	15
820	36	40	19	13
896	40	42	5	5
920	33	35	15	14
1105	40	40	16	12
1118	36	36	15	13
1173	36	37	8	8
C1182 (*O. ulmi.)*	33	30	24	27
C1184 (*O. novo-ulmi.)*	46	40	3	8
C1185 (*O. novo-ulmi.)*	43	43	0	0
C1186 (*O. ulmi.)*	38	39	12	15
C1187 (*O. novo-ulmi.)*	39	40	0	0

Replicate 2, Day 0

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	0	0	0	0
694	0	0	0	0
695	0	0	0	0
703	0	0	0	0
734	0	0	0	0
735	0	0	0	0
745	0	0	0	0
804	0	0	0	0
820	0	0	0	0
896	0	0	0	0
920	0	0	0	0
1105	0	0	0	0
1118	0	0	0	0
1173	0	0	0	0
C1182 (*O. ulmi.)*	0	0	0	0
C1184 (*O. novo-ulmi.)*	0	0	0	0
C1185 (*O. novo-ulmi.)*	0	0	0	0
C1186 (*O. ulmi.)*	0	0	0	0
C1187 (*O. novo-ulmi.)*	0	0	0	0

Replicate 2, Day 3

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	9	9	0	0
694	11	11	1	1
695	10	10	1	1
703	9	9	1	0
734	12	12	1	2
735	11	12	2	2
745	10	10	0	0
804	10	10	0	0
820	11	11	1	0
896	9	9	0	0
920	12	13	0	0
1105	13	11	2	2
1118	12	11	1	1
1173	11	10	0	0
C1182 (*O. ulmi.)*	8	8	8	8
C1184 (*O. novo-ulmi.)*	13	13	0	0
C1185 (*O. novo-ulmi.)*	14	15	0	0
C1186 (*O. ulmi.)*	9	9	7	7
C1187 (*O. novo-ulmi.)*	10	10	0	0

Replicate 2, Day 5

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	15	15	1	1
694	16	16	1	1
695	13	13	1	1
703	14	14	1	1
734	16	16	2	3
735	15	14	2	2
745	15	15	0	0
804	16	16	0	0
820	15	15	1	1
896	14	14	0	0
920	15	15	1	1
1105	16	16	1	1
1118	14	15	1	1
1173	15	14	1	1
C1182 (*O. ulmi.)*	14	14	9	9
C1184 (*O. novo-ulmi.)*	18	18	0	0
C1185 (*O. novo-ulmi.)*	19	20	0	0
C1186 (*O. ulmi.)*	18	18	8	8
C1187 (*O. novo-ulmi.)*	19	19	0	0

Replicate 2, Day 7

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	19	19	2	2
694	18	19	2	2
695	18	17	2	2
703	19	19	2	1
734	20	20	4	4
735	21	20	4	4
745	20	20	0	0
804	20	20	1	1
820	21	21	3	2
896	20	19	0	0
920	21	21	3	3
1105	22	22	4	4
1118	20	20	2	2
1173	20	21	2	2
C1182 (*O. ulmi.)*	18	18	11	11
C1184 (*O. novo-ulmi.)*	33	34	0	0
C1185 (*O. novo-ulmi.)*	32	25	0	0
C1186 (*O. ulmi.)*	22	23	10	10
C1187 (*O. novo-ulmi.)*	22	22	0	0

Replicate 2, Day 10

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	24	24	3	3
694	24	24	4	4
695	23	23	4	4
703	24	24	3	3
734	27	27	5	5
735	26	26	5	5
745	26	26	2	2
804	25	26	5	5
820	27	26	3	3
896	28	28	0	0
920	26	26	4	4
1105	27	27	5	5
1118	27	27	4	4
1173	27	26	4	4
C1182 (*O. ulmi.)*	22	22	14	15
C1184 (*O. novo-ulmi.)*	30	30	0	0
C1185 (*O. novo-ulmi.)*	30	29	0	0
C1186 (*O. ulmi.)*	22	24	10	10
C1187 (*O. novo-ulmi.)*	28	28	0	0

Replicate 2, Day 12

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	26	26	4	4
694	29	30	8	8
695	27	27	5	5
703	30	29	7	8
734	32	32	7	7
735	31	30	6	6
745	31	31	3	3
804	30	31	7	7
820	31	30	5	5
896	32	32	1	1
920	28	28	7	7
1105	28	27	7	7
1118	29	29	7	7
1173	31	30	4	4
C1182 (*O. ulmi.)*	25	25	18	18
C1184 (*O. novo-ulmi.)*	34	33	1	1
C1185 (*O. novo-ulmi.)*	30	31	0	0
C1186 (*O. ulmi.)*	30	31	15	16
C1187 (*O. novo-ulmi.)*	33	33	0	0

Replicate 2, Day 14

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	36	36	9	9
694	40	40	11	11
695	35	35	10	10
703	35	35	11	11
734	37	37	12	12
735	38	39	11	11
745	36	36	11	11
804	37	37	16	15
820	36	37	15	16
896	40	42	5	5
920	33	34	14	14
1105	40	40	12	12
1118	36	36	13	13
1173	36	35	8	8
C1182 (*O. ulmi.)*	33	32	24	25
C1184 (*O. novo-ulmi.)*	41	40	8	8
C1185 (*O. novo-ulmi.)*	43	43	0	0
C1186 (*O. ulmi.)*	38	39	15	15
C1187 (*O. novo-ulmi.)*	39	40	0	0

Replicate 3, Day 0

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	0	0	0	0
694	0	0	0	0
695	0	0	0	0
703	0	0	0	0
734	0	0	0	0
735	0	0	0	0
745	0	0	0	0
804	0	0	0	0
820	0	0	0	0
896	0	0	0	0
920	0	0	0	0
1105	0	0	0	0
1118	0	0	0	0
1173	0	0	0	0
C1182 (*O. ulmi.)*	0	0	0	0
C1184 (*O. novo-ulmi.)*	0	0	0	0
C1185 (*O. novo-ulmi.)*	0	0	0	0
C1186 (*O. ulmi.)*	0	0	0	0
C1187 (*O. novo-ulmi.)*	0	0	0	0

Replicate 2, Day 3

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	9	9	1	1
694	10	10	1	1
695	10	10	1	1
703	9	9	1	1
734	12	12	1	1
735	11	12	2	1
745	10	10	1	0
804	10	10	0	0
820	11	11	0	0
896	9	9	0	0
920	11	12	1	0
1105	13	12	2	2
1118	12	12	1	1
1173	11	11	0	0
C1182 (*O. ulmi.)*	9	9	9	9
C1184 (*O. novo-ulmi.)*	13	13	0	0
C1185 (*O. novo-ulmi.)*	15	15	0	0
C1186 (*O. ulmi.)*	9	9	8	8
C1187 (*O. novo-ulmi.)*	10	10	0	0

Replicate 3, Day 5

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	14	14	1	1
694	16	16	1	1
695	12	13	1	1
703	14	14	2	1
734	16	16	2	2
735	15	15	2	2
745	15	15	0	0
804	16	16	0	0
820	15	15	2	1
896	14	14	0	0
920	15	15	1	1
1105	17	16	5	5
1118	15	15	1	1
1173	15	15	1	1
C1182 (*O. ulmi.)*	13	14	9	9
C1184 (*O. novo-ulmi.)*	17	17	0	0
C1185 (*O. novo-ulmi.)*	18	20	0	0
C1186 (*O. ulmi.)*	18	18	8	8
C1187 (*O. novo-ulmi.)*	19	19	1	1

Replicate 3, Day 7

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	18	18	2	2
694	18	18	2	2
695	18	18	2	2
703	19	19	2	1
734	20	20	3	4
735	20	20	5	5
745	20	20	1	1
804	21	20	1	1
820	21	21	2	2
896	20	18	0	0
920	22	22	3	3
1105	22	22	5	5
1118	21	21	2	2
1173	20	20	2	2
C1182 (*O. ulmi.)*	17	17	12	12
C1184 (*O. novo-ulmi.)*	23	22	0	0
C1185 (*O. novo-ulmi.)*	22	22	0	0
C1186 (*O. ulmi.)*	22	20	9	9
C1187 (*O. novo-ulmi.)*	21	22	1	1

Replicate 3, Day 10

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	24	24	4	4
694	25	24	4	4
695	24	24	4	4
703	24	24	4	4
734	26	26	5	5
735	26	26	4	5
745	25	25	2	2
804	25	25	4	4
820	27	27	3	3
896	28	28	1	1
920	26	26	4	4
1105	26	26	5	5
1118	28	28	4	4
1173	27	27	4	4
C1182 (*O. ulmi.)*	23	23	14	14
C1184 (*O. novo-ulmi.)*	30	29	0	0
C1185 (*O. novo-ulmi.)*	31	29	0	0
C1186 (*O. ulmi.)*	22	23	10	10
C1187 (*O. novo-ulmi.)*	29	29	1	1

Replicate 3, Day 12

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	26	27	4	4
694	29	29	9	9
695	28	28	5	5
703	29	29	7	7
734	31	31	6	6
735	30	31	7	8
745	31	29	3	3
804	30	30	8	8
820	31	31	5	6
896	33	33	1	1
920	29	29	7	7
1105	29	29	7	7
1118	30	30	7	7
1173	31	31	4	4
C1182 (*O. ulmi.)*	25	25	20	20
C1184 (*O. novo-ulmi.)*	35	34	1	1
C1185 (*O. novo-ulmi.)*	34	33	0	0
C1186 (*O. ulmi.)*	30	32	15	15
C1187 (*O. novo-ulmi.)*	33	33	1	1

Replicate 3, Day 14

Sample Number	20°C, X-axis Growth (mm)	20°C, Y-axis Growth (mm)	30°C, X-axis Growth (mm)	30°C, Y-axis Growth (mm)
614	39	40	10	9
694	40	40	14	13
695	35	35	10	10
703	35	35	11	11
734	37	36	15	14
735	39	40	14	16
745	36	36	11	11
804	36	37	15	15
820	39	40	14	13
896	40	42	5	5
920	34	35	15	14
1105	40	40	11	12
1118	37	36	14	13
1173	37	37	8	8
C1182 (*O. ulmi.)*	33	32	20	22
C1184 (*O. novo-ulmi.)*	42	40	5	5
C1185 (*O. novo-ulmi.)*	43	43	0	0
C1186 (*O. ulmi.)*	40	39	15	15
C1187 (*O. novo-ulmi.)*	40	40	1	1