National Diagnostic Protocol

Dutch Elm Disease

caused by *Ophiostoma* spp.

NDP 37 V1

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at http://plantbiosecuritydiagnostics.net.au/sphd/sphd-reference-standards/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for Dutch Elm Disease is current as at the date contained in the version control box below.

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Further information

Inquiries regarding technical matters relating to this project should be sent to: sphds@agriculture.gov.au

Contents

1 INTRODUCTION

Dutch Elm Disease (DED) is a devastating disease of elms caused by two fungal species *Ophiostoma ulmi* and *Ophiostoma novo-ulmi*. The fungus spreads through the tissues of susceptible elms and blocks the supply of water. Wilt symptoms may appear in a small branch initially and then spread to the rest of the canopy, eventually killing the tree. Spores of the DED pathogen can be spread from diseased trees to healthy trees by the European elm bark beetle, *Scolytus multistriatus*, or through root grafts formed between neighbouring elms. Other Scolytid beetle vectors, *S. scolytus* and *Hylurgopinus rufipes* do not occur in Australia.

Ophiostoma ulmi was responsible for the first pandemic of the disease in Europe and North America in the 1920s-1940s, whilst the more aggressive *O. novo-ulmi* is responsible for the current second pandemic. As *O. novo-ulmi* spreads, it is replacing *O. ulmi*. *Ophiostoma novo-ulmi* is comprised of two subspecies: *O. novo-ulmi* subsp. *novo-ulmi* (formerly known as the Eurasian race), and *O. novo-ulmi* subsp. *americana* (formerly known as the North American race) (Brasier and Kirk 2001). A fourth taxon, *O. himal-ulmi,* has been described from elms in the Himalayas but has not yet been associated with naturally diseased elms, although its pathogenicity to *U. procera* has been demonstrated in inoculation studies (Brasier and Mehrotra 1995).

1.1 Host range

Most *Ulmus* species, particularly those from Europe and North America, are highly susceptible to DED, however some Asian elms have shown greater levels of resistance (Stipes and Campana 1981) (Appendix 8.1 Table 12). DED has also been recorded in another genus of the Ulmaceae, *Zelkova*, but the genus is highly resistant to DED (Stipes and Campana 1981).

Ophiostoma novo-ulmi is more pathogenic and has two separate and distinguishable subspecies, *O. novo-ulmi* subsp. *novo-ulmi* and *O. novo-ulmi* subsp. *americana* (Brasier, 2001). The two subspecies differ in several ways and initially had separate geographical distributions. The less-aggressive *O. ulmi* is rapidly facing extinction as North America and Europe are being colonised by the more aggressive *O. novo-ulmi* (Brasier and Mehrotra 1995). *Ophiostoma himal-ulmi* was described by Brasier and Mehrotra (1995) from bark beetle galleries of *U. wallichiana* in the Himalayas. Although not initially associated with wilt disease, the fungus has been shown by Brasier and Mehrotra (1995) to be pathogenic by artificial inoculation to *Ulmus procera*, and to produce very high levels of cerato-ulmin, and therefore may also represent a threat to cultivated elms.

2 TAXONOMIC INFORMATION

Kingdom: Fungi

Division: Ascomycota

Subdivision: Pezizomycotina

Class: Sordariomycetes

Subclass: Sordariomycetidae

Order: Ophiostomatales

Family: Ophiostomataceae

Names and Synonyms

Four taxa of *Ophiostoma* are associated with elms and make up the DED complex:

1. *Ophiostoma ulmi* (Buisman) Melin & Nannf.

   Synonyms:

   Ceratostomella ulmi Buisman

   Ceratocystis ulmi (Buisman) C. Moreau

   Graphium ulmi M.B. Schwarz *Pesotum ulmi* (M.B. Schwarz) J.L. Crane & Schokn.

   Pesotum ulmi (M.B. Schwarz) J.L. Crane & Schokn

2. *Ophiostoma novo-ulmi* subsp. *novo-ulmi* Brasier
3. *Ophiostoma novo-ulmi* subsp. *americana* Brasier & S.A. Kirk
4. *Ophiostoma himal-ulmi* Brasier & M.D Mehrotra

3 DETECTION

3.1 Symptoms

Wilting leaves are usually the first sign of a DED infection (Figure 1).

In early summer the leaves on one or more newly infected branches in the crown of an infected tree may wilt. These leaves can turn brown in about 10 days. Some may fall off, but most will remain attached, perhaps into winter, if left unattended. As the disease progresses these symptoms rapidly spread to other parts of the tree. Late season infections will cause wilting and yellowing of leaves, and may cause the leaves to prematurely leaf fall. Diseased trees may also form small or dwarfed leaves in spring if the infection has occurred very late in the previous autumn.

Figure 1. Wilting, and browning leaves, in a small branch infected with Dutch Elm Disease (Photo from Y.P. Tan, the Netherlands, 2010).

Brown longitudinal streaks can be found in the outer sapwood of infected branches, revealed by peeling back the bark (Figure 2). In cross section, the annual growth ring of infected branches may be totally discoloured, or show only intermittent dark spots (Figure 3A). Trees that survive may bury infections in growth rings of previous years.

Figure 2. Brown streaking in the sapwood of branch with the bark partially removed, a characteristic symptom of Dutch Elm Disease (Photo from Y.P. Tan, the Netherlands, 2010).

Figure 3. Discolouration of the annual ring caused by Dutch Elm Disease seen in (A) cross-section as compared to (B) the cross-section of a healthy branch (Photo from Y.P. Tan, the Netherlands, 2010).

3.1.1 Sites of infection

Infections most often occur in twigs, as newly emerged adult elm bark beetles feed in the crotches of twigs (maturation feeding) prior to mating. The pathogen spreads within the tree by movement of mycelium and spores through xylem vessels. In trees that become infected through root grafts, the wilting often begins in the spring in the lower branches and progresses upwards (LeBoldus *et al*. 2013).

3.1.2 Factors influencing occurrence

Infections most often occur in spring, when the newly emerged young adult beetles emerge to feed on the twigs of healthy trees. Infections that occur later in the season will result in the leaves to prematurely fall.

3.2 Detection method

Many of the symptoms described are not confined to DED. Care should be taken to identify other possible causes of these symptoms, such as wind breakage, possum damage, and insect attack. Figure 4 is a flow chart for responding to a report of DED-like symptoms.

Laboratory analysis is necessary to confirm DED.

3.3 Sampling

1) Prior to sampling, disinfect pole pruner head and secateurs with several sprays of 80% ethanol or 1% bleach ensuring good coverage so that there is no contamination from previous use.
2) Select one or more branches in the elm tree crown that have typical wilt symptoms and leaf browning and remove them. For large tree use a cherry picker or similar equipment to reach appropriate sites.
3) Select an older portion of the branch that is about 2 to 3 cm in diameter or larger.
4) Cut branch samples from the selected branch of about 15 cm in length. Keep samples as clean as possible. Ensure they are not contaminated with soil and do not place samples on soil.
5) Remove side twigs and leaves.
6) Check these samples for the presence of cambium layer staining under the bark by removing a 2.5 cm long section of bark near each end of the sample. If no staining is apparent retain the sample but also sample other branches that show symptoms. Leave the bark remaining on samples intact. Ensure the branch is not completely dead when sampled – the cambium should still be moist and green even if streaked with brown and the wood should be moist and soft enough to cut easily.
7) Collect 5 or 6 such samples per tree for submission to the laboratory. Ensure that the bark has not been removed on the major portion of these samples.
8) Wrap each sample piece in waxed paper and twist ends to ensure stem ends are covered. If bark beetles are present, collect these into a separate screw-topped container for submission with the branch sample. Place samples into a heavy duty paper bag and fully label the bag with precise collection details. Ensure the bag is taped or tied to securely contain the contents.
9) Place bags containing samples in an esky or similar insulated container to keep them cool. Samples should not be allowed to dry out over a weekend. If samples cannot be submitted immediately, they should be kept in a refrigerator.

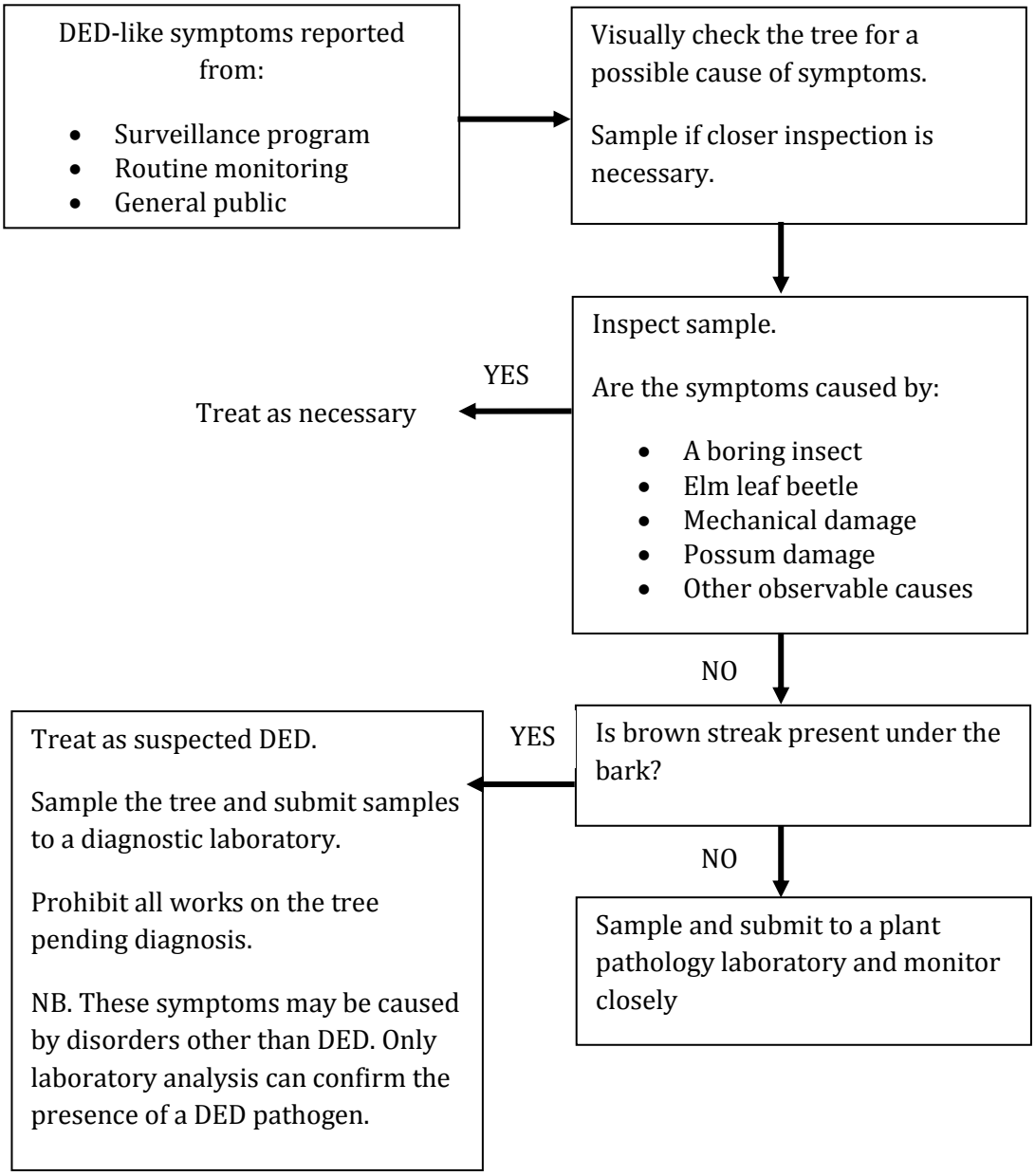

Figure 4. Flowchart for responding to a report of DED-like symptoms.

3.4 Other diseases with symptoms similar to DED

3.4.1 Bacterial wetwood

Bacterial wetwood is thought to be caused by the Gram-negative bacterium *Erwinia nimipressuralis* De Bary. It is characterised by a dark brown discoloration of the affected wood (Figure 4). The disease can be found in the trunk, branches, and roots. Generally, in grafted roots, wetwood is not observed. A cross section of an infected trunk usually shows a solid brown ring in the wood of one or more seasons. Wilted branches show streaking in the wood of the current season. In xylem tissue less than 10 years old, discolored tissue is often not easy to detect. Freshly cut wood has a foul odour and a water-soaked appearance.

Figure 4. Cross-section of an elm showing wetwood infection (Photo: W.A. Sinclair, Cornell University, Ithaca, NY, USA).

3.4.2 Dothiorella wilt

Dothiorella ulmi Verral & May is known to cause wilt in such elm species as *Ulmus americana*, *U. crassifolia*, *U. pumila*, and *U. rubra*. It is characterised by the wilting and yellowing of the leaves, followed by leaf shedding and gradual branch dieback at the top of the crown. It may take one to several years before a particular branch actually dies. The diseased bark becomes shrunken and reddish brown. On the surface of affected branches, flat cankers with dead bark can be observed. Dark pycnidia develop sparsely on newly dead twigs and bark, thereby partially masking the cankers. Infected branches show a brown discoloration pattern in the outer annual growth rings (sapwood and cambium).

3.4.3 Phloem necrosis

Elm phloem necrosis is caused by the elm yellow phytoplasms (*Candidatus* Phytoplasma ulmi) (EY) from the class Mollicutes. Signs of elm phloem necrosis become visible from late spring to early autumn, although EY infection in early autumn cannot be distinguished from normal autumn senescence. The inner phloem and the outer wood at the base of the stem turns lemon-yellow to butterscotch brown with or without flecks. The discolored area gives off a faint odor of wintergreen oil (methyl salicylate). In young trees, the discoloration may extend to the branches. As the phloem necrosis progresses, the leaves of EY infected elms will droop, curl, turn bright yellow then brown, and

premature defoliation takes place. Trees infected early in the growing season will usually die within a few weeks.

3.4.4 Verticillium wilt

Verticillium wilt is caused by *Verticillium albo-atrum* Reinke & Berth. and *V. dahliae* Kleb. Symptoms start with wilting of the leaves on one or several twigs on a branch. Although the external symptoms of Verticillium wilt resemble those of DED, crown involvement is somewhat less. Severely infected branches show foliage discoloration, sharply reduced twig growth, and partial leaf shedding. A characteristic symptom of Verticillium wilt is the sapwood discoloration observed in the roots, stems, and branches. This brown streaking runs with the grain of the wood.

4 IDENTIFICATION

4.1 Morphological methods

4.1.1 Isolation and storage

The fungus is relatively easy to isolate into agar culture, even from heavily contaminated bark material, because it is more tolerant of low concentrations of cycloheximide than many other fungi. The fungus can be readily isolated from infected xylem vessels of newly diseased twigs and from beetle breeding galleries in the bark of diseased trees. It is often possible to detect the fruiting bodies of the fungus (synnemata and ascomata) or to isolate from bark and frass in beetle breeding galleries and these should always be examined as a supplementary source of identification.

4.1.2 Media

Malt Extract Agar (MEA)

The use of 33 g of the Oxoid proprietary powder gives a final 2% malt concentration.

- 33 g MEA (Oxoid Australia)
- 20 g of bacteriological-grade agar
- 1 L of distilled water
- Autoclave at 121°C for 15 minutes
- Store in the dark at 5°C

Selective medium

- Dissolve 0.2 g of cycloheximide in 100ml of distilled water.
- Add 50 mL of this solution to 1 L of MEA and autoclave at 121°C for 15 minutes.
- Add 10 mL of a 1% solution of a mixture of streptomycin and penicillin (1:1) to make its final concentration 0.1% of the cooled liquid medium. Adding streptomycin alone at a final concentration of 0.1% is also reasonably effective.
- The medium should be poured cool (about 50°C) to avoid condensation.
- Store in the dark, preferably at 5°C.

Note: cycloheximide is a class 1A “extremely hazardous” substance, and due care should be taken in its handling.

Elm Sapwood Agar (ESA)

- Prepare 50g of ground elm sapwood.
- Add 15 g of agar and 500 mL of water.
- Autoclave at 121°C for 15 minutes.
- Stir during pouring to maintain dispersal of the sapwood.

Store in the dark, preferably at 5°C.

4.1.3 Isolation

1) To isolate from twigs:

   Cut away the bark and wood with a sharp scalpel (better results may be achieved by swabbing the bark with 70% ethanol before cutting) to expose the characteristic dark streaks in the xylem. Remove small slivers of infected xylem tissue, place on the selective agar surface and incubate at 20°C in darkness. Subculture to a fresh MEA plate as soon as mycelial growth appears.

2) To isolate from diseased bark:

   Remove small pieces of inner bark or frass with forceps or a scalpel, place on the selective agar surface and incubate similarly. Subculture to a fresh MEA plate as soon as mycelial growth appears.

3) Production of synnemata and perithecia on bark with beetle breeding galleries:
   Moist-incubate pieces of bark with galleries by placing on moist paper towel in a sealable plastic container (i.e. take-away food container). Incubate at room temperature checking daily for sporulation. Production of perithecia will take longer than synnematal production. Isolate by single spore isolation onto MEA, or by transfer of spore masses onto selective medium.

4.1.4 Storage of cultures

Because the fungus is likely to change rapidly in culture, careful storage is essential and only fresh isolates should be studied for identification. For short-term storage, (e.g. for identification or for experimental stocks), maintain unsealed cultures on MEA at 20°C in darkness, subculturing every 4 weeks, avoiding any abnormal sectors.

For long-term storage, there are 3 alternative methods:

1) Water – cut sections of agar (e.g. 4 x 4 mm) with mycelium from actively growing colony edges of cultures on MEA and store in a sterile McCartney bottle together with 5 mL of sterile distilled water. The bottles should be kept in a cool and dark location.
2) Glycerol – place sections of agar with mycelium in a sterile cryovial tube with sterile 15% v/v glycerol, and then store in a -80°C freezer.
3) Frozen twigs – place several pieces of sterile (autoclaved) split elm twigs (about 4 x 0.5 cm) in a sterile McCartney bottle together with 5 mL of sterile water. Inoculate the twigs and incubate until good synnematal production has occurred, then store in a freezer at -10 to -15°C. In this way, twigs can be removed for isolation when required, and isolates keep their original colony morphology for several years.

4.1.5 Growth rate and colony morphology

The pathogens, including *O. himal-ulmi*, can be identified by a combination of growth rate and colony morphology on MEA under carefully controlled conditions (Table 1).

- Dispense 20 mL of MEA per petri dish, allow the plate surfaces to dry.
- Inoculate the plates centrally with inoculum plugs about 2 mm in diameter with two replicates for each isolate.
- Incubate in darkness at 20°C.
- After 48 hours measure two colony diameters at right angles from the reverse of each plate.
- After 5 more days re-measure the colony diameters and calculate the mean radial growth rate (mm/day) over the final 5 day period for each isolate.

- Bring the plates to the laboratory bench at room temperature (20-25°C) in diffuse daylight for a further 10 days (minimum), and then examine the colony morphology. The initial 7 day dark period in the incubator and subsequent 10 day light period are important for bringing out the cultural characteristics of the two strains.

A further growth rate test at 33°C but with 10 days between the first and second growth rate measurements is essential to supplement the test at 20°C. At 33°C, growth of the *O. ulmi*, *O. novo-ulmi* subsp. *novo-ulmi* and *O. novo-ulmi* subsp. *americana* are very different. Therefore, the combination of the 20°C and 33°C tests provides a qualitative method of strain diagnosis.

Table 1. Summary of the cultural characteristics of DED pathogens.

Character	***Ophiostoma ulmi***	***Ophiostoma novo-ulmi* subsp. *novo-ulmi***	***Ophiostoma novo-ulmi* subsp. *americana***	***Ophiostoma himal-ulmi***
Growth rate (mm/day) 20°C 33°C	(1.5-) 2.0-3.1 (-3.5) 1.1-2.8	3.2-4.8 (-5.5) 0.1-0.5	3.1-4.4 (-4.8) 0-(-0.1)	3.0-4.9 n/a
Optimal temperature for growth	30°C	20-22°C	20-22°C	22-25°C
Upper limit for growth	35°C	32-33°C	32-33°C	33°C
Colony morphology[1]	From smooth and waxy to lawn of relatively undifferentiated aerial mycelium. Weak diurnal zonation.	Fibrous, striate, petaloid. Strong diurnal zonation	Less striate and petaloid, often lobed and uneven. Strong diurnal zonation.	Darkly pigmented and fibrous. Strong diurnal zonation
Pathogenicity on 4 year old clonal *Ulmus procera*[2]	10-35% (-40%) Recovery normal.	80-100% No recovery.	60-100% Recovery occasional.	n/a

[1] Colony morphology on MEA after 7 day incubation in darkness at 20°C and more than 10 days of incubation in diffuse daylight.

[2] % defoliation at 12 weeks

References: Brasier 1991, Braiser and Mehrotra 1995, Brasier and Kirk 2001.

4.1.6 Obtaining spore stages in the laboratory

Conidia

Conidia formed on most media at room temperature. Slide preparation of conidia requires careful attention to ensure the conidia are not confused with cells from the yeast-like phase. This requires use of a dissecting microscope at its maximum magnification. Choose aerial sporulation, which is powdery in appearance. Avoid areas that are slimy unless deliberately looking at the yeast-like phase.

Synnemata

Best formed on pieces of sterilised elm wood or on media containing elm bark or sapwood such as ESA. Inoculate the medium or the elm wood with a 2 mm^2 mycelial plug from MEA and incubate under lights at room temperature.

Perithecia

Like synnemata, perithecia are best obtained on sterilised elm wood or on media containing extracts of elm bark and sapwood such as ESA. For perithecial formation, ESA is best when the agar level is only 1-2 mm above the powdered wood. Both A and B mating types of the fungus are required for perithecial formation. Inoculate the medium or the elm wood with a 2 mm^2 mycelial plug from MEA and incubate under lights at room temperature. A good way to obtain perithecia is to use the method for testing for mating type or that for testing races of the aggressive strains (Braiser and Kirk 2001).

4.1.7 Evaluation of morphological characters

Identification is only achieved if cultural characters agree with the provided descriptions and illustrations (section 4.3.1, 4.3.2), if anamorphic morphology in culture agrees with the generic description (section 4.3.3) and if these results agree with molecular data. It is not essential to use synnematal and perithecial characters, although this should be attempted as they provide useful additional characters for confirmation. Any inconsistency between morphological and molecular data should be further investigated.

Ophiostoma is a genus of pleomorphic ascomycetes which have multiple life stage morphs, including hyphomycetous anamorphs, yeast-like anamorphs, synnematous anamorphs and perithecial teleomorphs.

All of the DED *Ophiostomas* share more or less identical anamorphic morphology, so anamorphic characters do not differentiate between taxa, but do provide an essential generic identification as a first step.

4.1.8 Anamorphic morphology in culture

Generic description of anamorphic characters in culture

Hyphae septate, *ca* 1-6 µm diameter, submerged hyphae sometimes up to 10 µm diameter; aerial hyphae often aggregated into strands. Mycelial conidia usually abundant, *Sporothrix*: conidiophores mostly lateral, *ca* 10-30 (-50) µm; conidia holoblastic, borne on short denticles of *ca* 0.5-1 µm, single celled, hyaline, very variable ellipsoid to elongate, often tapering and slightly curved, with a small attachment collar, 4.5-14 x 2-3 µm. Mycelial conidia often aggregated into mucilaginous droplets, also budding in a yeast-like fashion. Synnematal anamorph (*Graphium* or *Pesotum*) usually absent on MEA, generally produced only on sterilized elm sap wood (but abnormal synnemata may be produced on MEA by degenerate colonies); single or multiple, brown-black, slender, up to 1-2 mm tall. Attached to substratum by brown rhizoid-like hyphae and composed of parallel bundles of brown septate hyphae, flaring at the top to branch hyaline hyphae producing holoblastic single-celled hyaline ovoid to ellipsoid conidia *ca* 2-6 x 1-3 µm, aggregating into a cream-white mucilaginous spore drop. The holoblastic budding yeast-like anamorph is produced in liquid cultures, and on the surface of solid media.

Ophiostoma ulmi

Colonies on MEA after 7 days in darkness at 20°C and 10 days in diffused daylight are smooth, waxy to lawns of relatively undifferentiated or delicately striate aerial mycelium (Figure 5). Diurnal zonation usually weak to moderate. Colonies creamy-white to yellow-brown sometimes with purple or brown patches. Pigmented or non-pigmented sectors common. Growth on MEA at 20°C ranging from (1.5-) 2.0-3.1 (-3.5) mm/day; growth optimum *ca* (25-) 27.5-30°C; maximum 35°C. Note: colonies may become slow, irregular and/or dense grey-white felty to woolly due to virus-associated disease or to degeneration during storage (Brasier 1991).

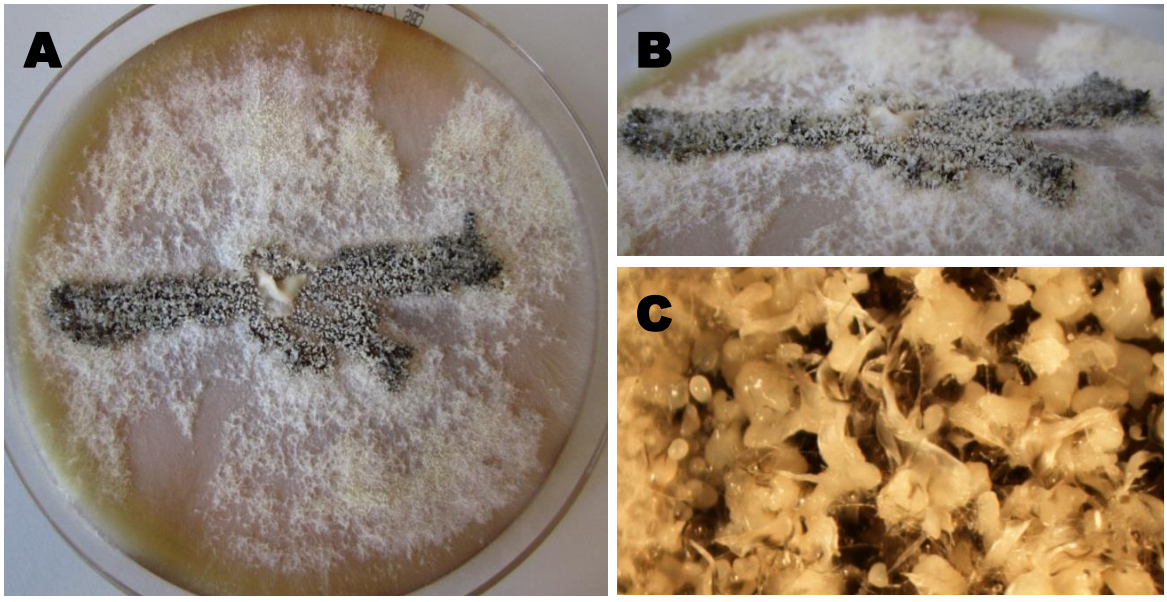

Figure 5. Colony morphology of *Ophiostoma ulmi* (isolate CBS 115.47). (Photo: Y.P. Tan). Plate photos are taken with Canon IXUS130 IS digital camera (A and B), while the close up is taken with Nikon SMZ1000 stereomicroscope at 18x magnification (C).

Ophiostoma novo-ulmi* subsp. *novo-ulmi

Colonies on MEA after 7 days in darkness at 20°C and 10 days in diffused daylight grayish-white to cream-white, uneven fibrous, striate or petaloid forms; commonly with moderate aerial mycelium aggregated into ropes to give a fibrous striate appearance or occasionally with less aerial mycelium with frosty to smooth colonies (Figure 6). Diurnal zonation moderate to strong. Growth on MEA at 20°C in darkness ranging from 3.1-4.4 mm/day; growth optimum *ca* 20-22°C; maximum 32-33°C. Note: colonies may become felty to dense woolly or slow growing degenerate-'amoeboid' looking due to virus associated disease or to degeneration during storage (Brasier 1991).

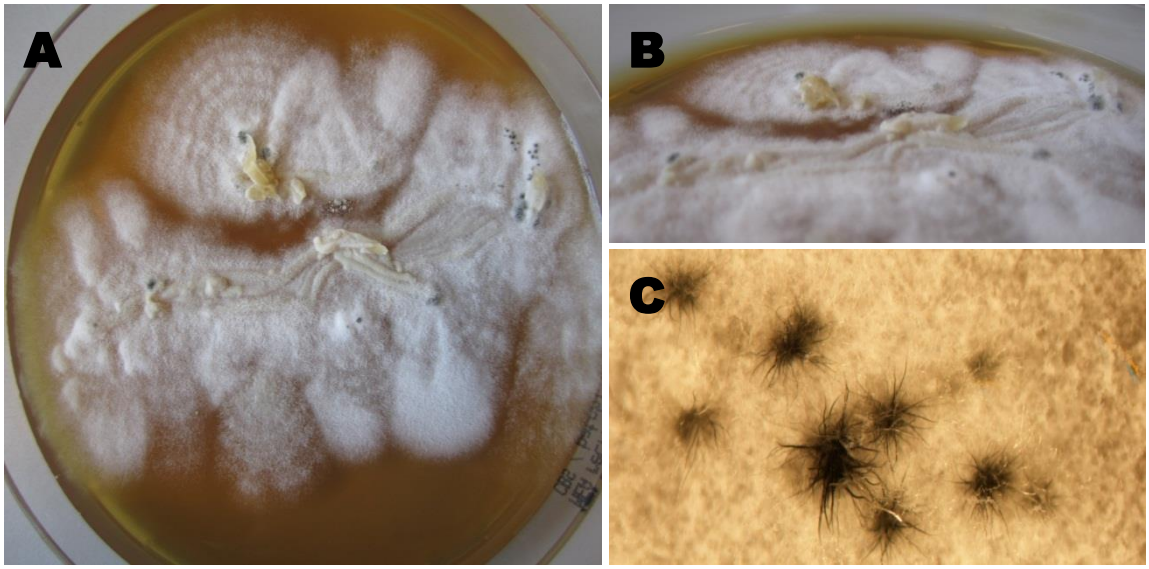

Figure 6. Colonial morphology of *Ophiostoma novo-ulmi* subsp. *novo-ulmi* (isolate CBS 116562). (Photo: Y.P. Tan). Plate photos are taken with Canon IXUS130 IS digital camera (A and B), while the close up is taken with Nikon SMZ1000 stereomicroscope at 18x magnification (C).

***Ophiostoma novo-ulmi* subsp. *americana* – additional descriptions**

Colonies on MEA after 7 days in darkness at 20°C and 10 days in diffused daylight grayish-white to cream-white regular fibrous-striate petaloid forms; commonly with moderate aerial mycelium aggregated into ropes to give a fibrous striate appearance or occasionally with less aerial mycelium with frosty to smooth colonies (Figure 7). Diurnal zonation moderate to strong. Growth on MEA at 20°C in darkness ranging from 3.2-4.8 mm/day; growth optimum *ca* 20-22°C; maximum 32-33°C. Note: colonies may become felty to dense woolly or slow growing degenerate-'amoeboid' looking due to virus associated disease or to degeneration during storage (Brasier and Kirk 2001).

Ophiostoma himal-ulmi

Colonies on MEA after 7 days in darkness at 20°C and 10 days in diffused daylight grey-black to cream-black fibrous, ranging from regular striate forms to regular lobed forms. Diurnal zonation moderate to strong. Growth on MEA at 20°C in darkness ranging from 3.0-4.9 mm/day; growth optimum *ca* 22-25°C; maximum 33°C (Braiser and Mehrotra 1995).

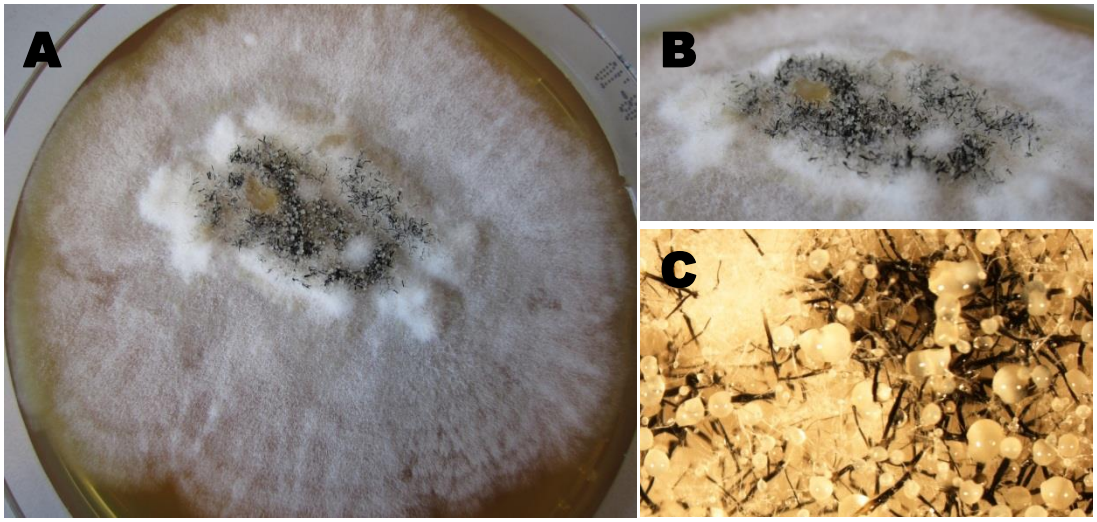

Figure 7. Colonial morphology of *Ophiostoma novo-ulmi* subsp. *americana* (isolate CBS 108928). (Photo: Y.P. Tan) Plate photos are taken with Canon IXUS130 IS digital camera (A and B), while the close up is taken with Nikon SMZ1000 stereomicroscope at 18x magnification (C).

4.1.9 Conidia and conidiophores

Ophiostoma ulmi

Conidiophores mostly lateral, *ca* 10-30 (-50) µm; conidia holoblastic, borne on short denticles of *ca* 0.5-1 µm, single celled, hyaline, very variable ellipsoid to elongate, often tapering and slightly curved, with a small attachment collar, 4.5-14 x 2-3 µm. Mycelial conidia often aggregated into mucilaginous droplets, also budding in a yeast-like fashion; aggregates of the mycelia conidia and budding conidia often coalescing to a yeast-like mass, conferring a waxy appearance to the colonies (Figure 5C, Figure 8).

Ophiostoma novo-ulmi* subsp*. novo-ulmi

Conidiophores mostly lateral, *ca* 10-30 (-50) µm; conidia holoblastic, borne on short denticles of *ca* 0.5-1 µm, single celled, hyaline, very variable ellipsoid to elongate, often tapering and slightly curved, with a small attachment collar, 4.5-14 x 2-3 µm. Mycelial conidia often aggregated into mucilaginous droplets, also budding in a yeast-like fashion.

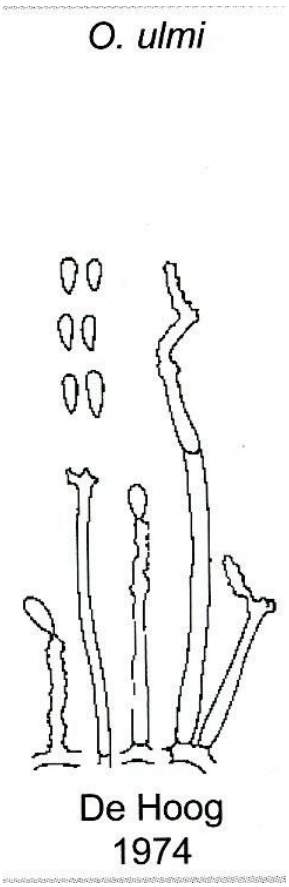

Figure 8. Generic drawing of *Ophiostoma* conidia and conidiophores (De Hoog 1974)

Ophiostoma novo-ulmi* subsp. *americana

Conidiophores mostly lateral, *ca* 10-30 (-50) µm; conidia holoblastic, borne on short denticles of *ca* 0.5-1 µm, single celled, hyaline, very variable ellipsoid to elongate, often tapering and slightly curved, with a small attachment collar, 4.5-14 x 2-3 µm. Mycelial conidia often aggregated into mucilaginous droplets, also budding in a yeast-like fashion (Figure 7C).

Ophiostoma himal-ulmi

Conidiogenous cells mostly lateral, *ca* 15-30(-50) µm; conidia holoblastic, borne on short denticles of ca 0.5-1.0 µm, single cells, hyaline very variable ellipsoid to elongate, often tapering and slightly curved, with a small attachment collar, mean 7.0 (±0-4) x 2.3 µm, range *ca* 4-14 x 2-3 µm. Mycelial conidia often aggregated into mucilaginous droplets also budding in a yeast-like fashion.

Only on sterilized elm sap wood (but abnormal synnemata may be produced on MEA by degenerate colonies); single or multiple, brown-black, slender, up to 1-2 mm tall. Attached to substratum by brown rhizoid-like hyphae and composed of parallel bundles of brown septate hyphae, flaring at the top to branch hyaline hyphae producing holoblastic single-celled hyaline ovoid to ellipsoid conidia *ca* 2-6 x 1-3 µm, aggregating into a cream-white mucilaginous spore drop.

Ophiostoma novo-ulm i* subsp. *novo- ulmi

Synnematal anamorph (*Graphium* or *Pesotum*) usually absent on MEA, generally produced only on sterilized elm sap wood (but abnormal synnemata may be produced on MEA by degenerate colonies); single or multiple, brown-black, slender, up to 1-2 mm tall. Attached to substratum by brown rhizoid-like hyphae and composed of parallel bundles of brown septate hyphae, flaring at the top to branch hyaline hyphae producing holoblastic single-celled hyaline ovoid to ellipsoid conidia *ca* 2-6 x 1-3 µm, aggregating into a cream-white mucilaginous spore drop.

Ophiostoma novo-ulm i* subsp. *americana

Synnematal anamorph (*Graphium* or *Pesotum*) usually absent on MEA, generally produced only on sterilized elm sap wood (but abnormal synnemata may be produced on MEA by degenerate colonies); single or multiple, brown-black, slender, up to 1-2 mm tall. Attached to 18 substratum by brown rhizoid-like hyphae and composed of parallel bundles of brown septate hyphae, flaring at the top to branch hyaline hyphae producing holoblastic single-celled hyaline ovoid to ellipsoid conidia *ca* 2-6 x 1-3 µm, aggregating into a cream-white mucilaginous spore drop.

Ophiostoma himal-ulm i

Synnematal anamorph (*Graphium* or *Pesotum*) usually plentiful on MEA, and abundant on sterilized elm sap wood; single or multiple, brown-black, slender, up to 1 mm tall. Attached to substratum by brown rhizoid-like hyphae and composed of parallel bundles of brown septate hyphae, flaring at the top to branch hyaline hyphae, producing holoblastic single-celled hyaline ovoid to ellipsoid conidia *ca* 3.7 (±0.2) x 2.3 µm, range ca 2-6 x 1-3 µm, aggregating into a cream-white mucilaginous spore drop.

4.2 Molecular methods

Laboratory requirements

- Protective gloves
- 2.0, 200, and 1000 µL sterile barrier pipette tips
- 2.0, 20, 200, and 1000 µL pipette tips
- Microcentrifuge
- Microcentrifuge tubes 1.5 mL
- Benchtop vortexer
- 0.2 mL PCR tubes
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and powerpack
- UV transilluminator
- Camera/gel documentation system

4.2.1 Extraction of DNA from cultures or infected plant tissues

Genomic DNA (gDNA) may be extracted from pure fungal cultures and infected plant tissues using suitable commercially available DNA extraction kits following manufacturer's instructions; e.g. QIAGEN Plant DNeasy kit, Bioline ISOLATE II Plant DNA extraction kit, etc. Store the gDNA at -20°C.

Note: For the development of this protocol, UltraClean® Microbial DNA Isolation Kit (Mo Bio Lab Inc., Carlsbad, California, USA) was used following the manufacturer's protocol.

This protocol was verified using gDNA extracted from cultures.

4.2.2 Conventional Polymerase Chain Reaction (PCR)

Primers and expected product sizes

For DNA quality evaluation, amplify the ITS region:

ITS1 (forward)	5'-TCCGTAGGTGAACCTGCGG-3' (White et al., 1990)
ITS4 (reverse)	5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990)

Expected product size: 580bp (approx..)

An alternative forward primer V9G (De Hoog & Gerrits 1998) is used as an alternative to the ITS1 in tests overseas and has been successfully used by the author, however this was not verified as part of this protocol.

V9G (forward) 5'-TTACGTCCCTGCCCTTTGTA-3' (De Hoog & Gerrits 1998)

For species-specific amplification of the 3' region of the *MAT-1-2* gene: (developed by author)

OulmiF (forward)	5'-CCCCTCGACTTAGTTTACTAGTTCTGTC-3'
OnovoF (forward)	5'-ATTTAGCCTTCCCCAAATGCTCA-3'
DEDR (reverse)	5'-CGATTTTAGCAACTGCTGCA-3'

Table 2: Expected product sizes for each primer combinations

	Primer pairs	
Isolates	OulmiF & DEDR	OnovoF & DEDR
O. ulmi	278 bp	no PCR product
O. novo-ulmi subsp. *novo-ulmi*	352 bp	248 bp
O. novo-ulmi subsp. *americana*	336 bp	230 bp
O. himal-ulmi	no PCR product	277 bp

PCR reagents

Any suitable PCR master mix can be used. However, the PCR test should be validated using the PCR master mix intended with relevant positive controls before adopting the test for diagnostic purposes.

Note: This protocol was developed using Taq DNA polymerase from Bioline (Luckenwalde,

Germany). This protocol was verified using a Platinum® Taq DNA polymerase (Invitrogen) and GoTaq® Green PCR Master Mix (Promega).

DNA gel running buffer

There are a number of buffers that can be used for gel electrophoresis. This protocol was developed using Tris-Borate-EDTA (TBE).

TBE gel running buffer can be purchased commercially in concentrated liquid form. Follow the manufacturer's instructions to dilute it to a 0.5X concentration. Alternatively, it can be made up from the following components:

	Per 1L	Final conc.
Tris base	27.0 g	0.4 M
Boric acid	13.75 g	0.05 M
0.5M EDTA pH 8.0	10.0 mL	0.001 M

Dissolve components in 1L distilled water. Store at room temperature.

Agarose gel

	Per 100 mL	Conc.
DNA grade agarose	1.5 g	1.5%
	3.0 g	3%
TBE	100 mL	0.51x

Dissolve the molecular biology-grade agarose in TBE buffer in a heat-proof glass container (e.g. beaker or Schott bottle) by heating in a microwave. Once the bottle is slightly cool to the touch, pour into the gel tray with comb. It will take approximately 30 mins to set at room temperature (20-22°C).

WARNING: The container and the content are extremely hot. Handle the container with care using heat-proof gloves.

DNA stains and DNA Loading dye

Either Ethidium Bromide or GelRed® may be used to stain double-stranded DNA to visualise it on an agarose gel. Any suitable loading dye can be used if required.

Note: This protocol was developed using ethidium bromide by adding 10 µL of a 10,000X concentration (in water) in 1 mL of DNA loading dye. Vortex well to mix, and stored in a dark container when not in use. This protocol was verified using Sybersafe by adding 5 µL of a 10,000X concentrate in 80 mL of 1X TAE agarose gel.

DNA quality evaluation PCR

Prepare the following PCR mix as described below for the number of test samples, a positive control, a blank, and one extra. A positive control can be any gDNA from an ascomycete fungi.

Component	**Volume (µL)**
Nuclease-free H_2O	8.95
10x PCR buffer ($MgCl_2$ free)	1.25
$MgCl_2$ (50 mM)	0.5
dNTP mix (5 mM of each dNTP)	0.7
Primer ITS1 or V9G (10 µM)	0.25
Primer ITS4 (10 µM)	0.25
Taq DNA Polymerase (5 Units/µL)	0.1
Total	12

Add 0.5 µL (need to add more if low concentration) of gDNA as template, place in a thermocycler using the following program:

Initial denature	95°C	4 mins	1 cycle
Denature	95°C	1 min	30 cycles
Annealing	55°C	30 secs	
Extension	72°C	1 min	
Final extension	72°C	5 mins	1 cycle
Hold	10°C		1 cycle

Load 5 µL PCR product with 1 µL 6x gel loading dye on a 1.5% agarose gel, and run the gel at 130 V for 1 hour. Follow the gel staining procedure as mentioned above and use appropriate gel imaging system. The samples should produce a PCR product of approximately 580 bp to indicate that the DNA quality is suitable for PCR test. If there is no amplification, the DNA extraction procedure needs to be repeated.

PCR-based method for identification

A PCR-based method for identification has been designed based on the differences observed in the 3' flanking region of the mating type (*MAT-1-2)* gene (Paoletti *et al.*, 2005). Prepare two PCR mixes separately for primer pairs OulmiF/DEDR and OnovoF/DEDR (as above) for the number of test samples, four positive controls, a blank, and one extra. The controls are gDNA from *O. ulmi*, *O. novo-ulmi* subsp*. novo-ulmi*, *O. novo-ulmi* subsp. *amerciana* and *O. himal-ulmi* (Table 2).

Add 0.5 µL DNA template and place in a thermocycler using the program:

Initial denaturation	95°C	2 mins	1 cycle
Denaturation	95°C	1 min	30 cycles
Annealing	58°C	30 secs	
Extension	72°C	1 min	
Final extension	72°C	5 mins	1 cycle
Hold	10°C		1 cycle

Load 5 µL PCR product with 1 µL 6x gel loading dye on a 3% agarose gel, and run the gel at 130 V for 1 hour. Follow the gel staining procedure as mentioned above and use appropriate gel imaging system.

Table 2. Reference gDNA used in the PCR-based method for identification available from CBS Fungal Biodiversity Centre (Utrecht, the Netherlands) or BRIP (Brisbane, QLD, Australia).

Strain	**CBS**	**BRIP**
Ophiostoma ulmi	102.63	53466
	103.63	53467
Ophiostoma novo-ulmi subsp. *novo-ulmi*	116560	53484
	116562	53486
Ophiostoma novo-ulmi subsp. *americana*	109212	53490
	116561	53485
Ophiostoma himal-ulmi	374.67	53491

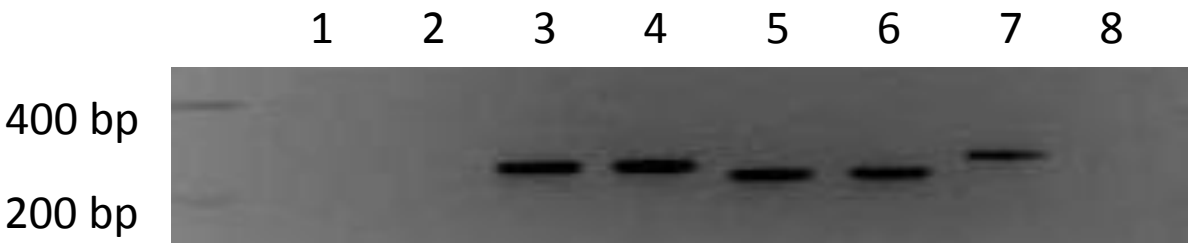

Figure 9. Example DNA electrophoresis gel from PCR using the primers OnovoF and DEDR. *Ophiostoma ulmi* (lanes 1 and 2), *O. novo-ulmi* subsp. *novo-ulmi* (lanes 3 and 4), *O. novo-ulmi* subsp. *americana* (lanes 5 and 6), *O. himal-ulmi* (lane 7), and the negative control (lane 8). The sizes of the ladder is indicated.

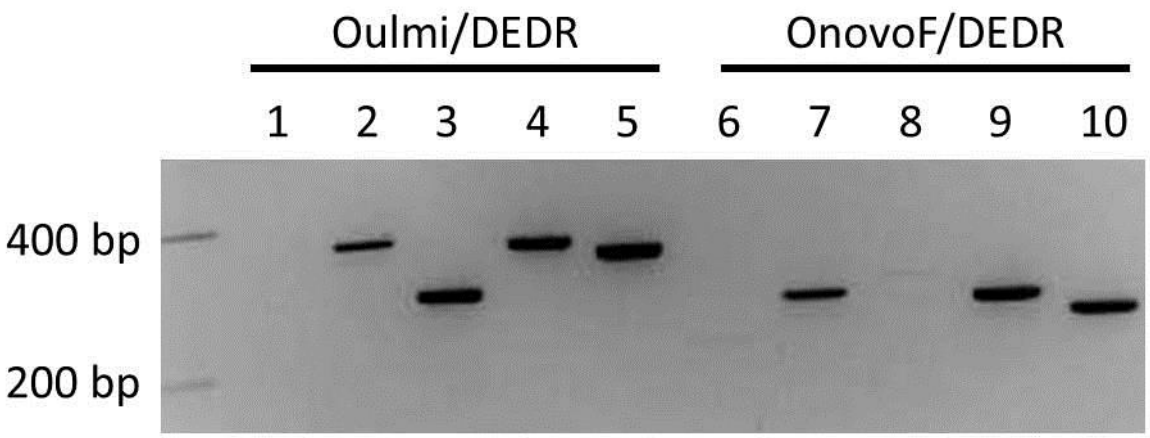

Figure 10. DNA electrophoresis gel from PCR using the primers Oulmi/DEDR and OnovoF/DEDR. Tissue from a twig of a healthy elm tree (*Ulmus* 'Homestead' hybrid) (lanes 1 and 6), tissue from an infected twig of elm tree (*Ulmus* x *hollandica* 'Pioneer' hybrid) (lanes 2 and 7), *Ophiostoma ulmi* (lanes 3 and 8), *O. novo-ulmi* subsp. *novo-ulmi* (lanes 4 and 9), *O. novo-ulmi* subsp. *americana* (lanes 5 and 10),. The sizes of the ladder is indicated.

Sequencing the 3' region of the MAT-1-2 region

The PCR *MAT-1-2* PCR should be sequenced to confirm the identity of the fungal species. The PCR products obtained with primer pairs OulmiF/DEDR and OnovoF/DEDR can be purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The cleaned-up PCR product can either be prepared for sequencing with ABI Big Dye (Applied Biosystems/Roche Applied Science) according to the manufacturer's instructions, or it can be submitted directly to a DNA sequencing facility (e.g. Australian Genome Research Facility or Macrogen Inc.). The raw sequences are compared against the reference sequences in Table 3.

Table 3. *MAT-1-2* gene reference sequences for comparison (Paoletti *et al.*, 2005).

Species	**GenBank**
Ophiostoma ulmi	AY887022
	AY887023
	AY887031
Ophiostoma novo-ulmi subsp. *novo-ulmi*	AY887027
	AY887028
	AY887029
Ophiostoma novo-ulmi subsp. *americana*	AY887025
	AY887026
Ophiostoma himal-ulmi	FJ959072

4.3 Pathogenicity testing

Ophiostoma ulmi and *O. novo-ulmi* are best differentiated on elms of moderate disease tolerance, such as *Ulmus procera*, *U. carpinifolia*, and *U. japonica*. Gibbs and Brasier (1973) showed that clones of 3-5 years old *U. procera* gave excellent differentiation under the conditions of northwest Europe. Inoculum of the yeast-like phase was prepared by shaking the culture for 3 days in 10 mL of liquid medium to give a spore concentration of about 1 x 10^5/mL. Trees 3-5 years old are inoculated by cutting into the xylem with a sharp scalpel halfway up the crown of the tree, applying two spore drops to the blade from a hypodermic syringe (three replicate trees per isolate) and scoring for symptoms (percentage defoliation) at 4-week intervals thereafter. In northwest Europe, the optimal time for inoculation is the first two or three weeks of June. In hotter, drier climates, moisture stress should be avoided if full symptoms are to develop, and the most susceptible period for inoculation may be earlier (May-June).

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8 APPENDICES

8.1 Host list

Table 12. A non-exhaustive list of hosts and their susceptibility

Host	Location	Susceptibility
Ulmus americana Planch. (American Elm)	Native to North America.	Highly susceptible to DED.
Ulmus crassifolia Nutt. (Cedar Elm)	Native to south central North America	Highly susceptible to DED.
Ulmus glabra Huds. (Scots Elm)	Native to Europe, Asia Minor, and the Caucasus.[1]	Highly susceptible to DED, but less favoured as a host by the elm bark beetles.
Ulmus laevis Pall. (European White Elm)	Native to Europe.	Highly susceptible to DED, but less favoured as a host by the elm bark beetles.
Ulmus minor (Mill.) (Field Elm)	Native to south Europe, and Asia Minor.	Highly susceptible to DED and is not now commonly cultivated.
Ulmus parvifolia Maxim., Franch. Et Savatier, Forbes & Hemsl., Shirasawa. (Chinese Elm)	Native to China, Japan, Korea and Vietnam.[3]	Highly resistant to DED.
Ulmus procera Salisb. (English Elm)	Thought to have originated from Italy. Populations of English Elm in Spain, Italy and the UK showed that they are all genetically identical, clones of a single tree thought to have been introduced to the British Isles by the Romans.[2]	Highly susceptible to DED due to its genetic homogeneity.
Ulmus pumila L. (Siberian or Asiatic Elm)	Native to Turkestan, eastern Siberia, Mongolia, northern China, northern India and Korea.[3]	Resistance to DED is variable.[4]
Ulmus rubra (Slippery Elm)	Native to North America.	Less susceptible to DED than other American elms.
Ulmus thomasii Sarg.	Native to Midwestern US.	Highly susceptible to DED.

(Rock Elm)		
Ulmus x hollandica Mills. (Dutch Elm)	Natural hybrid of *U. glabra* and *U. minor* is found where the location of both species overlap.	Varied susceptibility to DED.
Zelkova serrata (Thunb.) Makino (Keyaki or Japanese Zelkova)	Native to Japan, Korea, eastern China and Taiwan.[5]	Highly resistant to DED. Reactions of other *Zelkova* spp. to DED is unknown.

[1] Richens, 1984. [2] Gil *et al.* 2004. [3] Fu *et al.* 2002. [4] Smalley and Guries, 1993. [5] Andrews, 1994.