© Triveni Enterprises
Vikas Nagar, Lucknow, INDIA
editor@jeb.co.in
Full paper available on: www.jeb.co.in

J. Environ. Biol.
33, 897-901 (2012)
ISSN: 0254-8704
CODEN: JEBIDP

Morphometric and molecular characterization of fungus *Pestalotiopsis* using nuclear ribosomal DNA analysis

Author Details

Praveen Gehlot (*Corresponding author*) — Department of Botany, JNV University, Jodhpur - 342 001, India
e-mail: drpg73@rediffmail.com

S.K. Singh — Central Arid Zone Research Institute, Jodhpur - 342 003, India

Rakesh Pathak — Central Arid Zone Research Institute, Jodhpur - 342003, India

Publication Data

Paper received:
28 January 2011

Revised received:
05 May 2011

Accepted:
25 May 2011

Abstract

Taxonomy of the fungus *Pestalotiopsis* based on morphological characters has been equivocal. Molecular characterization of ten *Pestalotiopsis* species was done based on nuclear ribosomal DNA internal transcribed spacer (ITS) amplifications. Results of the analyses showed that species of genus *Pestalotiopsis* are monophyletic. We report ITS length variations, single nucleotide polymorphisms (SNPs) and insertions/ deletions (INDELS) among ten species of *Pestalotiopsis* that did not cause any phylogenetic error at either genus or species designation levels. New gene sequences have been assigned (Gen Accession numbers from HM 190146 to HM 190155) by the National Centre for Biotechnology Information, USA.

Key words

Pestalotiopsis, Morphology, Host association, Molecular phylogeny, rDNA sequencing

Online copy

Introduction

The genus *Pestalotiopsis* is a heterogeneous group of Coelomycetes fungi and cosmopolitan in distribution (Kang *et al.,* 1999). Most species are weak plant pathogens (Zhu *et al.,* 1991; Zhang *et al.,* 2003), some are saprobes in soil and in plant debris (Tang *et al.,* 2003). Its species infect many economically important plants such as mango, coconut, rice, tea *etc.* and are common endophytes isolated from many tropical and sub-tropical plants (Okane *et al.,* 1997; Guo, 2002; Joshi *et al*., 2009). *Pestalotiopsis* species have gained much attention and importance in recent years as they produce many important secondary metabolites with potential use on medical application and control of plant diseases (Strobel, 2002; Harper *et al.,* 2003; Kumar *et al.,* 2004; Ding *et al.,* 2009; Liu *et al.,* 2009).

The taxonomic affinities of *Pestalotiopsis* species have been equivocal and hampered by opinions regarding the basic criteria used in segregating the species. NagRaj (1993) classified the genus based on the morphology of conidia and on conidiogenesis. At present, inter-specific delimitation is still obscure (McParland and Cubeta, 1997; Morgan *et al.,* 1998).

Some species have also been identified based on their host occurrence (Kohlmeyer and Volkmann-Kohlmeyer, 2001; Chen *et al.,* 2002). Molecular studies have shown that the genus contain two distinct lineages again based on pigmentation of median cells and four distinct grouping based on morphology of apical appendages but the reliability of other phenotypic characters of this genus was not supported (Jeewon *et al.,* 2003).

In the recent years, precise assessment of diversity and identification of fungi had a great impact on fungal taxonomy due to rapid developments in molecular techniques (Phillips *et al.,* 2007; Zhu *et al.,* 2008; Thongkantha *et al.,* 2009). Fungal identification is more reliable when classical and molecular approaches are combined (Hyde and Soytong, 2008; Than *et al.,* 2008). Sequence analysis of the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA (rDNA) has been widely used for molecular identification and phylogenetic diversity studies of fungi (Egger, 1995; Lacap *et al.,* 2003; Promputtha *et al.,* 2005). The rDNA region contains both variable and conserved region allowing the comparison and discrimination of organisms at different taxonomic levels. The ITS polymorphism might occur at genus, species or individual level making it useful for phylogeny, evolution and bio-geographical diversity studies (Carvalho *et al.,* 2009). The DNA sequences of ITS I and ITS II regions of 5.8S rRNA gene evolves relatively quickly and can be useful in determining inter-species variations (Vogler and Bruns, 1998).

The aim of the present study was to molecularly identify, characterize and to find out phylogenetic relationships among predominant fungal species *Pestalotiopsis* prevalent in Aravali forest range of Rajasthan, India.

Materials and Methods

The infected twigs and leaves of eight plant species were collected from various localities of Aravali mountain range forest of Rajasthan, India. Pure cultures from the infected plant tissues were raised on potato dextrose agar (PDA) culture medium in petri dishes at 25 ± 2°C for 10 days. All the isolates were again sub-cultured in petri dishes for 10 days and colony morphology (colour, growth rate, texture, mycelium form and morphometric characters) of conidia of each isolate was recorded.

The genomic DNA was extracted from fresh cultures grown on PDA culture medium. Approximately, 100 mg of each fungal mycelium was crushed with a micro-pestle in conical 1.5 ml micro centrifuge tube with liquid nitrogen. DNeasy® plant minikit of Qiagen GMBH (Germany) was used and protocols suggested by Sambrook *et al.* (1989) and Birren and Lai (1993) were followed for DNA isolation. The quality of DNA was checked on 0.8% (w/v) agarose in a gel electrophoresis with 1× TAE buffer and analyzed after staining with ethidium bromide. The purity of the extracted DNA was checked at 260 nm and 280 nm (Pharma spec UV-1700, Shimadzu Company).

The polymerase chain reaction (PCR) primers ITS-1 and ITS-4 were used to amplify the ITS-I and ITS-II regions of ribosomal DNA, which encompasses the 5.8S rRNA gene (White *et al.,* 1990). The amplification was performed using PCR in a total volume of 50 ml in a thermal cycler (Corbett Research, USA) following standard PCR conditions (Kakani *et al.,* 2011). The PCR products were visualized on 1% (w/v) agarose gel in Tris-acetic acid EDTA (1× TAE) buffer at 60 V for 100 min. Agarose gel was stained with ethidium bromide and photographed under UV light using syngene gel documentation system.

Each amplified PCR product of ITS amplified region containing ITS1, 5.8S rRNA gene and ITS2 was directly sequenced using ITS-1 (forward primer) and ITS-4 (reverse primer) by big dye terminator method on ABI prism DNA sequencer. The sequence data obtained from ITS-4 reverse primer was inversed using Genedoc software and clubbed with sequence data of ITS-1 after deleting the common portions to obtain complete sequence of amplified ITS product. Nucleotide sequence comparisons were performed by using Basic Local Alignment Search Tool (BLAST) network services of the National Centre for Biotechnology Information (NCBI) database. The fungal species were designated to the sequenced analyses based on similarity with the best aligned sequence of BLAST search. Multiple sequence alignment was performed using clustal X 1.83 software. The complete rDNA sequences of ITS1 and ITS2 encompassing 5.8S of different

Table - 1: Morphometric characterization of *Pestalotiopsis* isolates collected from Aravali forests.

Isolate	Host	*Conidial length (µm)	Conidial width (µm)	*Type of median colour cells and length (µm)	*Size of appendages (µm) apical	basal
Ps 1	*Phoenix sylvestris*	22.9±0.9	6.0±0.6	VU 17.1±0.8	18.9±1.1	4.5±0.6
Ps 2	*Gravillea robusta*	27.1±0.7	6.0±0.8	Concolour16.8±0.7	17.5±0.9	4.0±0.8
Ps 3	*Cryptostegia grandiflora*	25.0±0.9	5.5±0.6	VF 17.5±0.7	21.6±1.3	5.4±0.7
Ps 4	*Mangifera indica*	24.9±0.8	6.3±0.9	VF 18.9±0.9	18.9±1.2	5.1±1.0
Ps 5	*Cycas circinalis*	22.9±1.2	6.2±0.9	VU 17.1±0.7	18.9±0.9	5.4±0.7
Ps 6	*Mangifera indica*	22.2±0.8	5.8±0.9	Concolour 16.2±1.0	23.1±0.7	4.9±0.8
Ps 7	*Butea monosparma*	26.2±0.8	6.9±0.7	Concolour 15.6±0.8	13.7±0.8	5.0±0.8
Ps 8	*Eugenia jambalana*	22.9±0.9	6.2±1.0	Concolour 17.1±1.2	22.9±0.7	4.9±0.7
Ps 9	*Psidium guajava*	27.1±0.7	6.7±1.2	VF 18.9±1.0	25.6±0.7	5.1±0.9
Ps 10	*Mangifera indica*	22.8±1.0	6.0±1.1	Concolour 14.8±0.6	25.6±1.2	6.7±01.0

VU = Versicolour umber, VF = Versicolour fuliginous; * = Mean values of 30 conidia per isolate

Table - 2: Nucleotide base pair lengths of internal transcribed spacer region encompassing 5.8S ribosomal DNA gene.

Isolate	Gen accession number	ITS-1 (bp)	5.8S gene (bp)	ITS-2 (bp)	Total (bp)	*Pestalotiopsis* species
Ps 1	HM190146	142	158	162	462	*P. clavispora* (Atk.) Stey.
Ps 2	HM190147	142	158	160	460	*P. diospyri* Syd.
Ps 3	HM190148	142	158	160	460	*P. oxyanthi* Thuem.
Ps 4	HM190149	142	158	162	462	*P. versicolor* Stey.
Ps 5	HM190150	141	156	160	457	*P. gracilis* (Kleb.)
Ps 6	HM190151	142	158	160	460	*P. virgatula* (Kleb.) Stey.
Ps 7	HM190152	194	158	166	518	*P. cocculi* Guba.
Ps 8	HM190153	142	158	162	462	*P. microspora* (Speg.) Guba.
Ps 9	HM190154	142	158	162	462	*P. sydowiana* Bres.
Ps 10	HM190155	142	158	162	462	*P. mangiferae* (P. Henn.) Stey

Online Copy

Pestalotiopsis species were submitted to NCBI database and Gen Accession numbers obtained. The phylogenetic relationships of *Pestalotiopsis* species were established by generating phylogram using tree view software (Page, 1996) based on single nucleotide polymorphisms in the ITS region of 5.8S ribosomal nuclear DNA.

Results and Discussion

The morphometric characterization of ten isolates of *Pestalotiopsis* isolated from eight host plants is presented in Table 1. All the ten *Pestalotiopsis* isolates were recorded with minor variations and homogenous conidial lengths (22.2 to 27.1 µm), conidial width (5.5 to 6.9 µm), length of median colour cells (14.8 to 18.9 µm) and size of basal appendages (4.0 to 6.7 µm). However, three types of median colour cells were recorded among the isolates *i.e.*, Ps 1 and Ps 5 (versicolour amber), Ps 2, Ps 6, Ps 7, Ps 8 and Ps 10 (concolour) and Ps 3, Ps 4 and Ps 9 (versicolour fuliginous) with variation in size of apical appendages (13.7 to 25.6 µm). Three isolates Ps 4, Ps 6 and Ps 10 representing three species of *Pestalotiopsis* were isolated from a single host *Mangifera indica*. The PCR amplified products of the ten isolates of *Pestalotiopsis* generated a single prominent band on agarose gel (Fig. 1).

The amplified product of ITS region (ITS1-5.8S-ITS2) of *Pestalotiopsis* isolates ranged between 500 to 550 bp. The nucleotide base pair lengths of internal transcribed spacer regions encompassing 5.8S nuclear rDNA of ten species of *Pestalotiopsis* are shown in Table 2. Seven species of *Pestalotiopsis* viz., *P. clavispora, P. diospyri, P. oxyanthi, P. gracilis, P. cocculi, P. microspora* and *P. sydowiana* were found associated with different hosts studied based on the maximum identities with the available sequences in the NCBI database. Whereas, three species viz., *P. mangiferae, P. versicolour* and *P. virgatula* were isolated from a single host *Mangifera indica*. The details of the ITS1-5.8S-ITS2 length of each *Pestalotiopsis* species and Gen Accession numbers are presented in Table 2. Interestingly, *P. cocculi* was recorded with the maximum gene length of 518 bp which was significantly higher than all other *Pestalotiopsis* species ranging between 457 to 462 bp. Besides ITS length variations, *i.e.*, ITS I (141-194 bp) and ITS II (160-166 bp), we report single nucleotide polymorphisms (SNPs) at number of sites by way of single to a few base pair substitution, insertions/ deletions (Indels) among *Pestalotiopsis* isolates studied. The new gene sequences were assigned Gen Accession numbers from HM 190146 to HM190155.

The multiple sequence alignment of all the ten *Pestalotiopsis* species generated a phylogram using tree view software (Fig. 2). The phylogram showed that all the *Pestalotiopsis* species prevailing in the Aravali forest arise from single branch and validated monophyletic association. The phylogram also showed that all the ten *Pestalotiopsis* species delineated into three clades. Clade 1 included six species of *Pestalotiopsis* in which *P. clavispora, P. versicolor, P. microspora* showed more genetic similarities with each other as compared to *P. mangiferae, P. sydowiana* and *P. oxyanthi.* However, *P. oxyanthi* was found distinctly related to other species of the sub-clade. The clade 2 included three species viz., *P. gracilis, P. cocculi* and *P. diospyri*. Clade 3 is represented by a single species *P. virgatula* and recorded the most distinct to rest of the *Pestalotiopsis* species studied. The species of *Pestalotiopsis* having uniform base pair length of 462 were grouped into same clade in the phylogram. Steyaert (1949) divided the genus *Pestalotiopsis* into different sections on the basis of the number of apical appendages. Under present investigation, all the species of *Pestalotiopsis* were recorded with 2-3 apical appendages. There were insignificant differences in size of conidia, size of apical appendages and could not be distinguished on the basis of this morpho-taximetrics. *P. clavispora, P. versicolor and P. gracilis* showed morphological similarities. Whereas, phylogram aligned these species into separate clades. We report no correlation between the morphological and genetical affinities of these species. Morphologically similar species were delineated into different clades upon genetic analysis and vice versa.

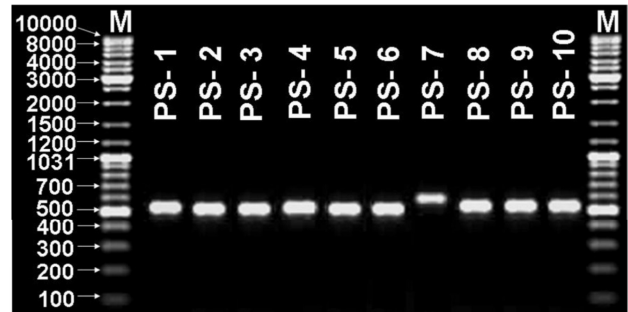

Fig. 1: ITS profiles of 10 *Pestalotiopsis* species

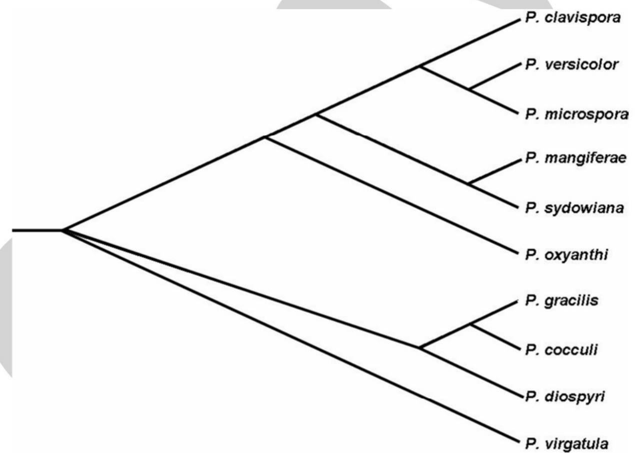

Fig. 2: Phylogram based on multiple sequence alignment of *Pestalotiopsis* species using tree view software

Out of ten species identified, five species *P. diospyri, P. virgatula, P. cocculi, P. microspora* and *P. mangiferae* were of concolour type and the species *P. clavispora* and *P. gracilis* were of versicolour umber type. While, *P. oxyanthi, P. sydowiana* and *P. versicolour* were recorded as versicolour fuliginous type. These morphological groups of species when compared with multiple sequence alignment of rDNA regions delineated the species with similar pigmentation type into different clades. Satya and Saksena

(1984) argued that colour contrast of median cells is not a dependable character for identification of *Pestalotiopsis* species.

The association with specific hosts has provided a convenient means for species separation and description of new species (Zhou and Hyde, 2001; Jeewon *et al.,* 2004). Whereas, under present investigation host *vis-a-vis Pestalotiopsis* species association did not provide any clue of designation of species. Rather, it resulted in ambiguity to identify species as the host *Mangiferae indica* was found to harbor three species of *Pestalotiopsis*. Earlier reports of Fungi of India also validate our findings. By contrast, Cannon and Simmons (2002) reported *Pestalotiopsis* species are host specific. The species of this genus are not host specific as they were also reported as facultative parasites, saprophytes and or endophytes (Suryanarayanan *et al.,* 2000; Yanna and Hyde, 2002; Wei and Xu, 2004; Keith *et al.,* 2006; Hu *et al.,* 2007; Joshi *et al.,* 2009). We observed that species isolated from same host were genetically grouped in different clades and the species relationship is independent to association with the host. Similar observations were recorded by Jeewon *et al.* (2004). Morgan *et al.* (1998) reviewed the taxonomy of this genus and explored the utility of artificial neural network to identify and characterize *Pestalotiopsis* species.

Our study revealed that classification of *Pestalotiopsis* based on conidial morphology, pigmentation, culture characters and or host affinities are not congruent and are often misleading. Whereas, the molecular identification based on rDNA gene sequences is a robust molecular tool to study phylogenetic relationship. Sequence data from rDNA, ITS region has been extensively used to designate species (Baayen *et al.,* 2002; Jeewon *et al.,* 2004; Than *et al.,* 2008). The PCR and DNA sequencing combined with morphological verification can provide accurate identification of fungal diversity by validating taxonomic identification. The results validate the use of rDNA gene sequencing as DNA barcode for species designation within the genus *Pestalotiopsis*.

Acknowledgments

First author is thankful to the Head, Department of Botany, Jai Narain Vyas University, Jodhpur for research support and Council for Scientific and Industrial Research, New Delhi for financial assistance under the Pool Scheme for Senior Research Associates.

References

Baayen, R.P., P.J.M. Bonants, G. Verkley, G.C. Carroll, A.A.H.A. Vander, M. De weerdt, I.R. Van Brouwershaven, G.C. Schutte, W.J.R. Maccheroni, C. Glienke De Blanco and J.L. Azevedo: Non-pathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as cosmopolitan endophyte of woody plants, *G. mangiferae (Phyllosticta capitalensis*). *Phytopathol.,* **92**, 464-467 (2002).

Birren, B. and E. Lai: Pulsed field gel electrophoresis: A practical guide. Academic Press, San Diego, CA. p. 253 (1993).

Cannon, P.F. and C.M. Simmons : Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. *Mycologia,* **94**, 210-220, (2002).

Carvalho, A., H. Guedes-Pinto and J. Lima Brito: Genetic diversity among old Portugese bread wheat cultivars and botanical varieties evaluated by ITS rDNA PCR-RFLP markers. *J. Genet.,* **88**, 363-367 (2009).

Chen, Y., X.G. Wei and W.P. Chen: New species of *Pestalotiopsis*. *Mycosystema,* **21**, 316-323 (2002).

Ding, G., Y. Li, S. Fu, J. Wei and Y. Che: Ambuic acid and torreyanic acid derivatives from the endolichenic fungus *Pestalotiopsis* sp. *J. Nat. Products,* **72**, 182-186 (2009).

Egger, K.N.: Molecular analysis of ectomycorrhizal fungal communities. *Can. J. Bot.,* **73**, 1415-1422 (1995).

Guo, L.D.: *Pestalotiopsis* besseyi, a new record of endophytic fungi from pine in China. *Mycosystema,* **21,** 455-456 (2002).

Harper, J.K., E.J. Ford, G.A. Strobel, A. Arif, D.M. Grant, J. Porco, D.P. Tomer and K. Oneill: Pestacin: A 1, 3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. *Tetrahedron,* **59**, 2471-2476 (2003).

Hu, H.L., R. Jeewon, D.Q. Zhou, T.X. Zhou and K.D. Hyde: Phylogenetic diversity of endophyic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp. evidence from rDNA and β-tubulin phylogenies. *Fungal Diversity,* **24**, 1-22 (2007).

Hyde, K.D. and K. Soytong: The fungal endophyte dilemma. *Fungal Diversity,* **33**, 163-173 (2008).

Jeewon, R., E.C.Y. Liew, J.A. Simpson, I.J. Hodgkiss and K.D. Hyde: Phylogenetic significance of morphological characters in the taxonomy of *Pestalotiopsis* species. *Mol. Phylo. Evol*., **27**, 372-383 (2003).

Jeewon, R., E.C.Y. Liew and K.D. Hyde: Phylogenetic evaluation of species nomenclature of *Pestalotiopsis* in relation to host association. *Fungal Diversity,* **17**, 39-55 (2004).

Joshi, S.D., R. Sanjay, U.I. Baby and A.K.A. Mandal: Molecular characterization of *Pestalotiopsis* spp. associated with tea (*Camellia sinensis*) in Southern India using RAPD and ISSR markers. *Ind. J. Biotechnol.,* **8**, 377-383 (2009).

Kakani, R.K., S.K. Singh, A. Pancholy, R.S. Meena, R. Pathak and A. Raturi: Assessment of genetic diversity in *Trigonella foenum-graecum* based on nuclear ribosomal DNA, Internal Transcribed Spacer and RAPD analysis. *Pl. Mol. Biol. Rep.,* **29**, 315-323 (2011).

Kang, J.C., K. Hyde and R.Y.C. Kong: Studies on Amphisphaeriales: The Amphisphaeriaceae (*Sensu stricto*). *Mycol. Res.,* **103**, 53-64 (1999).

Keith, L.M., E. Maile, Velasquez and F.T. Zee: Identification and characterization of *Pestalotiopsis* spp. causing scab disease of guava, *Psidium guajava* in Hawaii. *Plant Disease,* **90**, 19-23 (2006).

Kohlmeyer, J. and B. Volkmann-Kohlmeyer: Fungi on *Juncus roemerianu*. 16 more new coelomycetes, including *Tetranacriella,* gen. nov. *Botanica Marina,* **44**, 147-156 (2001).

Kumar, S.S., H.Y. Cheung, C.S. Lau, F. Chen and K.D. Hyde: *In-vitro* studies of endophytic fungi from *Tripterygium wilfordii* with anti-proliferative activity on human peripheral blood mononuclear cells. *J. Ethnopharmacol*., **94**, 295-300 (2004).

Lacap, D.C., K.D. Hyde and E.C.Y. Liew: An evaluation of the fungal morphotype concept based on ribosomal DNA sequences. *Fungal Diversity,* **12**, 53-66 (2003).

Liu, L., S. Liu, X. Chen, L. Gua and Y. Che: Pestalofones A-E, bioactive cyclohexanone derivatives from the plant endophytic fungus *Pestalotiopsis fici. Bioorganic and Medicinal Chem.,* **15**, 606-613 (2009).

McPartland, J.M. and M.A. Cubeta: New species, combinations, host associations and location records of fungi associated with hemp (*Cannabis sativa). Mycol. Res.,* **101**, 853-57 (1997).

Morgan, A., L. Bobby, E.M. Mordue and C.W. Morris: Evaluation of artificial neural networks for fungal identification, employing morphometric data from spores of *Pestalotiopsis* species. *Mycol. Res.,* **103**, 975-84 (1998).

NagRaj, T.R.: Coelomycetous anamorphs with appendage bearing conidia. Mycologue publications, Waterloo, Ontaria, Canada (1993).

Okane, I., A. Nagagiri and T. Ito: Preliminary study of endophytic fungi in evergreen plants from Ishigaki and Iriomote islands. The Institute for Fermentation, *Osaka Res. Commu*., **18**, 45-51 (1997).

Page, R.D.M.: Tree View: An application to display phylogenetic on personal computers. *Computer Appli. Biol. Sci.,* **12**, 357-358 (1996).

Online Copy

Phillips, A.J.L., P.W. Crous and A. Alves: *Diplodia seriata*, the anomorph of "*Botryo-sphaeria" obtusa. Fungal Diversity,* **25**, 141-55 (2007).

Promputtha, I., R. Jeewon, S. Lumyong, E.H.C. Mc Kenzie and K.D. Hyde: Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). *Fungal Diversity,* **20,** 167-186 (2005).

Sambrook, J., E.F. Fritsch and T. Maniatis: Molecular cloning: A laboratory manual. 2nd Edn., Cold Spring Harbor Laboratory Press, Plainview, New York USA (1989).

Satya, H.N. and B. Saksenas: Some aspects of taxonomy of the genus Pestalotia I- colour intensities of intermediate cells of spores. *In*: Proceeding of the International Symposium on Taxonomy of Fungi (Ed.: C. V. Subramanian). University of Madras, India (1984).

Steyaert, R.L.: Contributions a l'etude monographique de *Pestalotia* de Not. et. *Monochaetia* Sacc. (*Truncatella* gen. nov. et. *Pestalotiopsis* gen. nov.). *Bull. Jard. Bot. Etat Bruxelles,* **19**, 285-354 (1949).

Strobel, G.A.: Rainforest endophytes and bioactive products. *Critical Rev. Biotechnol.,* **22,** 315-333 (2002).

Suryanarayanan, T.S., G. Senthilarasu and V. Murugandamam: Endophytic fungi from *Cuscuta reflaxa* and its host plants. *Fungal Diversity,* **4**, 17-23 (2000).

Tang, A., K.D. Hyde and R.T.C. Corlett: Diversity of fungi on wild fruits in Hong Kong. *Fungal Diversity,* **14**, 165-185 (2003).

Than, P.P., R.G. Shivas, R. Jeewon, S. Pongsupasamit, T.S. Marney, P.W.J. Taylor and K.D. Hyde: Epitypification and phylogeny of *Colletotrichum acutatum* J.H. Simmonds. *Fungal Diversity,* **28**, 97-108 (2008).

Thongkantha, S., R. Jeewon, D. Vijayakrishna, S. Lumyong, E.H.C. Mc Kenzie and K.D. Hyde: Molecular phylogeny of *Magnaporthaceae* (Sordariomycetes) with a new species, *Ophioceras chiangdaoense* from *Dracaena loureiroi* in Thailand. *Fungal Diversity,* **34**, 157-173 (2009).

Vogler, D.R. and T.D. Bruns: Phylogenetic relationships among the pine stem rust fungi *Cronartium* and *Peridermium* spp. *Mycologia,* **90**, 244-257 (1998).

Wei, J.G. and T. Xu: *Pestalotiopsis kunmingensis* sp. nov., an endophyte from *Podocarpus macrophyllus*. *Fungal Diversity,* **15**, 247-254 (2004).

White, T.J., T. Burns, S. Lee and J.W. Taylor: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: A guide to methods and applications (Eds.: M.A. Innis, D.H. Gelfand, J.J. Sninsky and T. J. White). Academic Press, San Diego, California, pp. 315-322 (1990).

Yanna, H.O.W.H. and K.D. Hyde: Fungal succession on fronds of *Phoenix hanceana* in Hong Kong. *Fungal Diversity,* **10**, 185-211 (2002).

Zhang, J.X., T. Xu and Q.X. Ge: Notes on *Pestalotiopsis* from Southern China. *Mycotaxon,* **85**, 91-92 (2003).

Zhou, D.Q. and K.D. Hyde: Host- specificity, host exclusivity and host-recurrence in saprobic fungi. *Mycol. Res.,* **105**, 1449-1457 (2001).

Zhu, G.S., Z.N. Yu, Y. Gui and Z.Y. Liu: A novel technique for isolating orchid mycorrhizal fungi. *Fungal Diversity,* **33**, 123-137 (2008).

Zhu, P.L., Q.X. Ge and T. Xu: The perfect stage of *Pestalosphaeria* from China. *Mycotaxon,* **50**, 129-140 (1991).

Online Copy