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Phyllosticta capitalensis, a widespread endophyte of plants

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Abstract *Phyllosticta capitalensis* is an endophyte and weak plant pathogen with a worldwide distribution presently known from 70 plant families. This study isolated *P. capitalensis* from different host plants in northern Thailand, and determined their different life modes. Thirty strains of *P. capitalensis* were isolated as endophytes from 20 hosts. An additional 30 strains of *P. capitalensis* from other hosts and geographic locations were also obtained from established culture collections. Phylogenetic analysis using ITS, ACT and TEF gene data confirmed the identity of all isolates. Pathogenicity tests with five strains of *P. capitalensis* originating from different hosts were completed on their respective host plants. In all cases there was no infection of healthy leaves, indicating that this endophyte does not cause disease on healthy, unstressed host plants. That *P. capitalensis* is often isolated as an endophyte has important implications in fungal biology and plant health. Due to its endophytic nature, *P. capitalensis* is commonly found associated with lesions of plants, and often incorrectly identified as a species of quarantine importance, which again has implications for trade in agricultural and forestry production.

Keywords *Guignardia* · Leaf spot · Morphology · Molecular phylogeny · Quarantine

Introduction

Species in the genus *Phyllosticta* are mostly plant pathogens of a wide range of hosts and are responsible for diseases including leaf spots and black spots on fruits (Wulandari et al. 2009; Glienke et al. 2011; Wang et al. 2012). There are about 3,200 names listed for the genus *Phyllosticta* in Index

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Fungorum (http://www.indexfungorum.org/; accessed February 2013) and 3,340 names in MycoBank (http://www.mycobank.org/; accessed February 2013). The USDA Fungal Database lists 78 *Phyllosticta* records associated with plant hosts (http://nt.ars-grin.gov/fungaldatabases/; accessed February 2013).

Phyllosticta species may be associated with a "*Guignardia*-like" sexual state (van der Aa 1973; Wikee et al. 2011). For example, the sexual state of *Phyllosticta ampelicida* (Engelm.) Aa, the black rot pathogen of grapevine is *Guignardia bidwellii* (Ellis) Viala & Ravaz (van der Aa 1973; Ullrich et al. 2009). Leaf spots on *Morinda citrifolia* (*Rubiaceae*) commonly have both ascomata and pycnidia of *P. morindae* (Petr. & Syd.) Aa (Wulandari et al. 2010a, b). Likewise, both ascomata and pycnidia of *P. maculata* M.H. Wong & Crous are present on banana leaves with freckle disease (Wong et al. 2012).

Guignardia citricarpa Kiely (synonym of *P. citricarpa* (McAlpine) Aa), which causes black spot of citrus (e.g. oranges), is of quarantine concern in Europe (Baayen et al. 2002; Agostini et al. 2006), but *P. citriasiana* Wulandari, Crous & Gruyter, which causes brown spot of pomelo fruit (*Citrus maxima*) is not of quarantine concern as this fruit is not grown in Europe (Wulandari et al. 2009). A few species have also been reported as endophytes and saprobes (Van Der Aa et al. 2002; Baayen et al. 2002; Glienke et al. 2011). *Phyllosticta maculata* the cause of banana leaf freckle has also been isolated as an endophyte from healthy grapevine leaves (Kuo and Hoch 1996). *Phyllosticta capitalensis* Henn. is commonly isolated as an endophyte and is a widespread species (Glienke-Blanco et al. 2002; Silva and Pereira 2007; Silva et al. 2008).

Phyllosticta capitalensis was described by Hennings (1908) who found it associated with necrotic leaves of *Stanhopea* sp. (*Orchidaceae*) collected in Brazil. The supposed sexual morph, *G. mangiferae* A.J. Roy was later described from *Mangifera indica* L. (*Anacardiaceae*) in India (Roy 1968). However, there has been confusion with the identification and naming of the *P. capitalensis* sexual morph. Okane et al. (2003) stated that the teleomorph of *P. capitalensis* differed morphologically from *G. mangiferae* and that it was, in fact, *G. endophyllicola* Okane, Nakagiri & Tad. Ito. The latter fungus was described as a pathogen of several ericaceous plants by Okane et al. (2001). In the past there has also been confusion between *G. endophyllicola* and *G. citricarpa*. Both sexual names have been used for this fungus, for example, *G. endophyllicola* (Okane et al. 2003; Pandey et al. 2003) and *G. mangiferae* (Baayen et al. 2002; Glienke-Blanco et al. 2002; Guo et al. 2003; Suryanarayanan et al. 2004; Devarajan and Suryanarayanan 2006; Shaw et al. 2006). However, *G. citricarpa* is a distinct species and the cause of citrus black spot (Paul et al. 2005; Baayen et al. 2002).

Fungal endophytes colonise healthy plant host tissues but may become pathogenic when the plant host is stressed through environmental or biological factors (Petrini 1991; Hyde and Soytong 2008; Purahong and Hyde 2011) that induce the fungus to change from one life mode to another (Fisher and Petrini 1992). As with *Phyllosticta*, some species of other common genera such as *Bipolaris*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Diaporthe*, *Fusarium*, *Pestalotiopsis*, *Phoma* and *Verticillium* have been isolated as endophytes (Photita et al. 2001, 2004; Anderson et al. 2011; Bensch et al. 2012; Damm et al. 2012a, b; de Gruyter et al. 2013; Lima et al. 2012; Orlandelli et al. 2012), and some of these are also serious pathogens (Photita et al. 2004; Slippers and Wingfield 2007).

The present study provides an overview of the distribution and host range of *P. capitalensis* worldwide, through the application of multi-gene phylogeny to illustrate its widespread nature. Generally, *Phyllosticta* species are considered plant pathogens but it is still unclear whether they are generalists or host-specific. The distinction between a pathogen and a latent pathogen with endophytic nature is also unclear. In this study we isolated *Phyllosticta* species from northern Thailand, both as endophytes and as pathogens associated with leaf spots of various hosts. We also obtained a range of geographically diverse isolates of *P. capitalensis* from the CBS-KNAW Fungal Biodiversity Centre. All isolates were sequenced compared with sequences downloaded from GenBank.

Material and methods

Isolates

Thirty strains of *Phyllosticta* were isolated from leaf spots or as endophytes from healthy leaves of ornamental plants (Table 1). If pycnidia were present on diseased tissue then a single spore isolation procedure as described by Chomnunti et al. (2011) was used to obtain cultures. To obtain isolates of *Phyllosticta* from diseased leaves of host plants when fruit bodies were not present, the leaf was surface disinfected by wiping with 70 % ethanol. Small pieces of leaf were then cut from the interface between healthy and diseased tissue. These were surface sterilised in 70 % ethanol, and plated onto ½ strength potato dextrose agar (½PDA; Crous et al. 2009). For isolation of endophytes, healthy leaves were washed in tap water and surface disinfected with 70 % ethanol. They were then cut into small pieces (about 1×1 cm), suspended in 70 % ethanol (3 times for 15 min each) and washed in distilled water (3 times) before placing on ½PDA. All dishes were incubated at 27 °C for 1 week and observed daily. The growing tips of hyphae of *Phyllosticta* colonies that developed were cut out and transferred to fresh PDA dishes. Isolates are deposited in Mae Fah Luang

Table 1 Isolates of *Guignardia* and *Phyllosticta* used in the phylogenetic study

Strain	Code[1]	Host	Mode*	Country	Gene and GenBank No.		
					ITS	TEF1	ACT
G. bidwellii	CBS 111645	*Parthenocissus quinquefolia*	P	USA	JN692542	EU683653	JN692518
G. mangiferae	IMI 260576	*Mangifera indica*	E	India	JF261459	JF261501	JF343641
P. brazilianiae	LGMF 333	*Mangifera indica*	E	Brazil	JF343574	JF343595	JF343658
P. brazilianiae	LGMF 334	*Mangifera indica*	E	Brazil	JF343566	JF343587	JF343650
P. brazilianiae (ex-type)	LGMF 330 CBS 126270	*Mangifera indica*	E	Brazil	JF343572	JF343593	JF343656
P. capitalensis	CPC 20251	wild plant	P	Thailand	KC291333	KC342553	KC342530
P. capitalensis	CPC 20252	*Punica granatum*	P	Thailand	KC291334	KC342554	KC342531
P. capitalensis	CPC 20254	*Saccharum officinarum*	E	Thailand	KC291335	KC342555	KC342532
P. capitalensis	CPC 20255	*Arecaceae*	P	Thailand	KC291336	KC342556	KC342533
P. capitalensis	CPC 20256	*Ophiopogon japonicus*	P	Thailand	KC291337	KC342557	KC342534
P. capitalensis	CPC 20257	*Ficus benjamina*	P	Thailand	KC291338	KC342558	KC342535
P. capitalensis	CPC 20258	*Ophiopogon japonicus*	P	Thailand	KC291339	KC342559	KC342536
P. capitalensis	CPC 20259	*Orchidaceae*	P	Thailand	KC291340	KC342560	KC342537
P. capitalensis	CPC 20263	*Magnoliaceae*	E	Thailand	KC291341	KC342561	KC342538
P. capitalensis	CPC 20266	*Polyscias* sp.	E	Thailand	KC291342	KC342562	KC342539
P. capitalensis	CPC 20268	*Hibiscus syriacus*	E	Thailand	KC291343	KC342563	KC342540
P. capitalensis	CPC 20269	*Ophiopogon japonicus*	E	Thailand	KC291344	KC342564	KC342541
P. capitalensis	CPC 20270	*Tectona grandis*	E	Thailand	KC291345	KC342565	KC342542
P. capitalensis	CPC 20272	*Orchidaceae*	P	Thailand	KC291346	KC342566	KC342543
P. capitalensis	CPC 20275	*Polyalthia longifolia*	E	Thailand	KC291347	KC342567	KC342544
P. capitalensis	CPC 20278	*Euphorbia milii*	E	Thailand	KC291348	KC342568	KC342545
P. capitalensis	CPC 20423	*Philodendron* 'Xanadu'	P	Thailand	KC291349	KC342569	KC342546
P. capitalensis	LC 0002	*Alocasia* sp.	E	Thailand	KC291350	KC342570	KC342547
P. capitalensis	LC 0006	*Dieffenbachia* sp.	E	Thailand	KC291351	KC342571	KC342548
P. capitalensis	LC 0008	*Anthurium* sp.	E	Thailand	KC291352	KC342572	KC342549
P. capitalensis	LC 0009	*Sansevieria hyacinthoides*	E	Thailand	KC291353	KC342573	KC342550
P. capitalensis	LC 0010	*Tinospora craspa*	E	Thailand	KC291354	KC342574	KC342551
P. capitalensis	LC 0025	*Calophyllum* sp.	E	Thailand	KC291355	KC342575	KC342552
P. capitalensis	CBS 100175	*Citrus* sp.	E	Brazil	FJ538320	FJ538378	FJ538436
P. capitalensis	CBS 114751	*Vaccinium* sp.	P	New Zealand	EU167584	FJ538407	FJ538465
P. capitalensis	CBS 115046	*Myracrodruon urundeuva*	E	Brazil	FJ538322	FJ538380	FJ538438
P. capitalensis	CBS 115047	*Aspidosperma polyneuron*	E	Brazil	FJ538323	FJ538381	FJ538439
P. capitalensis	CBS 115049	*Bowdichia nitida*	E	Brazil	FJ538324	FJ538382	FJ538440
P. capitalensis	CBS 123373	*Musa paradisiaca*	E	Thailand	FJ538341	FJ538399	FJ538457
P. capitalensis	CBS 123404	*Musa paradisiaca*	E	Thailand	FJ538333	FJ538391	FJ538449
P. capitalensis	CBS 226.77	*Paphiopedilum callosum*	P	Germany	FJ538336	FJ538394	FJ538452
P. capitalensis	LGMF 03	*Citrus lalifolia*	P	Brazil	JF261452	JF261494	JF343634
P. capitalensis	LGMF 181	*Citrus reticulata*	P	Brazil	JF261447	JF261489	JF343629
P. capitalensis	LGMF 219	*Citrus sinensis*	E	Brazil	JF261448	JF261490	JF343630
P. capitalensis	LGMF 240	*Citrus sinensis*	E	Brazil	JF261443	JF261485	JF343625
P. capitalensis	LGMF 222	*Citrus sinensis*	E	Brazil	JF261450	JF261492	JF343632
P. capitalensis	LGMF 220	*Citrus sinensis*	E	Brazil	JF261446	JF261488	JF343628
P. capitalensis	LGMF 358	*Mangifera indica*	E	Brazil	JF261449	JF261491	JF343631
P. capitalensis (ex-epitype)	CPC18848	*Stanhopea graveolens*	P	Brazil	JF261465	JF261507	JF343647
P. citriasiana (ex-type)	CBS 120486	*Citrus maxima*	P	Thailand	FJ538360	FJ538418	FJ538476
P. citriasiana	CBS 123370	*Citrus maxima*	P	Vietnam	FJ538355	FJ538413	FJ538471

Table 1 (continued)

Strain	Code[1]	Host	Mode*	Country	Gene and GenBank No.		
					ITS	TEF1	ACT
P. citriasiana	CBS 123371	*Citrus maxima*	P	Vietnam	FJ538356	FJ538414	FJ538472
P. citriasiana	CBS 123372	*Citrus maxima*	P	Vietnam	FJ538357	FJ538415	FJ538473
P. citribraziliensis (ex-type)	CBS 100098	*Citrus* sp.	H	Brazil	FJ538352	FJ538410	FJ538468
P. citribraziliensis	LGMF09	*Citrus* sp.	H	Brazil	JF261436	JF261478	JF343618
P. citricarpa	CBS 102374	*Citrus aurantium*	P	Brazil	FJ538313	GU349053	FJ538429
P. citricarpa	CBS 120489	*Citrus sinensis*	P	Zimbabwe	FJ538315	FJ538373	FJ538431
P. citricarpa (ex-epitype)	CBS 127454	*Citrus limon*	P	Australia	JF343583	JF343604	JF343667
P. citricarpa	CBS 127452	*Citrus reticulata*	P	Australia	JF343581	JF343602	JF343665
P. citricarpa	CBS 127455	*Citrus sinensis*	P	Australia	JF343584	JF343605	JF343668
P. citrichinaensis	ZJUCC 200956	*Citrus reticulata*	P	China	JN791664	JN791515	JN791589
P. citrichinaensis	ZJUCC 200964	*Citrus maxima*	P	China	JN791662	JN791514	JN791582
P. citrichinaensis	ZJUCC 2010150	*Citrus maxima*	P	China	JN791620	JN791459	JN791533
P. citrichinaensis	ZJUCC 2010152	*Citrus sinensis*	P	China	JN791611	JN791461	JN791535
P. kerriae (ex-holotype)	MUCC 17	*Kerria japonica*	P	Japan	AB454266	KC342576	AB704209
P. hypoglossi	CBS 101.72	*Ruscus aculeatus*	P	Italy	FJ538365	FJ538423	FJ538481
P. hypoglossi	CBS 434.92	*Ruscus aculeatus*	P	Italy	FJ538367	FJ538425	FJ538483
P. hypoglossi	CBS 167.85	*Ruscus hypoglossum*	P	Italy	FJ538366	FJ538424	FJ538482
P. owaniana	CBS 776.97	*Brabejum stellatifolium*	P	South Africa	FJ538368	FJ538426	FJ538484
P. owaniana	CPC 14901	*Brabejum stellatifolium*	P	South Africa	JF261462	JF261504	JF343644
P. spinarum	CBS 292.90	*Chamaecyparis pisifera*	P	France	JF343585	JF343606	JF343669
P. podocarpi	CBS 111646	*Podocarpus falcatus*	P	South Africa	AF312013	KC357671	KC357670

[1] CBS: CBS-KNAW Fungal Biodiverstiy Centre, Utrecht, The Netherlands; CPC: working collection of Pedro Crous housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, LC: culture collection of Nilam F. Wulandari, Chiangmai, Thailand. LGMF: culture collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil, ZJUCC: Zhejiang University Culture Collection, Zhejiang, China

**P* pathogen, *E* endophyte

University Culture Collection (MFLUCC) and in the working collection of Pedro Crous (CPC) housed at the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands (CBS). Other fungal isolates of representative *Phyllosticta* spp. were obtained from the CBS (Table 1).

Morphology

Growth rates, cultural characteristics and morphology of the isolates were determined on culture media prepared according to Crous et al. (2009). All isolates were grown at 27 °C. To induce sporulation, isolates were grown on pine needle agar (PNA) and synthetic nutrient-poor agar (SNA), and incubated under near UV-light. Colony colour and growth rate were established on PDA, malt extract agar (MEA) and oatmeal agar (OA). Culture characteristics were assessed, and the colour of upper and lower surface of cultures was recorded after 14 days in the dark at 27 °C. Colony colour on MEA, OA and PDA were determined using the colour charts of Rayner (1970).

Molecular phylogeny

DNA extraction, amplification, and sequencing

Strains were grown on MEA at room temperature for 2–3 days, after which the mycelium was harvested. DNA was isolated using Ultraclean™ Microbial DNA kit (Mo Bio, Calsbad, CA, USA) following the manufacturer's protocol. Transcribed spacer-polymerase chain reaction (ITS-PCR) was performed with primers V9G (De Hoog and Gerrits van den Ende 1998) and ITS4 as described by White et al. (1990). Part of elongation factor 1-α gene (TEF) was amplified with forward primer EF1 and reverse primer EF2. The primers ACT-512F and ACT-783R were used to amplify part of the actin gene (ACT). Cycle sequencing of PCR products were performed in PCR condition. PCR products were separated by gel electrophoresis at 130 V for 20 min in 1 % agarose gel in 1× TAE running buffer and visualized under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK). Purified PCR products were sequenced using both PCR primers with a

BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) containing AmpliTag DNA Polymerase. The amplified products were analyzed on an automatic DNA sequencer (Perkin-Elmer, Norwalk, CN) and aligned using MEGA v5 software. Phylogenetic analyzing was executed by Phylogenetic analyses using parsimony; PAUP v4.0b10 (Swofford 2003) and MrBayes v. 3.0b4 (Huelsenbeck and Ronquist 2001) for Bayesian analyses. *Guignardia bidwellii* was chosen as outgroup for the phylogenetic tree. Representative sequences were deposited in GenBank.

Pathogenicity testing

Attached, young healthy leaves of five plant species (*Cordyline fruticosa*, *Dendrobium lindleyi, Ficus* sp., *Ophiopogon japonica*, *Punica granatum*) were washed with distilled water, wiped with 70 % ethanol and dried with sterile tissue paper. To complete the Koch's postulated the inoculation methods followed Than et al. (2008). Two to five leaves of each plant were wounded with a total of ten wounds. The leaves were wounded by pricking them with a pin. Both wounded and unwounded leaves were inoculated with plugs (0.7 mm diam) taken from the edge of 14 day-old colonies of test fungi growing on PDA; sterile agar plugs served as a control. All leaves were kept individually in moist chambers for 1 week and observed for symptom expression every other day. After 7 days, if positive, the fungus was reisolated from any tissue showing lesions and this isolate was considered to be pathogenic; absence of symptoms on leaves classified the isolate as non-pathogenic.

Results

Collection of *Phyllosticta* species

Thirty strains of *Phyllosticta capitalensis* were isolated from 20 host plants growing in the north of Thailand (Table 1, see also Fig. 1). No other species of *Phyllosticta* were isolated.

Morphological description of *Phyllosticta capitalensis* (Fig. 2)

On Punica granatum Pycnidia epiphyllous, globose, brown or black, 120–125 μm high, 135–140 μm wide, wall 12–15 μm thick. *Conidiogenous cells* lining wall of pycnidium, phialidic, cylindrical, hyaline, 2–2.2×2.2–3 μm. *Conidia* ellipsoidal, hyaline, 1-celled, smooth-walled, 8–11×5–6 μm, surrounded by a mucilaginous sheath, bearing a single apical appendage, usually 5–8 μm long.

In culture On SNA, *ascomata* forming on and under media in 3 days, black, globose, 69–74×104–119 μm ($\bar{x} = 73 \pm 2 \times 109 \pm 5$; $n=10$), wall composed of a single layer, 7–9 μm thick ($\bar{x} = 8 \pm 1$; $n=10$), brown. *Asci* bitunicate, containing 6–8 ascospores, irregularly biseriate, clavate, 36–80×7–15 μm ($\bar{x} = 51 \pm 1 \times 11 \pm 2$, $n=10$). *Ascospores* ellipsoid to broadly fusoid, widest in the middle, hyaline, smooth, thin-walled, 12–22×5–10 μm ($\bar{x} = 16 \pm 2 \times 7 \pm 1$, $n=50$), 1-celled, surrounded by mucilaginous sheath. On OA, colonies appear flat with an irregular margin, initially hyaline with abundant mycelium, gradually becoming greenish after 3–4 days. On MEA, colonies appear woolly, flat, irregular, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 days with white hyphae on the undulate margin, eventually turning black; reverse dark green to black. At 27 °C, in the dark, mycelium reached the edge of the Petri-dish in 20 days with a growth rate of 0.4 cm per day. On PDA, colonies appear woolly, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 days with white hyphae on the undulate margin, eventually turning dark green to black; reverse black. After 10 days in the dark at 27 °C, mycelium reached the edge of the Petri-dish with a growth rate of 0.9 cm per day.

Material examined All CPC collected by Saowanee Wikee and LC by Nilam F. Wulandari, from June 2010 to November 2011, Chiang Rai, Thailand. From leaf spots of unknown wild plant CPC 20251; from leaf spots *Punica granatum,* CPC 20252; from healthy leaf of *Saccharum officinarum* CPC 20254; from leaf spots of *Arecaceae* CPC 20255; from leaf spots of *Ophiopogon japonica* CPC 20256, CPC 20258 and CPC 20269; from leaf spots of *Ficus benjamina* CPC 20257; from leaf spots of *Orchidaceae* CPC 20259 and CPC 20272; from healthy leaf of *Magnoliaceae* CPC 20263; from healthy leaf of *Codiaeum variegatum* CPC 20265; from healthy leaf of *Polyscias* sp. CPC 20266; from healthy leaf of *Hibiscus syriacus* CPC 20268; from healthy leaf of *Tectona grandis* CPC 20270; from healthy leaf of *Poloalthia longifolia* CPC 20275; from healthy leaf of *Euphorbia milli* CPC 20278; from healthy leaf of *Philodendron* sp. CPC 20423; from healthy leaf of *Alocasia* sp. LC 0002; from healthy leaf of *Dieffenbachia* sp. LC 0006; from healthy leaf of *Anthurium* sp. LC 0008; from healthy leaf of *Sansevieria hyacinthodes* LC 0009; from healthy leaf of *Tinospora craspa* LC0010; from healthy leaf of *Calophyllum* sp. LC 0025.

Phylogenetic analysis

Phylogenetic relationships among the *Phyllosticta capitalensis* isolates from various hosts and locations were investigated in this study using MP and Bayesian phylogenetic analyses. The analysis of combined ITS, TEF and ACT genes of the

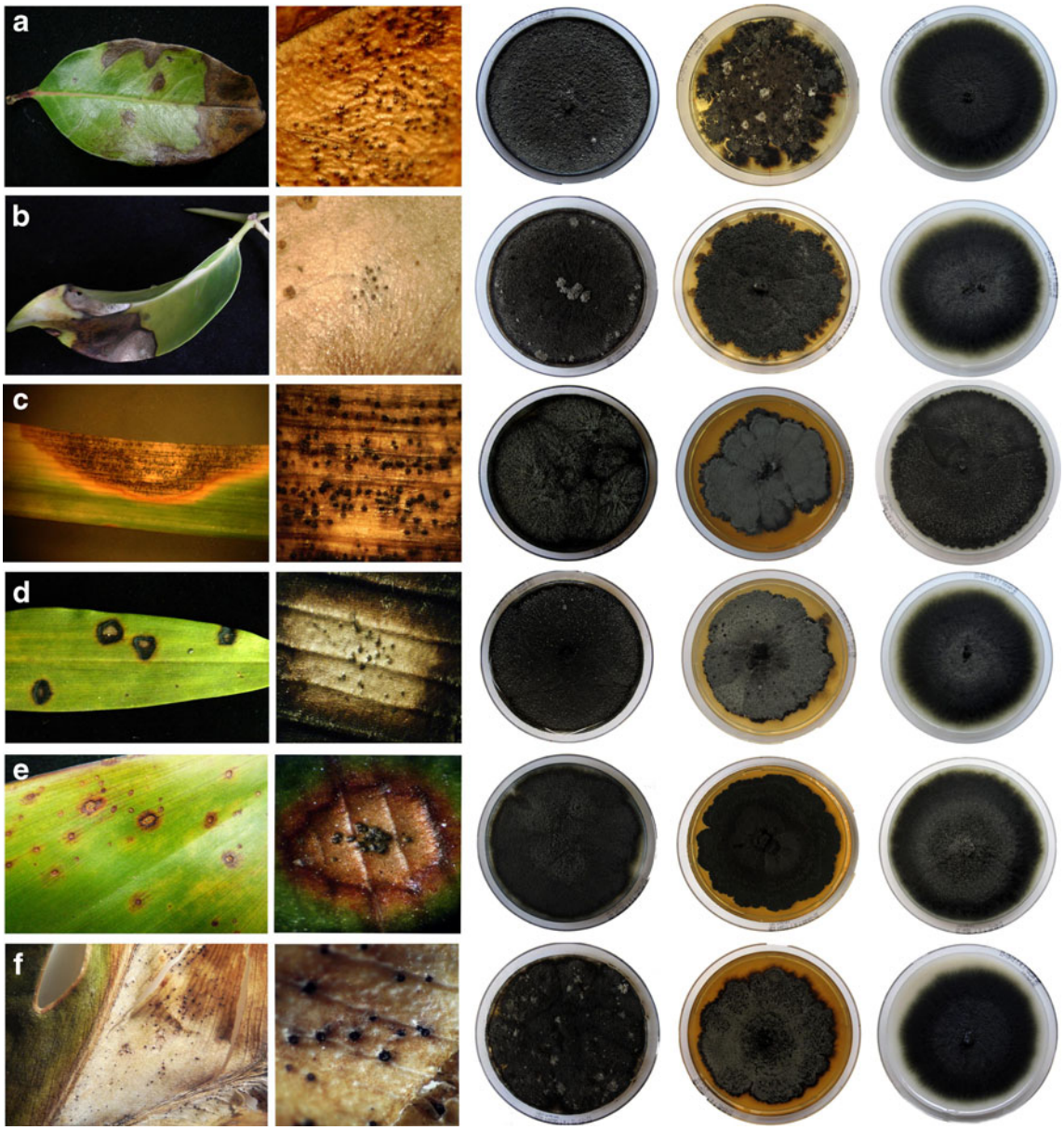

Fig. 1 Leaf spot symptoms on living leaves of hosts and cultures characteristic of *Phyllosticta capitalensis* on PDA (*left*), MEA (*middle*) and OA (*right*). **a** *Punica granatum* (CPC20252; MFLUCC11-0053) **b** *Ficus* sp. (CPC20257; MFLUCC11-0058) **c**. *Ophiopogon japonica* (CPC20258; MFLUCC11-0059) **d**. *Dendrobium lindleyi* (CPC20259; MFLUCC11-0064) **e**. *Cordyline fruticosa* (CPC20273; MFLUCC10-0135) **f**. *Philodendron* 'Xanadu' (CPC20423; MFLUCC 12–0232)

Phyllosticta strains newly sequenced in this study and 67 strains of *Phyllosticta* obtained from GenBank and Mei University, Japan (Table 1) were aligned and used to construct their phylogeny. The combined dataset of 64 strains (including the out-group) consisted of 974 characters, of which 483 characters were constant, and 148 characters were variable and parsimony-uninformative. Parsimony analysis generated 48 trees, of which the best one is shown in Fig. 3 (TL = 873, CI = 0.804, RI = 0.963, RC = 0.774). In the parsimony tree (Fig. 3) bootstrap values and Bayesian analysis of combined data are given at the nodes.

In the phylogenetic tree 12 clades representing various *Phyllosticta* species are evident. *Guignardia bidwellii* was chosen as out-group. The representative strain of *G. mangiferae* (IMI 260576) fell outside the *P. capitalensis s. str.* clade. The isolates in the *P. capitalensis s. str.* clade were from different hosts and different continents. *Phyllosticta brazilianiae* was isolated from an orchid in Brazil; *P. citricarpa* was isolated from *Citrus* sp. and *P. citriasiana* was isolated from *Citrus maxima*, Vietnam; *P. spinarum* was isolated from *Chamaecyparis pisifera*, France; *P. kerriae* was isolated from *Kerria japonica,* Japan; *P. citribraziliensis* was

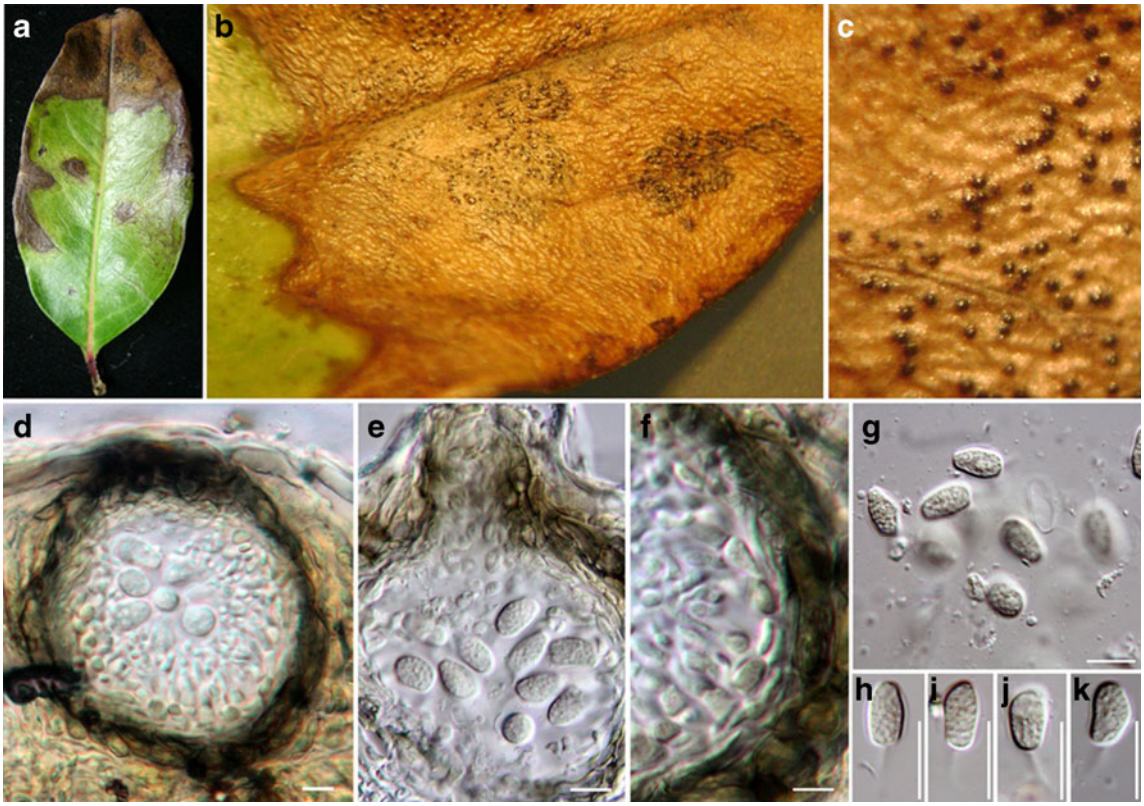

Fig. 2 *Phyllosticta capitalensis* on *Punica granatum* (CPC 20252). **a**–**c** Leaf spots on host plant **d**–**f**. *Vertical* section through pycnidia showing developing conidia **g**–**k**. Conidia (**d**, *bar*=20 μm, **g**–**k** *bars*=10 μm)

isolated from citrus, Brazil; *P. hypoglossi* was isolated from *Ruscus aculeatus*, Italy; *P. citrichinaensis* was isolated from *Citrus maxima*, China; *P. podocarpi* was isolated from *Podocarpus falcatus*, South Africa and *P. owaniana* from *Brabejum stellatifolium*, South Africa.

Pathogenicity testing with *Phyllosticta capitalensis*

The ability of *Phyllosticta capitalensis* strains isolated from leaf spots of five hosts in Thailand to induce leaf spot symptoms on these host species was tested through inoculating mycelium plugs onto attached wounded and unwounded living leaves. In all cases there was no infection of the young healthy plant leaves.

Discussion

This study reviews previous data on *Phyllosticta capitalensis* and provides additional data on host infection and distribution in Thailand. Many factors such as environmental conditions, host and non-host organisms, and plant defence mechanisms (e.g. secondary metabolite, specific and non-specific protein expression and hydrogen peroxide residue) play an important role in response to microbial infection.

Phyllosticta capitalensis has been repeatedly isolated worldwide from healthy plant tissues as an endophyte and rarely from leaf spots as a pathogen, and has been recorded from almost 70 plant families (Baayen et al. 2002; Okane et al. 2003; Motohashi et al. 2009, Tables 1 and 2, Fig. 4, this study). The fact that it is isolated so often as an endophyte has important implications to studies of fungal biology including plant pathology methodology, ecological results of endophyte studies and screening for novel compounds from endophytes.

Implications to plant pathology methodology

A standard protocol used for isolating plant pathogens involves cutting segments from the leading edge of lesions, which are then surface sterilized and plated onto media (Crous et al. 2009). The rationale is that the causative agent grows out from the lesions and can be isolated as a pure culture. Testing can then be undertaken to establish pathogenicity, while the colony can be identified using morphology. This standard methodology (Koch's postulate) has been long used by plant pathologists to determine the identity of non-sporulating pathogens ad infinitum (Phoulivong et al. 2010; Thompson et al. 2010; Wikee et al. 2011).

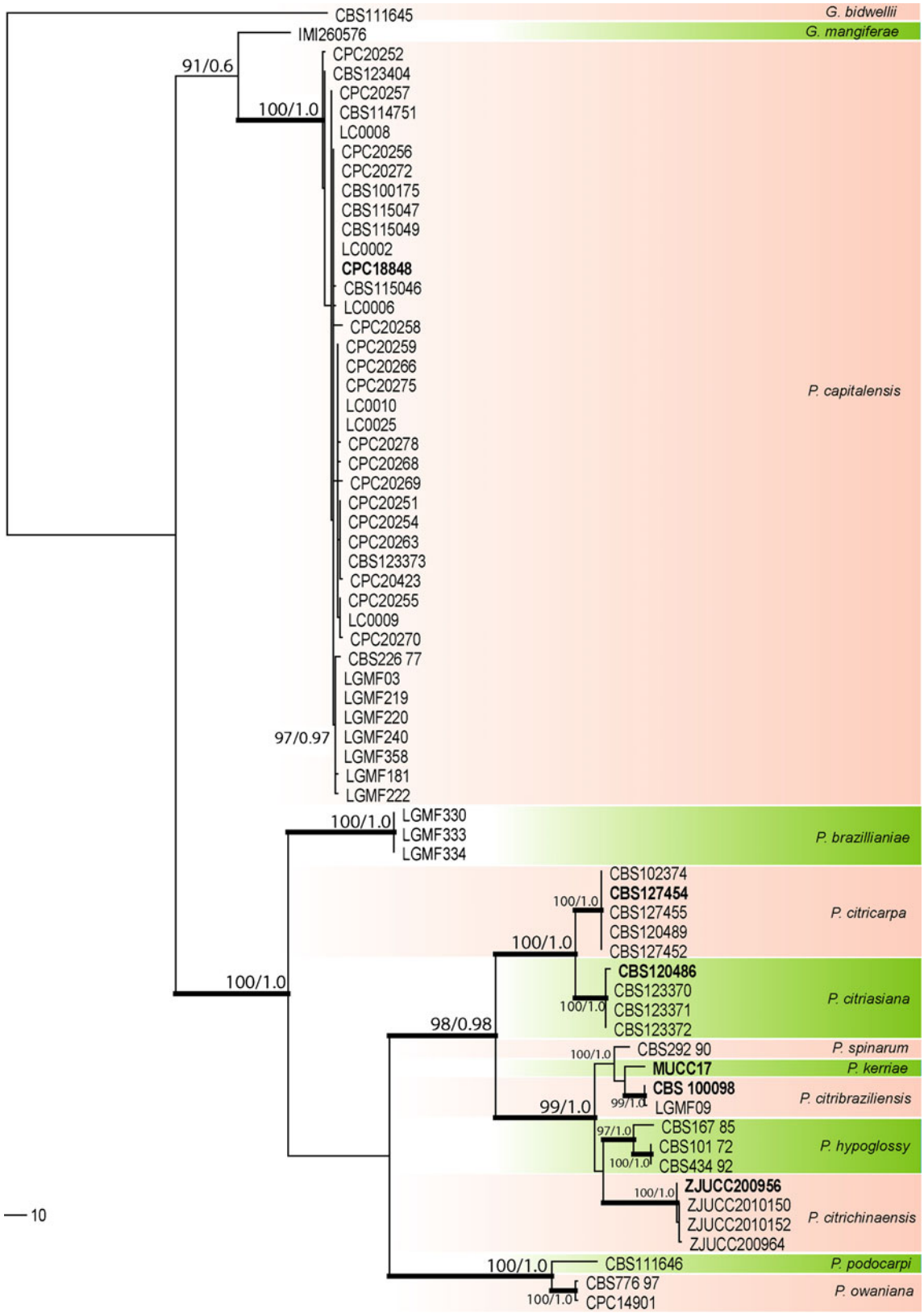

Fig. 3 Phylogenetic tree generated from 1000 replicates bootstrap values parsimony analysis/Bayesian analysis based on combined ITS rDNA, TEF1 and ACT sequence data. The tree is rooted with *Guignardia bidwellii* (CBS 111645)

Table 2 Hosts and countries from which *Phyllosticta capitalensis* has been isolated, usually as an endophyte, rarely as a pathogen (P) (See also Fig. 1)

Plant family	Plant genus	Country	Reference
Acanthaceae	*Mackaya*	South Africa	
Anacardiaceae	*Anacardium*	Brazil	Glienke et al. 2011
	Comocladia	Puerto Rico	
	Loxostylis	South Africa	
	Mangifera	Brazil	
		Ghana	Baayen et al. 2002
	Myracrodruon	Brazil	Glienke et al. 2011
	Rhus	South Africa	Baayen et al. 2002
	Sclerocarya	South Africa	
	Spondias	Brazil	
Annonaceae	*Monanathotaxis*	South Africa	
	Polyalthia	Thailand	Present study
Apocynaceae	*Aspidosperma*	Brazil	Glienke et al. 2011
	Secamone	South Africa	
	Cerbera	Japan	Okane et al. 2003
	Nerium	Japan	Motohashi et al. 2009
Aquifoliaceae	*Ilex*	USA	
		Japan	Okane et al. 2003
	Cerbera	Japan	Okane et al. 2003
Araliaceae	*Cussonia*	South Africa	
	Hedera	South Africa	
	Polyscias	Puerto Rico	
	Schefflera	Costa Rica	Baayen et al. 2002
	Polyscias	Thailand	Present study
Araceae	*Alocasia*	Thailand	Present study
	Anthurium	Thailand	Present study
	Dieffenbachia	Thailand	Present study
	Livistona	Thailand	Present study
	Spathiphyllum	Japan	Motohashi et al. 2009
	Philodendron	Thailand	Present study
Asparagaceae	*Sansevieria*	Thailand	Present study
	Ophiopogon (P)*	Thailand	Present study
Boraginaceae	*Cordia*	South Africa	
Calophyllaceae	*Calophyllum*	Thailand	Present study
Capparaceae	*Maerua*	South Africa	
Chrysobalanaceae	*Parinari*	South Africa	
Combretaceae	*Combretum*	South Africa	
Convolvulaceae	*Ipomoea*	Malaysia	Present study
Cornaceae (Nyssaceae)	*Curtisia*	South Africa	Baayen et al. 2002
	Davidia	Japan	Motohashi et al. 2009
Celastraceae	*Putterlickia*	South Africa	Baayen et al. 2002
Cercidiphyllaceae	*Cercidiphyllum*	Japan	Motohashi et al. 2009
Ebenaceae	*Diospyros*	South Africa	
	Euclea	South Africa	
Ericaceae	*Rhododendron*	Japan	Okane et al. 2003
	Enkianthus	Japan	Okane et al. 2001
	Vaccinium	New Zealand	Glienke et al. 2011
Fabaceae	*Bowdichia*	Brazil	Glienke et al. 2011

Table 2 (continued)

Plant family	Plant genus	Country	Reference
	Cercis	Japan	Motohashi et al. 2009
Fagaceae	*Lithocarpus*	Japan	Motohashi et al. 2009
Ginkgoaceae	*Ginkgo*	Japan	Motohashi et al. 2009
Lamiaceae	*Vitex*	Malaysia	Present study
Lauraceae	*Cinnamomum*	Japan	Okane et al. 2003
	Ocotea	South Africa	
Lecythidaceae	*Barringtonia*	South Africa	Baayen et al. 2002
Leguminosae	*Caesalpinia*	Japan	Okane et al. 2003
Loganiaceae	*Stychnos*	South Africa	
	Anthocleista	South Africa	
Lythraceae	*Punica* (P)	Thailand	Present study
Malvaceae	*Hibiscus*	Thailand	Present study
Meliaceae	*Ekebergia*	South Africa	
	Trichilia	South Africa	Baayen et al. 2002
Menispermaceae	*Cocculus*	USA	
Moraceae	*Artocarpus*	Thailand	Baayen et al. 2002
	Ficus (P)	Thailand	Present study
	Morus	Thailand	
Magnoliaceae	*Michelia*	Thailand	Present study
	Magnolia	Thailand	Glienke et al. 2011
		USA	
Menispermaceae	*Tinospora*	Thailand	Present study
Euphorbiaceae	*Clutia*	South Africa	Baayen et al. 2002
	Croton	South Africa	
	Codiaeum	Thailand	Present study
	Ctenomeria	South Africa	
	Euphorbia	Thailand	Present study
Flacourtiaceae	*Dovyalis*	South Africa	
Iteaceae	*Itea*	USA	
Lamiaceae	*Tectona*	Thailand	Present study
Musaceae	*Musa*	Thailand	Okane et al. 2003
		Indonesia, USA	Glienke et al. 2011
Myrtaceae	*Eucalyptus*	Brazil, South Africa	Glienke et al. 2011
	Psidium	Brazil	Baayen et al. 2002
Oleaceae	*Ligustrum*	Japan	Motohashi et al. 2009
	Schrebera	South Africa	
Ophioglossaceae	*Botrychium*	USA	
Orchidaceae	*Arundina*	Japan	Okane et al. 2003
	Coelogyne	Thailand	
	Dendrobium	Thailand	Present study
	Paphiopedilium	Germany	Okane et al. 2001
Orchidaceae	*Rhynchostylis* sp.	Malaysia	Williams & Liu 1976, Singh 1980
	Stanhopea	Brazil	Glienke et al. 2011
Pittosporaceae	*Pittosporum*	Hawaii	Baayen et al. 2002
Poaceae	*Saccharum*	Thailand	Present study
Podocarpaceae	*Podocarpus*	South Africa	
Proteaceae	*Leucospermum*	Hawaii	
	Protea	Hawaii	
	Telopea	Australia	

Table 2 (continued)

Plant family	Plant genus	Country	Reference
Pittosporaceae	*Pittosporum*	Japan	Motohashi et al. 2009
Pteridophta	*Pteridophytes*	Japan	Okane et al. 2003
Rhamanaceae	*Scutia*	South Africa	
	Zizyphus	South Africa	
Rhizophoraceae	*Kandelia*	Japan	Okane et al. 2003
Rosaceae	*Cliffortia*	South Africa	
	Rubus	Japan	Okane et al. 2003
	Prunus	Japan	Okane et al. 2003
	Eriobotrya	Japan	Motohashi et al. 2009
Rubiaceae	*Canthium*	South Africa	
	Coprosma	Hawaii	Baayen et al. 2002
	Gardenia	South Africa	
	Pavetta	South Africa	
	Rauvolfia	South Africa	
	Rothmannia	South Africa	
Rutaceae	*Zanthoxylum*	Japan	Okane et al. 2003
	Citrus (P)	Argentina, Australia, Brazil, China, Hong Kong, New Zealand, South Africa, Taiwan, Thailand, USA	Glienke et al. 2011; Wang et al. 2012
	Fortunella	USA	
	Vitex	South Africa	
	Zanthoxylum	Pueto Rico	Baayen et al. 2002
Sapindaceae	*Allophylus*	South Africa	
	Dodonaea	Hawaii	
	Litchi	South Africa	
	Nephelium	USA	Glienke et al. 2011
	Paullinia cupana	Brazil	Baayen et al. 2002
Smilacaceae	*Smilax*	South Africa	Glienke et al. 2011
Solanaceae	*Capsicum*	Dominican	Glienke et al. 2011
Stangeriaceae	*Stangeria*	South Africa	Baayen et al. 2002
Sterculiaceae	*Sterculia*	Puerto Rico	
Theaceae	*Camellia*	USA	Baayen et al. 2002
Tiliaceae	*Grewia*	South Africa	
Trimeniaceae	*Xymalos*	South Africa	
Ulmaceae	*Trema*	South Africa	
Veronicaceae	*Hebe (Veronica)*	South Africa	
Viscaceae	*Viscum*	South Africa	
Vitaceae	*Ampelopsis*	USA	Baayen et al. 2002
	Cryphostemma	South Africa	
	Rhoicissus	South Africa	
Zamiaceae	*Encephalartos*	South Africa	
Zingiberaceae	*Amomum*	Thailand	Okane et al. 2003
	Zingiber	Thailand	Okane et al. 2003

*(P) = Leaf spot

Recent studies on *Phyllosticta* causing freckle disease of banana and disease of other hosts have shown that extreme caution must be applied when using the above standard plant pathology approach (Wong et al. 2012). Conidia of *Phyllosticta* rarely germinate in culture and thus with many species it is impossible to obtain single spore cultures

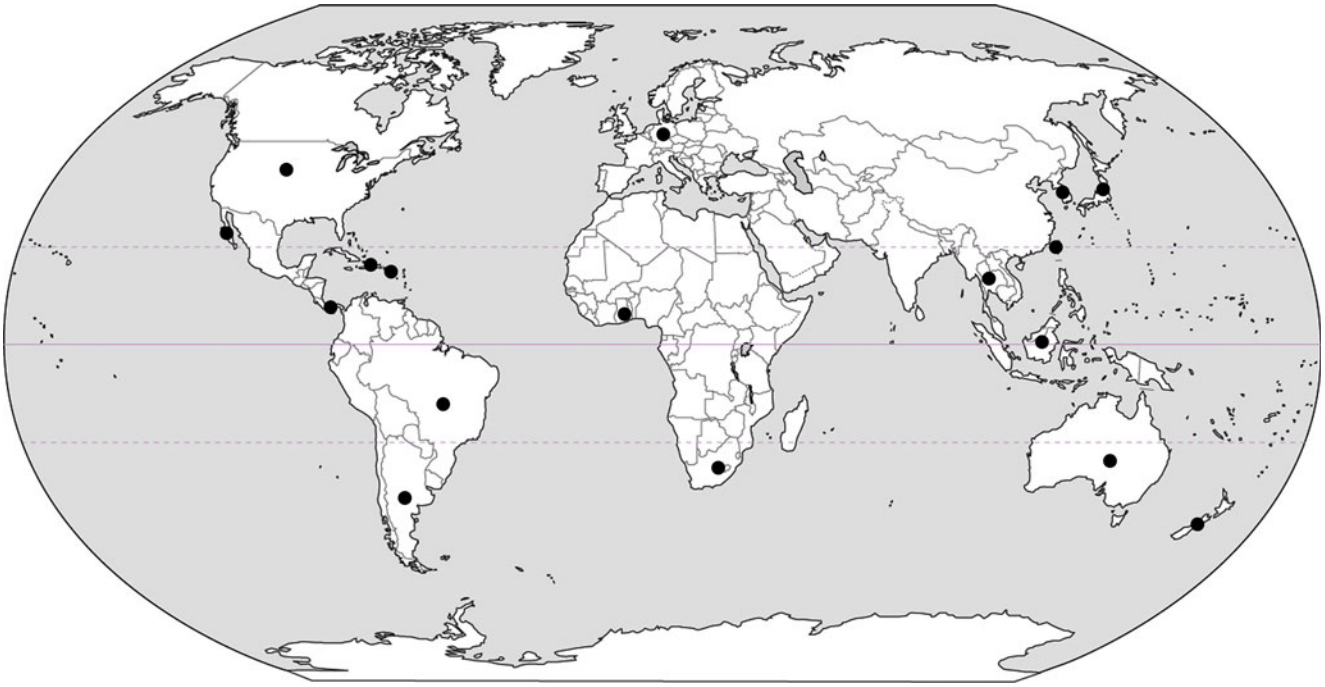

Fig. 4 World distribution of *Phyllosticta capitalensis* (the *dots* represent countries)

(Chomnunti et al. 2011). If freckle infected banana tissues are surface sterilized and plated on agar, *P. capitalensis* invariably grows out and, therefore, is concluded to be the pathogen, which is not the case. If these strains of *P. capitalensis* are used in pathogenicity testing they may also be weak pathogens and thus "substantiate" the record as the causal agent. However, Wong et al. (2012) carefully dissected whole ascomata from freckle diseased banana tissues. They then surface sterilized the ascomata and plated them out to obtain "single ascomata cultures". In this way they were able to establish that freckle disease was caused by more than one species of *Phyllosticta* and discerned the causal agent of freckle in Queensland as *P. cavendishii* M.H. Wong & Crous (Wong et al. 2012). *Phyllosticta citricarpa*, which causes citrus black spot (CBS) is widespread in some citrus-producing countries but is absent from EU and USA, where it is a regulated pathogen. CBS has been often misdiagnosed on citrus fruit and many of the lesions are, in fact, colonised by *P. capitalensis*. Traditional methods of diagnosis are time consuming and involve incubation of infected material, morphological examination of the fungus, and perhaps dissecting and plating of lesion pieces. Misdiagnosis of CBS may result in significant financial loss to farmers and exporters. An acurate and less time consuming method to verify and identify *Phyllosticta* species on citrus fruit is essential for both the producer and regulatory authorities (Meyer et al. 2012).

Further careful research of this type in other banana growing regions is likely to reveal other species causing freckle disease. The above example serves to illustrate how a Koch's postulate can result in incorrect data concerning the identity of causal agents of disease, particularly with *Phyllosticta* species. Besides banana disease we suspect that many diseases caused by *Phyllosticta* (and "*Guignardia*"), unless directly identified via sporulating structures, e.g. *Guignardia candeloflamma* K.D. Hyde, on a species of *Pinanga* in north Queensland, Australia and an unidentified palm in Irian Jaya (Fröhlich and Hyde 1995), may be wrongly attributed to *P. capitalensis*. Future studies must take this problem of protocol into account. Whether this phenomenon applies to other fungal genera needs future investigation.

Endophyte study protocols

There are many definitions of an endophyte and these have been summarized by Hyde and Soytong (2008). A standard definition is "organisms that colonize plant organs in some period of time in plant life cycle without causing obvious harm on the host" (Petrini 1984; 1991). The standard methodology for isolating endophytes has been reviewed in numerous instances (e.g. Guo et al. 1998, 2001; Photita et al. 2004, 2005) and has been criticised for being biased towards fast growing fungal strains (Hyde and Soytong 2007). However, in principle the method is the same as that used by plant pathologists for isolating pathogens from diseased tissue, albeit that endophyte researchers use healthy leaves. The problem with the protocol mentioned above concerning isolating *P. capitalensis* rather than the *Phyllosticta* causal agent may also occur in endophyte

studies. *Phyllosticta capitalensis* is a quick growing species; in culture the colony covers a 9 cm Petri-dish in 10 days. Other species grow more slowly, e.g. *P. yuccae* reaches 3–5 cm diam in 15 days (Bissett 1986), while growth of *P. vaccinii* can be as low as 0.4 mm/day. Four species of *Phyllosticta* (*P. citriasiana*, *P. capitalensis*, *P. citricarpa* and *P. citrichinaensis*) were recently isolated from *Citrus* in China (Wang et al. 2012) and *P. citrichinaensis* grew at 3.8± 0.34 mm per day at 24 °C on PDA. Therefore, it is highly likely that *P. capitalensis* will be isolated in endophyte studies, while others species which are probably also endophytes, will not be isolated. This will skew the results considerably and the resulting endophyte lists, percentages and statistics may have little scientific meaning.

If this phenomenon of isolating *P. capitalensis* for the reasons mentioned above is happening in the case of *Phyllosticta* it may also be happening in other genera such as *Colletotrichum*, *Diaporthe, Fusarium* or *Pestalotiopsis* (Promputtha et al. 2005; Udayanga et al. 2011; Summerell et al. 2010; Maharachchikumbura et al. 2011; Damm et al. 2012a, b). To determine this fact we took the common ubiquitous endophytes *Colletotrichum siamense* Prihastuti, L. Cai & K.D. Hyde, *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., and *Pestalotiopsis adusta* (Ellis & Everh.) Steyaert and blasted the ITS sequence data from the epitype strains against GenBank accessions and established the percentage of them that were isolated as endophytes. Twelve strains of *Colletotrichum* in GenBank had 100 % similarity with the ITS sequence data of *C. siamense* (Prihastuti et al. 2009) and 50 % of these strains were isolated as endophytes. The ITS sequence of ex-isotype of *D. phoenicicola* (CBS161.64, Udayanga et al. 2012) was subjected to a standard BLAST search in GenBank to analyze the homology of sequences. Among the first 10 results of highly similar sequences (100 or 99 % similarity) of retrieved data, eight were isolated as endophytes from a wide range of hosts. This is not surprising as *Diaporthe* is a commonly isolated genus of fungal endophytes (Botella and Diez 2011; Sun et al. 2011; Hofstetter et al. 2012). Eleven strains of *Pestalotiopsis* in GenBank had 100 % similarity with the ITS sequence data of *P. adusta* (Maharachchikumbura et al. 2012) and 73 % were endophytes. Again this is not surprising as *Pestalotiopsis* species are often isolated as endophytes (Aly et al. 2010; Debbab et al. 2011, 2012; Maharachchikumbura et al. 2011). Therefore, it seems that certain taxa in these genera are widespread endophytes and this needs further study.

Screening endophytes for novel compounds

It has been common practice to isolate endophytes from medicinal plants using the premise that strains will be isolated that can produce bioactive compounds similar to those produced by the plant (Krohn et al. 2007; Huang et al. 2008; Kumaran et al. 2008; Xu et al. 2010; Zhao et al. 2010). The fungi are thought to have obtained the mechanisms of production of natural products from the plant by so called horizontal gene transfer (Strobel et al. 2004); whether this premise is correct or pure speculation is open to debate (Schulz et al. 2002; Selim et al. 2012) and in fact may be false (Heinig et al. 2013). The isolation of endophytes may provide a large diversity of highly creative fungi for screening (Aly et al. 2010; Xu et al. 2011; Debbab et al. 2011; 2012). The findings of the present study indicate that there are problems with the above approach. It is clear in the case of *Phyllosticta* that *P. capitalensis* will probably be the only endophyte species isolated. Therefore, we recommend that researchers screening for novel compounds should study the saprobes and pathogens as well as the endophytes. This will give a higher fungal diversity and higher likelihood of isolating rare and unusual species, and thus a higher likelihood of discovering greater chemical diversity.

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