# *Phyllosticta* species on orchids (Orchidaceae), introducing *Phyllosticta speewahensis* sp. nov. (Phyllostictaceae, Ascomycota) from northern Australia

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Several species of *Phyllosticta* (syn. *Guignardia*) have been described from orchids worldwide. A new species, *Phyllosticta speewahensis*, is proposed for a specimen isolated from leaf spots on a hybrid *Vanda* orchid in northern Queensland, Australia. Phylogenetic analysis of the nrDNA internal transcribed spacer region (ITS) and partial translation elongation factor 1-alpha (TEF1) gene sequences showed that *P. speewahensis* is most closely related to *P. hostae*. The likelihood that orchids harbour further cryptic species of endophytic and pathogenic *Phyllosticta* species is discussed.

Keywords: cryptic species, *Phyllosticta*, molecular phylogeny, taxonomy, Orchidaceae.

*Phyllosticta* (syn. *Guignardia*) species are widely distributed as plant pathogens, endophytes and saprobes on a range of hosts (van der Aa 1973; van der Aa & Vanev 2002; Wikee *et al*. 2011, 2013). Several species of *Phyllosticta* were originally described from orchids (Orchidaceae) based on their distinct morphology and host association (Tab. 1). Van der Aa & Vanev (2002) revised *Phyllosticta* based on the literature and examination of type specimens, accepting only *P. aplectri* and *P. capitalensis* amongst the taxa described from orchids.

Recently, several new species of *Phyllosticta* have been discovered from a wide range of plants, using phylogenetic analysis of DNA gene sequences, supported by morphology and host association (Wulanderi *et al*. 2009, Wang *et al*. 2012, Wong *et al*. 2012, Su & Cai 2012, Shivas *et al*. 2012, Crous 2012), including *P. bifrenariae* O. L. Pereira, Glienke & Crous from the orchid *Bifrenaria harrisoniae* in Brazil (Glienke *et al*. 2011). In 2012, a cultivated orchid (*Vanda* hybrid 'Waxy Blue') in northern Queensland was found to have leaf spots colonized by a *Phyllosticta* sp. Morphology and molecular analysis of

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sequence data could not match this taxon with any known species, and thus, it is introduced as new to science in this paper.

**Tab. 1.** Species originally described as *Phyllosticta* from orchids (Orchidaceae).

| Species, authority and year of publication a | Substrate and/or orchid host b | Country of type | Reference |
|---|---|---|---|
| *P. aplectri* Ellis & Everh. 1894 | *Aplectrum hyemale* | USA | van der Aa (1973) |
| *P. capitalensis* Henn. 1908 | leaves of *Stanhopea* sp. | Brazil | Hennings (1908) |
| *P. cymbidii* Sawada 1943[a] | leaves of *Cymbidium aloifolium* | Taiwan | van der Aa (1973) |
| *P. himantoglossi* Petr. 1959[b] | dying leaves of *Himantoglossum hircinum* | Austria | Petrak (1959) |
| *P. imbricata* B. R. D. Yadav & V. G. Rao 1981 | living leaves of *Pholidota imbricata* | India | van der Aa & Vanev (2002) |
| *P. odontoglossi* Verplancke & Claessens 1934[c] | living leaves of *Odontoglossum* | Belgium | van der Aa & Vanev (2002) |
| *P. phalenopsidis* Marchal & Verplancke 1926[d] | living leaves of *Dendrobium phalaenopsis* | Belgium | van der Aa & Vanev (2002) |
| *P. phalenopsidis* var. *vanillae* Verplancke & Claessens 1934[d] | living leaves of *Vanilla luja* | Belgium | van der Aa & Vanev (2002) |

[a] an invalid name (van der Aa 1973)
[b] probably a *Phoma* (van der Aa & Vanev 2002)
[c] probably an *Asteromella* or *Phoma* (van der Aa & Vanev 2002)
[d] excluded from *Phyllosticta* (van der Aa & Vanev 2002)

## Materials and methods

### Isolates and morphology

Isolates were maintained on 2% potato dextrose agar (PDA) and incubated for 14 d under 12 h ultraviolet light / 12 h dark cycle following Wulanderi *et al.* (2009). Fungal structures were mounted in lactic acid on glass slides and examined by Nomarski interference contrast microscopy. Conidial measurements are given as follows: (minimum) mean – standard deviation × mean + standard deviation (maximum) rounded to 0.5 µm for 30 conidia. Other morphological measurements reflect the range of dimensions observed. Colony colours were determined by Rayner's (1970) colour charts. A representative isolate is deposited as ex-type culture in BRIP 58044.

### DNA isolation, amplification and phylogenetic analysis

DNA was extracted from fungal mycelium with the Gentra Puregene kit (Qiagen, Melbourne, Australia) according to the manufacturer's instructions. PCR amplification was conducted using the Phusion High-Fidelity PCR

**Tab. 2.** Isolates used in this study. Newly deposited sequences are in bold.

| Taxon | Culture Accession No.[a] | GenBank Accession No. | | Reference |
|---|---|---|---|---|
| | | ITS | TEF | |
| *Guignardia bidwellii* | CBS 11645 | EU683672 | EU683653 | Wikee *et al.* (2011) |
| *Guignardia gaultheriae* | CBS 447.70 | JN592543 | JN692531 | Su & Cai (2012) |
| *Phyllosticta bifrenariae* | CBS 128855[b] | JF343565 | JF343586 | Glienke *et al.* (2011) |
| *Phyllosticta brazilianiae* | CBS 126270 | JF343572 | JF343593 | Glienke *et al.* (2011) |
| *Phyllosticta capitalensis* | CBS 128856 | JF261465 | JF261507 | Glienke *et al.* (2011) |
| *Phyllosticta citriasiana* | CBS 120486 | FJ538360 | FJ538418 | Wulandari *et al.* (2009) |
| *Phyllosticta citribraziliensis* | CBS 100098 | FJ538352 | FJ538410 | Glienke *et al.* (2011) |
| *Phyllosticta citricarpa* | BRIP 52614<br>CBS 127454 | JF343583 | JF343604 | Glienke *et al.* (2011) |
| *Phyllosticta citrichinaensis* | CBS 130529 | JN791597 | JN791452 | Wang *et al.* (2012) |
| *Phyllosticta hostae* | CGMCC 3.14355 | JN692535 | JN692523 | Su & Cai (2012) |
| *Phyllosticta hubeiensis* | CGMCC 3.14986 | JX025037 | JX025042 | Zhang *et al.* (2012) |
| *Phyllosticta ilicis-aquifolii* | CGMCC 3.14358 | JN692538 | JN692526 | Su & Cai (2012) |
| *Phyllosticta kerriae* | MAFF 240047<br>MUCC 0017 | AB454266 | KC342576 | Motohashi *et al.* (2009) |
| *Phyllosticta schimae* | CGMCC 3.14354 | JN692534 | JN692522 | Su & Cai (2012) |
| *Phyllosticta styracicola* | CGMCC 3.14985 | JX025040 | JX025045 | Zhang *et al.* (2012) |
| *Phyllosticta speewahensis sp. nov.* | BRIP 58044 | **KF017269** | **KF017268** | This study |
| *Saccharata capensis* | CBS 122693 | EU552130 | EU552095 | Marincowitz *et al.* 2008 |

[a] BRIP: Plant Pathology Herbarium, Brisbane, Australia; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CGMCC: China General Microbial Culture Collection; MAFF: National Institute of Agrobiological Sciences, Tsukuba, Ibaraki Prefecture, Japan; MUCC: Laboratory of Plant Pathology, Mie University, Tsu, Mie Prefecture, Japan

[b] not an ex-type/ex-epitype isolate

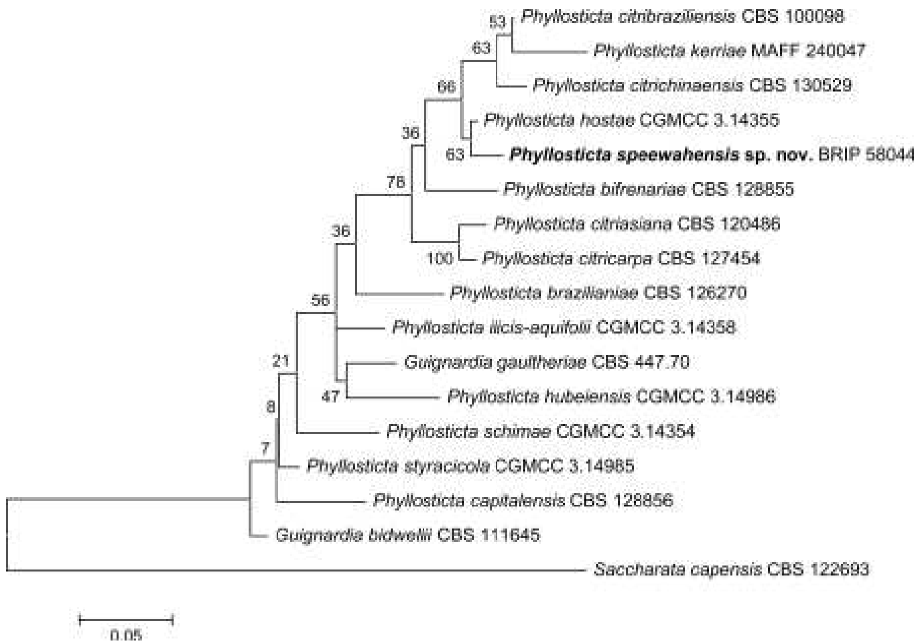



**Fig. 1.** Maximum likelihood tree, with bootstrap (1000 replicates) values incorporated of nucleotide sequences of the ITS and TEF gene regions. The scale bar shows the number of substitutions per site. The tree was rooted to *S. capensis* (CBS 122693). The alignment is deposited in TreeBASE (S14236). All sequences are from ex-type cultures with the exception of *P. bifrenariae* (Tab. 2).

Master Mix (Thermofisher Scientific, Scoresby, Vic., Australia), which consisted of 12.5 µL of 2 × Master Mix with HF Buffer, 0.5 µL each of 10 mM of forward and reverse primers, and 1 µL of DNA template. The internal transcribed spacer (ITS) region was amplified with primers V9G (de Hoog & Gerrits van den Ende 1998) and ITS4 (White *et al.* 1990), and part of the translation elongation factor 1-α (TEF) was amplified with primers EF1-728 F (Carbone & Kohn 1999) and EF2 (O'Donnell *et al.* 1998). PCR products were amplified in a Bio-Rad C1000 thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) using the following conditions: 98 °C for 30 s, 30 cycles at 98 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s, followed by a 5 min final extension at 72 °C. The products were sequenced by Macrogen Incorporated (Seoul, Korea) using the AB 3730xl DNA Analyser (Applied Biosystems, Forster City, California, USA). The ITS and TEF sequences of BRIP 58044 were deposited in GenBank as KF017269 and KF017268, respectively.

The ITS and TEF sequences from isolate BRIP 58044 were assembled using Vector NTI Advance v.11 (Life Technologies, Mulgrave, Vic., Australia) and aligned against published sequences from GenBank. The ITS and TEF sequences of ex-type cultures from 16 taxa, along with sequences from *P.*

*bifrenariae* were included in the alignment (Tab. 2). *Saccarata capensis* was selected as the outgroup taxon as it has been shown to reside in the same clade as *Phyllosticta* within the family Phyllostictaceae (Liu *et al*. 2012). The ITS and TEF sequences were initially aligned separately using ClustalW in MEGA 5.2 (Tamura *et al*. 2011) and manually adjusted where needed. The most suitable maximum likelihood (ML) nucleotide substitution model for each gene was determined using the model test function in MEGA 5.2, and then used for the phylogenetic analysis of the ITS and TEF sequences individually, as well as for the concatenated ITS and TEF data. A ML tree using the concatenated data was generated in MEGA 5.2 using the Kimura-2 parameter substitution model with Gamma distribution.

## Results

The ITS and TEF alignments contained 573 and 211 bases, respectively. Individual analysis of ITS and TEF resulted in ML trees with the same topology (data not shown), therefore the sequence data for both genes were concatenated. The generated ML tree shows the *Phyllosticta* species described in this study clusters close to *P. hostae* Y. Y. Su & L. Cai, but is nonetheless a distinct species (Fig. 1). *Phyllosticta hostae* was recently described from a leaf of *Hosta plantaginea* (Liliaceae) in China (Su & Cai 2012).

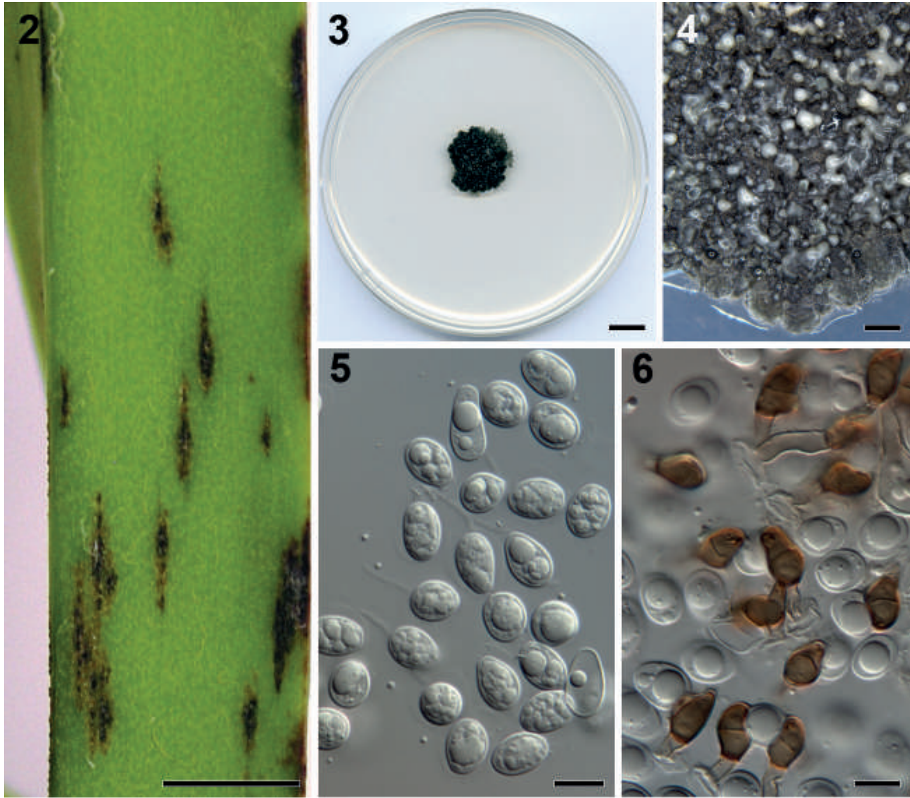
## Taxonomy

***Phyllosticta speewahensis*** R. G. Shivas, Y. P. Tan & K. R. E. Grice, **sp. nov.** – Figs. 2–6.
MycoBank no.: MB 804255

Description. – Leaf spots amphigenous, narrowly linear, widest in the middle, up to 2 cm long and 1 mm wide, pale to dark reddish brown at the margins, becoming black in the centre, solitary, or in clusters of up to 5, with raised, black, solitary pycnidia, arranged along the central axis. Pycnidia 50–80 µm diam., globose, wall composed of layers of *textura angularis*, outer layer dark reddish brown. Conidiophores reduced to conidiogenous cells. Conidiogenous cells terminal, hyaline, smooth-walled, cylindrical, 5–20 × 2–4 µm. Conidia subglobose, broadly ellipsoidal or obovoid, with a truncate to rounded base and rounded apex, hyaline, (9) 10–13.5 (15) × 7.5–9 (9.5) µm, aseptate; wall uniformly 0.5 µm thick, enclosed in a mucilaginous sheath up to 3 µm wide, with an apical hyaline tapered appendage 5–17 µm long. Appressoria single, medium brown, ellipsoidal to clavate, or irregularly lobed, mostly curved, 7–14 × 5–8 µm, with a horizontal median septum, or aseptate, apex rounded, smooth-walled. Teleomorph not observed.

Holotypus. – AUSTRALIA, Queensland, Speewah, in leaf spots on *Vanda* hybrid 'Waxy Blue', 12 September 2012, *leg*. K. R. E. Grice, BRIP 58044, sequences ex-type in GenBank KF017269 (ITS), KF017268 (TEF).

Culture characteristics (after 4 d in the dark on PDA and a further 2 wk under 12 h ultraviolet light / 12 h dark cycle, at 23 °C). – Colonies




**Figs. 2-6.** *Phyllosticta speewahensis* (holotype). **2.** In leaf lesions on cultivated *Vanda* hybrid (bar = 1 cm). **3–4.** Colony on PDA after 14 d (bars 1 cm and 1 mm, respectively). **5.** Conidia (bar 10 µm). **6.** Conidia (hyaline) and appressoria (brown) (bar 10 µm).

1.5 to 2 cm diam., flat, without aerial mycelium, Greenish Black (Rayner 1970); reverse Dark Slate Blue (Rayner 1970).

Etymology. – Named after the town nearest to the location of the fungus.

Host plant. – *Vanda* hybrid (Orchidaceae).

Distribution. – Speewah, Queensland, Australia.

*Phyllosticta speewahensis* has similar sized conidia to *P. bifrenariae* (10–16 × 7–9 µm) and is morphologically and culturally similar to this species. Two morphological differences in the description of these species are that appressoria were found in cultures of *P. speewahensis* but not *P. bifrenariae*; and spermatia formed in cultures of *P. bifrenariae* but not *P. speewahensis*. The presence or absence of these morphological features is clearly an unreliable character in species differentiation as their production will be influenced by media and other environmental conditions. *Phyllosticta speewahensis* can only be reliably differentiated from *P. bifrenariae* by DNA

sequence data. *Phyllosticta speewahensis* has larger or wider conidia, (9) 10–13.5 (15) × 7.5–9 (9.5) µm, than the two other species reported from orchids, namely *P. aplectri* (5–8 × 4–6 µm) and *P. capitalensis* (10–14 × 5–7 µm) (van der Aa 1973, Glienke *et al*. 2011).

## Discussion

Of the numerous species of *Phyllosticta* that have been described from orchids, only *P. bifrenariae* has been demonstrated to be a pathogen (Silva *et al*. 2008 as *P. capitalensis*). Leaf spots caused by *Phyllosticta* spp. are common on a wide range of orchid genera and species (Cash and Watson 1955 as *P. pyriformis*, van der Aa 1973 as *P. capitalensis*). *Phyllosticta capitalensis sensu* Glienke *et al*. (2011) is now generally recognized as an endophyte of a wide range of plants from around the world (Wikee *et al*. 2013), although it was first described from an orchid (*Stanhopea*). There are likely to be a number of cryptic species of *Phyllosticta*, both endophytes and pathogens, yet to be discovered on orchids, as molecular phylogenetic methods are applied to culture specimens (Ko-Ko *et al*. 2011). The discovery of these hidden species of *Phyllosticta* spp. will certainly precede knowledge about their pathogenicity, distribution and biosecurity importance.

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