Polyphasic characterisation of three new Phyllosticta spp.

Y.Y. Su¹, L. Cai¹

Key words  molecular morphology phylogeny systematics taxonomy

Abstract  Three new species of Phyllosticta, P. hostae on Hosta plantaginea (China), P. schimae on Schima superba (China), and P. ilicis-aquifolii on Ilex aquifolium (UK), are described and illustrated in this study. They are compared with morphologically similar and phylogenetically closely related species. A polyphasic approach using phylogeny, host association, disease symptoms, colony and morphological characteristics, is employed to justify the introduction of the new taxa. Phylogenetic relationships of the new species with other Phyllosticta species are revealed by DNA sequence analyses based on the nrDNA-internal transcribed spacer (ITS) regions and a combined multilocus alignment of the ITS, partial translation elongation factor 1-alpha (TEF1), actin (ACT), and glyceraldehyde 3-phosphate dehydrogenase (GPDH) gene regions.

Article info  Received: 14 December 2011; Accepted: 2 April 2012; Published: 27 April 2012.

INTRODUCTION

Many Phyllosticta (teleomorph Guignardia) species cause plant diseases such as leaf spots, leaf blotch, as well as black spots and lesions on fruits of various plants (van der Aa & Vanev 2002). These plant pathogenic fungi may cause serious damage to the host plant through reduced photosynthetic ability and premature leaf or fruit fall (Glienke-Blanco et al. 2006, Baldasari et al. 2008). Phyllosticta species have also been recorded as endophytes and saprobes on a wide range of host plants (Baayen et al. 2002, van der Aa & Vanev 2002, Okane et al. 2003, Wulandari et al. 2009, Glienke et al. 2011).

The generic circumscription of Phyllosticta as defined by van der Aa (1973) has been widely accepted (Bissett 1979, 1986, Yip 1989, Crous et al. 2006, Motohashi et al. 2008, 2009, Glienke et al. 2011, Wikee et al. 2011). The main characters are: pycnidial mella, mycelium, and lesions on fruits of various plants (van der Aa & Vanev 2002). According to these criteria, van der Aa & Vanev (2002) reconsidered 2,936 names in Phyllosticta, accepting 141 species based on original literature and a re-examination of herbarium specimens. About 50 % of the species were reclassified in Phoma, 20 % in Asterina, 5 % in Phomopsis and c. 18 % in other coelomycetous genera or other taxonomic groups. Some Phyllosticta species have been linked to their teleomorph states, for example, P. ampelicida is the anamorph of G. bidwillii (van der Aa 1973), but most appear to be asexual. Recent changes to the rules that govern fungal nomenclature require that only one name for a single biological species should be used instead of different names for different morphs (Hawksworth et al. 2011, Wingfield et al. 2012). The earlier and well-known generic name Phyllosticta (Persoon 1818), thus has priority over Guignardia (Viala & Ravaz 1892), as followed by Glienke et al. (2011). The systematics of Phyllosticta species has long been problematic because of the limited morphological characters and the unreliable use of host-association based nomenclature. Polyphasic approaches combining morphological characters and phylogenetic relationships can resolve species relationships, based on which a natural classification could be established (Wulandari et al. 2009, Glienke et al. 2011). Although the rDNA-internal transcribed spacer (ITS) locus has some resolution at species level, it is insufficient for separating cryptic species in Phyllosticta (Wulandari et al. 2009, Glienke et al. 2011). Therefore, multilocus phylogenetic analyses have been increasingly used for species discrimination in this genus (Wulandari et al. 2009, Glienke et al. 2011, Wang et al. 2012). For example, it was shown that G. mangiferae is a distinct taxon from P. capitalensis, which is a species complex awaiting more detailed phylogenetic study (Glienke et al. 2011).

In the present study, three new species of Phyllosticta are described based on morphological characters and phylogenies derived from ITS and combined multilocus gene sequences.

MATERIALS AND METHODS

Isolates
Phyllosticta species were isolated from diseased leaves of ornamental or forest plant species from China and the United Kingdom. Infected leaves were incubated in moist chambers at room temperature to induce sporulation. Pure cultures were obtained by single spore isolation as described by Choi et al. (1999). Alternatively, 5 × 5 mm pieces of surface-sterilised tissue were taken from the margin of leaf lesions and were consecutively immersed in 70 % ethanol solution for 1 min, sodium hypochlorite solution with 3 % available chlorine for 2 min, rinsed in sterile distilled water, blotted dry in sterile paper towels and incubated on 2 % potato-dextrose agar (PDA) (Cai et al. 2009).

Morphology
Cultures were grown on PDA for microscopic examination. Fungal structures were mounted on glass slides in clear lactic acid, and studied by means of a light microscope. Colony morphologies were assessed after 7 d growth on PDA, and colours rated according to the colour charts of Rayner (1970).

¹ State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, P.R. China; corresponding author e-mail: mrcailei@gmail.com.
DNA extraction, PCR amplification and sequencing

Mycelial discs were taken from actively sporulating areas near the growing edge of 10 d old cultures and transferred to PDA. Genomic DNA was extracted with a Biosa Genomic DNA Extraction Kit (Bioneer Technology Co., Ltd., Hangzhou, P.R. China) according to the manufacturer’s protocol. Quality and quantity of DNA were estimated visually by staining with GelRed after 1 % agarose gel electrophoresis. The ITS1 and ITS4 primer pair (White et al. 1990) was used to amplify the ITS region following the procedure described by White et al. (1990). The primers EF1-728F and EF1-986R (Carbone & Kohn 1999) were used to amplify a partial fragment of the translation elongation factor 1-α gene (TEF1); the primers ACT-512F and ACT-783R (Carbone & Kohn 1999) were used to amplify a partial fragment of the actin gene (ACT); the primers GDF1 (Guerber et al. 2003) and Gpd2-LM (Myllys et al. 2002) or GDR1 (Guerber et al. 2003) were used to amplify a partial fragment of the glyceraldehyde 3-phosphate dehydrogenase gene (GPDH). Amplification conditions followed Arzanlou et al. (2008). DNA sequencing was performed at the SinoGenoMax Company Limited, Beijing.

Sequence alignment and phylogenetic analyses

Sequences from forward and reverse primers were aligned to obtain a consensus sequence. Sequences of our isolates, together with reference sequences obtained from GenBank (Table 1), were aligned using Clustal X (Thompson et al. 1997). The separate ITS and the combined multilocus alignments were subjected to phylogenetic analyses. Phylogenetic analyses were performed using PAUP v. 4.0b10 (Swofford 2003). Ambiguously aligned regions were excluded from all analyses. An unweighted parsimony (UP) analysis was performed. Trees were inferred using the heuristic search option with TBR branch swapping and 1 000 random sequence additions, branches of zero length were collapsed and all equally most parsimonious trees were saved. Descriptive tree statistics such as tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], and homoplasy index [HI], were calculated for trees generated. Clade stability was assessed in a bootstrap analysis with 1 000 replicates, each with 10 replicates of random stepwise addition of taxa. A Shimodaira-Hasegawa test (SH test) (Shimodaira & Hasegawa 1999) was performed in order to determine whether trees were significantly different. Trees were visualised in TreeView v. 1.6.6 (Page 1996).

For the Bayesian analyses, the models of evolution were estimated by using MrModeltest v. 2.3 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0.4 (Huelsenbeck & Ronquist 2001), under the estimated model of evolution. Six simultaneous Markov chains were run for 1 000 000 generations and trees were sampled every 100th generation (resulting in 10 000 total trees). The first 2 000 trees, representing the burn-in phase of the analyses, were discarded and the remaining 8 000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree. Novel sequence data were deposited in GenBank (Table 1), alignments in TreeBASE (www.treebase.org, submission no.: 12430), and taxonomic novelties in MycoBank (Crous et al. 2004).

Table 1 Sources of isolates and GenBank accession numbers used in this study. The newly generated sequences in this study are shown in bold.

| Species               | Strain no. | ITS Accession number | TEF1 Accession number | ACT Accession number | GPDH Accession number |
|-----------------------|------------|----------------------|-----------------------|----------------------|-----------------------|
| Guignardia bidwellii  | CBS 111645 | JN692542             | JN692530              | JN692518             | –                     |
| G. gattii             | CBS 447.70 | JN692543             | JN692531              | JN692519             | JN692508              |
| G. mangiicorna       | IMI 260576 | JF261459             | JF261501              | JF343641             | JF343748              |
| G. sanseveriana      | CBS 120428 | JN692544             | JN692532              | JN692520             | JN692509              |
| Phyllosticta bifrenariae | VIC 305668 | JF343565             | JF343566              | JF343565             | JF343578              |
| P. braziliicorna     | LGMF 330*  | JF343572             | JF343593              | JF343566             | JF343578              |
| P. capitellis         | CBS 123373 | JF343566             | JF343587              | JF343560             | JF343572              |
| P. citriasiana       | CBS 120486; PD 05/1969753* | JF343560         | JF343566              | JF343560             | JF343572              |
| P. citribraziliensis | CBS 120488 | JN692545             | JN692533              | JN692521              | –                     |
| P. cucsonoi          | CBS 100098* | JF343555             | JF343523              | JF343519              | JF343507              |
| P. ciferica          | LGMF08     | JF261435             | JF261477              | JF343617             | JF343692              |
| P. cifericapa        | CBS 122442 | JF343572             | JF343593              | JF343566             | JF343578              |
| P. ciferica           | CBS 127454* | JF343585             | JF343604              | JF343667             | JF343771              |
| P. cucsonoi          | CPC 14875 | JF343578             | JF343599              | JF343662             | JF343764              |
| P. hostae            | CPC 14875* | JF343579             | JF343600              | JF343663             | JF343765              |
| P. hypoglossii       | CGMCC 3.14355* | JN692535            | JN692523              | JN692511              | JN692503              |
| P. hypoglossii       | CGMCC 3.14356 | JN692536            | JN692524              | JN692512              | JN692504              |
| P. hypoglossii       | CGMCC 3.14357 | JN692537            | JN692525              | JN692513              | JN692505              |
| P. ilicis-aquifolii  | CGMCC 3.1435* | JF343585             | JF343605              | JF343667             | JF343771              |
| P. ilicis-aquifolii  | CGMCC 3.14358* | JF343585            | JF343604              | JF343667             | JF343771              |
| P. ilicis-aquifolii  | CGMCC 3.14359 | JN692539            | JN692527              | JN692515              | –                     |
| P. ilicis-aquifolii  | CGMCC 3.14360 | JN692540            | JN692528              | JN692516              | –                     |
| P. oweniana           | CBS 776.97 | JF343560             | JF343626              | JF343684             | JF343767              |
| P. schimae           | CBS 120425 | JF343561             | JF343622              | JF343684             | JF343767              |
| P. spinarin           | CBS 292.90 | JF343565             | JF343606              | JF343669             | JF343773              |
| P. yuccae            | CBS 117136 | JN692541             | JN692529              | JN692517              | JN692507              |

1 ATCC: American Type Culture Collection, Virginia, USA; CBS: CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; CGMCC: China General Microbial Culture Collection; CPC: Culture collection of P.W. Crous, housed at CBS; IFO: Institute for Fermentation, Osaka, Japan; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakenham Lane, UK; LGMF: Culture collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brasil; PD: Plant Protection Service, Wageningen, The Netherlands; VICT: Culture collection of Federal University of Viçosa, Viçosa, Brazil.

2 Indicates the ex-type cultures.
3 ITS: Internal transcribed spacers 1 and 2 together with 5.8S rDNA; TEF1: partial translation elongation factor 1-α gene; ACT: partial actin gene; GPDH: partial glyceraldehyde-3-phosphate dehydrogenase gene.
RESULTS

Phylogenetic relationships were inferred using the ITS alignment, and the combined ITS, TEF1, GPDH, and ACT sequence alignment. The 67 ITS sequence dataset from 52 taxa comprised 517 characters after alignment. Of these, 252 characters were parsimony informative, 47 were variable and parsimony-uninformative, and 218 were constant. Parsimony analysis generated two trees, and one of the equally most parsimonious trees with shorter tree length (TL = 1051, CI = 0.669, RI = 0.841, RC = 0.562, HI = 0.331) was selected and shown in Fig. 2. For the Bayesian analyses, the best-fit model (GTR+I+G) was selected in MrModeltest 2.3. The branches with significant Bayesian posterior probability (≥ 95 %) were thickened in the phylogenetic tree. Similarly all three species appear in distinct lineages (Fig. 2).

The combined datasets of ITS, TEF1, GPDH, and ACT contained 32 combined sequences from 18 taxa and comprised 1 791 characters after alignment. Of these, 407 characters were parsimony informative; 129 were variable and parsimony-uninformative, and 218 were constant. Parsimony analysis generated three equally most parsimonious trees and the one of the equally most parsimonious trees with shorter tree length (TL = 935, CI = 0.539, RI = 0.827, RC = 0.446, HI = 0.461) was selected and shown in Fig. 1. For the Bayesian analyses, model (GTR+I+G) was selected in Modeltest 2.3. The branches with significant Bayesian posterior probability (≥ 95 %) were thickened in the phylogenetic tree. All three species described as new in this manuscript appear in distinct lineages (Fig. 1).
Table 2

| Phyllosticta species | Pycnidial size (µm) | Pycnidial wall | Conidiogenous cells (µm) | Conidia (µm) | Sheath diam (µm) | Appendage size (µm) | References |
|----------------------|---------------------|----------------|--------------------------|--------------|-----------------|---------------------|------------|
| P. aspidistria       | ×86                 | 61–118         | ×1.2–2.5                 | 9.5–12.5     | 8.5–10          | ×17–24.5           | Motohashi et al. (2008) |
| P. cruenta           | ×2–4                | 12–8           | –                        | 4–17         | ×21             | 5–10, mostly 16–19 | van der Aa (1973) |
| P. crypta            | 45–95               | 4–14 μm thick  | 2–3.5                    | 5–12         | 3.8–6.2         | 0.3–1              | Bissett (1979) |
| P. cumminsii         | 75–140              | 4–19 μm thick  | 3.5–14                   | 6.7–10.5     | 1.6–4           | –                  | Bissett (1979) |
| P. hemerocallidis    | 84–139              | –              | –                        | 8–13         | ×3.5            | –                  | van der Aa & Vanev (2002) |
| P. hostae            | 40–150              | 2–3 layers     | 2–3.5                    | 5–8          | 4–13            | 2.5–6              | Present study |
| P. hypoglossi        | 120–250             | 2–4 cells, 12–30 µm thick | 4–10       | 3–4.5         | 4–13            | 2.5–6              | Present study |
| P. subefusa          | 90–120              | 5–12 µm thick  | –                        | 5–8          | –               | –                  | Present study |
| P. yuccae            | 90–150              | 3–7 layers, 14–38 µm thick | 5.4–8          | 2–7.6         | –               | –                  | Present study |

**References**

- Motohashi et al. (2008)
- van der Aa (1973)
- Bissett (1979)
- Present study
- van der Aa & Vanev (2002)
- Motohashi et al. (2008)
- van der Aa (1973)
- Present study
- van der Aa & Vanev (2002)
- Motohashi et al. (2008)
- van der Aa (1973)
- Present study
- van der Aa & Vanev (2002)
- Motohashi et al. (2008)
- van der Aa (1973)
- Present study
- van der Aa (1973)

**Leaf spots**

Leaf spots elliptoid or circular to somewhat irregular, yellow to pale brown, surrounded by dark brown border. Pycnidia black, subepidermal, globose, 40–150 µm diam. Pycnidial wall composed of depressed or irregular cells in 2–3 layers, brown to dark brown, darker around ostiole, hyaline or pale and flattened towards the inside. Conidigenous cells 7–22 x 2–5 µm, holoblastic, phialidic, cylindrical, subcylindrical to ampulliform, hyaline, thin-walled, smooth. Conidia 8–15 x 5–9 µm (T = 10.9 ± 1.4 x 7.6 ± 0.8, n = 30), unicellular, thin- and smooth-walled, ellipsoid, subglobose to ovoid, with a large central gullet, truncate at the base when young, rounded at both ends, enclosed in a 1–3 µm thick mucilaginous sheath, and bearing a hyaline, mucoid apical appendage, 4–8 x 1–3 µm, straight to flexible, unbranched, tapering towards an acute tip.

**Culture characteristics**

Colonies on PDA flat, surface greenish grey in centre, white-grey at margin when young, becoming leaden-grey in centre, lavender-grey at margin after 2 wk.
Notes — *Phyllosticta hostae* was isolated from *Hosta plantaginea* (Liliaceae), which is grown as a common ornamental plant in China and many Asian countries. There are several reports of fungal pathogens isolated from *H. plantaginea*, e.g., *Alternaria asphodeli* (Zhang 1999), *Botrytis cinerea* (Zhang 2006), and *Colletotrichum omnivorum* (Cho & Shin 2004). To date, *P. hostae* is the only species of *Phyllosticta* described from the plant genus *Hosta*. However, nine *Phyllosticta* species are currently known on *Liliaceae*, i.e. *P. aspidistrica*, *P. cruenta*, *P. crypta*, *P. cumminsii*, *P. hemerocallidis*, *P. hypoglossi*, *P. subeffusa*, *P. uvulariae*, and *P. yuccae* (van der Aa & Vanev 2002, Motohashi et al. 2008). A comparison of their morphological characters with *P. hostae* is given in Table 2.

The phylogenetic tree generated from a multilocus sequence alignment showed that the three strains of *P. hostae* constituted a distinct lineage with 100 % bootstrap support (Fig. 2). DNA sequence analysis showed that *P. hostae* was most closely related to *P. citribraziliensis*, *P. cussonia*, *P. hypoglossi*, *P. spi-
narum*, and *P. vaccinii* (teleomorph *Guignardia vaccinii*). Of these species, *P. vaccinii* is morphologically most similar, but the ex-type strain (CBS 126.22) shares only 94 % identity to *P. hostae* in ITS sequence. *Phyllosticta vaccinii* was isolated from the leaves of *Vaccinium arboretum*. The pycnidia of *P. vaccinii* are larger (80–175 μm vs 40–150 μm) than that of *P. hostae*, and the conidia are slightly smaller (8–12 × 5–8 μm vs 8–15 × 5–9 μm). In addition, the appendages of *P. vaccinii* can be up to 17 μm long, while that of *P. hostae* is less than 8 μm (van der Aa 1973).

*Phyllosticta schimae* Y.Y. Su & L. Cai, sp. nov. — MycoBank MB564905; Fig. 4

Etymology. Named after its host, *Schima superba*.

Leaf spots circular, somewhat irregular, yellow to pale brown, surrounded by dark brown borders, fruiting bodies not observed. Pycnidia on PDA grey to black, aggregated, superficial to
eruptent, globose to ampulliform, 150–200 μm diam. Conidiogenous cells 8–30 × 2–4 μm, holoblastic, phialidic, short cylindrical, subcylindrical to ampulliform, hyaline, thin-walled, smooth. Conidia 7–13 × 4–7 μm (X = 9.5 ± 1.1 × 6.2 ± 0.4, n = 30), unicellular, thin- and smooth-walled, globose, ellipsoid to obovoid, truncate at the base when young, later rounded at both ends, enclosed in a mucilaginous sheath, and bearing a hyaline, mucoid apical appendage, 4–10 × 1–3 μm, straight to flexible, unbranched, tapering towards an acute tip. Spermatogenous cells subcylindrical to ampulliform, 11–25 × 2–4 μm. Spermatia aseptate, dumbbell-shaped, 7–11 × 1–2.5 μm (X = 8.3 ± 1.4 × 1.4 ± 0.3, n = 30).

Culture characteristics — Colonies on PDA flat, brown-black, with moderate aerial mycelium.

Specimens examined. CHINA, Zhejiang, Gutianshan Nature Reserve, on leaf of Schima superba, 18 Aug. 2010, Y.-Y. Su, HMAS242923 (holotype); ex-type culture CGMCC3.14354.

Notes — Schima superba is one of the dominant tree species in evergreen broad leaf subtropical forests in China. Currently there are only two unnamed Phyllosticta species reported from Schima (Theaceae) (Kobayashi 2007). Only two species, P. plurivora and P. theacearum, were recorded in the plant family Theaceae (van der Aa & Vanev 2002), and the former has been considered a synonym of P. theacearum (van der Aa & Vanev 2002). Phyllosticta theacearum produces shorter conidiogenous cells (4–6 μm vs 8–30 μm) than that of P. schimae (van der Aa 1973, van der Aa & Vanev 2002). Phyllosticta schimae appears closely related to P. ampelicida (teleomorph G. bidwellii) (92 % identity in ITS sequence) (Fig. 1, 2), which was isolated from the leaves of Ampelopsis auinquefolia (van der Aa 1973). Morphologically, P. schimae produces larger pycnidia (150–200 μm vs 70–180 μm), and longer conidiogenous cells (8–30 × 2–3 μm vs 6 × 3 μm) than P. ampelicida (van der Aa 1973).

Phyllosticta ilicis-aquifolii Y.Y. Su & L. Cai, sp. nov. — MycoBank MB564906; Fig. 5

Etymology. Named after its host, Ilex aquifolium.

Leaf spots ellipsoid or circular to somewhat irregular, grey to pale brown, about 7 mm diam, surrounded by dark brown border. Pycnidia amphigenous, subepidermal, single, 70–230 μm diam. Pycnidial wall composed of depressed or irregular cells of 2–4 layers, brown to dark brown, darker around ostiole, hyaline or pale and flattened towards the inside. Conidiogenous cells (8–)12–17(–19) × (2–)3–4 μm, holoblastic, phialidic, cylindrical, subcylindrical to ampulliform, hyaline, thin-walled, smooth. Conidia 10–18 × 6–9 μm (X = 13.4 ± 1.8 × 7 ± 0.7, n = 30), unicellular, thin- and smooth-walled, globose, ellipsoid.
to obovoid, with a large central guttule, truncate at the base when young, later rounded at both ends, enclosed in a thick mucilaginous sheath, 1–3 μm thick, and bearing a hyaline, mucoid apical appendage, (9–)12–17(–30) x 2–3 μm, straight to flexible, unbranched, tapering towards an acute tip. Spermatoogenous cells subcylindrical to ampulliform, 5–17 x 1–4 μm. Spermatia aseptate, dumbbell-shaped, 5–8 x 1.5–2.5 μm (\( \bar{X} = 6.7 \pm 0.7 \times 1.9 \pm 0.2, n = 30 \)).

Culture characteristics — Colonies on PDA flat, with irregular margin, surface white-grey when young; leaden-grey in centre, and white-grey at margin after 2 wk.

Specimens examined. UK, England, London, on leaf of Ilex aquifolium, 15 Aug. 2010, L. Cai, HMAS242922 (holotype), ex-type culture CGMCC3.14358; ibid. SYY590, culture CGMCC3.14359; ibid. SYY591, culture CGMCC3.14360. Three duplicate strains were deposited in IMI.

Notes — Phyllosticta ilicis-aquifolii was isolated from the common ornamental and hedge plant Ilex aquifolium. It is characterised by its large conidia that have a long mucoid appendage, which is distinct from most Phyllosticta species. Other Phyllosticta species reported from Aquifoliaceae include P. llimonae and P. concentrica (van der Aa & Vanev 2002). Phyllosticta ilicis-aquifolii differs from P. llimonae in producing

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**Fig. 5 Phyllosticta ilicis-aquifolii.** a. Appearance of conidiomata on host leaf surface; b. vertical section of pycnidium in leaf tissue; c. vertical section through the peridium; d. colony on PDA 7 d after inoculation; e. colony on PDA 1 mo after inoculation; f. pycnidia forming on PDA; g. conidium; h. i. conidiogenous cells giving rise to conidia; j, k. conidia; l. spermatogenous cells producing spermatia; m. spermatia. — Scale bars: b, c, k = 20 μm, f = 50 μm, g, h–j, l, m = 10 μm.
shorter conidiogenous cells (12–17 vs 28–32 μm) (Bertault 1982), and from *P. concentrica* (teleomorph *Guignardia philo-

pra*) in producing larger spermatia (6–15 x 1.5–3 μm vs 5–8 x 1.5–2.5 μm) (van der Aa 1973). In addition, the ex-type strains *Phyllosticta ilicis-asciiolfii* and *G. philopora* shared 94 % identity in ITS sequence and clustered in different clades in the ITS phylogenetic tree (Fig. 1). Although there are 26 synonyms listed under *P. concentrica* (http://www.mycobank.org), the con-

idial sizes in these species descriptions are all smaller (shorter than 12 μm) than that of *P. ilicis-asciiolfii*, except for *Sphaeria
taxi* (20–22 x 10 μm vs 10–18 x 6–9 μm in *P. ilicis-asciiolfii*) and *Phoma ilicis* (12–15 x 3 μm vs 10–18 x 6–9 μm).

*Phyllosticta ilicis-asciiolfii* appears most closely related to *P. gautheriae* (teleomorph *G. gautheriae*) (94 % identity in ITS sequence) and *P. pyraola* (95 % identity in ITS sequence) (Fig. 1). Morphologically, *P. ilicis-asciiolfii* can be distinguished from these species by its larger conidia (10–18 x 6–9 μm vs 4–9 x 4–7 μm in *P. gautheriae* and 4.5–7.5 x 4–9 μm in *P. pyraola*) (van der Aa 1973).

**DISCUSSION**

In this paper we have described and named three new *Phyllosticta* species based on morphological and molecular charac-
ters. Each has morphological characters typical for *Phyllosticta*, i.e., stromatic conidiomata, holoblastic conidiogenesis, one-
celled conidia provided with a surrounding mucoid layer and an apical appendage (van der Aa 1973, van der Aa & Vanen 2002).

Plant pathogenic *Phyllosticta* species are usually specific to host species or genera (van der Aa 1973, van der Aa & Vanen 2002, Motohashi et al. 2008, 2009, Wikree et al. 2011). Morpho-

logical comparisons of *Phyllosticta* spp. are often made with species reported from congeneric hosts (van der Aa & Vanen 2002, Motohashi et al. 2006, Wulandari et al. 2009, Glienke et al. 2011, Wang et al. 2012). In our study, the new species were compared with other species reported from the same host fam-

ily and species that are morphologically and phylogenetically closely related. These results showed that the three species were distinct, representing novel taxa.

Jin (2011) reported that the conidial appendages of some *Phyllosticta* species might disappear with time or elongate when mounted in water. Therefore, fresh cultures were used to study the ex-type strains. In this study, the morphological comparisons were made mainly based on other characters, e.g., the shape and size of conidia, pycnidia, and conidigenous cells.

Although the generic concept of *Phyllosticta* as defined by van der Aa (1973) is extensively accepted, the identification of spe-
cies is still difficult due to limited morphological characters that can be used for comparison. Recent molecular studies have revealed the ambiguity of taxonomy based on morphologi-
cal characters and host associations (Wulandari et al. 2009, Glienke et al. 2011). Multilocus phylogenetic analysis has been shown to be more useful in predicting natural species relations-

ships in the genus (Motohashi et al. 2009, Wulandari et al. 2009, Glienke et al. 2011). Traditionally applied phenotypic characters (host, symptom, colony characteristics, and morphology) should therefore be re-evaluated for their taxonomic usefulness in light of phylogenetic relationships (Hyde et al. 2010).

**Acknowledgements** This work was financially funded by CAS KSCX2-

YWZ-1026 and NSFC 31110103906. Drs Kevin D. Hyde and Roger G. Shivas are thanked for providing valuable comments on this manuscript.

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