*Phytopathol. Mediterr.* (2011) 50 (Supplement), S112–S126

# Characterization of *Cadophora luteo-olivacea* and *C. melinii* isolates obtained from grapevines and environmental samples from grapevine nurseries in Spain

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**Summary.** Fifty-eight *Cadophora luteo-olivacea* and three *C. melinii* isolates were recovered from grapevines showing black vascular streaking and decline symptoms characteristic of Petri disease, and from different stages of the grapevine nursery process in Spain. The isolates were studied by means of phenotypical characterization, DNA analysis and pathogenicity tests. The morphological characters studied included conidiophore, phialide and conidial morphology. Colony characters and pigment production on MEA, PDA and OA were also examined. Phenotypical data were subjected to cluster analysis, which clearly separated *C. luteo-olivacea* isolates into four groups. Mating tests were performed on all possible combinations for each *Cadophora* species but no sexual fruiting bodies were produced. Partial sequences of the nuclear ribosomal internal transcribed spacer (ITS), beta-tubulin (BT) and the elongation factor 1α (EF) were analysed, but no genetic variation occurred within the *C. luteo-olivacea* isolates or within the *C. melinii* isolates in any of the regions studied. Pathogenicity tests were conducted on 1-year-old grapevine cuttings of four different rootstocks using four *C. luteo-olivacea* isolates and one isolate of *C. melinii*. All *Cadophora* isolates except the *C. melinii* isolate caused significantly longer lesions in the xylem of grapevine rootstocks than in the controls.

**Key words**: *Cadophora* species, grapevine nurseries, *Vitis vinifera*, young vineyards.

## Introduction

The genus *Cadophora* (*C.*) Lagerberg & Melin was established by Lagerberg *et al*. (1927), with the type species *Cadophora fastigiata* Lagerberg & Melin, which causes stains in softwood timber. Melin and Nannfeldt (1934) added five new species to the genus: *C. americana* Nannf., *C. obscura* Nannf., *C. lagerbergii* Melin & Nannf., *C. melinii* Nannf. and *C. richardsiae* Nannf. Davidson (1935) described two more species, isolated from stained wood or pulpwood products: *C. brunnescens* R.W. Davidson and *C. repens* R.W. Davidson. Subsequently, Conant (1937) determined that *Phialophora* (*P.*) Medlar and *Cadophora* were congeneric and transferred the eight species of *Cadophora* to *Phialophora*. This researcher also considered *C. americana* to be a synonym of *P. verrucosa* Medlar. Since then a very broad concept of *Phialophora* has been retained, even in the major revision by Schol-Schwarz (1970).

Gams (2000) proposed that the phialophora-like anamorphs with affinities to the Dermateaceae in the *Helotiales*, such as *Mollisia* (Fr.) P. Karst. and *Pyrenopeziza* Fuckel, should be accommodated in the *Cadophora*, the species of which have pale to hyaline collarettes on top of their phialides with the vegetative hyphae more or less pigmented. This author suggested the following binomials for the genus *Cadophora*: *C. fastigiata*, *C. melinii*, *C. repens* and *C. malorum* (Kidd & Beaum.) W. Gams. Harrington and McNew (2003) used mor-

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phology and rDNA sequences to determine which phialophora-like species might have affinities to the *Helotiales,* and to confirm the connection of *Cadophora* to the Dermateaceae. They found that the pigmentation of the species studied was often quite variable between species, proposed new combinations in *Cadophora* and also reviewed the taxonomy of the recognized species. The genus *Cadophora* then comprised *C. fastigiata* (the type species), *C. malorum*, *C. melinii*, *C. lagerbergii*, *C. finlandia* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew, *C. gregata* (Allington & D.W. Chamb.) T.C. Harr. & McNew, and *C. luteo-olivacea* (F.H. Beyma) T.C. Harr. & McNew. The inclusion of *P. repens* R.W. Davidson in the *Cadophora* group was not supported by ITS sequence analysis. *P. atra* F.H. Beyma and *C. heteroderae* F.H. Beyma were synonymized with *C. malorum*, and *P. goidanichii* Delitala with *C. luteo-olivacea* by Harrington and McNew (2003).

The known *Cadophora* species and their relatives occur in many habitats such as decaying wood (Nilsson, 1973; Morrell and Zabel, 1985; Blanchette *et al.,* 2004; Held *et al.,* 2005; Arenz *et al*., 2006), soil (Kerry, 1990; Aislabie *et al*., 2001; Arenz *et al*., 2006; Hujslová *et al*., 2010) or plants. *Cadophora* species on plants are mainly associated with decay symptoms in vines and fruit-rotting in kiwi [*Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson var. *deliciosa*] (Di Marco *et al*., 2004; Johnston *et al*., 2005; Haegi *et al*., 2006; Riccioni *et al*., 2007; Di Marco and Osti, 2008; Prodi *et al*., 2008). Species of *Cadophora* have also been recovered from grapevines showing decline symptoms in South Africa, Pennsylvania, California and Uruguay (Halleen *et al.,* 2005; Overton *et al*., 2005; Rooney-Latham, 2005; Abreo *et al*., 2008). Halleen *et al*. (2007) confirmed that *C. luteo-olivacea* is a vascular pathogen of grapevines in South Africa and can also be recovered from healthy nursery cuttings. Recently, Manning and Mundy (2009) have isolated *C. luteo-olivacea* and *C. melinii* from esca-diseased grapevines in New Zealand.

Over the last few years, *Cadophora* spp. have frequently been isolated from nursery grapevines and Petri-diseased grapevines in Spain. The species identity of these isolates was unknown prior to this study. Limited information is available on the phenotypical and molecular variability of *Cadophora* isolates obtained from grapevine and on their role in the decline of young vines. Therefore, the objective of this research was to study these *Cadophora* isolates by means of phenotypical characterization, DNA analysis and pathogenicity tests.

## Materials and methods

### Fungal isolates

In this study we used 58 *C. luteo-olivacea* and three *C. melinii* isolates representative of different locations in Spain and recovered in 2007 and 2008 (Table 1). Some of the isolates were recovered from vines in young vineyards, others from planting material showing black vascular streaking and decline symptoms characteristic of Petri disease. Sections were cut from affected areas, washed under running tap water, surface-disinfected for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces of discoloured or decayed tissue were plated on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, Hants, England) supplemented with 0.5 g $L^{-1}$ streptomycin sulphate (MEAS) (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated for 10–15 days at 25ºC in the dark.

Additionally, some isolates were obtained from nursery samples at three stages of the propagation process: samples from pre-grafting hydration tanks, washings from scissors and washings from grafting tools. Approximately 100 mL of water was sampled from tanks in which cuttings had been soaked for 72 hours. Scissors and grafting tools were washed with a 0.2% sterile solution of Tween-20, and 30 to 60 mL of every washing was placed in a sterile tube. Water samples from the hydration tanks, washings from the scissors and washings from the grafting tools were filtered as described by Eskalen and Gubler (2001). Water samples (10 mL) were forcibly passed through 5 μm and 0.45 μm pore-size syringe filters (Sartorius Stedium Biotech, Göttingen, Germany) attached in tandem to sterile 20 mL syringes (BD Biosciences, Madrid, Spain). The 5 μm filters trapped debris and large fungal spores. The 0.45 μm filters trapped small particulates and smaller spores, including spores of *Cadophora* species. The filters were rinsed with 4 mL of sterile water to backwash any trapped spores and collected in microfuge tubes. Aliquots of 1 mL were then spread

Table 1. Isolation details and GenBank accession numbers of the *Cadophora* isolates studied.

| Species/ Isolate | Year | Geographical origin | | | GenBank accessions[b] | | |
|---|---|---|---|---|---|---|---|
| | | Town | Province | Source of collection[a] | ITS | β-tubulin | EF |
| *C. luteo-olivacea* | | | | | | | |
| Clo-1 | 2007 | Ayelo | Valencia | Tempranillo/110 R | HQ661084 | HQ661054 | HQ661069 |
| Clo-2, CBS 128571 | 2007 | Ciudad Real | Ciudad Real | Tempranillo/110 R | HQ661085 | HQ661055 | HQ661070 |
| Clo-3 | 2007 | Ciudad Real | Ciudad Real | Tempranillo/110 R | | | |
| Clo-4 | 2007 | Ayelo | Valencia | Grafting machine | HQ661086 | HQ661056 | HQ661071 |
| Clo-5, CBS 128572 | 2007 | Ayelo | Valencia | Hydration tank | | | |
| Clo-6 | 2007 | Ciudad Real | Ciudad Real | Tempranillo/110 R | | | |
| Clo-7, CBS 128573 | 2007 | Ciudad Real | Ciudad Real | Tempranillo/110 R | HQ661087 | HQ661057 | HQ661072 |
| Clo-8 | 2007 | Ayelo | Valencia | Hydration tank | | | |
| Clo-9 | 2007 | Ayelo | Valencia | Grafting machine | | | |
| Clo-10 | 2007 | Ayelo | Valencia | Pruning scissors | | | |
| Clo-11 | 2007 | Ayelo | Valencia | Grafting machine | | | |
| Clo-12 | 2007 | Ayelo | Valencia | Grafting machine | | | |
| Clo-13 | 2007 | Ayelo | Valencia | Grafting machine | | | |
| Clo-14 | 2007 | Ayelo | Valencia | Hydration tank | | | |
| Clo-15, CBS 128574 | 2007 | Ayelo | Valencia | Hydration tank | HQ661088 | HQ661058 | HQ661073 |
| Clo-16 | 2007 | Ayelo | Valencia | Grafting machine | | | |
| Clo-17 | 2007 | Ayelo | Valencia | Grafting machine | | | |
| Clo-18, CBS 128575 | 2007 | Ciudad Real | Ciudad Real | Tempranillo/110 R | HQ661089 | HQ661059 | HQ661074 |
| Clo-19 | 2007 | Olivenza | Badajoz | Syrah | | | |
| Clo-20 | 2007 | Requena | Valencia | Tempranillo/110 R | | | |
| Clo-21 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-22 | 2007 | Ayelo | Valencia | 1103 P | HQ661090 | HQ661060 | HQ661075 |
| Clo-23 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-24 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-25 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-26 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-27 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-28 | 2007 | Ayelo | Valencia | 1103 P | HQ661091 | HQ661061 | HQ661076 |
| Clo-29 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-30 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-31 | 2007 | Ayelo | Valencia | 1103 P | | | |
| Clo-32 | 2007 | Ayelo | Valencia | 110 R | | | |
| Clo-33, CBS 128576 | 2007 | Ayelo | Valencia | 110 R | HQ661092 | HQ661062 | HQ661077 |
| Clo-34, CBS 128577 | 2007 | Ayelo | Valencia | 110 R | | | |
| Clo-35 | 2007 | Ayelo | Valencia | 110 R | | | |
| Clo-36 | 2007 | Ayelo | Valencia | 110 R | | | |
| Clo-37 | 2007 | Ayelo | Valencia | 110 R | | | |
| Clo-38 | 2007 | Ayelo | Valencia | 110 R | | | |
| Clo-39 | 2007 | Ayelo | Valencia | 110 R | | | |
| Clo-40 | 2007 | Socuéllamos | Ciudad Real | Garnacha/110 R | HQ661093 | HQ661063 | HQ661078 |
| Clo-41 | 2007 | Socuéllamos | Ciudad Real | Airen/1103 P | | | |
| Clo-42 | 2008 | Ayelo | Valencia | 1103 P | | | |
| Clo-43 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-44 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-45 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-46, CBS 128578 | 2008 | Ayelo | Valencia | 110 R | HQ661094 | HQ661064 | HQ661079 |
| Clo-47 | 2008 | Ayelo | Valencia | 110 R | | | |

*continues*

Table 1. *Continued*

| Species / Isolate | Year | Geographical origin | | | GenBank accessions[b] | | |
|---|---|---|---|---|---|---|---|
| | | Town | Province | Source of collection[a] | ITS | β-tubulin | EF |
| Clo-48 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-49 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-50 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-51 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-52 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-53 | 2008 | Ayelo | Valencia | 110 R | | | |
| Clo-54 | 2008 | Cariñena | Zaragoza | Syrah/110 R | | | |
| Clo-55 | 2008 | Sa Punta | Mallorca | Merlot/110 R | HQ661095 | HQ661065 | HQ661080 |
| Clo-56 | 2008 | Sa Punta | Mallorca | Merlot/110 R | | | |
| Clo-57 | 2008 | Petra | Mallorca | Manto Negro | | | |
| Clo-58 | 2008 | Petra | Mallorca | Manto Negro | | | |
| CBS 141.41 | 1939 | Munksund | Sweden | Waste water of Schleiferei Byske | | | |
| *C. melinii* | | | | | | | |
| Cme-1 | 2007 | Olivenza | Badajoz | Syrah | HQ661096 | HQ661066 | HQ661081 |
| Cme-2 | 2007 | Olivenza | Badajoz | Syrah | HQ661097 | HQ661067 | HQ661082 |
| Cme-3 | 2007 | Olivenza | Badajoz | Syrah | HQ661098 | HQ661068 | HQ661083 |
| CBS 268.33 | 1933 | - | Sweden | - | | | |

[a] Isolates were collected from *Vitis vinifera* plants (indicated as rootstock or scion/rootstock combinations) or from different stages in the grapevine nursery process (hydration tanks, grafting machines or pruning scissors).
[b] The sequences of a selection of isolates have been deposited in GenBank.

on a semi-selective culture medium adapted from Tello *et al*. (2009) [2% MEA amended with 1.5 g $L^{-1}$ streptomycin sulphate; 0.02 g $L^{-1}$ Folpet (Aragonesas Agro, Madrid, Spain); and 0.015 g $L^{-1}$ ampicillin (Laboratorio Reig Jofré S.A., Sant Joan Despí, Spain)]. Plates were air dried under a laminar flow hood with the lids partially lifted for 30 min. Plates were then sealed with parafilm and incubated at 25ºC in darkness for one month. Petri dishes were inspected daily for mycelial growth and re-isolation. All isolates were transferred to potato dextrose agar (2% PDA; Biokar-Diagnostics, Beauvais, France). The isolates were single-spored by serial dilution prior to use (Dhingra and Sinclair, 1995) and were stored in a 15% glycerol solution at -80ºC in 1.5 mL cryovials. Reference isolates of *C. luteo-olivacea* (CBS 141.41) and *C. melinii* (CBS 268.33) were obtained from the Centraalbureau voor Schimmelcultures in the Netherlands (CBS) (Table 1).

**Morphological and cultural characterization**

Morphological characters used in this study included conidiophore, phialide and conidial morphology. Colony characters and pigment production were determined on MEA, PDA (2% PDA; Biolab, Midrand, South Africa) and oatmeal agar (OA; 60 g oatmeal; 12.5 g agar; Difco, Osi, Maurepas, France) (Gams *et al*. 2007) incubated at 25ºC for 8 and 16 d. Colony colours were determined using the colour charts of Rayner (1970). Cardinal temperatures for growth were determined by incubating three MEA plates per isolate in the dark at temperatures ranging from 5 to 40ºC at 5ºC intervals. Radial growth was measured after 8 d at 25ºC on MEA.

Aerial mycelium of colonies cultivated on MEA were inspected under the microscope or by a slide culture technique (Arzanlou *et al*., 2007). Slide cultures were set up in Petri dishes containing 2 mL of sterile water, into which a U-shaped glass rod was placed, extending above the water surface. A block of an actively growing fungal colony, approx. 1 cm square, was placed on a sterile microscope slide, covered with a somewhat larger, sterile glass covering slip, and incubated in a moist chamber. Fungal sporulation was monitored, and when it was optimal, images

of the sporulating fungus were photographed with a Nikon camera system (Digital Sight DXM 1200, Nikon Corporation, Japan). Slides were mounted in lactic acid, and 30 measurements (×1000 magnification) of the relevant structures were made. The 5th and 95th percentiles were defined for all measurements with the extremes given in parentheses.

Mating tests with all *C. luteo-olivacea* and *C. melinii* isolates were performed as described by Mostert *et al*. (2003). *Cadophora* spp. isolates were grown on MEA plates for 2 weeks, using 5 plates per isolate. Conidia were dislodged from the agar surface with a glass rod, and suspensions were prepared in 5 mL sterile distilled water. Two aliquots of 100 mL each, representing two different isolates, were pipetted onto twice-autoclaved pieces of grapevine canes placed on 2% water agar plates (GWA) (Difco, France). Each *Cadophora* species was mated in all possible combinations. Controls consisted of a 200 mL aliquot of one isolate only. Plates were incubated at 20ºC and 25ºC under continuous white light (Philips TLD18W/33) and microscopically examined at weekly intervals for fruiting bodies. Each pair of isolates was replicated three times. The experiment was repeated.

To group *C. luteo-olivacea* isolates by their phenotypical characters, cluster analysis was conducted on the Euclidean distance matrix with the unweighted pair group method based on arithmetic averages (UPGMA). The morphological characters used in the analysis were: colony colour on MEA and PDA as nominal variables; the maximum number of guttules in conidia and the optimum growth temperatures as symmetric-binary variables; conidophores, phialides, collarettes and conidia measurements and radial growth as interval variables; and yellow pigmentation on PDA and OA as asymmetric-binary variables. The clustering method and similarity coefficient were tested using the NCSS 2007 procedure (Statistical Solutions Ltd, Cork, Ireland).

**Molecular analysis**

Fungal mycelium and conidia were scraped from pure cultures grown on PDA for 2 weeks at 25ºC in the dark and ground to a fine powder with liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Norcross, GA, USA) following manufacturer's instructions. DNA was visualized on 0.7% agarose gels (agarose D-1 Low EEO, Conda, Madrid, Spain) stained with ethidium bromide. The DNA was stored at –20ºC.

The internal transcribed spacers 1 and 2 as well as the 5.8S ribosomal RNA gene of approximately 595 bp for *C. luteo-olivacea* isolates and 555 bp for *C. melinii* isolates was amplified with the primers ITS1 and ITS4 (White *et al*., 1990). Oligonucleotide primers BT1a and BT1b were used to amplify the partial β-tubulin (BT) gene of approximately 480 bp for the *C. luteo-olivacea* isolates and 300 bp for the *C. melinii* isolates (Petit and Gubler, 2005). Amplification of the partial translation elongation-factor 1α (EF) gene of approximately 555 bp for *C. luteo-olivacea* isolates and 525 bp for *C. melinii* isolates was done with the primers EF1-688F and EF1-1251R (Alves *et al*., 2008).

Each PCR reaction contained 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.4 μM of each primer, 1 U of DNA *Taq* polymerase (Dominion MBL, Córdoba, Spain), and 1 μL of template DNA. The PCR reaction mix was adjusted to a final volume of 25 μL with water (Chromasolv Plus, Sigma-Aldrich, Steinheim, Germany). PCR amplifications were performed on a Peltier Thermal Cycler-200 (MJ Research, Waltham, USA). The program consisted of an initial step of 4 min at 94°C, followed by 30 cycles (35 cycles for BT) of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and an elongation at 72°C for 1.5 min. A final extension was performed at 72°C for 10 min. The PCR products were visualized in 1.5% agarose gels. A 100 bp ladder was used as a molecular weight marker (Dominion MBL). PCR products were purified with the High Pure PCR product purification kit (Roche Diagnostics, Germany) and sequenced in both directions by the DNA Sequencing Service of the Universidad Politécnica de Valencia-CSIC.

The sequences were aligned using the MAFFT sequence alignment program version 6 (Katoh and Toh, 2008) followed by manual adjustments of the alignments in the Sequence Alignment Editor v. 2.0a11 (Rambaut, 2002). Reference sequences of the ex-type cultures of the relevant *Cadophora* species and reference sequences used by Harrington and McNew (2003) were obtained from GenBank. Phylogenetic analysis of the aligned

sequence data was performed with Phylogenetic analysis using parsimony (PAUP) v. 4.0b10 (Swofford, 2003). Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analysis, alignment gaps were treated as missing data and all characters were unordered and of equal weight.

Maximum parsimony analysis was performed using the heuristic search option with 10 random simple taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm with the option of saving no more than 10 trees with a score greater than or equal to 5 (Harrison and Langdale, 2006). Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications. Tree length (TL), the consistency index (CI), the retention index (RI) and the rescaled consistency index (RC) were calculated. Sequences derived in this study were lodged at GenBank (Table 1) and the alignment and phylogenetic tree in TreeBASE (number S10849).

#### Pathogenicity tests

One isolate of *C. luteo-olivacea* of each of the four morphological groups defined by cluster analysis and one isolate of *C. melinii* were used. Pathogenicity tests were conducted on 1-year-old grapevine cuttings of four rootstocks (140 Ruggeri, 161-49 Couderc, 1103 Paulsen and 110 Richter). In total 96 dormant cuttings of each rootstock were cut into equal lengths containing four to five buds, and then hot-water treated at 53ºC for 30 min to eliminate any fungal trunk pathogens (Gramaje *et al.,* 2009). In order to enhance callusing and rooting, dormant cuttings were buried in sterilized peat moss in plastic boxes, and placed in a callusing room at 25ºC and 100% humidity for 4 weeks. After callusing and rooting, the cuttings were wounded between the two upper internodes with a 5 mm cork borer. A 5 mm mycelium agar plug from a 2-week-old culture was placed in the wound. Wounds were wrapped with parafilm. Eight cuttings per fungal isolate were used for each rootstock. Eight cuttings of each rootstock were inoculated with 5 mm non-colonized PDA agar plugs from two different plates for negative controls. Inoculated cuttings were planted immediately in individual pots, placed in a greenhouse at 25ºC and watered every 3 days or as needed. Plants were arranged in a completely randomized design. The experiment was repeated.

Cuttings were collected after 14 weeks and inspected for lesion development. The extent of vascular discolouration was measured upwards and downwards from the inoculation point. Additionally, shoot dry weight was evaluated for the shoots above the inoculation point. Small pieces (0.5 to 1 cm) of necrotic tissue from the edge of each lesion were cut and placed on MEAS in an attempt to recover the inoculated fungi and fulfill Koch's postulates. Fungi were identified as previously described.

Lesion length and shoot dry weight data were subjected to analysis of variance using SAS v. 8.1 (SAS Institute, Cary, North Carolina, USA) and Student's t-test for Least Significant Difference was calculated at the 5% significance level to compare the treatment means for the different fungal species and grapevine rootstocks.

## Results

#### Morphological characterization

The isolates were identified as *C. luteo-olivacea* or *C. melinii* based on morphological characters (Cole and Kendrick, 1969; Schol-Schwarz, 1970; Gams, 2000; Harrington and McNew, 2003). Conidiophore, phialide and conidial morphology were similar in all *C. luteo-olivacea* isolates (Figure 1). Aerial mycelium on MEA consisted of branched, septate hyphae occurring singly or in bundles of up to 5; hyphae tuberculate with warts up to 3 μm diam, verruculose to smooth, medium brown and 2.5-4 μm wide. Conidiophores were mostly short, usually unbranched, arising from aerial or submerged hyphae, erect to flexuous, up to 7-septate, pale brown, (9–)11.5–65(–69) (av.=26) μm long and 2–2.5 (av.=2) μm wide. Phialides were terminal or lateral, mostly monophialidic, smooth to verruculose, hyaline, with collarettes 2.5–3 μm long and 2–2.5 μm wide, mostly cylindrical, some elongate-ampulliform, attenuated at the base or navicular, (4–)8.5–26(–31) × 1.5–3 (av.=5×2) μm. Conidia were hyaline, with up to 3 guttules, ovoid or oblong ellipsoidal, (3–)3.5–7.5×2–3 (av.=4.5×2) μm, L/W=2.1.

Some differences were found in colony characters and pigment production of the *C. luteo-*

*olivacea* isolates studied (Figure 2 and Table 2). Colonies on MEA were flat, felty, with even edge and varying in colour from white to greenish-olivaceous (23'"b). Colonies on PDA were flat, felty and cottony in the middle, with an even edge and varying in colour from white to grey-olivaceous (21'"i). Colonies on OA were flat, felty and cottony in the middle, with an even edge and varying in colour from white to olivaceous-buff (21'"d). For all isolates, colonies reached a radius of 15.5–17.2 mm after 8 d at 25ºC. The minimum temperature for growth was 5ºC, optimum 20–25ºC and maximum 30ºC. Some *C. luteo-olivacea* isolates produced yellow pigmentation on PDA and/or MEA plates.

The *C. luteo-olivacea* isolates were different from the type description since they had predominantly monophialidic phialides, which were thinner. The conidial shape and the diversity of the colony colours on the media studied also differed. Van Beyma (1940) described colonies of *C. luteo-olivacea* as having thick phialides often in bunches, conidia ellipsoid or elongate, and colonies olive-yellow with white mycelium in the middle.

A comparison based on the morphological and colony characters of the 58 *C. luteo-olivacea* isolates studied are shown in a dendrogram (Figure 3). Analysis revealed that isolates were distributed in two clusters at a Euclidean distance with a cut off value of 1.26. Four distinct groups were formed. Cluster A comprised group I. Cluster B was divided into three groups, II, III and IV. The cophenetic correlation value was 0.9419. Group I contained 6 isolates. This group was the most diverse, with a dissimilarity ranging from 0 to 0.92. Group II contained 21 isolates and had a dissimilarity ranging from 0 to 0.88. Isolate CBS 141.41 was included in this group and joined the other isolates of the same group at a dissimilarity of 0.88. Group III contained 23 isolates with a dissimilarity ranging from 0 to 0.81. Group IV contained 9 isolates and was the most homogeneous (dissimilarity ranging from 0 to 0.51). Colony characters and pigment production were the most useful parameters distinguishing these groups (Figure 2 and Table 2).

The majority of *C. luteo-olivacea* isolates were from grapevines. Seven isolates were from the grafting machines, four from the hydration tank and one from pruning scissors.

The morphological and colony characters of *C. melinii* isolates are shown in Figure 4. Aerial mycelium on MEA consisted of branched, septate hyphae occurring singly or in bundles of up to 13; hyphae tuberculate with warts up to 2.5 µm diam, verruculose to smooth, medium brown and 3–3.5 µm wide. Conidiophores mostly short, usually branched, arising from aerial or submerged

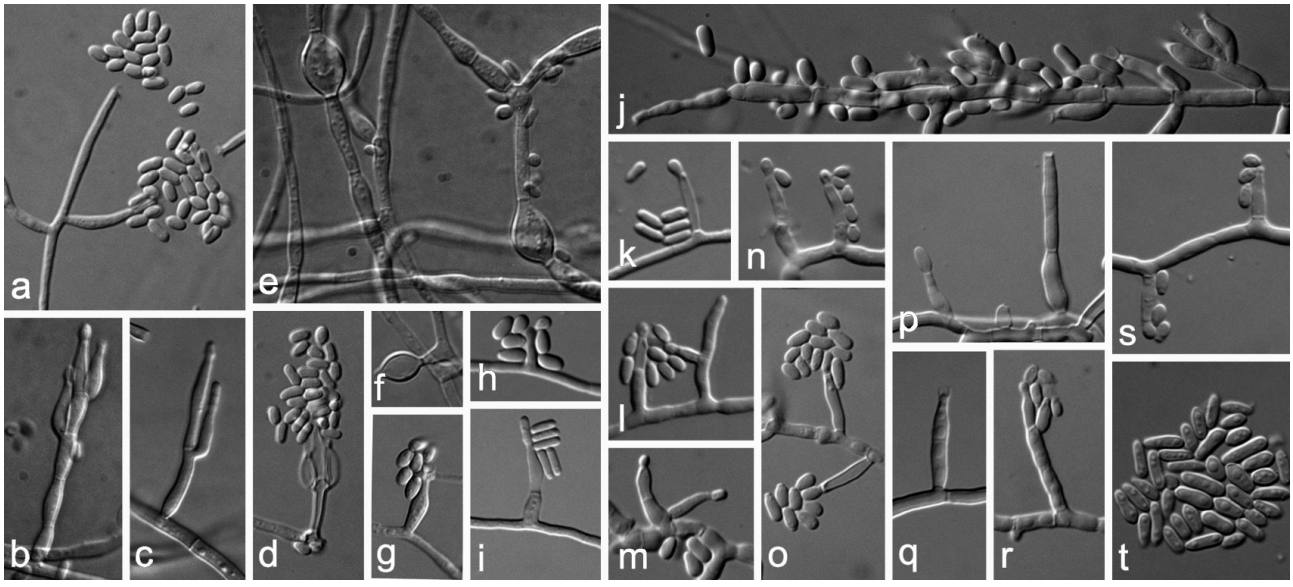


Figure 1. *Cadophora luteo-olivacea*. a–t, Aerial structures on MEA; a-d, conidiophores; e-f, hyphal swellings; g-s, conidiophores and phialides; t, conidia. Scale bar: a–t=10 µm. Scale bar for a also applies to b–t.

hyphae, erect to flexuous, up to 4-septate, pale brown, (11–)11.5–50.5(–65) (av.=24.5) µm long and 2–3 (av.=2.5) µm wide. Phialides terminal or lateral, mostly monophialidic, smooth to verruculose, hyaline, with 1.5–3.5 µm long, 2–3 µm wide, subcylindrical or navicular collarettes, (4–)5–13.5(–15)×1.5–3(–4) (av.= 8×2.5) µm. Conidia hyaline, with up to 2 guttules, mostly ovoid, (3–)4–5.5×1.5–2.5 (av.=4.5×2) µm, L/W=2.1.

Regarding cultural characteristics, colonies reached a radius of 12–14.5 mm after 8 d at 25ºC. The minimum temperature for growth was 5–10ºC, the optimum 20–25ºC and the maximum 30ºC. Colonies on MEA were flat, felty, with an even edge; after 16 d, olivaceous-black (27””k) to grey-olivaceous (23””i) from the top, olivaceous-black (27””k) from the bottom. Colonies on PDA were flat, felty, with an even edge; after 16 d, white to olivaceous-buff (21”’f) from the top and the bottom. Colonies on OA were raised with striating furrows,

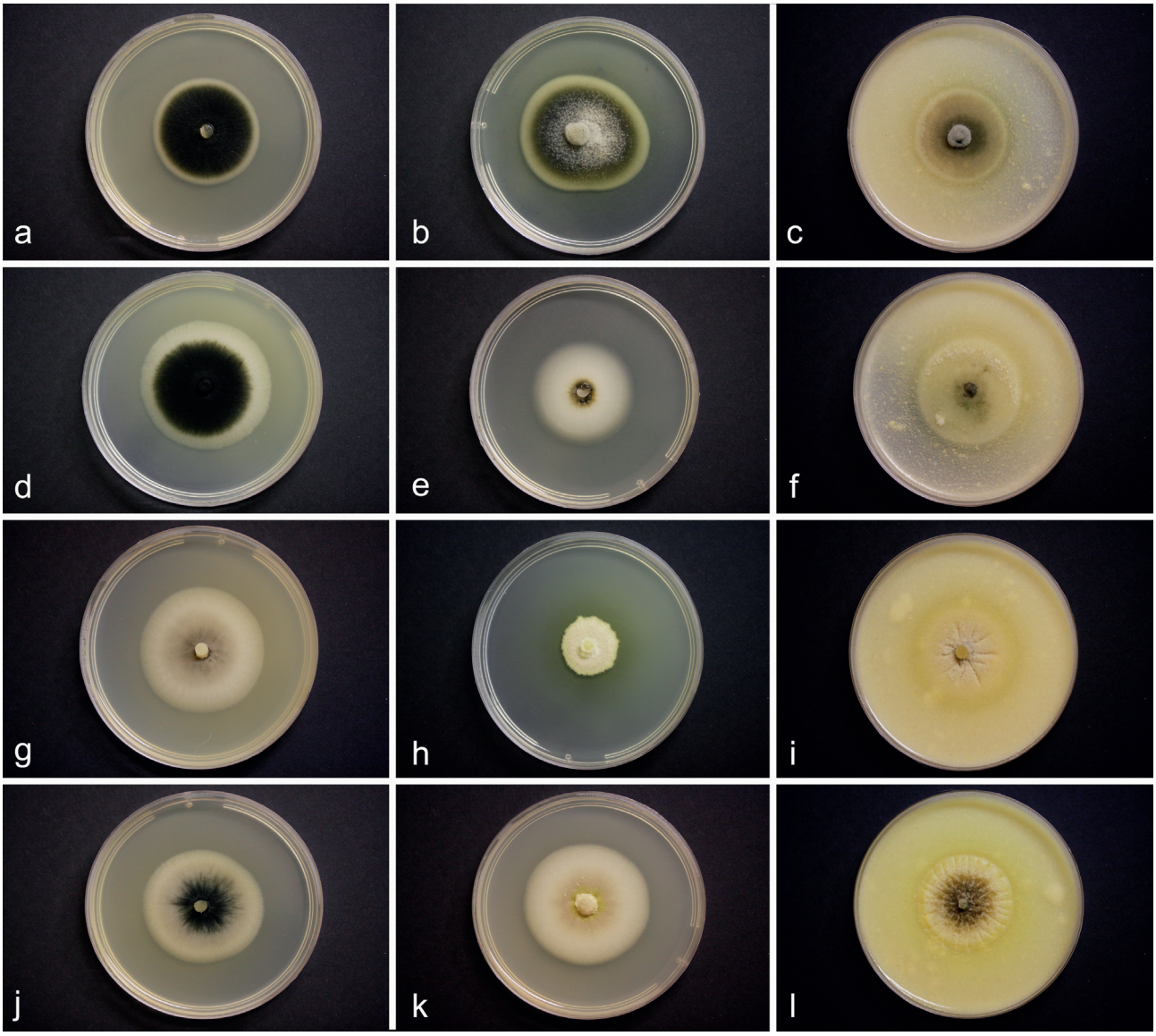


Figure 2. *Cadophora luteo-olivacea*. Group 1: a–c. Sixteen-day-old colonies on MEA (a), PDA (b) and OA (c). Group 2: d-f, Sixteen-day-old colonies on MEA (d), PDA (e) and OA (f). Group 3: g-i. Sixteen-day-old colonies on MEA (g), PDA (h) and OA (i). Group 4: j-l. Sixteen-day-old colonies on MEA (j), PDA (k) and OA (l).

Table 2. Summary of colony colour useful for the identification of each group of *Cadophora luteo-olivacea*.

| *C. luteo-olivacea* group | Colony colour on MEA | Colony colour on PDA | Colony colour on OA | Yellow pigmentation on PDA | Yellow pigmentation on OA |
|---|---|---|---|---|---|
| Group 1 | Greenish olivaceous (23’’’b) to olivaceous buff (21’’’b) above and in reverse | Olivaceous black (27’’’’k) to grey olivaceous (21’’’’i) above and in reverse | Olivaceous (21’’k) to olivaceous buff (21’’’d) above | No | No |
| Group 2 | Olivaceous black (25’’’’k) to white towards the margin above and in reverse | White to greenish olivaceous (21’’’i) close to the centre | White to olivaceous buff (21’’’d) close to the centre above | No | No |
| Group 3 | White to grey olivaceous (21’’’’i) close to the centre above and in reverse | White to straw (19’f) above, straw (19’f) topure yellow (21’b) in reverse | Olivaceous buff (21’’’d) to straw (21’d) above | Yes | Yes |
| Group 4 | White to oliveaceous black (25’’’’k) close to the centre above and in reverse | White to olivaceous buff (23’’’b) close to the centre above and in reverse | Greenish olivaceous (23’’’b) to straw (21’f) above | No | Yes |

woolly when close to the centre, with an even edge; after 16 d, they were pale olivaceous-grey (21””’d) to olivaceous-black (27””’k) from the top.

The *C. melinii* isolates differed from the type description in hyphal colour and in phialide shape. Cole and Kendrick (1969) described colonies of *C. melinii* as having hyphae light-brown to hyaline, with the phialides subcylindrical or flexuous. A useful feature of *C. melinii* not observed in that work but mentioned by Cole and Kendrick (1973) and by Domsch *et al*., (1980) was that the collarettes were inwardly curved.

The isolates did not correspond to the type description of *C. fastigiata* in that very few of the multiple branched or verticillate conidiophores characteristic of *C. fastigiata* were seen. *C. fastigiata* also had globose and subglobose conidia. Such conidia were very rarely found in the *C. luteo-olivacea* and *C. melinii* isolates.

After 2 months of evaluation, no sexual fruiting bodies were produced by crossing *C. luteo-olivacea* with each other, or *C. melinii* isolates with each other.

**Molecular analysis**

The ITS phylogeny (Figure 5) clearly showed that the isolates were either grouped with the reference sequences of *C. luteo-olivacea,* with a bootstrap support of 85%, or with the reference sequences of *C. melinii,* with a bootstrap support of 72%. No BT or EF sequences of any *Cadophora* species were available on GenBank, therefore, no analyses were made with these sequences. Minor differences were found among the *C. luteo-olivacea* sequences with 3 nucleotides varying in the ITS region, 3 nucleotides in the BT region, and 2 nucleotides in the EF region. The *C. melinii* isolates from grapevines were very similar, with 1 nucleotide varying in the ITS region, 2 nucleotides in the BT region, and 2 nucleotides in the EF region. Sequences in the *C. melinii* clade exhibited more variation when the isolates came from European silver fir or kiwi.

**Pathogenicity tests**

The assays on lesion length and on shoot dry weight gave similar results ($P$=0.4224 and $P$=0.5180 respectively) so that these results were combined in a single analysis. Analysis of variance on the lesion length of the rootstocks detected a significant treatment effect ($P$<0.001; ANOVA not shown). All *Cadophora* isolates except *C. melinii* caused lesions in the rootstock xylem that were significantly longer than the lesions in the con-

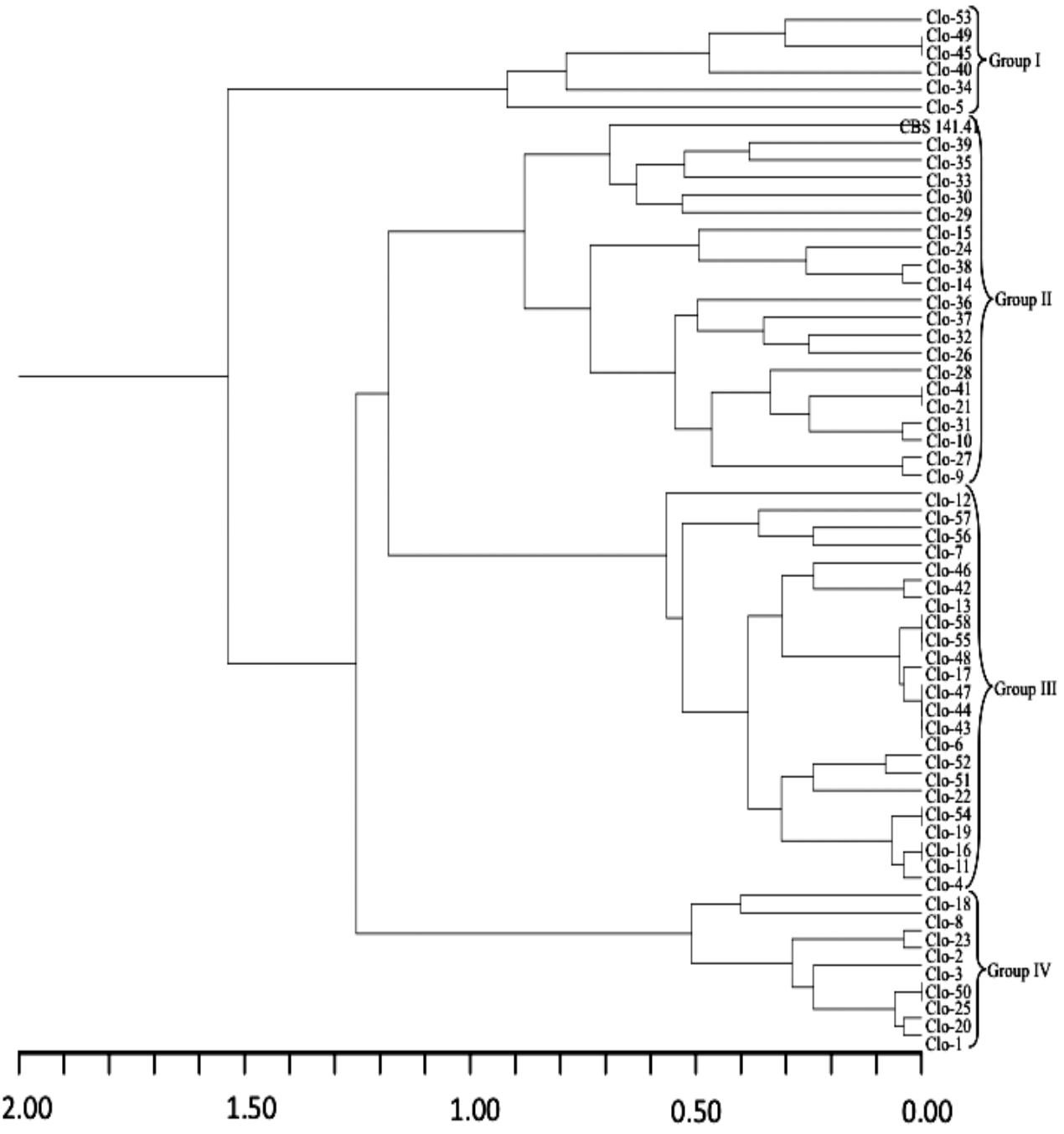


Figure 3. Dendrogram showing phenotypic relatedness, based on morphological and colony characters of 59 *Cadophora luteo-olivacea* isolates. Cluster analysis was performed using the Unweighted pair group with arithmetic average method in the NCSS program. The correlation cophenetic value (*r*) was 0.9419.

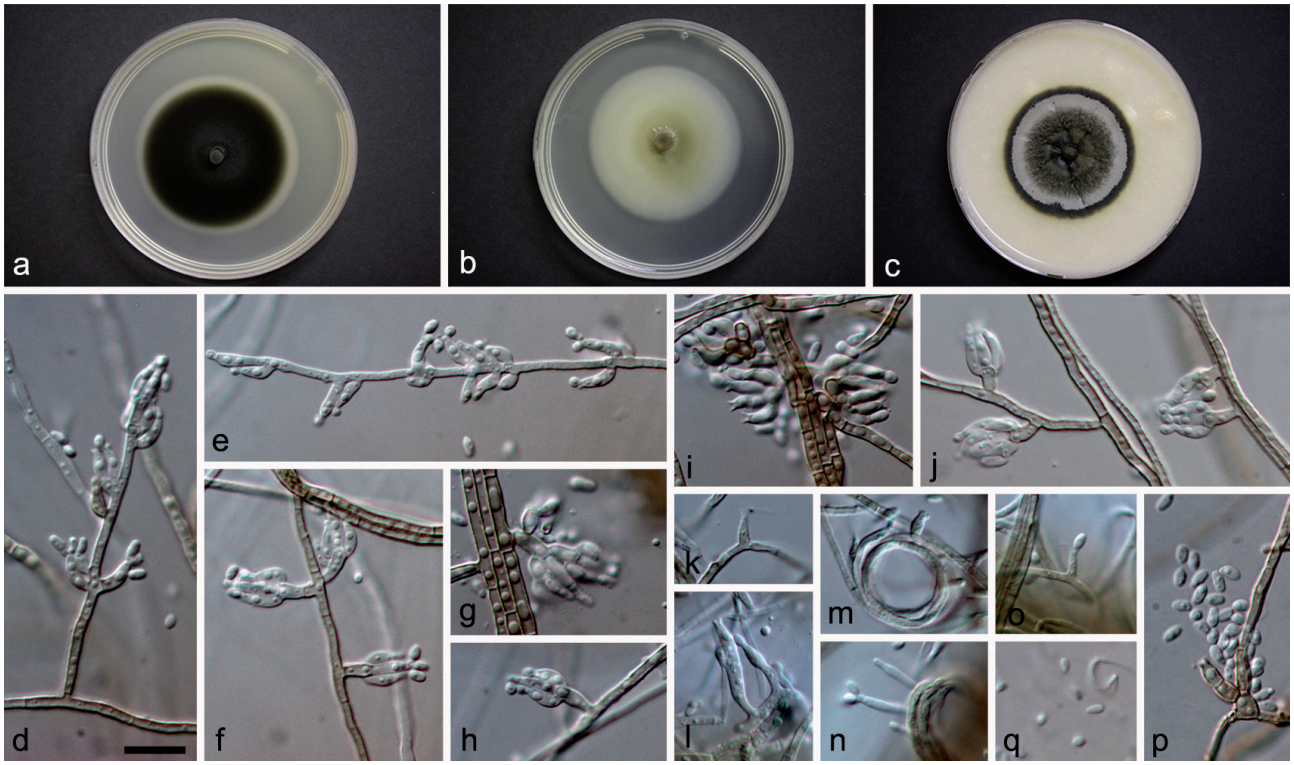


Figure 4. *Cadophora melinii*. a–c. Sixteen-day-old colonies on MEA (a), PDA (b) and OA (c). d-p. Aerial structures on MEA; d-p, conidiophores and phialides; q. conidia. Scale bar: d–q=10 μm. Scale bar for d also applies to e–q.

trols (Table 3). The lesion length of the *C. luteo-olivacea* isolates did not differ between rootstock 140 Ru and rootstock 161-49 C. On rootstock 1103 P, isolate Clo-1 caused the longest lesions, but the differences with Clo-5 and Clo-33 were not significant. In rootstock 110 R, isolate Clo-54 caused the longest lesions, but they did not differ significantly from those of Clo-1 or Clo-5.

Analysis of variance of the shoot dry weight on grapevine rootstocks detected a significant treatment effect on rootstocks 140 Ru and 110 R ($P<0.001$; ANOVA not shown). There was no significant treatment effect on rootstocks 161-49 C and 1103 P ($P>0.05$; ANOVA not shown). In both rootstock 140 Ru and rootstock 110 R, the shoot dry weight of *Cadophora*-inoculated vines was significantly lower than that of the control vines.

All fungi were reisolated from inoculated grapevine rootstocks on MEAS fulfilling Koch's postulates. No *Cadophora* species was isolated from any of the control vines.

## Discussion

*Cadophora luteo-olivacea* and *C. melinii* isolates from grapevines showing black vascular streaking and decline symptoms characteristic of Petri disease, and from different stages of the grapevine nursery process were characterized.

DNA analysis indicated that there was little genetic variation within the isolates of *C. luteo-olivacea* and within the isolates of *C. melinii*. In each species, selected genotypes were dominant. Similar findings were reported by Rooney-Latham (2005), who characterized 16 *C. luteo-olivacea* isolates from affected grapevines in California by molecular analysis of the ITS. The occurrence of the same genotype in different vineyards and production areas suggests that the long-range dispersal of inoculum by airborne spores or the transport of infected plant material has an important role in causing the spread of the fungus. The overall low level of genetic variation confirmed that asexual reproduction was dominant in the field. Attempts

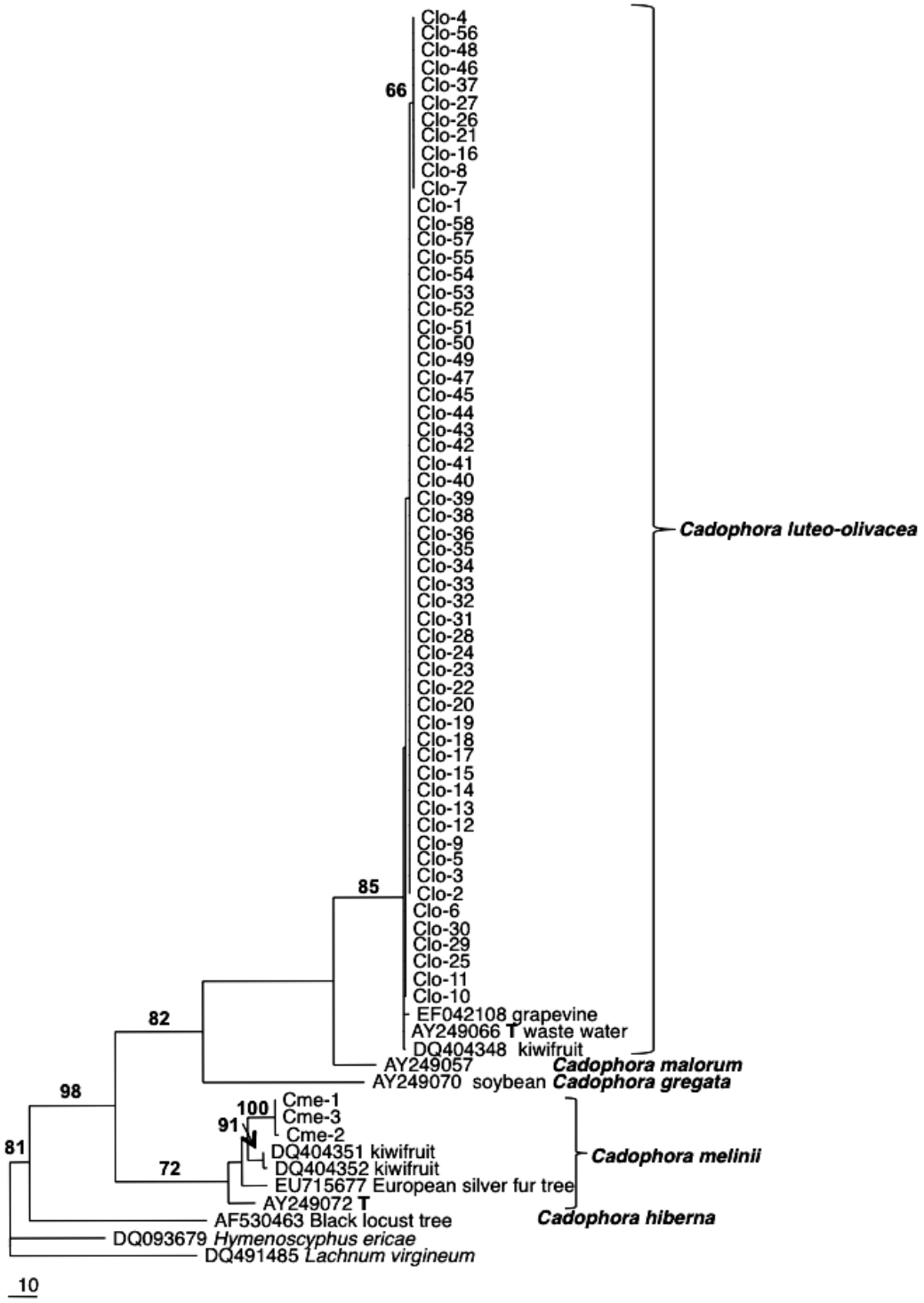


Figure 5. One of the 24 most parsimonious trees obtained from heuristic searches of ITS sequences (Length=312 steps; CI=0.853; RI=0.892; RC=0.761). Bootstrap support values above 65% are shown at the nodes as well as the probability values. *Hymenoscyphus ericae* and *Lachnum virgineum* were used as outgroups. Sequences marked with Clo and Cme were generated in this study and were isolated from grapevines. A 'T' indicates the sequence of the ex-type culture of the species.

Table 3. Means lesion length and shoot dry weight from grapevine rootstocks 14 weeks after inoculation with *C. luteo-olivacea* and *C. melinii* isolates.

| Isolate | Mean lesion length (mm)[a] | | | | Shoot dry weight (g)[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | 140 Ru | 161-49C | 1103 P | 110 R | 140 Ru | 161-49C | 1103 P | 110 R |
| Clo-1 | 91.9 a[b] | 74.9 a | 81.5 a | 66.1 ab | 0.13 b | 0.57 | 1.14 | 0.60 c |
| Clo-5 | 90.0 a | 76.1 a | 71.6 ab | 71.9 ab | 0.71 b | 0.33 | 0.96 | 1.53 b |
| Clo-33 | 90.9 a | 72.6 a | 69.6 ab | 52.1 b | 0.90 b | 0.59 | 0.76 | 1.3 bc |
| Clo-54 | 90.3 a | 69.4 a | 67.6 b | 78.5 a | 0.97 b | 1.55 | 0.96 | 0.77 bc |
| Cme-2 | 8.4 b | 4.5 b | 6.3 c | 9.5 c | 0.52 b | 1.28 | 1.02 | 0.67 bc |
| Control | 4.1 b | 1.4 b | 2.8 c | 3.5 c | 2.05 a | 0.93 | 1.92 | 2.6 a |
| LSD ($P$<0.05) | 9.3 | 9.1 | 11.2 | 17.7 | 0.80 | nd | nd | 0.74 |

[a] Means followed by the same letter are not significantly different ($P$<0.05).
[b] Average of two groups of 8 cuttings each.
nd, Not determined.

to induce teleomorph production from *C. luteo-olivacea* or *C. melinii* isolates were unsuccessful.

Based on their phenotypic characters, *C. luteo-olivacea* isolates clustered into four groups. These isolates did not group according to their geographical origin or their place of collection. Different morphological groups occurred in the same vineyard or grapevine nursery. Colony characters and pigment production were the most useful parameters by which these groups could be distinguished. Even when isolates of *C. luteo-olivacea* had different colony colours and textures, the conidiophore and conidial dimensions were quite homogeneous. The relevance of these morphological groups is doubtful since little variation was found in the three gene areas sequenced. Rooney-Latham (2005), who also studied the phenotypical variation of *C. luteo-olivacea* isolates from California, reported that on MEA after 14 days all these isolates varied only in colour, from cream to buff. In our study, the cardinal temperatures for the growth of isolates of *C. luteo-olivacea* and *C. melinii* were minimum 5ºC, maximum 30ºC and optimum 20-25ºC. This result was consistent with Rooney-Latham (2005), who found that *C. luteo-olivacea* did not grow at temperatures at or above 35ºC.

*Cadophora luteo-olivacea* is closely related to *C. malorum,* resembling it in its morphology, biology and ITS sequence (Harrington and McNew, 2003). Both *C. luteo-olivacea* and *C. malorum* also occur as endophytes, the former in grapevines (Halleen *et al*., 2007), and the latter in kiwifruit (Prodi *et al*., 2008), and they therefore need to be carefully distinguished. Schol-Schwarz (1970) considered *C. luteo-olivacea* (as *P. luteo-olivacea* F.H. Beyma) to be a synonym of *C. malorum* (as *P. malorum* Kidd & Beaum.); however, the present work and Harrington and McNew (2003) demonstrated that these species are distinct by their ITS sequence data.

In the pathogenicity tests, the Spanish isolates of *C. luteo-olivacea* caused lesions in the xylem of all grapevine rootstocks inoculated and reduced shoot weight in rootstocks 140 Ru and 110 R. These results are consistent with Halleen *et al*. (2007), who found that this species when artificially inoculated colonized grapevine pruning wounds and caused trunk lesions, and was therefore potentially a pathogen. In contrast, *C. melinii* did not cause significant lesions in the xylem of any rootstock, although it reduced shoot weight in both rootstock 140 Ru and rootstock 110 R. *C. melinii* severely affects kiwi plants in Italy (Prodi *et al*., 2008); however, there is no convincing report that it significantly affects grapevines.

The occurrence of viable propagules of *C. luteo-olivacea* at different stages of the propagating process also demonstrates that grapevine nurseries are an important source of inoculum for this spe-

cies. There is therefore a need to develop measures to control *C. luteo-olivacea* in nurseries. Gramaje *et al.* (2010) investigated *in vitro* the sensitivity of *C. luteo-olivacea* to hot-water treatments and found that conidial germination of this fungus was inhibited at temperatures above 51ºC, while treatments up to 54ºC for 60 min were necessary to inhibit mycelial growth.

The frequency of isolation of *C. luteo-olivacea* is increasing in vine-growing areas worldwide (Halleen *et al.,* 2005; Overton *et al*., 2005; Rooney-Latham, 2005; Abreo *et al.*, 2008; Manning and Munday, 2009). Further research on the distribution and epidemiology of this pathogen is therefore important and should aid in finding an effective way to control this pathogen.

## Acknowledgements

This research was financially supported by the Projects AGL2006-11884-C04-01 (Ministerio de Educación y Ciencia, Spain), RTA2007-00023-C04-03 and RTA2010-00009-C03-03 (Programa Nacional de Recursos y Tecnologías Agrarias, Ministerio de Educación y Ciencia, Spain), and the European Regional Development Fund (ERDF). We thank V. Garrigues for technical assistance.

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*Accepted for publication: March 11, 2011*