



Munkovalsaria donacina from grapevines and Desert Ash in Australia

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Abstract

A bitunicate ascomycete bearing brown 1-septate ascospores consistent with the botryosphaeriaceous genera *Dothiorella* and *Spencermartinsia* was observed on discarded grapevine (*Vitis vinifera* L.) canes, and in culture was purported to yield a coelomycetous asexual morph in *Spencermartinsia*. However, morphological studies and amplification and sequencing of the internal transcribed spacer (ITS) region and portions of the small (18S) and large (28S) subunits of the nuclear ribosomal RNA gene identified the ascomycete as *Munkovalsaria donacina* (Niessl) Aptroot, and revealed that the fungus is neither connected to an asexual morph in *Spencermartinsia*, nor related to the Botryosphaeriaceae (Botryosphaeriales). Instead, these findings provide the first report of *M. donacina* from grapevines and Desert Ash (*Fraxinus angustifolia* subsp. *angustifolia* Vahl), extending the range of the taxon to 38 species in 24 families.

Keywords – Botryosphaeriaceae – Botryosphaeria dieback – grapevine trunk diseases

Introduction

In November 2009 during an industry survey of vineyards in Western Australia, an unidentified ascomycete bearing bitunicate asci comprising eight brown, thick-walled, 1-septate ascospores, consistent with those of the grapevine (*Vitis vinifera* L.) trunk disease pathogens *Dothiorella* and *Spencermartinsia*, was observed on discarded grapevine canes (Pitt et al. 2013). Following transfer of ascospores to pure culture, a coelomycete with brown, thick-walled, two-celled conidia, pigmented and septate prior to discharge from conidiogenous cells developed in culture. This species was similar to, but distinct from the generic type, *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips et al., both morphologically and phylogenetically. However, at the time of our original study, we did not introduce a novel combination for this species because supposed ascospores of the fungus lacked the terminal apiculi Phillips et al. (2008) used to typify the genus.

In the interim, three new species have been described in *Spencermartinsia* from hosts originating from Iran, New Zealand and Spain (Abdollahzadeh et al. 2014), all circumscribed based on phylogeny and morphology of asexual morphs. However, sexual morphs were not observed for any of these species and it remains unclear whether apiculate ascospores are a consistent character of the genus (Phillips et al. 2013). In an attempt to clarify the nature of the association between the

bitunicate ascomycete and the *Spencermartinsia* sp. that arose from our original survey, additional field collections were undertaken in September 2013, with the ascomycete collected once again on discarded grapevine canes as well as fallen branches of Desert Ash (*Fraxinus angustifolia* subsp. *angustifolia* Vahl), a popular deciduous tree commonly found in gardens and streetscapes. However, in this instance, the transfer of ascospores to culture did not reveal an asexual morph in *Spencermartinsia*, and instead gave rise to a fungus that was morphologically and phylogenetically unrelated to Botryosphaeriaceae.

The aim of this study was to identify the aforementioned bitunicate ascomycete and resolve the nature of the association between this fungus and the *Spencermartinsia* sp. that arose from our original collections from diseased grapevines in Western Australia.

Materials and Methods

Fungal isolates

Axenic single spore cultures of an ascomycete observed both on discarded grapevine canes and fallen branches of Desert Ash were obtained on 1.5% water agar (BD Difco technical agar; Benton, Dickinson and Company, North Ryde, Australia) according to Crous (2000). After incubating overnight at 25°C, single germinating ascospores were then transferred to potato dextrose agar supplemented with 50 µg/mL of streptomycin sulfate (PDA-Strep; Sigma-Aldrich, Castle Hill, Australia).

Morphology and culture characteristics

Perithecial contents from ascomata were mounted in water, and microscopic examinations thereof conducted with a Zeiss Axiophot compound microscope with DIC optics and captured using a Nikon D700 digital camera. Digital images of fungal strains including the lengths and widths of ascospores were recorded using Image-Pro© Plus software (v4.5.1.29, Media Cybernetics, Inc.). Mean, standard deviation and 95% confidence intervals were recorded for at least 50 ascospores per specimen and are given in µm, with extremes in brackets. To study colony morphology, cultures were maintained in incubators under controlled conditions of 12/12 hour photoperiod (fluorescent light) at 25°C for eight weeks. Colony colour (Rayner 1970) and growth rates (Sánchez et al. 2003) were determined on PDA. Material comprising ascomata and cultures of the fungus were submitted to the Plant Pathology Herbarium (DAR), Orange, Australia (DAR80529, DAR82454, DAR82455).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from seven day-old pure cultures grown in potato dextrose broth (PDB; Oxoid Ltd., Basingstoke, Hampshire, England) incubated at room temperature as described by Pitt et al. (2013). Amplification and sequencing of the internal transcribed spacer (ITS) region was performed as described by Pitt et al. (2013) using primer pairs ITS1 and ITS4 (White et al. 1990), while portions of the small subunit (18S) nuclear ribosomal RNA gene (SSU) and large subunit (28S) nuclear ribosomal RNA gene (LSU) were amplified and sequenced according to Zhang et al. (2009) using primer pairs, NS1 and NS4 (White et al. 1990) and LR5 and LROR (Vilgalys & Hester 1990, Rehner & Samuels 1994), respectively. DNA sequences of the ITS, SSU and LSU regions from isolate DAR82455 were submitted to GenBank (KJ628375, KJ628376, KJ628377).

Results

Amplification and sequencing of the ITS, SSU and LSU regions from an asexual morph that developed from single ascospores of a bitunicate ascomycete collected from grapevines in Western Australia displayed high similarity to the didymosporous genera *Phaeodothis* Syd. & P. Syd. (95-99% homology over the three regions), *Montagnula* Berl. (88-99%) and *Munkovalsaria* Aptroot (88-97%).

When examined, material collected from discarded grapevine canes and fallen branches of Desert Ash bore immersed, gregarious, clypei encircled ascomata. Asci were clavate, bitunicate with a long pedicel and small ocular chamber and comprised eight, ellipsoid ascospores per ascus, arranged irregularly to biserially, initially hyaline, thin-walled and unicellular, but becoming brown, thick-walled and unequally 1-septate with age. Ascospores were strongly constricted at the septum with the upper cell wider and pointed and lower cell longer and rounded, and morphologically were conspecific in all respects with those of the generic type, *Munkovalsaria donacina* (Niessl) Aptroot (Fig.1).

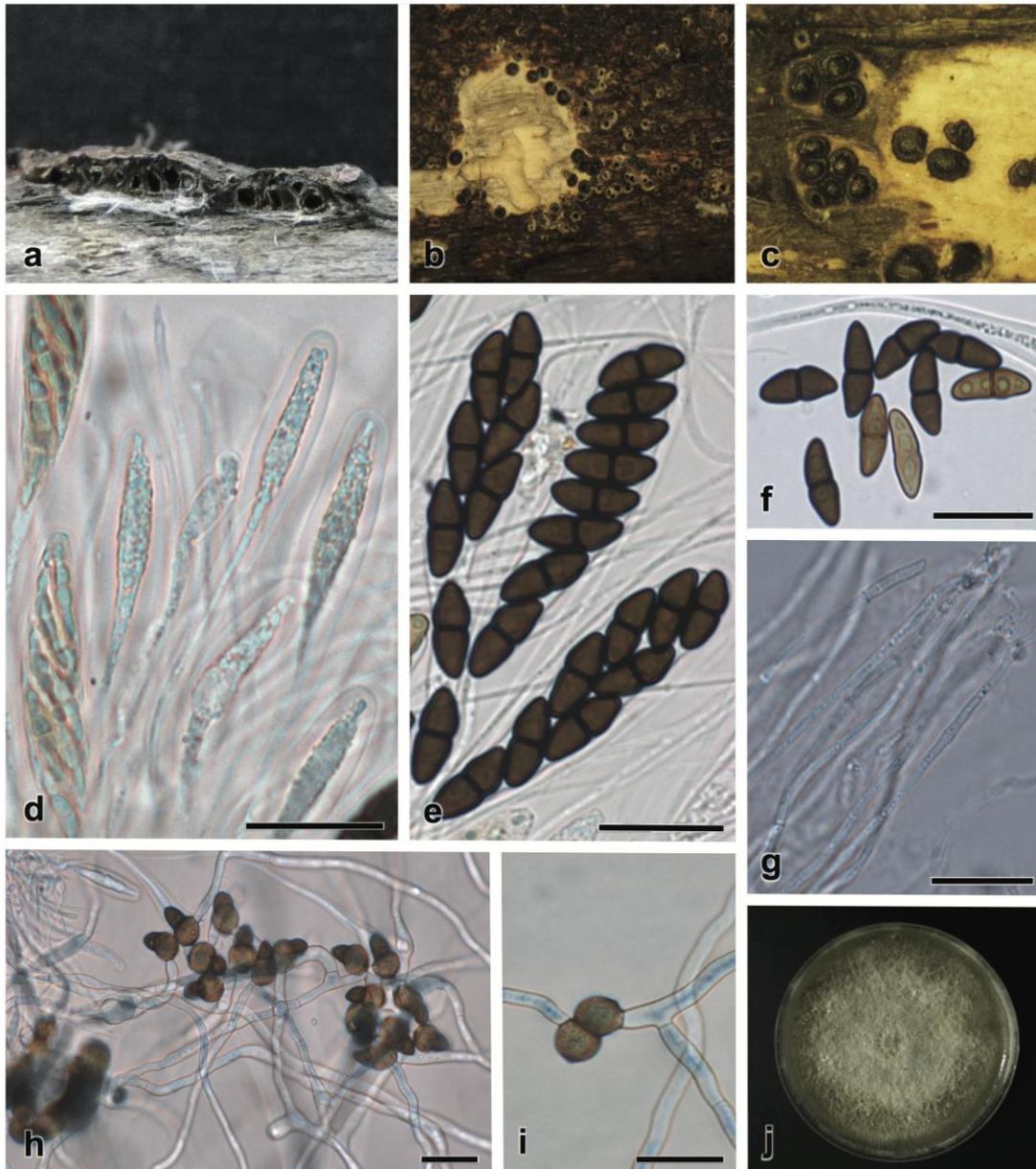


Fig. 1 – *Munkovalsaria donacina*. a) ascomata erumpent on surface of fallen branches of Desert Ash. b) emerging pseudothecial ostioles surrounded by clypeus on discarded grapevine canes. c) aggregated pseudothecial cavities on discarded grapevine canes. d) immature bitunicate asci with long pedicel and small ocular chamber. e) mature asci bearing eight brown 1-septate ascospores arranged biserially. f) immature hyaline and mature brown 1-septate ascospores. g) septate pseudoparaphyses. h) tight clusters of eight germinating ascospores originating from single ascus. i) single germinating ascospore. j) 28 day-old colony of *M. donacina* (DAR82455) on PDA. — Scale bars: d, e, h, i=50 μ m; f, g=20 μ m.

Taxonomy

Munkovalsaria donacina (Niessl) Aptroot, Nova Hedwigia 60(3-4): 346 1995.
MycoBank 413489

Fig. 1

- ≡ *Microthelia donacina* Niessl in Thümen, *Contrib. Flor. Mycol. Lusitan.*: no. 536 1879.
- ≡ *Didymosphaeria donacina* (Niessl) Sacc., *Syll. Fung.* 1: 715 1882.
- ≡ *Didymosphaerella donacina* (Niessl) Cooke, *Grevillea* 18(86): 29 1889.

The material is consistent with *M. donacina*, with immersed to erumpent, black, globose to pyriform, pseudothecial ascomata, arising singly or gregarious with clypei surrounding the ostioles. Asci clavate, bitunicate with a long pedicel and small ocular chamber. Ascospores ellipsoid, eight per ascus, arranged irregularly to biserially, initially hyaline, thin-walled, unicellular, becoming brown, thick-walled, and unequally 1-septate and strongly constricted at the septum, with the upper cell wider and pointed and lower cell longer and rounded, measuring (13.6) 14.8–15.2 (17.3) × (6.6) 7.5–7.7 (8.3) μm, with mean length and width of 15 ± 0.7 × 7.6 ± 0.4 μm, and an average length to width ratio of 2 ± 0.1 (n = 100). Pseudoparaphyses trabeculate, septate and ramified, up to ~3 μm in width.

Cultural characteristics – On PDA colonies filamentous, creamy white with raised dense cottony mycelium in the centre, becoming light to pale citrate (21k) at the margin after 28 days under near ultraviolet light (UVB, 315–280 nm; 12/12 hour photoperiod) at 25°C. Cardinal temperatures for growth: between 5 and 30°C, with an optimum of 25°C, at which colonies averaged 27 mm on PDA after 5 days. Asexual morph unknown.

Hosts – Plurivorous.

Known distribution – AUSTRALIA; BRAZIL; CENTRAL AFRICAN REPUBLIC; CHINA; COLOMBIA; EQUADOR; FRANCE; INDIA; ITALY; JAPAN; NAMIBIA; PAPUA NEW GUINEA; PARAGUAY; PHILIPPINES; PORTUGAL; REPUBLIC OF THE UNION OF MYANMAR; SIERRA LEONE; UNITED STATES.

Material examined – AUSTRALIA, Western Australia, Upper Swan, Noack Road, 300m east of the Great Northern Highway, 31°46'55.30" S, 116.01'40.74" E, 10m asl., discarded canes of *V. vinifera* cv. Cabernet Sauvignon, November 2009, leg. F.P. Trouillas (ascomata on host material, DAR80529); New South Wales, Cessnock, Wine Country Drive, 3.1km north of Cessnock, 32°48'25.66" S, 151°20'57.83" E, 66m asl., fallen branches of *F. angustifolia* subsp. *angustifolia*, September 2013, leg. W.M. Pitt (living culture and ascomata on host material, DAR82454); Pokolbin, Pokolbin Mountain Road, 650m west of McDonalds Road, 32°48'23.30" S, 151°16'29.20" E, 133m asl., discarded canes of *V. vinifera* cv. Shiraz, September 2013, leg. W.M. Pitt (living culture, DAR82455).

Discussion

Previously, we described a bitunicate ascomycete from grapevines that had brown 1-septate ascospores consistent with *Dothiorella* and *Spencermartinsia*, two botryosphaeriaceous genera known to be associated with *Botryosphaeria* dieback of grapevines (Pitt et al. 2013). At the time, the fungus was purported to be connected with an asexual morph in *Spencermartinsia* and we implied, albeit tentatively, that we had observed a new asexual morph-sexual morph relationship in this genus. However, additional morphological studies and DNA sequence comparisons described here have now identified the ascomycete as *Munkovalsaria donacina* and revealed that the fungus is neither connected to an asexual morph in *Spencermartinsia*, nor related to the Botryosphaeriaceae (Botryosphaeriales), and in fact resides in a different family and order (Dacampiaceae, Pleosporales).

Munkovalsaria was originally introduced by Aptroot (1995) to accommodate the redistribution of two species from *Didymosphaeria* Fuck., including *Didymosphaeria donacina* (Niessl.) Sacc. recombined as *M. donacina*, the generic type, and a second unnamed species from Frisullo et al. (1989) that the author described as *Munkovalsaria rubra* Aptroot, Van der Aa & O. Petrini. The genus *Munkovalsaria* is characterized by clavate bitunicate asci comprising eight brown, thick-

walled, 1-septate ascospores not unlike the sexual morphs of *Spencermartinsia*, and currently comprises three species following the addition of *Munkovalsaria appendiculata* Aptroot (Aptroot 2004). Yet, while the sexual morphs of these species, especially those of *M. donacina*, are found readily on the stems and branches of numerous woody hosts (Aptroot 1995), their asexual morphs remain elusive, and rather than coelomycetous, are considered hyphomycetous in character (Aptroot 1995, 2004, Gams 2000), and hence, could not be connected with a sexual morph in *Spencermartinsia*.

While every effort was made to confirm what we believed to be a genuine asexual morph-sexual morph connection in *Spencermartinsia*, including the use of an industry standard technique for the establishment of single ascospore cultures (Crous 2000), it is not uncommon for several different species to be present on a single host, nor given the method, for the propagules of such, to lay together in one spot, making them difficult to separate. Thus, in retrospect there seems little doubt that pycnidia of a *Spencermartinsia* sp. were also present on host material collected during the initial study, and no doubt that conidia of such were discharged along with ascospores of *M. donacina* onto awaiting plates. Thereafter, germinating conidia were obviously confused with ascospores of similar size, shape, colour and degree of septation. However, our previous report (Pitt et al. 2013) was not the first to connect *Munkovalsaria* to a coelomycete, nor to an asexual morph in the Botryosphaeriaceae, with Anahosur (1971) linking *M. donacina*, then *D. donacina*, to *Diplodia*.

In that study, from single ascospore cultures obtained from Wild Sage (*Lantana camara* L.), the author reported a pycnidial fungus resembling *Diplodia* with dark-brown, two-celled conidia of dimensions 16–20 × 2–4 µm. While this description and the photographs provided by the author more closely resemble what we now know as *Dothiorella*, the widths of conidia did not conform to any *Diplodia*, nor *Dothiorella* species currently known from culture (Phillips et al. 2013), and the only specimen of *Didymosphaeria* traceable to the author during this period arose from Indigoberry (*Randia dumetorum* Lam.), since re-described as *Mycomicrothelia subfallens* (Müll. Arg.) D. Hawksw. (Aptroot 1995). Furthermore, the only specimens from Sage were *Calospora lantanae* Anahosur and *Tryblidaria maharastrensis* Anahosur (Anahosur 1969). Still, if Anahosur (1971) did isolate asexual morphs of *Diplodia* or *Dothiorella* in association with *Munkovalsaria*, not unlike we did from grapevines (Pitt et al. 2013), then perhaps these genera merely occupy similar hosts and environments. Conversely, Anahosur (1971) may have isolated instead, one of a number of other quite similar species, or actually meant ‘*Diplodia*-like’, when he referred to the appearance of the conidia.

Munkovalsaria donacina is plurivorous and pantropical to nearly cosmopolitan in distribution, and prior to this study had been isolated from as many as 36 species within 22 families (Aptroot 1995, Hyde et al. 1999, Wang et al. 2004, Thuang 2008). Early reports also linked the fungus to leaf spot of Cluster Yam (*Dioscorea dumetorum* (Kunth) Pax) in Nigeria (Emua & Fajola 1981) and to stem death of Sage in India (Anahosur 1971), but specimens from Yam were re-examined and transferred to *Phaeodothis winteri* (Niessl) Aptroot (Aptroot 1995), and those from Sage could not be verified. In contrast, *M. donacina* has only been reported on one prior occasion in Australia, with Hyde et al. (1999) isolating the fungus from climbing palm (*Calamus australis* Mart.) in Queensland. To our knowledge, this is the first report of *M. donacina* from grapevines and Desert Ash, extending the range of the fungus to 38 species within 24 families.

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