

1 **Characterization of *Cylindrocarpon* Species, the Cause of Black Foot Disease of**
2 **Grapevine in California**

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11 **ABSTRACT**

12 This study investigated genetic, morphological, and pathogenic variation among
13 *Cylindrocarpon* (teleomorph: *Neonectria*) isolates associated with black foot disease of
14 grapevine (*Vitis* sp.) in California. To assess genetic variation, we sequenced the internal
15 transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA), partial beta-tubulin (BT)
16 gene introns and exons, and the small subunit mitochondrial rDNA. Isolates associated with
17 black foot disease belonged to two paraphyletic species, *Cylindrocarpon destructans* and *C.*
18 *macrodidymum*. The morphology of these isolates was in agreement with published
19 descriptions of both species. We found that *C. macrodidymum* isolates were reliably
20 distinguishable from *C. destructans* isolates in culture, by a unique orange-dark brown colony
21 color on 2% malt extract agar and genetically, by a species-specific 52 bp DNA insertion in
22 the BT region. Selected isolates of each species inoculated onto grapevine rootstock 5C

- 1 caused typical black foot disease symptoms. This is the first report of *C. macrodidymum* in
- 2 California.

1 Petit, E., and W.D. Gubler 2005. Characterization of *Cylindrocarpon* species, the cause of
2 black foot disease of grapevine in California. Plant Dis. 89:000-000.

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4 Additional key words: mtSSU rDNA, Nectriaceae, phylogeny, vineyards

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6 Black foot disease is a recently identified but worsening problem in California
7 vineyards (24). Roots of symptomatic grapevines (*Vitis* sp.) show black, sunken, necrotic
8 lesions (25). In cross section, the base of the trunk appears necrotic and xylem vessels are
9 plugged with black inclusions and tyloses (25). Above ground, leaves of infected vines
10 appear to be scorched by water stress, and the entire vine becomes stunted, and frequently
11 dies (25). Young vines up to eight-years-old are primarily affected by the disease (6,18).
12 Because diseased plants must be removed, the disease causes substantial economic losses due
13 to replanting costs. First reported in France in 1961 (18), the disease occurs in all the major
14 viticulture regions throughout the world, including Italy (10,11), Portugal (22), Spain (1),
15 South Africa (7), New Zealand (12), and Australia (28).

16 *Campylocarpon fasciculare* Schroers, Halleen and Crous, *Campylocarpon*
17 *pseudofasciculare* Halleen, Schroers and Crous, *Cylindrocarpon destructans* (Zinssm)
18 Sholten, *Cylindrocarpon macrodidymum* Halleen, Schroers and Crous, and *Cylindrocarpon*
19 *obtusisporum* (Cooke & Harness) Wollew. have all been described as the fungal causal agents
20 of black foot disease in grape-growing regions outside of California. *Campylocarpon*
21 *fasciculare* and *Campylocarpon pseudofasciculare* were recently shown to cause black foot
22 disease in South Africa (12). Since *C. destructans* was first reported in 1961 in France (18), it
23 has been isolated from diseased vines in Sicily (10), Portugal (22), Australia (28), New

1 Zealand (12) and South Africa (7). *C. macrodidymum* has been recently reported in Australia,
2 New Zealand, and South Africa (12). *C. obtusisporum* has been detected in Sicily in 1975
3 (11). In California, only *C. destructans* and *C. obtusisporum* have been reported to cause
4 black foot disease (25). Given the association of several different fungi on grapevines with
5 the disease outside of California, it is important to identify the *Cylindrocarpon* species that
6 cause the disease in California.

7 The genus *Cylindrocarpon* (teleomorph: *Neonectria*) contains approximately 125
8 described species (19). Both the anamorph and teleomorph can be used to classify
9 *Cylindrocarpon* species. The teleomorph of *C. destructans* was observed in France on
10 grapevines (18), and *in vitro* sexual crosses for *C. macrodidymum* isolates from South Africa
11 and Australia have been successful (12). Nevertheless, the sexual stage of *Cylindrocarpon*
12 spp. has never been observed in California vineyards or in the laboratory. Therefore, only the
13 anamorphic characteristics have been used to identify *Cylindrocarpon* species in California
14 (24). These characteristics are traditionally morphological and include colony pigmentation,
15 growth rate, production of chlamydo-spores, and microconidial/macroconidial shape and size
16 (2). Past research showed that *Cylindrocarpon* isolates associated with black foot disease in
17 California showed great variation in morphology, and it is unclear whether this variation
18 reflects intra or inter-species differences (20).

19 Although morphology is used extensively in fungal identification, it is often
20 insufficient to differentiate species when used alone (29). Recently, genetic information has
21 been used to supplement morphological descriptions (29). Often, fungi that were previously
22 identified as a single species when classified with morphology, were found to correspond to
23 several distinct species when described by multigene genealogy (29). This has been the case

1 for *C. destructans* isolates on ginseng (27). Distinct species may vary in important
2 phenotypic characters, including longevity in the field, host range, aggressiveness, and
3 susceptibility to different disease control treatments. Therefore, a complete knowledge of
4 species involved in a disease could help researchers improve disease control. The objective of
5 this study was to identify the species causing black foot disease of grapevine in California
6 using multigene phylogeny, morphological characteristics, and pathogenicity.

7 **MATERIALS AND METHODS**

8 **Isolates.** We collected a total of 31 *Cylindrocarpon* isolates, from 19 vineyards in
9 five counties throughout California between 1998 and 2003 (Table 1). Isolates were
10 recovered from roots and lower rootstock of grapevines exhibiting symptoms typical of black
11 foot. The margin of the symptomatic region was surface sterilized in 10% bleach for 1 min.
12 Tissue pieces, about 2 mm in diameter, were placed on potato dextrose agar (PDA)(DIFCO
13 Inc, BD Micro Biology Systems, Franklin Lakes, NJ) amended with tetracycline
14 hydrochloride (0.01%) (SIGMA-ALDRICH, Inc., St Louis, MO) and incubated at room
15 temperature for four days. Isolates were identified as *Cylindrocarpon* spp. based on colony
16 morphology and conidial characteristics. Single spore cultures were initiated using a
17 previously described method (13). For long-term storage, cultures were transferred to #1
18 Whatman® filter papers (Whatman, Whatman International Ltd., Maidstone, England)
19 overlaid on PDA and, after colonization, the filters were dried and stored at –20°C. For
20 phenotypic and genetic comparison, we included *Cylindrocarpon* isolates from grapevines
21 from Chile (CH103), France (FR102), and South Africa [*C. destructans* (CBS112602) and *C.*
22 *macrodidymum* (CBS112605)] in our collection (University of California, Davis) (Table 1).

1 **DNA extraction, PCR, sequencing and multigene phylogenies.** Fungal mycelium
2 and spores were collected from PDA plates after two weeks of growth. Genomic DNA was
3 extracted using QIAGEN DNAeasy plant minikit (QIAGEN Inc., Valencia, CA) following the
4 manufacturer's protocol. DNA concentrations were quantified with commercial standards on
5 agarose gels stained with ethidium bromide. Purified DNA was stored at -20°C .

6 We analyzed three regions of the genome: the internal transcribed spacer of ribosomal
7 DNA (ITS rDNA), partial sequences of the beta tubulin (BT) gene, and the mitochondrial
8 small subunit ribosomal DNA (mtSSU rDNA). Templates of the ITS region (comprising
9 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2)
10 were amplified using primers ITS1 and ITS4 (31). Partial sequences of the BT gene, BT1 and
11 BT2, were amplified using primers BT1a/BT1b and BT2a/ BT2b, respectively (9). Partial
12 sequences of the mtSSU rDNA were amplified using primers NMS1 and NMS2 (16). ITS
13 rDNA and BT1 regions were amplified and sequenced for all isolates ($n=35$). Based on the
14 phylogeny from these regions, as described below, we amplified the other regions for only ten
15 selected isolates. PCR reactions contained $200\ \mu\text{M}$ of dNTP (Applied Biosystems,
16 Warrington, UK), $0.375\ \mu\text{M}$ of each primer, 0.5 units of *Taq* DNA polymerase (QIAGEN,
17 Valencia, CA), 1xPCR buffer supplied together with the *Taq*, and 10 ng of template DNA
18 adjusted with milliQ water to a final volume of $20\ \mu\text{L}$. Amplifications were carried out in a
19 thermalcycler (PTC-200, MJ Research, Watertown, MA) as follows: 94°C for 4 min followed
20 by 35 cycles at 94°C for 60s, 58°C (62°C for the mtSSU DNA) for 60s, 72°C for 90 s, and a
21 final extension at 72°C for 5 min. PCR products were purified using the QIAquick PCR
22 Purification Kit (QIAGEN, Valencia, CA). Each region was sequenced in both directions by

1 the University of California, Davis, DBS DNA sequencing facility using a capillary
2 electrophoresis genetic analyzer (ABI 3730, Applied Biosystems, Foster City, CA).

3 Sequence contigs were edited using Sequencher 4.1 (Gene codes, Ann Arbor, MI).

4 Sequences were aligned using Clustal X Version 1.8 (30). Whenever possible, sequences were
5 aligned with published GenBank sequences for comparison (Table 2). The alignment was
6 corrected by visual inspection and any ambiguously aligned characters were deleted in
7 MacClade Version 4.03 (17). Separate analyses were run for the combined BT1 and ITS
8 datasets, for the combined BT2 and ITS datasets, for the ITS dataset alone, and for the mtSSU
9 dataset alone. *Nectria cinnabarina* was used as an outgroup for the ITS and the mtSSU
10 dataset analysis. In order to determine whether the BT and ITS sequence data could be
11 combined, the partition homogeneity test, as implemented in PAUP 4.0b10 (Sinauer
12 Associates, Inc. Publishers; Sunderland, MA), was run with 1,000 replicates. Maximum
13 parsimony analyses were performed in PAUP Version 4.0b10 using the heuristic search
14 option, 1,000 random addition sequence replicates, and the tree-bisection-reconnection
15 algorithm. Bootstrap values (5) were calculated using a heuristic search and 1,000 bootstrap
16 pseudoreplications. A Bayesian analysis was also performed under the likelihood model
17 GTR+I+G determined by Modeltest (21) using Mr. Bayes 2.01 (15). The maximum
18 likelihood model employed six substitution types with base frequencies estimated from the
19 data. Rate variation across sites was modeled using a gamma distribution. The Markov
20 Chain Monte Carlo search was run with four chains for 150,000 generations, with trees
21 sampled every 1,500 generations (the first 20 trees were discarded as "burnin"). Branch
22 lengths of sampled trees were saved. The posterior probabilities of trees and tree parameters
23 were estimated from this distribution.

1 **Morphology.** All *Cylindrocarpon* isolates (n=35) were cultured on Spezieller
2 Nahrstoffarmer agar plus yeast extract (SNAY) (3), incubated at 25°C in a 12-hour light/dark
3 cycle, illuminated by fluorescent strip lights and near-ultraviolet light (366 nm), and
4 examined after six days. Length and width of each type of conidia (e.g., microconidia and
5 macroconidia that were one-septate, two-septate and three-septate) were measured at 400X
6 magnification using a compound microscope. Means and standard deviations were calculated
7 from 30 observations per isolate. Data were analyzed using the GLM procedure in SAS (SAS
8 system, Version 8.1; SAS Institute, Cary, NC). An analysis of variance (ANOVA) was used
9 to determine the effects of species on conidial dimensions.

10 To determine the effect of temperature on colony growth, a mycelial disk (4 mm
11 diameter) from the growing margin of a 7-day-old colony was placed in the center of each
12 potato sucrose agar (PSA) plate (3). Inoculated plates were incubated at temperatures of 5,
13 10, 15, 20, 25, and 30°C in the light conditions described above. Each isolate had three
14 plates. Colony diameter was measured after 1, 3, and 6 days of incubation, and data were
15 converted to radial growth in centimeters per day. The experiment was repeated twice. Data
16 were analyzed using the GLM procedure in SAS (SAS system, Version 8.1; SAS Institute,
17 Cary, NC). An ANOVA was used to determine the effects of species on radial growth at
18 25°C after 6 days.

19 The color of the reverse of the colony, grown at 25°C for six days in the light
20 conditions described above, was observed on both PSA and 2% malt extract agar
21 (MEA)(DIFCO Inc, BD Micro Biology Systems, Franklin Lakes, NJ).

22 **Pathogenicity.** The pathogenicity of selected isolates (USME116, USST148,
23 USSO150, US40, USNA136 and USSOL152), representative of species previously

1 determined by the multigene phylogeny, was tested. Conidia of *Cylindrocarpon* isolates
2 grown on corn meal agar (DIFCO Inc, BD Micro Biology Systems, Franklin Lakes, NJ) for
3 two weeks were harvested into sterile water and adjusted to 10^6 conidia/ml. Six-month old
4 rootings of grapevine rootstock cv. 5C (*V. berlandieri* × *V. riparia*) grown in pro-mix BX
5 (Premier Horticulture-Oceanside, CA) in 8 cm diameter pots, were drench inoculated
6 individually with 25 ml of spore suspension, or sterile water as a control. The plants were
7 incubated for four months in the greenhouse and watered three times a week with Hoagland's
8 solution (14). Leaf symptoms of individual plants were rated on the following scale: 0=no
9 symptomatic area on leaves, 1= 0 to 10% of necrotic leaf area, 2= 10 to 25 % of necrotic leaf
10 area, 3= 25% to 50% of necrotic leaf area, 4= 50 % of necrotic leaf area and 5 was 100%
11 necrotic leaf area. Root symptoms, determined using the gridline intersection method (8),
12 were expressed as the percentage of root length having lesions. Symptomatic roots were
13 aseptically plated on PDA amended with tetracycline hydrochloride (0.01%) to re-isolate the
14 fungus, as described above. Each isolate treatment was replicated four times in a random
15 complete block design with one plant per pot. The experiment was repeated twice.
16 Greenhouse temperatures ranged between 20 and 25°C. Data were analyzed using the GLM
17 procedure in SAS (SAS system, Version 8.1; SAS Institute, Cary, NC). An ANOVA was
18 used to determine the effects of isolate on root and leaf symptoms. Tukey's tests were used
19 for treatment means comparisons.

20 **RESULTS**

21 **Multigene phylogenies.** ITS and BT1 sequences from 35 isolates of *Cylindrocarpon*
22 species from grapevines were combined after the partition homogeneity test was found to be
23 not significant ($P=0.31$). Of the 890 nucleotides analyzed, 48 were phylogenetically

1 informative. Maximum parsimony analyses yielded three equally parsimonious trees
2 (length=62, CI=0.984, RI=0.999, RC= 0.244, HI=0.616). Both Bayesian and maximum
3 parsimony analyses strongly supported (100%) the existence of two divergent species: *C.*
4 *destructans* and *C. macrodidymum* (Fig. 1). The *C. destructans* clade consisted of 17 isolates
5 from South Africa, France, and five counties (Mendocino, Napa, Santa Clara, Sonoma, and
6 Stanislaus) in California with nearly identical sequences (Fig. 1). The 18 isolates in the *C.*
7 *macrodidymum* clade, which originated from South Africa, Chile, and four counties
8 (Mendocino, Napa, Solano, and Sonoma) in California had almost identical sequences (Fig.
9 1). Moreover, a 52 bp insertion in the BT1 beta tubulin sequence, which was unique and
10 conserved in all *C. macrodidymum* isolates, was not found in *C. destructans*.

11 ITS and BT2 datasets were concatenated after the partition homogeneity test was
12 found to be not significant ($P=0.49$). Sequences of *Cylindrocarpon* isolates that originated in
13 our study were aligned with published GenBank sequences of three *Cylindrocarpon* species.
14 Of the 750 nucleotides analyzed, 78 were phylogenetically informative. Maximum parsimony
15 analyses produced three equally parsimonious trees (length=122, CI=0.803, RI=0.883, RC=
16 0.710, HI=0.197). Both Bayesian and maximum parsimony analyses identified three groups:
17 *C. destructans*, *C. macrodidymum*, and a third group containing the type species of
18 *Cylindrocarpon*, *C. cylindroides* (Fig. 2). All three groups were highly supported (100%). In
19 accordance with the previous tree (Fig. 1), grapevine isolates fell into two different clusters,
20 *C. destructans* and *C. macrodidymum* (Fig. 2). Isolates of *C. destructans* had nearly identical
21 sequences and formed a remote and highly supported (100%) group within the *C. destructans*
22 cluster (Fig. 2). The *C. macrodidymum* clade was exclusively composed of isolates from
23 grapevines, all having nearly identical sequences (Fig. 2).

1 ITS sequences of the *Cylindrocarpon* isolates from grapevines were aligned with
2 GenBank sequences from six *Cylindrocarpon* species, with *Nectria cinnabarina* (CBS
3 279.48) as an outgroup. Of the 435 nucleotides analyzed, 40 were parsimony informative.
4 Maximum parsimony analyses yielded 24 equally parsimonious trees (length=103, CI=0.873,
5 RI=0.943, RC= 0.823, HI=0.127). The results of the Bayesian analysis, with probability from
6 maximum parsimony and Bayesian analysis of each clade, are shown in Figure 3. The
7 structure of this tree confirmed the topology of tree in Figure 2. Isolates from grapevines
8 belonged to two different clades, *C. destructans* and *C. macrodidymum*, both supported at
9 100% (Fig. 3). *C. destructans* from grapevines, with nearly identical sequences, formed a
10 highly supported (99%) monophyletic sister clade within the *C. destructans* complex (Fig. 3).
11 The *C. destructans* isolates found on grapevines diverged (2.3 to 3%) from *C. destructans*
12 isolates found in numerous hosts including *Alnus glutinosa*, *Arbutus menziesii*, *Malus* sp.,
13 *Panax quinquefolius*, and *Cornus floridae* (Fig. 3). Furthermore, *C. macrodidymum* isolates
14 had almost indistinguishable sequences and were solely from grapevines (Fig. 3). Also, the
15 monophyly of the clade *C. macrodidymum* was highly supported (100%) (Fig. 3). Sister clade
16 of the rest of the *Cylindrocarpon* taxa, the monophyletic group containing the type-specimen
17 *C. cylindroides* and *C. obtusisporum* CBS 183.36 was moderately supported at 67% (Fig. 3).

18 The mtSSU sequences from our grapevine isolates were aligned with GenBank
19 sequences from 13 species representative of the *Neonectria/Cylindrocarpon* genus, with
20 *Nectria cinnabarina* (GJS 91-111) as an outgroup. Of the 657 nucleotides analyzed, 95 were
21 phylogenetically informative. Maximum parsimony analyses resulted in 100 equally
22 parsimonious trees (length=277, CI=0.852, RI=0.914, RC= 0.779, HI=0.148). The results of
23 the Bayesian analysis, with probability from maximum parsimony and Bayesian analysis of

1 each clade, are shown in Figure 4. The topology of the tree recognized four clades within the
2 *Neonectria/Cylindrocarpon* genus all supported at 100%: the clade containing species of the
3 group *Neonectria mammoidea*, the *C. destructans* clade, the *C. macrodidymum* clade and the
4 clade containing *C. cylindroides* (Fig. 4). Isolates from grapevines were found in two
5 paraphyletic clades, both very well supported (100%): the *C. destructans* clade and the *C.*
6 *macrodidymum* clade (Fig. 4). The *C. destructans* isolates from grapevines, with nearly
7 identical sequences, formed a monophyletic clade within the *C. destructans* clade (Fig. 4).
8 These *C. destructans* isolates from grapevines diverged (1.51% to 1.7%) from *C. destructans*
9 isolates found in numerous hosts including *Prunus persica*, *Pseudotsuga mensiezii*, and *Alnus*
10 *rubra* (Fig. 4). Also, the *C. macrodidymum* isolates, solely originating from grapevines, had
11 identical sequences (Fig. 4).

12 **Morphology.** For each type of conidia and for each species, the mean and standard
13 deviation of the length and width are given in Table 3. The statistical analysis conducted on
14 more than 17 isolates allowed us to see that the dimensions of spores of *C. macrodidymum*
15 were significantly bigger than of *C. destructans* ($P < 0.001$). However, the mean values are
16 close and the distribution of the width and of the length of the spores largely overlapped
17 (Table 3). Overall, the morphology of Californian isolates of *C. destructans* and *C.*
18 *macrodidymum* were conform to published descriptions (2,12,23). Indeed, all of the isolates
19 had microconidia and macroconidia that were one-septate, two-septate, and three-septate (Fig.
20 5). Moreover, *C. destructans* produced chlamydospores abundantly, and its conidial
21 dimensions (Table 3) were in concordance with previous studies (2,23). Also, *C.*
22 *macrodidymum* produced less chlamydospores than *C. destructans* and its conidial sizes
23 (Table 3) agreed with the published description (12).

1 All isolates grew at temperatures of 5, 10, 15, 20, and 25°C, but the optimal
2 temperature was 25°C. After 6 days at 25°C, *C. destructans* isolates (n=17) grew
3 significantly ($P<0.001$) faster (radial growth of 0.34 ± 0.05 cm/day) than *C. macrodidymum*
4 isolates (n=18) (radial growth of 0.30 ± 0.04 cm/day). Again, given that these means are very
5 close and the standard deviations overlapped, a single isolate could not be identified to the
6 species level based on these criteria.

7 Reverse colony colors on PSA after six days were variable (data not shown), and
8 could not be used to distinguish groups of isolates. However, reverse colony color on 2%
9 MEA consistently discriminated *C. destructans* from *C. macrodidymum*: *C. destructans*
10 isolates had a buff colony whereas *C. macrodidymum* isolates had a orange-dark brown
11 colony (Fig. 6).

12 **Pathogenicity.** A preliminary analysis of the data showed no significant trial-
13 treatment interactions; therefore, the data from the two trials were combined. All Californian
14 *C. destructans* and *C. macrodidymum* isolates tested were pathogenic to grapevine cv. 5C.
15 All isolates caused significant root rot on grapevine ($p=0.0047$), and there was no significant
16 level of variation between different isolates or different species (Table 4). Only the *C.*
17 *destructans* isolate USME 116 produced significant leaf symptoms ($p=0.05$) (Table 4). All
18 isolates were recovered from inoculated plants (data not shown).

19 **DISCUSSION**

20 Based on the sequence variation of three independent DNA regions, we identified two
21 species responsible for black foot in California: *C. destructans* and *C. macrodidymum*. In
22 agreement with this work, these two species have each been found to cause black foot of
23 grapevine in other regions of the world (12). In our study, we confirmed the presence, in

1 California grapevines, of *C. destructans* (24) and, for the first time, the occurrence of
2 pathogenic *C. macrodidymum* isolates.

3 Our study showed that the *C. destructans* complex, particularly large in the ITS
4 phylogeny, included numerous isolates from various hosts with remarkably variable
5 sequences. The broad variation (4.6% to 5.29%) found among isolates identified as the single
6 species *C. destructans* was in concordance with previous findings (27). A more complete
7 sampling of isolates of this *C. destructans* complex from other hosts will be necessary to fully
8 resolve the number of species within the *C. destructans* lineage. Our conclusion that isolates
9 of *C. destructans* from grapevines formed a monophyletic clade within this complex is
10 consistent with the conclusion of Halleen et al. based on partial sequences of ITS, BT and the
11 large subunit rDNA (12). Nevertheless, no morphological characteristics allowed us to
12 segregate these grapevine isolates from the rest of *C. destructans* isolates from various hosts
13 as described by Samuels and Brayford (4). Interestingly, we discovered that these isolates,
14 from extremely diverse geographical origins including France, South Africa and five counties
15 in California, had nearly no sequence variation.

16 In this work, the recently described species *C. macrodidymum*, exclusively composed
17 of isolates from grapevines, formed a monophyletic clade in all phylogenies. This conclusion
18 agrees with the work of Halleen et al. (12), who described *C. macrodidymum* as a new species
19 based on partial sequences of ITS, BT and the large subunit rDNA. Our morphological
20 analysis of isolates of *C. macrodidymum* was also in concordance with the description of
21 Halleen (12). Surprisingly, we found very little DNA variation in the *C. macrodidymum* clade
22 contrasting with the wide range of their geographical origin, which includes South Africa,
23 Chile and four counties in California.

1 Based solely on published phenotypic characteristics including
2 microconidial/macroconidial shape and size, growth rate, production of chlamydo­spores, and
3 colony pigmentation (2,12,26), it was difficult to tell apart *C. macrodidymum* from *C.*
4 *destructans*. Indeed, between these two species, the mean values were close and the
5 distribution of the sizes of the spores largely overlapped. Even though we confirmed that *C.*
6 *macrodidymum* formed relatively fewer chlamydo­spores than *C. destructans*, this character
7 was too inconsistent to be used accurately to differentiate these species. Although Halleen et
8 al. (12) distinguished these two species based on their macroconidia, we could not
9 systematically distinguish the slightly bent apical cell of *C. macrodidymum* from the
10 analogous *C. destructans* generally rounded cells. In addition, we found that the growth rate
11 at optimal temperature of 25°C did not distinguish these species either. Furthermore, in
12 agreement with Halleen et al. (12), our pathogenicity tests with various isolates showed that
13 both species induced root rot. There was no relationship between isolates belonging to a
14 specific clade and their degree of aggressiveness. After we further investigated the
15 morphology to find particular phenotypic variations paralleling genetic variations, we finally
16 discovered that colony color on 2% malt extract agar could reliably discriminate isolates from
17 the two species. Furthermore, by comparing the BT1 sequences, we found a conserved 52 bp
18 insertion unique to *C. macrodidymum* that provided a consistent species-specific DNA
19 marker.

20 Our results based on a large collection of grapevine *Cylindrocarpon* found in
21 California contradict an earlier report stating that *C. obtusisporum* is a causal agent of black
22 foot in California (25). Indeed, none of the ITS sequences of *Cylindrocarpon* isolates from
23 grapevines used in our study clustered with the ITS sequence of *C. obtusisporum* CBS 183.36

1 from *Solanum tuberosum*. *Cylindrocarpon* isolates identification, in this previous study, was
2 based only on morphological and growth characteristics (25), which, as we reported, does not
3 allow accurate identification of species. We hypothesize that the *C. macrodidymum* isolates
4 were wrongfully identified as *C. obtusisporum*.

5 In conclusion, multigene phylogeny, morphological studies, and pathogenicity tests
6 revealed that both *C. destructans* and *C. macrodidymum* are the cause of black foot disease in
7 California. This is the first report of *C. macrodidymum* associated with black foot disease in
8 California. In addition, we demonstrated that one can consistently distinguish *C. destructans*
9 from *C. macrodidymum* based on colony color on 2% malt extract agar. Furthermore, one
10 can accurately identify *C. macrodidymum* based on a unique DNA marker found in the BT
11 gene. This information adds to our currently limited knowledge of *Cylindrocarpon* on
12 grapevines and may contribute to the development of more effective disease management
13 strategies.

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Table 1. *Cylindrocarpon* isolates from grapevines (*Vitis* sp.) used in this study

Species ^a	Isolate	Geographic origin	Source ^b	Year ^b	Host ^c	
<i>C. destructans</i>	CBS 112602	Worcester, South Africa	F. Halleen	1999	99 R	
	FR 102	Champagne, France	P. Larignon	n/a	<i>Vitis</i> sp.	
	USME 116	Philo, Mendocino Co., CA	E. Petit	2003	5C	
	USNA 93	Napa, Napa Co., CA	H. Scheck	1999	<i>Vitis</i> sp.	
	USNA 98	St Helena, Napa Co, CA	H. Scheck	1999	<i>Vitis</i> sp.	
	USSC 20	Santa Clara, Santa Clara Co., CA	H. Scheck	1998	5C	
	USSO 54	Sonoma, Sonoma Co., CA	E. Petit	2003	101-14 Mgt	
	USSO 77	Sonoma, Sonoma Co., CA	H. Scheck	1999	V	
	USSO 2	Hopland, Sonoma Co., CA	H. Scheck	2002	<i>Vitis</i> sp.	
	USSO 131	Geyserville, Sonoma Co., CA	E. Petit	2003	101-14 Mgt	
	USSO 143	Geyserville, Sonoma Co., CA	E. Petit	2003	101-14 Mgt	
	USSO 126	Sonoma, Sonoma Co., CA	E. Petit	2003	101-14 Mgt	
	USSO 150	Sonoma, Sonoma Co., CA	E. Petit	2004	101-14 Mgt	
	USSO 78	Sebastopol, Sonoma Co., CA	H. Scheck	1998	<i>Vitis</i> sp.	
	USST 114	Ceres, Stanislaus Co., CA	E. Petit	2003	Freedom	
	USST 148	Ceres, Stanislaus Co., CA	E. Petit	2003	Freedom	
	USST 113	Hugson, Stanislaus Co., CA	E. Petit	2003	Freedom	
	<i>C. macrodidymum</i>	CBS 112605	Wellington, South Africa	F. Halleen	2000	Harmony
		CH 103	Chile	n/a	n/a	143B Mgt
US 40		CA	H. Scheck	n/a	<i>Vitis</i> sp.	
US 138		CA	E. Petit	2003	<i>Vitis</i> sp.	
USME 115		Philo, Mendocino, Co., CA	E. Petit	2003	101-14 Mgt	
USME 149		Philo, Mendocino Co., CA	E. Petit	2003	101-14 Mgt	
USNA 136		St Helena, Napa Co., CA	E. Petit	2003	3309 C	
USSL 152		Woodland, Solano Co., CA	E. Petit	2003	1103 P	
USSO 130		Sonoma, Sonoma Co., CA	E. Petit	2003	1103 P	
USSO 74		Windsor, Sonoma Co., CA	H. Scheck	1999	<i>Vitis</i> sp.	
USSO 133		Geyserville, Sonoma Co., CA	E. Petit	2003	44-53	
USSO 140		Geyserville, Sonoma Co., CA	E. Petit	2003	101-14 Mgt	
USSO 119		Sonoma, Sonoma Co. CA	E. Petit	2003	101-14 Mgt	
USSO 124		Sonoma, Sonoma Co. CA	E. Petit	2003	101-14 Mgt	
USSO 107		Healdsburg, Sonoma Co. CA	E. Petit	2003	3309 C	
USSO 151		Sonoma, Sonoma Co. CA	E. Petit	2003	44-53	
USSO 117		Sonoma, Sonoma Co., CA	E. Petit	2003	44-53	
USSO 146	Sonoma Co., CA	E. Petit	2003	44-53		

^a Species was determined from combined internal transcribed spacer, beta-tubulin, and mitochondrial small subunit ribosomal RNA sequence data.

^b n/a indicates not available.

^c Hosts are varieties of grapevine rootstock.

Table 2. GenBank sequences of *Neonectria* /*Cylindrocarpon* species used in the phylogenetic analysis

Species	Isolate	Host ^a	GenBank Accession no.		
			ITS ^b	BT2 ^c	mtSSU ^d
<i>N. lucida</i>	CTR 72-71	n/a			AY380925
<i>N. discophora</i>	GJS 91-116	n/a			AF315207
<i>N. viridispora</i>	AR 2690	n/a			AY380923
<i>N. phaeodisca</i>	CTR 71-60	n/a			AY380920
<i>N. fuckeliana</i>	GJS 92-42	n/a			AY380919
<i>N. veuillotiana</i>	GJS 91-116	<i>Quercus</i> sp.			AF315197
<i>N. rugulosa</i>	GJS 86-222	n/a			AF315208
<i>N. coronata</i>	CTR 71-19	n/a			AF315198
<i>N. westlandica</i>	GJS 85-45	n/a			AY380922
<i>N. trachosa</i>	GJS 92-95	n/a			AY380921
<i>C. destructans</i>	1557	<i>Panax quinquefolius</i>	AY295329	AY297195	
	IMI 375719	<i>Malus</i> sp.	AJ007356		
	CTR 71-322	n/a	AF220969		
	JAT 1378	<i>Cornus floridae</i>	AY295328		
	IMI 376408	<i>Arbutus menziesii</i>	AJ007352		
	IMI 376403	<i>Alnus glutinosa</i>	AJ007351		
	JAT 1551	<i>Prunus persica</i>			AF315202
	FMd2.1	<i>Alnus rubra</i>			AF315204
	RTDF 14	<i>Pseudotsuga mensiezii</i>			AF315203
<i>C. destructans</i> var. <i>coprosmae</i>	CTR 73-152	<i>Cosmospora</i> sp.	AF220970		
	GJS 85-162	<i>Metrosideros</i> sp.	AY295326		
<i>C. cylindroides</i>	CR 6	<i>Pseudotsuga mensiezii</i>	AY295301	AY297172	
	Cacun2ab2	<i>Pseudotsuga mensiezii</i>			AF315201
	P3p3n12cb	<i>Pseudotsuga mensiezii</i>			AF315200
	P4c2n22ad	<i>Pseudotsuga mensiezii</i>			AF315199
<i>C. magnusianum</i>	CBS 730.87	<i>Hypocrea pachybasioides</i>	AJ279446		
<i>C. didymum</i>	CCFC 185212	<i>Pyrus</i> sp.	AY295303	AY297216	
<i>C. obtusisporum</i>	CBS 183.36	<i>Solanum tuberosum</i>	AY677292		
<i>C. heteronenum</i>	CBS 100318	<i>Malus domestica</i>	AJ228662		
	JR0609B-2	<i>Malus pumila</i>			AF315205
	GBA1	<i>Malus pumila</i>			AF315206
<i>Nectria cinnabarina</i>	CBS 279.48	<i>Acer pseudoplatinus</i>	AF163025		
	GJS 91-111	<i>Acer</i> sp.			AF315209

^a n/a indicates not available^b ITS: Internal transcribed spacer^c BT2: Beta-tubulin 2^d mtSSU: mitochondrial small subunit ribosomal RNA

Table 3. Sizes of fungal structures of *Cylindrocarpon* isolates from grapevines grown on Spezieller Nährstoffarmer agar plus yeast extract, incubated at 25°C in a 12-hour light/dark cycle, illuminated by fluorescent strip lights and near-ultraviolet light (366 nm) and examined after six days.

Species ^a	Length × width (µm) ^b			
	Microconidia	Macroconidia		
		One-septate	Two-septate	Three-septate
<i>C. destructans</i>	7-12 × 2.7-4.1	11-18 × 3-5	18-26 × 4-5.5	22-31 × 4.5-6
<i>C. macrodidymum</i>	8-16 × 3.2-5.0	14-27 × 4-6	26-35 × 5-7.5	31-41 × 6-8

^a Species was determined from combined internal transcribed spacer, beta-tubulin, and mitochondrial small subunit ribosomal RNA sequence data.

^b Averages plus or minus standard deviation were derived from 30 observations per isolate for 17 *Cylindrocarpon destructans* isolates and for 18 *Cylindrocarpon macrodidymum* isolates.

Table 4. Effect of *Cylindrocarpon* isolates on symptom expression of six-month old grapevine rootstock Teleki 5C after 4 months incubation in a greenhouse

Species	Isolate	Leaf symptoms ^{a b}	Root rot index ^{a c}
<i>Cylindrocarpon destructans</i>	USME116	1.125 a	23.2 a
	USST148	0.250 ab	14.2 a
	USST150	0.250 ab	17.0 a
<i>Cylindrocarpon macrodidymum</i>	US40	0.750 ab	21.4 a
	USNA136	0.875 ab	25.5 a
	USSOL152	0.375 ab	14.4 a
	Control	0.000 b	00.0 b

^a Values represent the mean of 8 replications for each isolate. Means in a column followed by the same letter are not significantly different according to Tukey's test ($P = 0.05$).

^b Leaf symptoms were rated using the following scale: 0=no symptomatic area on leaves, 1=0 to 10% of necrotic leaf area, 2=10 to 25 % of necrotic leaf area, 3=25% to 50% of necrotic leaf area, 4=50 % of necrotic leaf area and 5 was 100% necrotic leaf area.

^c Root rot index is the percentage of root with rot.

1 **Figure Captions.**

2 **Fig. 1.** Bayesian phylogeny obtained using internal transcribed spacer 1, 5.8S ribosomal
3 RNA gene, and internal transcribed spacer 2, and beta-tubulin 1 DNA sequence data. The
4 numbers above branches are probabilities of clades obtained with the Bayesian analyses. The
5 numbers below branches are the bootstrap values obtained with the maximum parsimony
6 analyses. Isolates from grapevines are marked with an asterisk. FR: France, CH: Chile, SA:
7 South Africa, US: United States, USSO: United States Sonoma County, USSL: United States
8 Solano County, USME: United States Mendocino County, USNA: United States Napa
9 County, USST: United States Stanislaus County, USSC: United States Santa Clara.

10

11 **Fig. 2.** Bayesian phylogeny obtained using internal transcribed spacer 1, 5.8S ribosomal
12 RNA gene, and internal transcribed spacer 2 and beta-tubulin 2 DNA sequence data. The
13 numbers above branches are probabilities of clades obtained with the Bayesian analyses. The
14 numbers below branches are the bootstrap values obtained with the maximum parsimony
15 analyses. Isolates from grapevines are marked with an asterisk. FR: France, CH: Chile, SA:
16 South Africa, US: United States, USSO: United States Sonoma County, USSL: United States
17 Solano County, USME: United States Mendocino County, USNA: United States Napa
18 County, USST: United States Stanislaus County.

19

20 **Fig. 3.** Bayesian phylogeny obtained using internal transcribed spacer 1, 5.8S ribosomal
21 RNA gene, and internal transcribed spacer 2. *Nectria cinnabarina* (CBS 279.48) was used as
22 an outgroup. The numbers above branches are probabilities of clades obtained with the
23 Bayesian analyses. The numbers below branches are the bootstrap values obtained with the

1 maximum parsimony analyses. Isolates from grapevines are marked with an asterisk. FR:
2 France, CH: Chile, SA: South Africa, US: United states, USSO: United States Sonoma
3 County, USSL: United States Solano County, USME: United States Mendocino County,
4 USNA: United States Napa County, USST: United States Stanislaus County.

5
6 **Fig. 4.** Bayesian phylogeny obtained using mitochondrial small subunit ribosomal DNA
7 sequence data. *Nectria cinnabarina* (GJS 91-111) was used as an outgroup. The numbers
8 above branches are probabilities of clades obtained with the Bayesian analyses. The numbers
9 below branches are the bootstrap values obtained with the maximum parsimony analyses.
10 Isolates from grapevines are marked with an asterisk. FR: France, CH: Chile, SA: South
11 Africa, US: United states, USSO: United States Sonoma County, USSL: United States Solano
12 County, USME: United States Mendocino County, USNA: United States Napa County,
13 USST: United States Stanislaus County.

14
15 **Fig. 5.** Conidia of (A) *Cylindrocarpon destructans*, and (B) *Cylindrocarpon macrodidymum*.
16 Isolates were grown on Spezieller Nährstoffarmer agar plus yeast extract, incubated at 25°C
17 in a 12-hour light/dark cycle, illuminated by fluorescent strip lights and near-ultraviolet light
18 (366 nm) and examined after six days. Photographs were taken at 400X.

19
20 **Fig. 6.** Reverse of plates of colonies grown on 2% malt extract agar for six days at 25°C in a
21 12-hour light/dark cycle. A, *Cylindrocarpon destructans*. B, *Cylindrocarpon macrodidymum*.

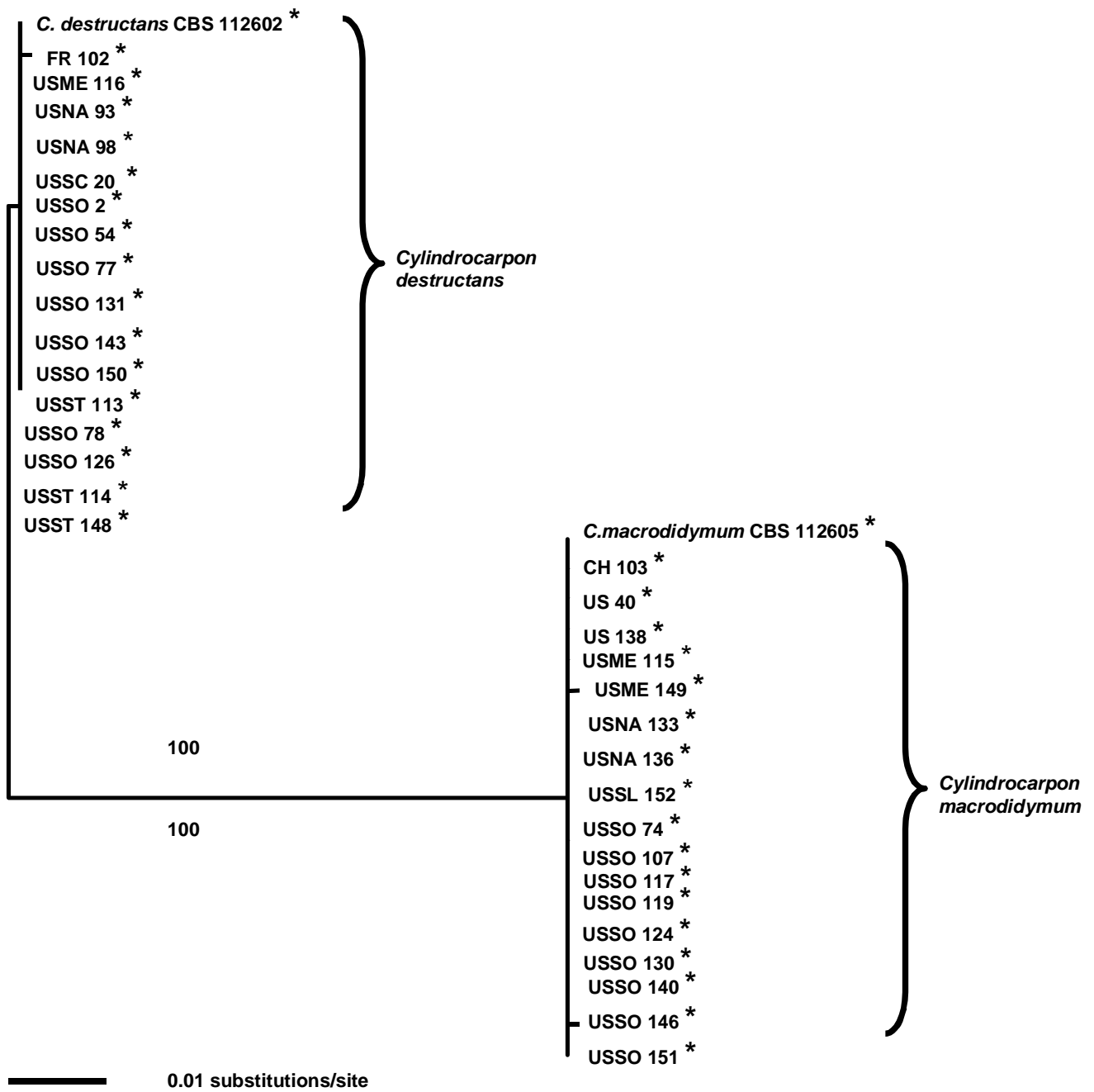


Figure 1, Petit, *Plant Disease*

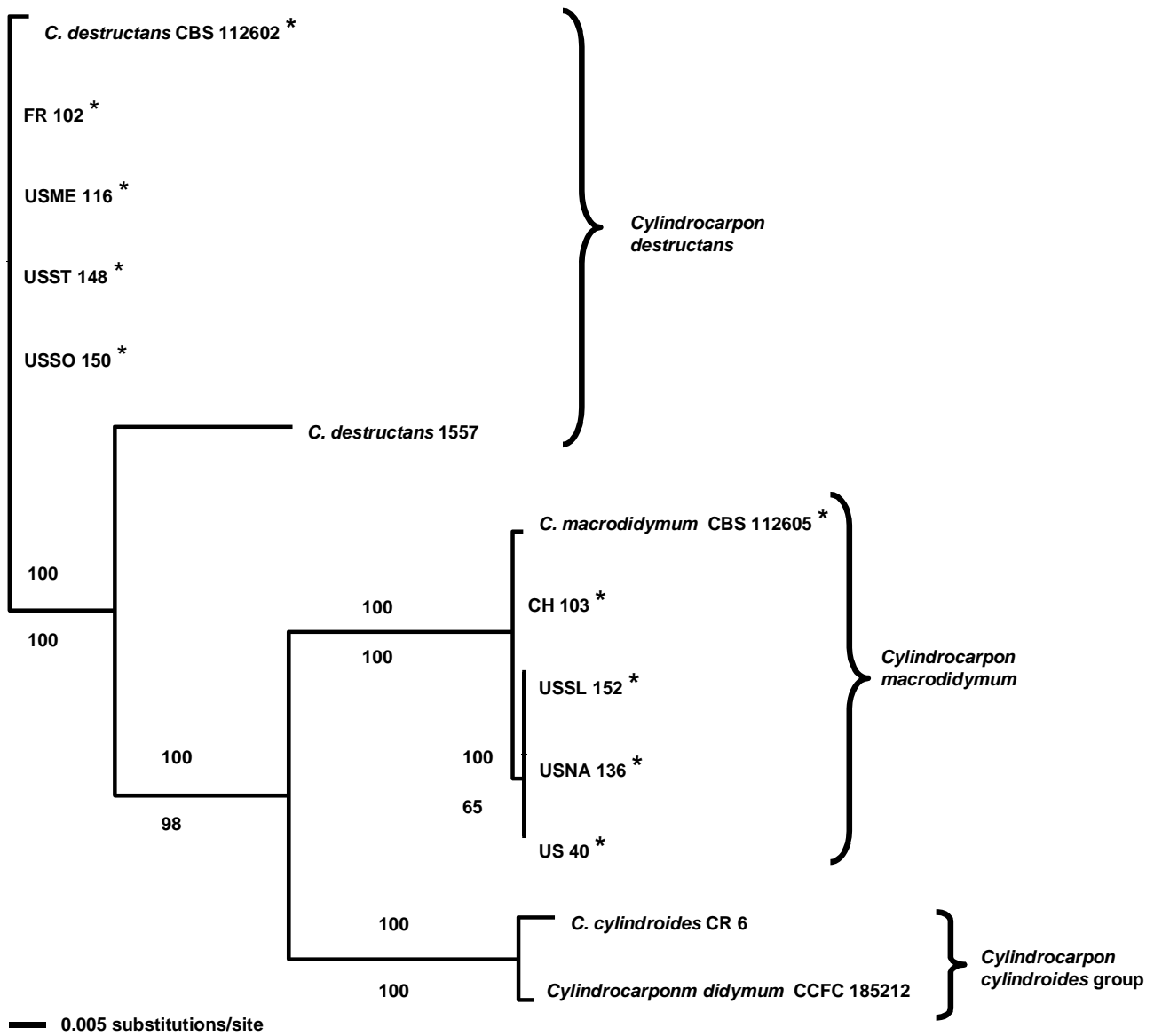


Figure 2, Petit, *Plant Disease*

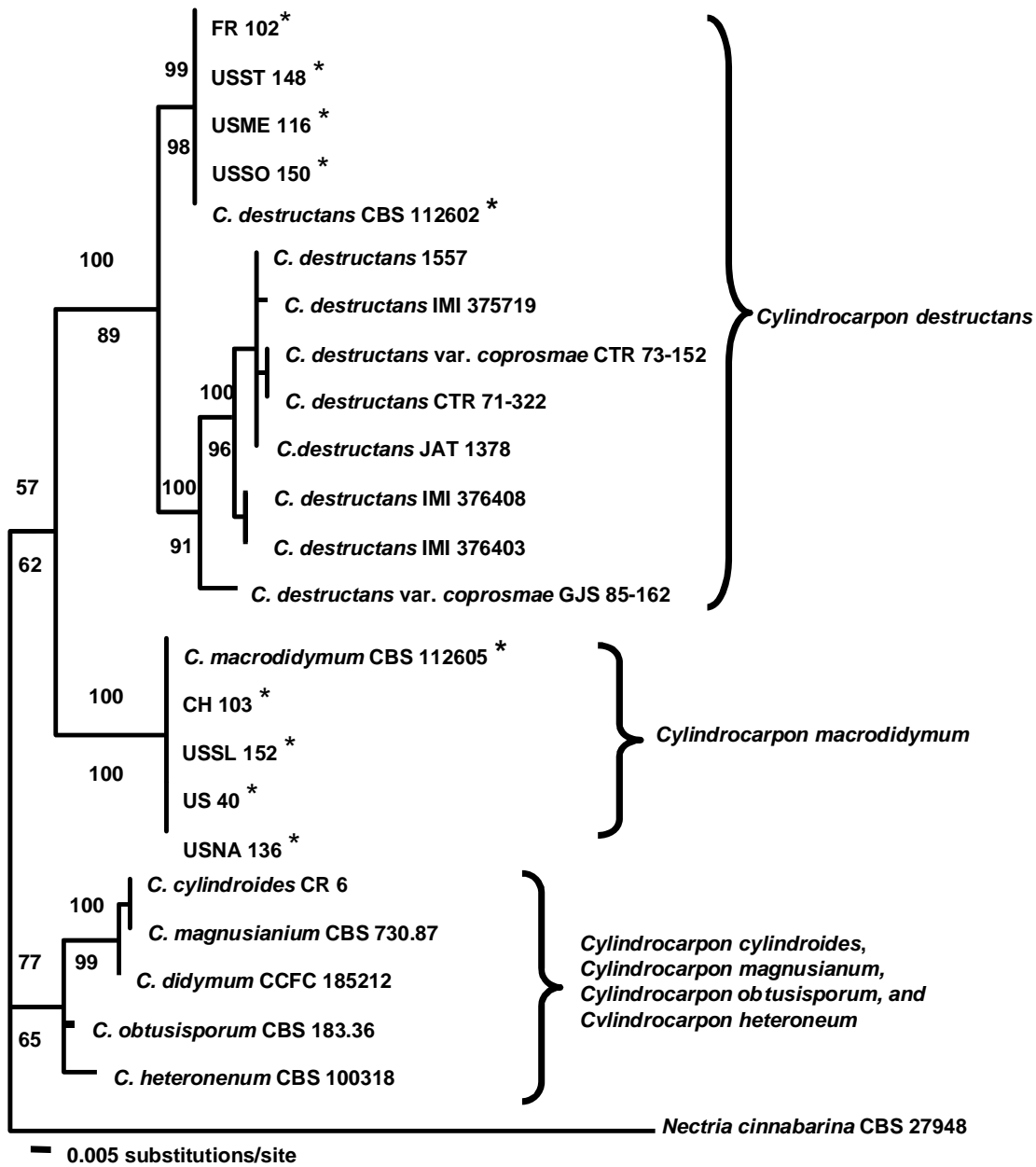


Figure 3, Petit, *Plant Disease*

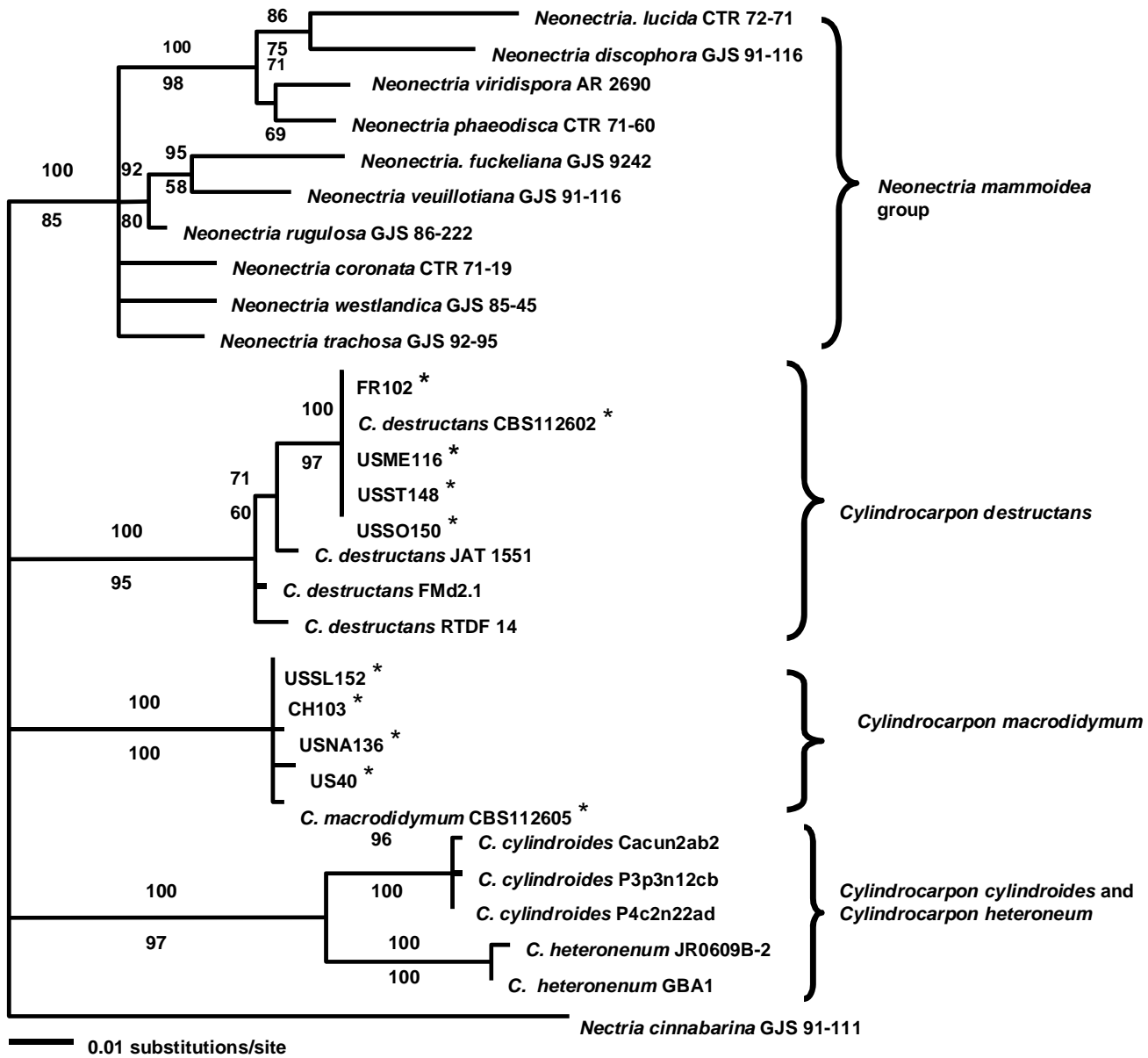


Figure 4, Petit, *Plant Disease*

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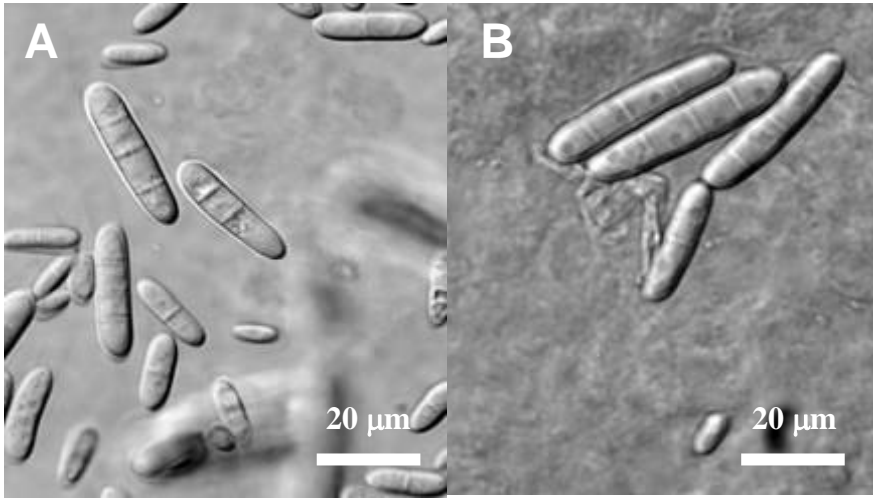


Figure 5, Petit, *Plant Disease*

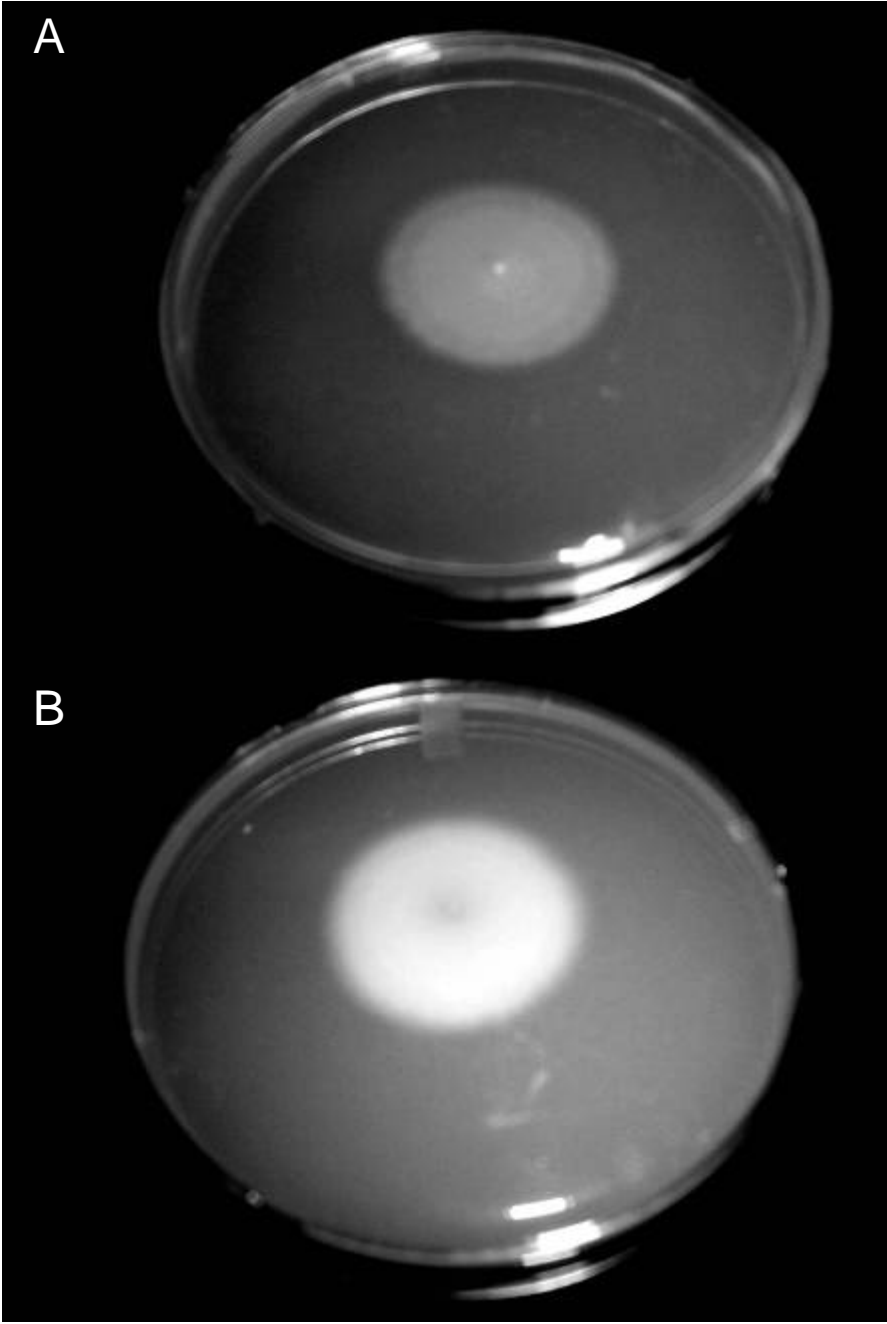


Figure 6, Petit, *Plant Disease*