CSIRO PUBLISHING
www.publish.csiro.au/journals/app *Australasian Plant Pathology*, 2004, **33**, 295–299

SHORT RESEARCH NOTE

Cryptovalsa ampelina, a forgotten shoot and cane pathogen of grapevines

L. Mostert[A,D], *F. Halleen*[B], *M. L. Creaser*[C] *and P. W. Crous*[A]

[A]Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands.
[B]ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch, 7599, South Africa.
[C]South Australian Research and Development Institute, GPO Box 397, Adelaide 5001, Australia.
[D]Corresponding author; email: mostert@cbs.knaw.nl.

Abstract. A diatrypaceous ascomycete with polysporous asci and reniform to allantoid, pigmented ascospores was isolated from grapevine canes in South Africa and Australia. The fungus was identified as *Cryptovalsa ampelina* based on its characteristic morphology. Subsequent phylogenetic analyses confirmed this species to belong to the Diatrypaceae. Pathogenicity was also confirmed by means of wound inoculations on grapevine canes.

Additional keywords: Canker pathogen, *Eutypa lata*, ITS rDNA sequence data.

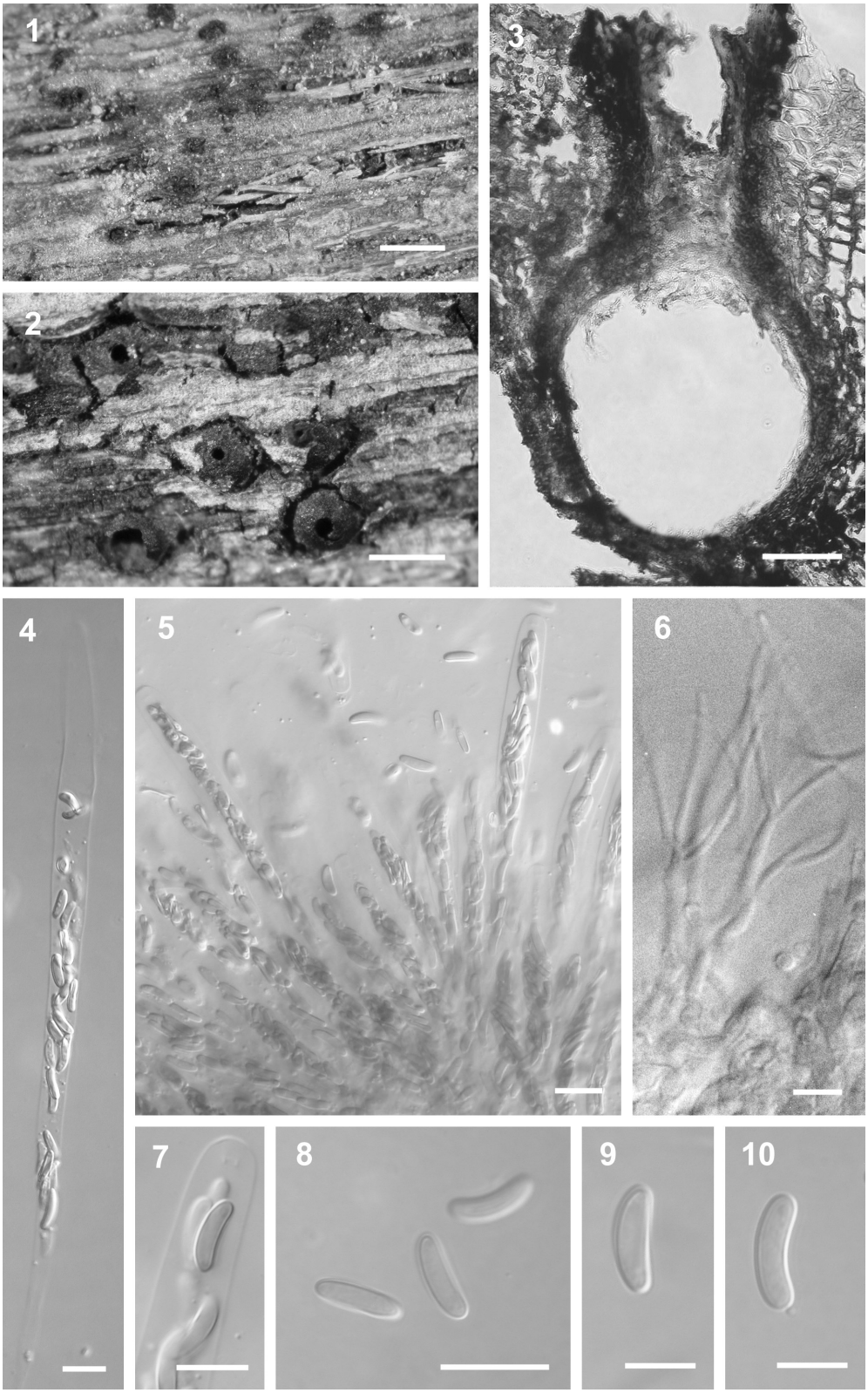
As part of an ongoing study of cane and trunk pathogens of grapevines (*Vitis vinifera*), several collections of a rather unusual ascomycete fungus were obtained from vines in Australia (two vineyards in Coonawarra and one in Eden Valley) and South Africa (five vineyards in the Western Cape) from 1999 to 2002. In his search for stromata of *Eutypa lata* (Pers.: Fr.) Tul & C. Tul. on grapevines in South Africa, Ferreira (1987) encountered another ascomycete which was 'quite abundant' on vine canes and wood. Although apparently similar to the Eutypa dieback pathogen, *Eutypa lata*, it could be distinguished by its characteristic polysporous, long-stipitate asci, and pigmented ascospores, which determined this fungus to be a member of the genus *Cryptovalsa* Ces. & De Not. (Glawe and Rogers 1984).

Although there are several *Cryptovalsa*-like fungi known to occur on grapevines, the two commonly acknowledged species of *Cryptovalsa* from this host are *Cryptovalsa ampelina* (Nitschke) Fuckel (Saccardo 1882) and *Cryptovalsa protracta* (Pers.) De Not. (Pantidou 1973). The species occurring on vines in South Africa has previously been identified as *Cryptovalsa* cf. *ampelina* (Ferreira and Augustyn 1989). By using strains of this fungus to inoculate apricot (*Prunus armeniaca*) branches, Ferreira (1987) concluded that it induced internal wood discoloration symptoms similar to that caused by *E. lata*. *E. lata* is the causal organism of apoplexy, gummosis or dieback of apricots (Carter 1957) and Eutypa dieback of grapevines (Carter 1988). Inoculations of vine pruning stubs with ascospores of *C. ampelina* demonstrated its ability to infect wounds shortly after pruning (Ferreira 1987). However, it was unclear whether infections would lead to external dieback symptoms typically associated with Eutypa dieback.

Grapevine shoots containing perithecia of *C. ampelina* from South Africa and Australia were collected from 1999 to 2002. Canes were soaked in water for 20 min and the perithecia then removed with a sterile scalpel. Squash mounts were made on glass slides in lactic acid to examine asci and ascospores. Dilution plates on 2% malt-extract agar (MEA; Biolab, Midrand, Johannesburg) were made to obtain single-ascospore isolates. Vertical sections were made of perithecia using a Leica CM1100 freezing microtome. Sections (10 μm) were mounted in lactic acid for examination. Structures were measured at 1000× magnification. The fungus was plated on divided plates with potato-dextrose agar (PDA; Biolab, Midrand, Johannesburg) on the one side, and water agar (WA; Biolab, Midrand, Johannesburg) with double autoclaved grapevine canes pieces, on the other. Plates were maintained at 25°C under continuous near-ultraviolet light. Although the anamorph formed after 33 days, no teleomorph was observed after 2 months of incubation under the conditions described above, and as far as we could establish, no teleomorph of this genus has yet been induced in culture (Glawe and Rogers 1984).

Cryptovalsa ampelina (Nitschke) Fuckel, in Fuckel, Symbolae Mycologicae, Figs 1–10
Beiträge zur Kenntnis der Rheinischen Pilze, p. 212. 1870.
Basionym: *Valsa ampelina* Nitschke, Pyrenomycetes Germanici **1**, 156, 1867.
Anamorph: *Libertella* sp.

 10.1071/AP03095 0815-3191/04/020295



Figs 1–10. Morphology of *Cryptovalsa ampelina*. (1–2) Perithecia on grapevine cane. (3) Vertical section through perithecium with neck and two-layered peridium. (4) Spindle-shaped ascus. (5) Squash mount with asci. (6) Conidiophores with conidiogenous cells and conidia. (7) Ascus tip with subapical ring. (8–10) Allantoid to reniform ascospores. Bar: Figs 1–2 = 1 mm; Fig. 3 = 100 μm; Figs 4–8, = 10 μm; Figs 9–10 = 5 μm.

Perithecia embedded in bark, singly erumpent, in rows or in small groups (Figs 1–2). *Stromata* poorly developed, visible around and between perithecia and immersed in bark.

Ascomatal venter 300–630 µm high, 300–500 µm diam, flask-shaped, with protruding necks, up to 420 µm long and up to 250 µm wide (Fig. 3); single erumpent perithecial necks periphysate, with outer layer of ascomatal neck consisting of dark melanised cells. *Peridium* layer up to 65 µm wide, comprising an inner layer of hyaline, compressed cells of *textura angularis*, 15–30 µm, and an outer layer of thick-walled brown cells of *textura globulosa*, 13–35 µm. *Asci* (70–) 89–104 (–125) × (7–) 8 (–9) µm (n = 24), polysporous, spindle-shaped, long-stipitate, tapering to the peduncle, with a subapical ring (Figs 4, 5, 7). *Ascopores* (7–) 8–9 (–11) × 2 µm, allantoid to reniform, non-septate, pale brown, smooth (Figs 8–10). *Conidiophores* hyaline, subcylindrical, 1–3-septate, 7–23 × 2 µm, branched above (Fig. 6). *Conidiogenous cells* hyaline, subcylindrical, 3–13 × 1–1.5 µm, with one to two conidia forming sympodially per conidiogenous cell. *Conidia* (17–) 20–21 (–23) × 1 µm, hyaline, smooth, filiform, slightly curved to hamate.

Specimens examined: SOUTH AFRICA: Western Cape Province, Stellenbosch, Uiterwyk Estate, on canes of *Vitis vinifera* (Cabernet Sauvignon), 2000, F. Halleen (herb. CBS 6582, culture CBS 112326); Hartenberg Estate, on canes of Cabernet Sauvignon, 2001, L. Mostert, herb. CBS 6597, cultures STE-U 5621 and 5622. AUSTRALIA: South Australia, Coonawarra from *Vitis vinifera* (Shiraz), Dec. 1999, M.L. Creaser (herb. CBS 6583, culture CBS 112247).

Nitschke (1867) separated *C. protractra* and *C. ampelina* on the basis that *C. ampelina* had globose perithecia, whereas those of *C. protracta* were more ovoid. Perithecia on all specimens collected in the present study are globose. Furthermore, perithecia of *Valsa protracta* are often arranged in groups of four (Nitschke 1867), which is in contrast to our material, and to *C. ampelina*. Ascospore dimensions of the Australian and South African collections also correspond with that of *C. ampelina* (9–10 × 2.5 µm), and not *C. protracta* (10–12 × 2.5–3 µm) (Nitschke 1867). *C. ampelina* readily produces an anamorph on grapevine canes, as well as in culture. Although the anamorph was already noted by Nitschke (1867), it was only later named by Saccardo (1884) as *Cytospora ampelina* Sacc. *C. ampelina* has a *Libertella* Desm. anamorph. However, the conidial dimensions (17–) 20–21 (–23) × 1 µm do not overlap with those given by Saccardo for *C. ampelina* (16 × 2.5 µm). Because many *Cytospora*-like coelomycetes occur on vines, it is possible that the anamorph-teleomorph connection reported by Saccardo (1884) is incorrect. We, therefore, refrain from proposing a formal recombination in *Libertella* until type material of the anamorph has been located and re-examined.

To determine the phylogeny of this fungus, and confirm that the Australian and South African cultures were indeed the same, genomic DNA was extracted from CBS 112326, STE-U 5621, 5622 (South Africa), and CBS 112247 (Australia), using the isolation protocol of Lee and Taylor (1990). The 5.8S nuclear rRNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) were amplified and sequenced using primers ITS1 and ITS4 (White *et al.* 1990). A consensus sequence was computed from the forwards and reverse sequences with SeqMan from the Lasergene package (DNAstar, Madison, WI). The sequences were consequently manually aligned in Sequence Alignment Editor version 2.0a11 (Rambaut 2002). Additional sequences from different genera within the Diatrypaceae family as well as from the Xylariaceae (closely related to Diatrypaceae) were retrieved from GenBank. Phylogenetic analyses conducted with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000) clearly illustrated that *C. ampelina* belongs to the Diatrypaceae (Fig. 11). The *Cryptovalsa* sequences were deposited in GenBank (AY307106–9) and the alignment in TreeBase S961. As far as we could establish, no other sequences of *Cryptovalsa* species have previously been published or released on GenBank.

To confirm the pathogenicity of *C. ampelina*, wound inoculations were conducted on 1-year-old potted grapevine plants (grafted nursery plants with scion cultivar *Chenin blanc*). The wounds were made on the dormant canes between the graft union and distal end of the scion using a sterile cork borer (4 mm diameter). Colonised mycelial plugs of the same diameter (CBS 112326) were inserted into the wounds and sealed with Parafilm. Uncolonised PDA plugs were used for control inoculations. The experiment was evaluated after 12 months by measuring the lesion length and confirming the presence of fungus by re-isolating from the lesion margins. A complete randomised design experiment was performed in a glasshouse with two treatments randomly allocated to 16 pot plants. The data were subjected to a one-way analyses of variance using SAS version 8.2 (SAS 1999). Shapiro-Wilk's test was performed to test for non-normality (Shapiro and Wilk 1965). Because there was no evidence against normality (P = 0.786), no transformation was needed. Lesions caused by the fungus were dark brown in colour and extended both up and downwards from the point of inoculation. Stem discoloration was observed on all inoculated plants. However, lesions were significantly longer (mean lesion length, 22.96 mm, s.e. ± 1.488 mm) than the pale brown lesions extending from the control inoculations (mean lesion length 11.67 mm, s.e. ± 0.655 mm) (P < 0.01), confirming that *C. ampelina* was pathogenic to grapevines. The paler, smaller lesions associated with control inoculations could not be attributed to fungal infections, and resembled those found in other, similar studies (Sparapano 2001). The fungus was re-isolated from all inoculated plants, but no fungi were isolated from the controls. Presently *C. ampelina* does not

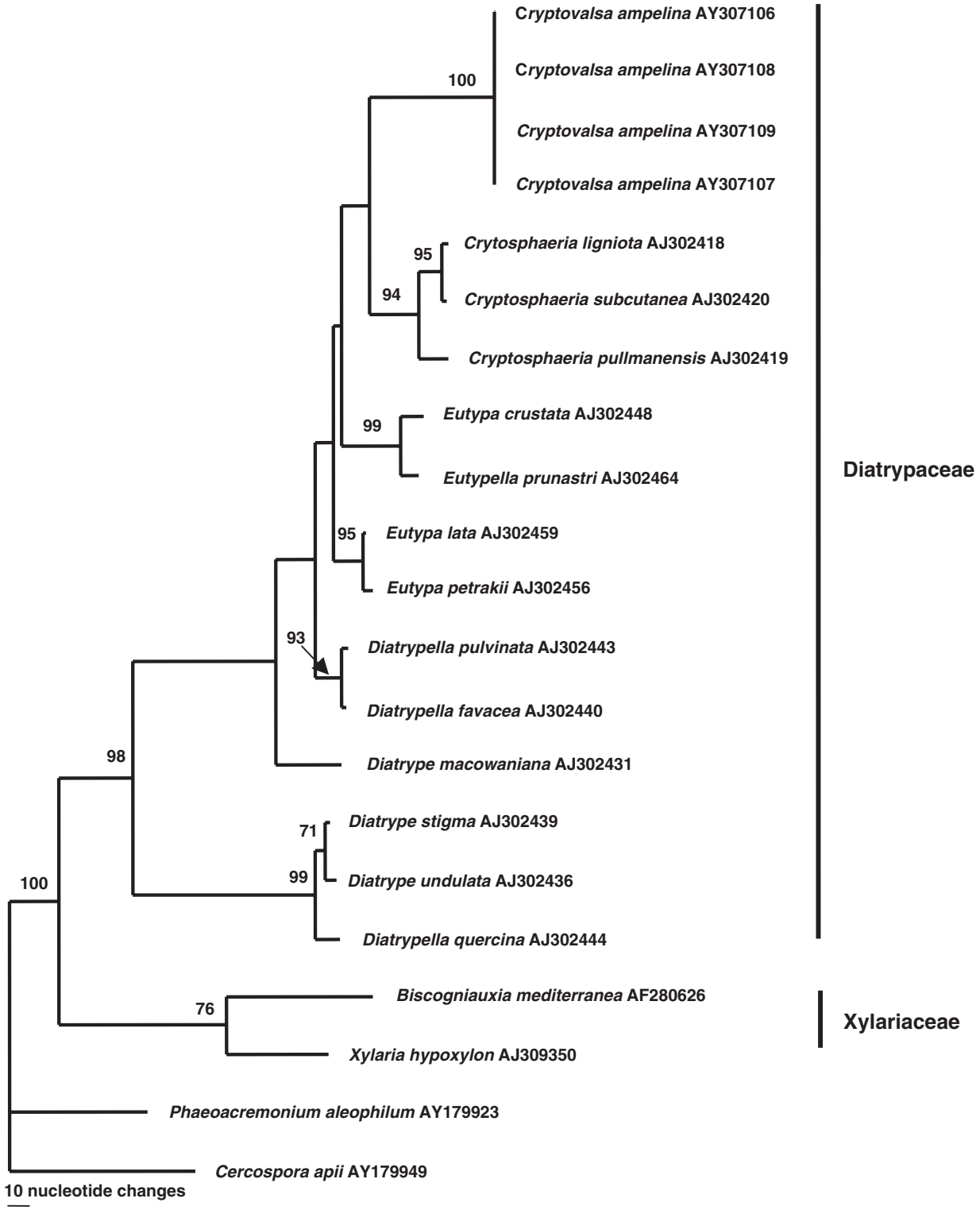


Fig. 11. Most parsimonious tree (length 623 steps, consistency index = 0.724, retention index = 0.698, rescaled consistency index = 0.505) obtained from a heuristic search using the 5.8S rRNA gene and flanking ITS1 and ITS2 regions. Bootstrap support values from 1000 replicates are shown above the nodes. *Phaeoacremonium aleophilum* and *Cercospora apii* were used as outgroups.

appear to be a highly virulent pathogen of grapevines, which is in accordance with our field observations. Its occurrence, relevance and ecological role in vineyards, however, still need to be determined.

Acknowledgements

The authors gratefully acknowledge Jan van Niekerk (Dept. of Plant Pathology, Univ. of Stellenbosch) and Sieyaam Safodien (ARC-Infruitec, Stellenbosch), who provided some of the isolates and sequence data used in the present study.

References

Carter MV (1957) *Eutypa armeniacae* Hansf. and Carter, sp. nov., an airborne vascular pathogen of *Prunus armeniaca* L. in southern Australia. *Australian Journal of Botany* **5**, 21–35.

Carter MV (1988) Eutypa dieback. In 'Compendium of grape diseases'. (Eds RC Pearson, AC Goheen) pp. 32–35. (APS Press: St Paul, USA)

Ferreira JHS (1987) Dieback of grapevines in South Africa. PhD Thesis, University of the Western Cape, South Africa.

Ferreira JHS, Augustyn OPH (1989) Differentiation between *Eutypa lata* and *Cryptovalsa* cf *ampelina* by means of cellular fatty acid analysis. *South African Journal of Enology and Viticulture* **10**, 18–22.

Glawe DA, Rogers JD (1984) Diatrypaceae in the Pacific Northwest. *Mycotaxon* **20**, 401–460.

Lee SB, Taylor JW (1990) Isolation of DNA from fungal mycelia and single spores. In 'PCR protocols: a guide to methods and applications'. (Eds MA Innis, DH Gelfand, JJ Sninsky, TJ White) pp. 282–287. (Academic Press: New York, USA)

Nitschke TH (1867) Pyrenomycetes Germanici. (Verlag von Eduard Trewendt: Breslau, Germany)

Pantidou ME (1973) 'Fungus–host index of Greece.' (Benaki Phytopathogical Institute: Kiphissia, Athens)

Rambaut A (2002) 'Sequence alignment editor. Version 2.0.' (Department of Zoology, University of Oxford: Oxford)

Saccardo PA (1882) Pyrenomycetum. *Sylloge Fungorum* **1**, 187.

Saccardo PA (1884) Sphaeropsidearum et melanconiearum. *Sylloge Fungorum* **3**, 257.

SAS (1999) 'SAS/STAT user's guide. Version 8, 1st printing, Vol. 2.' (SAS Institute Inc.: North Carolina)

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591–611.

Sparapano L, Bruno G, Graniti (2001) Three-year observation of grapevines cross inoculated with esca-associated fungi. *Phytopathologia Mediterranea* **40**, S376–S386.

Swofford DL (2000) 'PAUP* 4.0: phylogenetic analysis using parsimony.' (Sinauer Associates Inc.: Massachusetts)

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In 'PCR protocols: a guide to methods and applications'. (Eds MA Innis, DH Gelfand, JJ Sninsky, TJ White) pp. 315–322. (Academic Press: New York, USA)

Received 20 June 2003, accepted 8 October 2003