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Characterisation and pathogenicity of *Pestalotiopsis uvicola* and *Pestalotiopsis clavispora* causing grey leaf spot of mango (*Mangifera indica* L.) in Italy

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Abstract During 2009 and 2010, twenty-one isolates of *Pestalotiopsis* spp. Associated with grey patches on the leaves, twigs, and panicles of mango were collected in six orchards located in Sicily (Italy). Morphological characteristics of colony (colour and mycelium appearance), and conidia (size, shape, septation, length and the number of apical and the basal appendages) as well as phylogenetic analysis of the Internal Transcribed Spacer (ITS) region (ITS1, 5.8S gene, and ITS2) of six representative isolates revealed the occurrence of *P. uvicola* and *P. clavispora*. The representative isolates of both species were pathogenic to the artificially inoculated detached mango leaves cv. Kensington Pride and showed significant variation in lesion size. This is the first report worldwide of *P. uvicola* and *P. clavispora* causing grey leaf spot of mango.

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The mango (*Mangifera indica* L., family Anacardiaceae) is grown throughout a wide range of frost-free climates and is one of the world's most important fruit crops (Litz 1998). *Pestalotiopsis mangiferae* (Henn.) Steyaert (syn. *Pestalotia mangiferae* Henn.) is the casual agent of grey leaf spot and stem-end rot of mango fruit in several countries (Mordue 1980; Lim and Khoo 1985; Johnson 1994; Ko et al. 2007). Grey leaf spot, although common on mango in the tropics, is usually a minor problem (Lim 1998). *P. mangiferae* is a weak pathogen that usually requires wounding in order to infect mango. Other *Pestalotiopsis* species were described on mango plants. *P. mangifolia* (Guba) J. Xiang Zhang & T. Xu was founded associated to leaf spot and *P. versicolor* (Speg.) Steyaert was detected on flowers (Lim 1998). In addition, *P. glandicola* (Castagne) Steyaert was reported as causal agent of a post-harvest disease of mango in Bangalore (Ullasa and Rawal 1989). Since 1990, mango cultivation was introduced to the southern Italy (Sicily) and the main diseases present in this area are not recognised. Recently, during a survey conducted in mango plantations in Sicily, severe symptoms of grey leaf spot were detected in all orchards and observed on leaves, twigs, and panicles. Grey leaf spot symptoms were widespread with approximately 70 to 100 % symptomatic plants per field. As a consequence, the

vegetative growth and the fruit production of young mango plants were reduced (Fig. 1). Thus, the objectives of this work was to determine the etiology of the grey leaf spot of mango in southern Italy.

Surveys were performed during 2009 and 2010 in six mango orchards cv. Kensington Pride located in Catania (2 orchards), Messina (2 orchards), Palermo (1 orchard), and Ragusa (1 orchard). Twenty five samples of symptomatic leaves, twigs, and panicles were collected. Plant materials were surface disinfected by sequential washing in 70 % EtOH for 30 s and 10 % bleach solution (0.5 % NaOCl) (for 1 min), and then rinsed in distilled sterile water (DSW). Small pieces between the healthy tissues and infected ones were cut into 2–4 mm^2 and placed in 9-cm-diameters Petri dishes containing potato dextrose agar medium (PDA) (Difco Laboratories, Detroit, MI)) amended with streptomycin sulphate (0.1 gl^{-1}) to inhibit any bacterial growth. Plates were incubated at 25±2 °C in the dark for 5 days until fungal growth appeared. The most prevalent fungal species associated with grey leaf spot were *Pestalotiopsis* spp. Twenty-one pure cultures of *Pestalotiopsis* spp. were grown on PDA at 25 °C in the dark for 7–10 days (Table 1) and were identified on the basis of morphological criteria such as conidia size, septation, number of appendages as well as colony texture and colour (Keith et al. 2006; Espinosa et al. 2009; Sutton 1980). The isolates were initially separated into two groups according to their appearance on culture (Fig. 1). In all *Pestalotiopsis* isolates five-celled conidia were observed, of which apical and basal cells were thin-walled hyaline to pale olivaceous and the three median cells were thick-walled light to dark

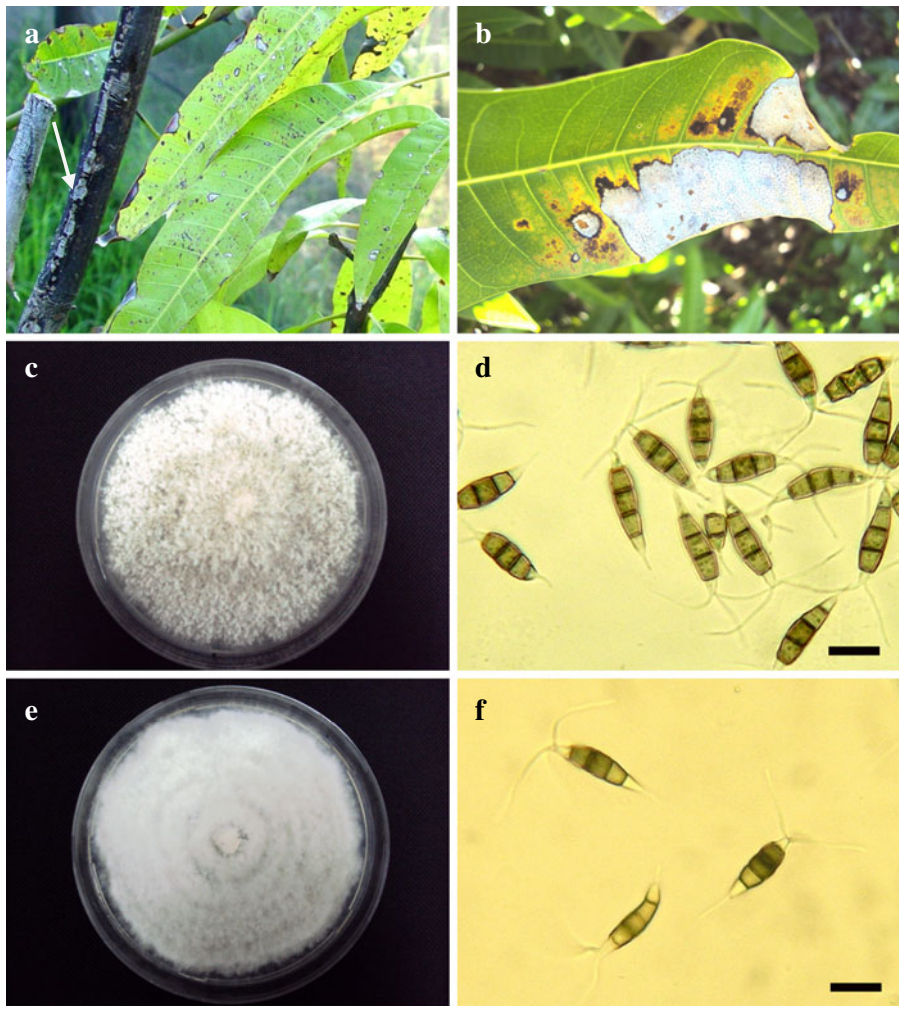

Fig. 1 Common observed symptoms of grey leaf spot during a survey in mango plantation. **a**, large grey necrotic lesions along the branch (*arrow*) **b** lesions coalesce to form grey patches and abundant black acervuli were formed on the necrotic tissues. Colony and conidia of *P. uvicola* (**c**, **d**) and *P. clavispora* (**e**, **f**). Scale bars: **d**-**f**=20 μm

Table 1 *Pestalotiopsis* isolates obtained in this study and their origin

Isolates (GeSA No°)[a]	Plant organ	Origin (Province)
P. uvicola		
2, 3, 4, 5[b], 6	twigs	Catania-orchard 1
7[b], 9	leaves	Catania-orchard 1
15, 17	twigs	Catania-orchard 2
16	leaves	Catania-orchard 2
25[b]	leaves	Ragusa-orchard 3
26	twigs	Ragusa-orchard 3
P. clavispora		
10[b], 11, 12, 13, 14	twigs	Catania-orchard 2
18[b]	twigs	Palermo-orchard 1
20, 21	panicles	Messina-orchard 1
22[b]	leaves	Messina-orchard 2

[a]GeSA: Dipartimento di Gestione dei Sistemi Agroalimentari e Ambientali, Catania, Italy

[b]Isolates used for molecular analysis and pathogenicity test

brown. In the first group of the isolates resembling to *P. uvicola* conidia were smooth, fusiform, straight, measuring 24.6±1.6 to 25.1±1.7 μm mean length and 7.2 to 7.9±0.6 μm mean width. Two to three hyaline apical appendages and one basal appendage were usually observed. The apical appendages lengths ranged from 14.1 to 19.5 μm mean length, while the basal appendages measured 6.4 to 7.2 μm mean length. The colony was initially white to greenish (front side of plates) and pale saffron colour in the centre of colony diffused beyond colony margins (reverse side). The acervuli were black and appeared moderately concentrated in the centre of the plate. In the second group of the isolates resembling to *P. clavispora*, conidia were smooth, fusiform, and wider at the middle than apex and base measuring, 22.3±1.4 to 24.7±1.9 μm mean length and 7.6±0.5 to 9.6±0 μm mean width. Two to four (three being the most frequently observed) apical hyaline straight and slightly swollen at apex appendages measuring 21.8 to

Table 2 *Pestalotiopsis* isolates retrieved from the GenBank used for phylogenetic analysis

Species	Culture No.	Location	Host	ITS GenBank Accession No.
Pestalotiopsis matildae	CBS118155	South Africa	*Thamnochortus spicigerus*	DQ278916
Pestalotiopsis matildae	CBS118143	South Africa	*T. spicigerus*	DQ278917
Pestalotiopsis microspora	TC-13	India	*Terminalia chebula*	AY924287
Pestalotiopsis microspora	AZ-71	India	*Azadirachta indica*	AY924291
Pestalotiopsis disseminata	CY236	USA-Texas	*Cyphomyrmex wheeleri*	HQ608049
Pestalotiopsis disseminata	CY152	USA-Texas	*Cyphomyrmex wheeleri*	HQ607992
Pestalotiopsis heterocornis	Endo358	China	*Podocarpus macrophyllus*	AY681489
Pestalotiopsis heterocornis	Endo391	China	*Podocarpus macrophyllus*	AY681491
Pestalotiopsis theae	Endo80	China	*Lucuma nervosa*	DQ813433
Pestalotiopsis theae	Endo46	China	*Dracontomelon duperreanum*	DQ813432
Pestalotiopsis clavispora	P6	Canada	*Argania spinosa*	HQ414541
Pestalotiopsis clavispora	PALUC-12	Chile	*Persea americana*	HQ659767
Pestalotiopsis uvicola	UCD2568AR	USA	*Vitis vinifera*	HQ288239
Pestalotiopsis uvicola	Isolate 173189	China	*Taxus chinensis*	JN198506
Guignardia mangiferae	CBS 115051	Brazil	*Spondias mombin*	FJ538325
Guignardia citricarpa	CBS 102374	Brazil	*Citrus aurantium*	FJ538313

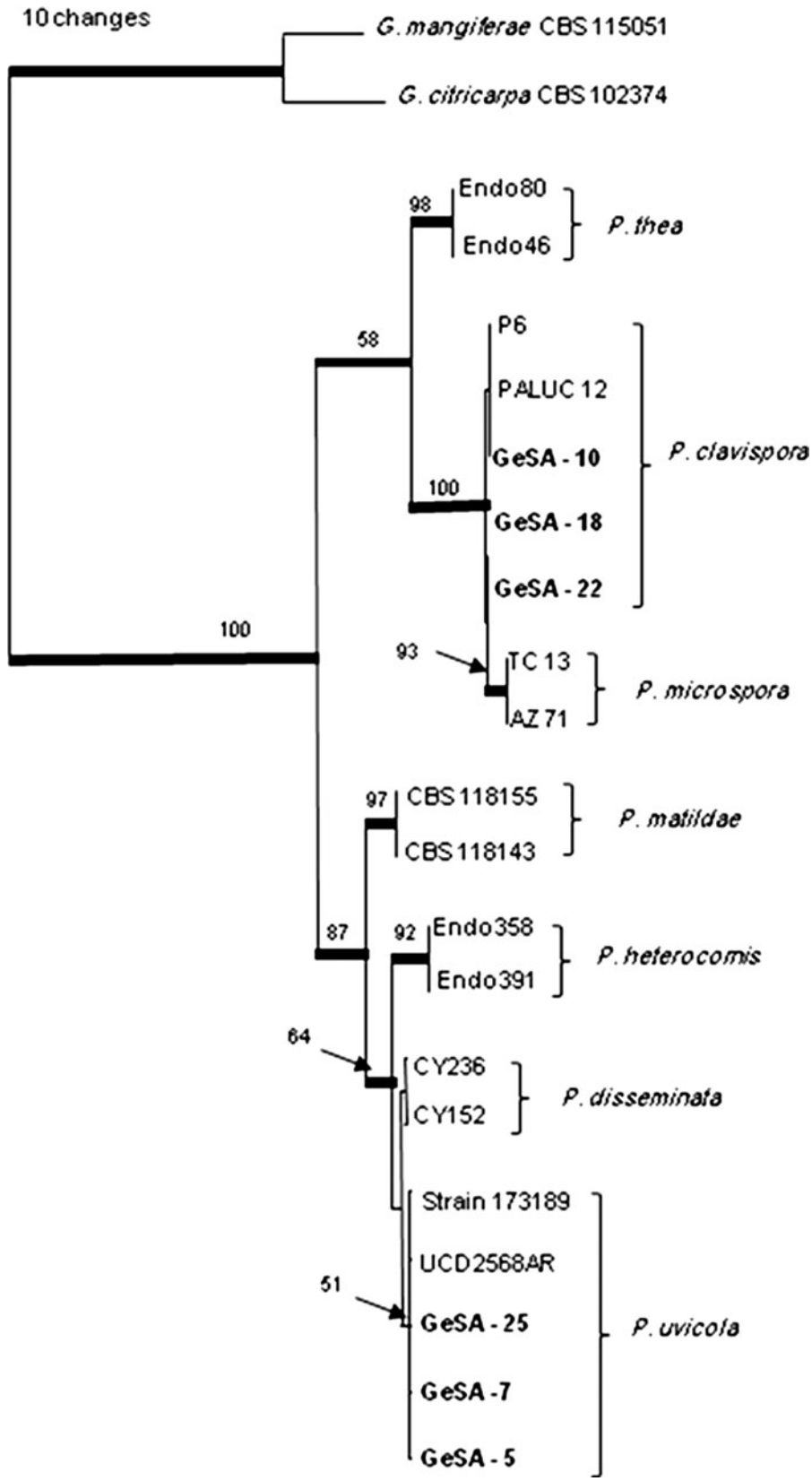

Fig. 2 One of the 17 consensus trees obtained by PUAP maximum parsimony analysis from partial ITS region (ITS1, 5.8S gene and ITS2) of rDNA data sequence (TL=252, CI= 0.921, RI=0.957, RC= 0.881, HI=0.079). Parsimony bootstrap values from 1,000 replicates are indicated on the branches nodes. The tree was rooted to outgroup taxa *Guignardia mangiferae* CBS115051 and *Guignardia citricarpa* CBS102374. Isolates in bold are obtained from mango

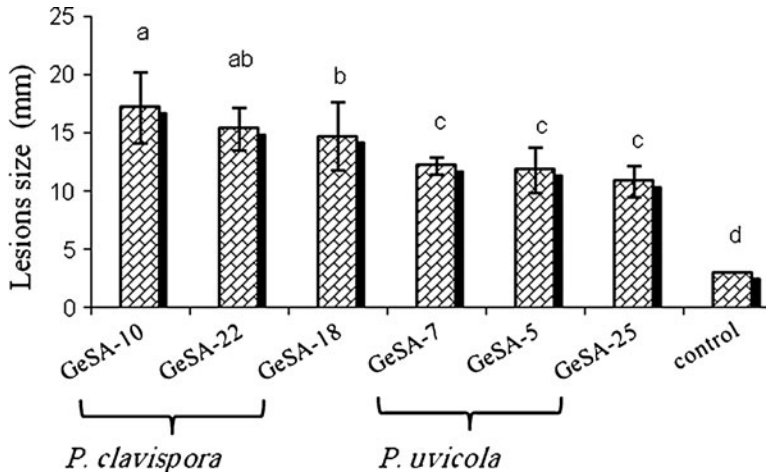

Fig. 3 Lesions induced by inoculation of the six representative isolates of *P. uvicola* and *P. clavispora* on detached mango leaves cv. Kensington Pride. Data within columns are the mean of six lesions developed on two detached mango leaves. Means values within columns followed by same letter are not significantly different according to Least Significant Different test (LSD) at $P<0.05$

33.9 μm mean length and one basal appendage measuring 5.5 to 8.1 μm mean length. The colony was white to creamy with dense mycelium, circular growth appearance (front side) and pale luteos to saffron colour diffused in the medium (reverse side). The acervuli were black and appeared throughout the entire plate.

Six representative isolates (GeSA-5, GenBank No JX875592; GeSA-7, GenBank No JX875593; GeSA-10, GenBank No JX875594; GeSA-18, GenBank No JX875595; GeSA-22, GenBank No JX875596 and GeSA-25, GenBank No JX875597), were selected and total DNA was extracted using Puregene® Genomic DNA Purification Kit following the manufacturer's instructions (Gentra Systems, Minnesota. U.S.A). Part of the internal transcribed spacer (ITS) region of the ribosomal DNA operon was amplified with the universal primers ITS1 and ITS4 (White et al. 1990). PCR reaction was carried out in 25 μl containing 1× PCR buffer (Invitrogen-Life Technologies), 1 mg/ml BSA, 2 mM $MgCL_2$, 1 mM dNTPs, 0.5 μM of each primer, 1U *Taq* polymerase and 1 μl of DNA template. After an initial hot start (94 °C for 3 min), 35 PCR cycles were performed on a Gene Amp®. PCR System 9700 thermocycler (Foster City, USA) using the following conditions: a denaturation step of 94 °C for 30 s followed by annealing at 50 °C for 50 s and extension at 72 °C for 1 min followed by final extension at 72 °C or 10 min. PCR products of the ITS region (ITS1, 5.8S gene, and ITS2) were sequenced using the same primers (ITS1 and ITS4) of the initial amplification. DNA purification and sequencing was performed at BMR Genomics (Padova, Italy) according to the manufacturer's protocol. Sequence data matrix was aligned using ClustalW option in *MEGA* version 5 (Tamura et al. 2011) and corrected the alignment where necessary. The phylogenetic relationship was determined using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002). Maximum parsimony was performed using the heuristic search option with 1,000 random taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. MAXTREE set to 1,000, branches of zero length were collapsed and equally parsimonious trees were saved. Bootstrap support values were evaluated using 1,000 bootstrap replicates to determine tree length (TL), consistency index (CI), rescaled consistency index (RC), retention index (RI), and the homoplasy index (HI) (Hillis and Bull 1993). PCR amplicons of all isolates were ~510 to 600 bp. The manually aligned and adjusted ITS data set contained 22 sequences of which six sequences were obtained from mango in this study and 18 were retrieved from GenBank (Table 2). From total 533 characters including gaps, 337 were constant and 35 variable characters were parsimony-uninformative while, 161 were parsimony-informative characters. After heuristic search, maximum parsimony analysis of the 161 informative characters resulted in 17 trees (TL=252, CI=0.921, RI =0.957, RC= 0.881, HI=0.079). Results of the phylogenetic analysis indicated that *Pestalotiopsis* species obtained from *M. indica* can be assigned in two well-separated clades of which, *P. clavispora* isolates were clustered with strains (P6 and PALUC 12) obtained from *Argania spinosa* and *Persea*

americana respectively, in a clade strongly supported with bootstrap value 100 %; whereas, *P. uvicola* isolates from mango were sub-grouped in a clade poorly supported with bootstrap value 51 % within isolates (Isolate 173189 and UCD2568AR) obtained from *Taxus chinensis and Vitis vinifera* respectively (Fig. 2).

The representative six isolates of *Pestalotiopsis* spp. were tested for pathogenicity on detached mango leaves cv. Kensington Pride disinfected by 10 % bleach solution (0.5 % NaOCl) for 2 min. Three leaves were used for each isolates. A 6-mm of mycelium plugs from a 7-days-old culture was placed on three wounded points. Leaves inoculated with only PDA plugs served as controls. Inoculated leaves were maintained in high relative humidity, and incubated at 25 °C. Mean lesion sizes were registered after 7 days of incubation. The trial was repeated once. Symptoms consisted of light to dark brown lesions surrounding the inoculation sites with irregular dark brown margins, later, become greyish in colour and numerous acervuli were developed on the necrotic tissues. To fulfill Koch's postulates, diseased tissues were placed on PDA. *Pestalotiopsis* spp. were re-isolated with 100 % frequency and the colonies were morphologically identical to those from original isolates. All the tested isolates of *Pestalotiopsis* were proven to be pathogenic to mango leaves. *P. clavispora* isolates produced significantly larger lesion sizes than *P. uvicola* isolates (Fig. 3).

Since the introduction of mango in Italy, little is known concerning the presence of fungal diseases and their etiology. In the present study we reported the occurrence on mango orchards of *P. uvicola* and *P. clavispora* identified on the basis of morphological features as well as molecular analysis and we demonstrated the pathogenicity of both species on mango leaves. *Pestalotiopsis* spp. generally are not host-specific and can infect a wide range of plants (Steyaert 1953; Suto and Kobayashi 1993; Taguchi et al. 2001). *P. uvicola* was previously reported in Sicily as the causal agent of leaf spot and stem blight of bay laurel (Vitale and Polizzi 2005). Moreover, *P. clavispora* was reported in China and in Chile as responsible for leaf spot, canker and twig dieback of blueberry (Luan et al. 2008; Espinoza et al. 2009) and more recently causing post-harvest stem end rot of avocado in Chile (Valencia et al. 2011). To our knowledge, this is the first report worldwide of *P. uvicola* and *P. clavispora* causing grey leaf spot on mango orchards.

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References

Espinoza, J. G., Briceno, E. X., Keith, L. M., & Latorre, B. A. (2009). Canker and twig die back of Blueberry caused by *Pestalotiopsis* spp. and a *Truncatella* sp. in Chile. *Plant Disease, 92*, 1407–1414.

Hillis, D. M., & Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology, 42*, 182–192.

Johnson, G. I. (1994). Stem-end rots. In R. C. Ploetz, G. A. Zentmyer, W. T. Nishijima, K. G. Rohrbach, & H. D. Ohr (Eds.), *Compendium of tropical fruit diseases* (pp. 39–41). St. Paul: American Phytopathological Society.

Keith, L. M., Velasquez, M. E., & Zee, F. T. (2006). Identification and characterization of *Pestalotiopsis* spp. causing scab disease of guava, *Psidium guajava*, in Hawaii. *Plant Disease, 90*, 16–23.

Ko, Y., Yao, K. S., Chen, C. Y., & Lin, C. H. (2007). First Report of Gray Leaf Spot of Mango (*Mangifera indica*) Caused by *Pestalotiopsis mangiferae* in Taiwan. *Plant Disease, 91*, 1684.

Lim, T. K. (1998). Part III. Mango. In R. C. Ploetz, G. A. Zentmyer, W. T. Nishijima, K. G. Rohrbach, & H. D. Ohr (Eds.), *Compendium of tropical fruit diseases* (p. 36). St. Paul: American Phytopathological Society.

Lim, T. K., & Khoo, K. C. (1985). *Diseases and disorders of mango in Malaysia*. Kuala Lumpur: Tropical Press.

Litz, R. E. (1998). Part III. Mango. In R. C. Ploetz, G. A. Zentyer, W. T. Nishijima, K. G. Rohrbach, & H. D. Ohr (Eds.), *Compendium of tropical fruit diseases* (pp. 33–34). St. Paul: American Pathological Society.

Luan, Y. S., Shang, Z. T., Su, Q., Feng, L., & An, L. J. (2008). First report of *Pestalotiopsis* sp. causing leaf spot of blueberry in China. *Plant Disease, 92*, 171.

Mordue, J. E. M. (1980). *Pestalotiopsis mangiferae*. Commonwealth Mycological Institute. *Descriptions of Pathogenic Fungi and Bacteria, 676*, 1–2.

Steyaert, R. L. (1953). New and old species of *Pestalotiopsis*. *Transactions of the British Mycological Society, 36*, 81–89.

Suto, Y., & Kobayashi, T. (1993). Taxonomic studies on the species of *Pestalotiopsis*, parasitic on conifers in Japan. *Transactions of the Mycological Society of Japan, 34*, 323–344.

Sutton, B. C. (1980). *The Coelomycetes: Fungi imperfecti with Pycnidia, Acervular and Stromata*. Kew: Commonwealth Mycological Institute.

Swofford, D. L. (2002). *PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b10*. Sunderland: Sinauer Associates.

Taguchi, Y., Watanabe, H., Akita, S., & Hyakumachi, M. (2001). Occurrence and control of ripe rot symptoms of persimmon fruit (in Japanese). *Japanese Journal of Phytopathology, 67*, 33–41.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA 5: molecular evolutionary

genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*, 2731–2739.

Ullasa, B. A., & Rawal, R. D. (1989). Occurrence of new postharvest disease of mango due to Pestalotiopsis glandicola. *Acta Horticulturae, 231*, 540–543.

Valencia, A. L., Torres, R., & Latorre, B. A. (2011). First report of *Pestalotiopsis clavispora* and *Pestalotiopsis* spp. causing postharvest stem end rot of avocado in Chile. *Plant Disease, 95*, 492.

Vitale, A., & Polizzi, G. (2005). Occurrence of *Pestalotiopsis uvicola* causing leaf spots and stem blight on bay laurel (*Laurus nobilis*) in Sicily. *Plant Disease, 89*, 1362.

White, T. S., Bruns, T., Lee, S., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, H. H. Sninsky, & T. J. White (Eds.), *PCR Protocols: A Guide to Methods and Applications* (pp. 315–322). New York: Academic Press Inc.