CZECH MYCOLOGY 65(1): 79–88, JUNE 10, 2013 (ONLINE VERSION, ISSN 1805-1421)

Multigene sequence-based identification of *Colletotrichum cymbidiicola*, *C. karstii* and *C. phyllanthi* from India

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Sharma G., Shenoy B.D. (2013): Multigene sequence-based identification of *Colletotrichum cymbidiicola*, *C. karstii* and *C. phyllanthi* from India. – Czech Mycol. 65(1): 79–88.

In this study, we employed multilocus phylogenetic analysis for species identification of six *Colletotrichum* isolates belonging to the *C. boninense* species complex from India. Maximum parsimony analysis of the ITS/ 5.8S RNA, partial *act*, *cal*, *chs1*, *gapdh*, *his3* and *tub2* gene regions identified morphologically similar species, *C. cymbidiicola*, *C. karstii* and *C. phyllanthi* from diverse plant samples of Indian origin. Morphological description and photographic illustrations of *C. phyllanthi* from freshly collected material are provided, as the ex-type culture of *C. phyllanthi* deposited in CBS is in non- sporulating state. This is the first report of *C. cymbidiicola* and *C. karstii* from India. We are also reporting two new hosts: *Bauhinia variegata* (Orchid tree) and *Bougainvillea glabra* (Paper flower) for *C. phyllanthi*, and one new host: *Olea dioica* (Rose sandalwood) for *C. karstii*.

Key words: anthracnose, *Colletotrichum*, identification, phylogeny, systematics.

Sharma G., Shenoy B.D. (2013): Identifikace druhů *Colletotrichum cymbidiicola*, *C. karstii* a *C. phyllanthi* z Indie založená na multigenové sekvenaci. – Czech Mycol. 65(1): 79–88.

V článku je použita multigenová sekvenace pro druhové určení šesti indických izolátů rodu *Colletotrichum* patřících do okruhu *C. boninense*. Je popsána metodika, pomocí níž byly určeny morfologicky podobné druhy *C. cymbidiicola*, *C. karstii* a *C. phyllanthi*. Jsou též publikovány nové druhy hostitelů těchto hub.

INTRODUCTION

Colletotrichum spp. can cause diseases in evolutionarily diverse plant hosts (Dean et al. 2012). Species identification in *Colletotrichum* can be challenging due to the presence of cryptic species in several *Colletotrichum* lineages (Cannon et al. 2012). *Colletotrichum boninense* sensu lato is an important species complex whose members have been reported from various economically important plant hosts belonging to families such as *Amaryllidaceae*, *Orchidaceae*, *Proteaceae*,

and *Solanaceae* (Moriwaki et al. 2003, Damm et al. 2012a, Farr et al. 2006, Farr & Rossman 2012, Lubbe et al. 2004, Yang et al. 2009).

Identification strategy for *Colletotrichum* species has been transformed by recent multilocus phylogenetic studies (Cannon et al. 2012; Damm et al. 2012a, b; Weir et al. 2012). In a major taxonomic reorganization, Damm et al. (2012a) identified 18 distinct clades and 17 phylogenetic species within the *C. boninense* species complex based on multilocus phylogenetic analysis, leading to a narrow definition of *C. boninense*. In the present study, we performed multigene sequence-based species identification for six *Colletotrichum* isolates belonging to the *C. boninense* species complex associated with various hosts from different geographical locations in India.

MATERIAL AND METHODS

Fungal isolates and DNA extraction. Our study included six *Colletotrichum* isolates, which were initially identified as "*C. gloeosporioides*" or *Colletotrichum* sp. based on morpho-taxonomic characters. Four of them were procured from three culture collections in India (Tab. 1), while MTCC 11391 and MTCC 11394 were isolated as endophytes following the isolation method described by Cai et al. (2009). The fungal isolates were subcultured on potato dextrose agar (PDA), potato carrot agar (PCA) (Himedia, India) and synthetic nutrient agar (SNA, Nirenberg 1976) media and grown at 20 °C for 7 days. To induce sporulation, cultures were grown in a 12-hour photoperiod under near-ultraviolet light, with pieces of autoclaved filter paper on PCA and SNA medium (Damm et al. 2012a). Genomic DNA from fresh mycelia was isolated using a DNA isolation kit (Zymo Research, USA, Catalogue number D6005) and stored at –20 °C.

Morphological characterisation. Morphological characterisation was carried out based on the 7-day old cultures grown at 20 °C on PDA (Damm et al. 2012a). Micro-morphological characters such as shape, size and colour of acervuli, conidia, and conidiogenous cells were observed as described by Cai et al. (2009) and photographed using a trinocular upright microscope (Olympus U-CMAD3, Japan) equipped with an Olympus camera and differential interference contrast (DIC). For each isolate, length and width of 100 randomly chosen conidia were measured using the CellB image analysis software (Olympus, Japan). The colony diameter was measured after 7 days to determine the growth rate (mm/day).

PCR amplification and DNA sequencing. Polymerase chain reactions (PCRs) were performed in a 50 µl reaction volume to amplify the ITS, partial *act*, *cal*, *chs1 gapdh*, *his3* and *tub2* gene regions. The reactions were carried out in an Eppendorf Mastercycler with the cycling parameters and primers as speci-

fied in previous papers [ITS, *act*, *gapdh*, *tub2*, *his3*, *chs1* (Damm et al. 2009) and *cal* (Prihastuti et al. 2009)]. The PCR products were run on a 1% Tris-Acetate-EDTA agarose gel stained with ethidium bromide (0.5 µg/ ml) at 100 V, 400 mA for 45 minutes to check the presence of the desired band. The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Catalogue number 28106). The purified PCR products were quantified using a ND-1000 Nanodrop Spectrophotometer (Thermo). The PCR products were sequenced using respective primers with the ABI Big Dye v3.1 Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems) using the manufacturer's protocol. The samples were purified to remove excess salt, denatured with HiDi-Formamide at 95 °C for 3 minutes and analysed using a 3730 DNA Analyzer (Applied Biosystems) at the central DNA sequencing facility of the Institute of Microbial Technology, Chandigarh.

Sequence alignment and phylogenetic analysis. The forward and reverse sequences obtained for each strain were aligned using Sequencher version 4.10.1 (Gene Codes Corp., Ann Arbor, Michigan, USA) to generate a consensus sequence. A multigene dataset comprising ITS, partial *act*, *cal*, *chs1*, *gapdh*, *his3* and *tub2* gene regions for the six *Colletotrichum* isolates and selected reference sequences from Damm et al. (2012a) was generated using SequenceMatrix version 1.7.8 (Vaidya et al. 2011). A maximum parsimony analysis of the multigene dataset was performed using PAUP version 4.0b10 (Swofford 2003). Ambiguously aligned regions were excluded from the analysis. The gaps in the alignment were treated as missing data. All the characters in the analysis were unordered and had equal weight. Trees were inferred using the heuristic search option with 20 random sequence additions and tree bisection and reconstruction (TBR) as the branch swapping algorithm. Maxtrees were set to 10,000; branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics [Tree Length (TL), Consistency Index (CI), Retention Index (RI), Related Consistency Index (RCI), Homoplasy Index (HI)] were calculated for the generated trees. The robustness of the trees was measured by 100 bootstrap replicates (Felsenstein 1985) and addition of 10 random sequences. Kishino-Hasewaga tests (Kishino & Hasewaga 1989) were performed in order to determine whether trees were significantly different. Trees were figured in Treeview (Page 1996) and edited in MEGA version 5 (Tamura et al. 2011) and Microsoft PowerPoint version 2007 (Microsoft Corp., USA). The sequences generated in this study have been deposited in NCBI-GenBank with accession numbers as listed in Tab. 1. The alignment files are deposited in TreeBase (www.treebase.org; Study ID: 13773).

Tab. 1. List of strains studied with strain information and GenBank accession numbers of the newly generated sequences.

Isolate code*	Taxon	Host	Substrate	Geographic location	Collected by	ITS	*gapdh*	*his3*	*tub2*	*act*	*chs1*	*cal*
MTCC 11391	*C. phyllanthi*	*Bauhinia variegata* (Orchid tree)	Leaf	IMTECH campus, Chandigarh	Gunjan Sharma, CSIR-IMTECH, Chandigarh	JN24 8677	JX57 6718	JX57 6703	JX57 6715	JX57 6709	JX86 3683	JX86 3677
MTCC 11394	*C. phyllanthi*	*Bougainvillea glabra* (Paper flower)	Leaf	IMTECH campus, Chandigarh	Gunjan Sharma, CSIR-IMTECH, Chandigarh	JN24 8690	JX57 6719	JX57 6704	JX86 3674	JX57 6710	JX86 3684	JX86 3678
MTCC 11392 = ITCC6178	*C. karstii*	*Passiflora edulis* (Passion fruit)	Fruit	Mizoram	Dr. K.A. Pathak, ICAR Research Complex for NEH Region, Kolasib, Mizoram	JN39 0872	JX57 6720	JX57 6705	JX57 6716	JX57 6711	JX86 3685	JX86 3679
MTCC 11393 = ITCC6328	*C. cymbidiicola*	*Cymbidium* sp. (Boat orchid)	?	Sikkim	?	JN39 0879	JX57 6723	JX57 6706	JX57 6717	JX57 6712	JX86 3687	JX86 3680
MTCC 6948	*C. karstii*	*Carica papaya* (Papaya)	Leaf	Rehman Khera, Uttar Pradesh	Dr. Om Prakash, CISH, Lucknow, Uttar Pradesh	JN39 0926	JX57 6721	JX57 6707	JX86 3675	JX57 6713	JX86 3686	JX86 3681
NFCCI1611	*C. karstii*	*Olea dioica* (Rose sandalwood)	Leaf spot	Mahabaleshwar, Maharashtra	Dr. Rajesh Kumar, NFCCI, Pune	JN39 0940	JX57 6722	JX57 6708	JX86 3676	JX57 6714	JX86 3688	JX86 3682

*Abbreviations: **ITCC** – Indian Type Culture Collection, New Delhi; **MTCC** – Microbial Type Culture Collection and Gene Bank, Chandigarh; **NFCCI** – National Fungal Culture Collection of India, Pune.

RESULTS

Multilocus phylogeny

There were a total of 2799 positions in the multilocus dataset. The gene boundaries in the multigene dataset included: *chs1*: 1–281, ITS: 282–848, *act*: 849–1127, *cal*: 1128–1585, *gapdh*: 1586–1899, *his3*: 1900–2296, and *tub2*: 2297–2799. The analysis involved 55 nucleotide sequences including the outgroup, *C. gloeosporioides* CBS 112999. Eighty-one characters from the ambiguous regions were excluded from the analysis. Out of the remaining 2718 characters, 1882 characters were constant, 607 characters were parsimony-informative and 229 characters were parsimony-uninformative. The maximum parsimony analysis resulted in 2 trees and, based on the KH test, these trees were not significantly different (details not shown). One of the two trees (TL = 1479, CI = 0.722, RI = 0.904, RC = 0.653, HI = 0.278) generated during the MP analysis is shown in Fig. 1. The bootstrap support for the observed branching pattern is shown next to the branches.

In the MP tree shown in Fig. 1, *Colletotrichum* isolates MTCC 11391 and MTCC 11394 clustered with the ex-isotype strain of *C. phyllanthi* (CBS 175.67) with strong bootstrap support. *Colletotrichum* isolate MTCC 11393 clustered with the *C. cymbidiicola* clade, while isolates MTCC 11392, MTCC 6948 and NFCCI 1611 grouped with members of *C. karstii* (Fig. 1).

Morphological characterisation

Morphology of the isolates MTCC 11391 (*C. phyllanthi*), MTCC 11393 (*C. cymbidiicola*) and MTCC 6948 (*C. karstii*) was studied and compared with the descriptions provided in Damm et al. (2012a) (data not shown). The conidial characteristics were within the range for all three species as already described (Damm et al. 2012a). Although we were not able to observe the sexual stage *Glomerella* in the cultures, conidia and conidiogenous cells were widely observed. However, in Damm et al. (2012a) the morphological description of *C. phyllanthi* was based on the original description by Pai (1966) due to the inability of the holotype culture to sporulate. Thus, we present the morphological data for *C. phyllanthi* in this paper.

C. phyllanthi (MB560746) is a new combination proposed by Damm et al. (2012a), who sequenced the ex-type strain of *Glomerella phyllanthi* CBS 175.67 [i.e. original collection from anthracnose symptoms of leaves of *Phyllanthus acidus* in Pune (= Poona), India; Pai 1966, 1970] and described it as *C. phyllanthi*, a combination of *Glomerella phyllanthi* and *Colletotrichum heveae* (presumed anamorph of the former, Pai 1970). However, Damm et al. (2012a) could not present a morphological description from the holotype culture as it failed to sporulate. In this study, MTCC 11391 and MTCC 11394 were isolated from *Bauhinia*

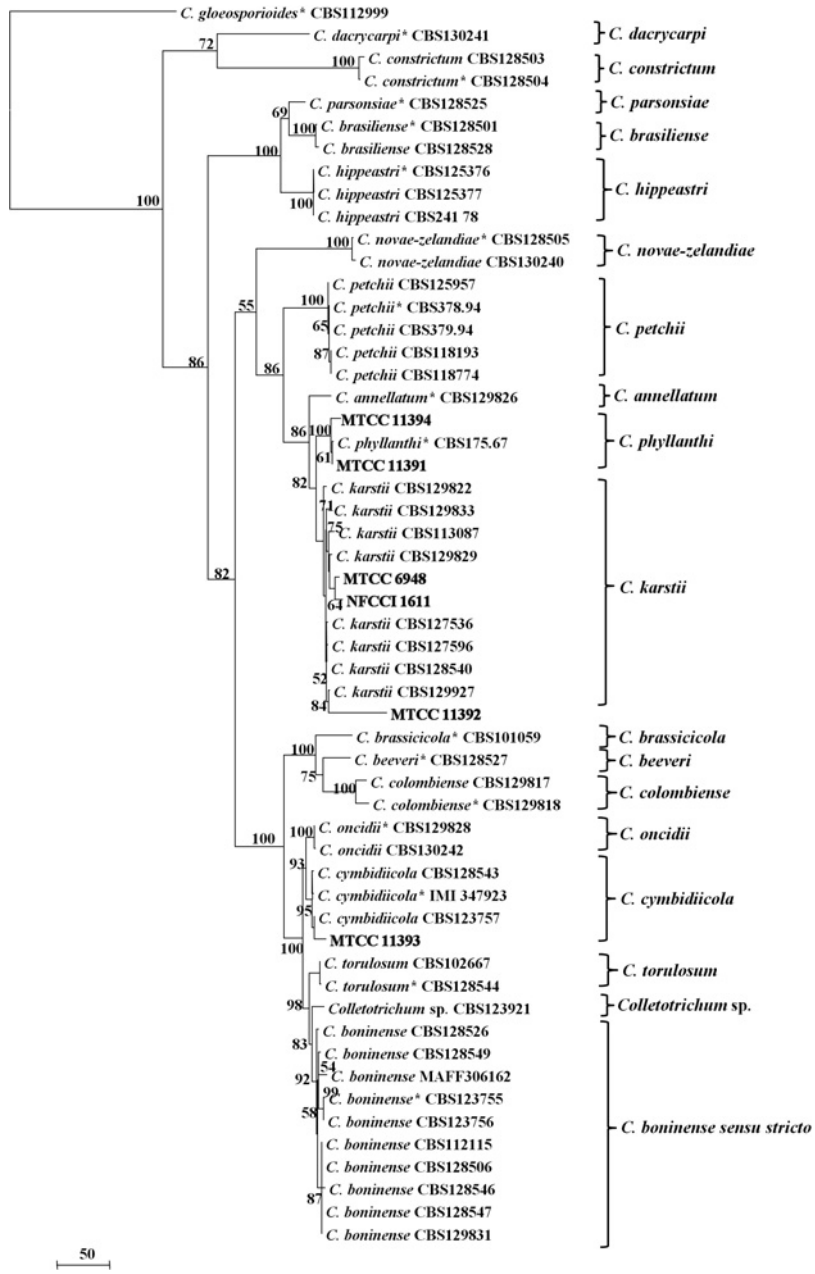

Fig. 1. One of the two most parsimonious trees showing phylogenetic affinities of six fungal isolates (highlighted by bold letters) from India, obtained from heuristic search of the multigene dataset. *Colletotrichum gloeosporioides* CBS 112999 is used as outgroup, and bootstrap support values more than 50 %, for 100 replicates, are shown at the nodes.

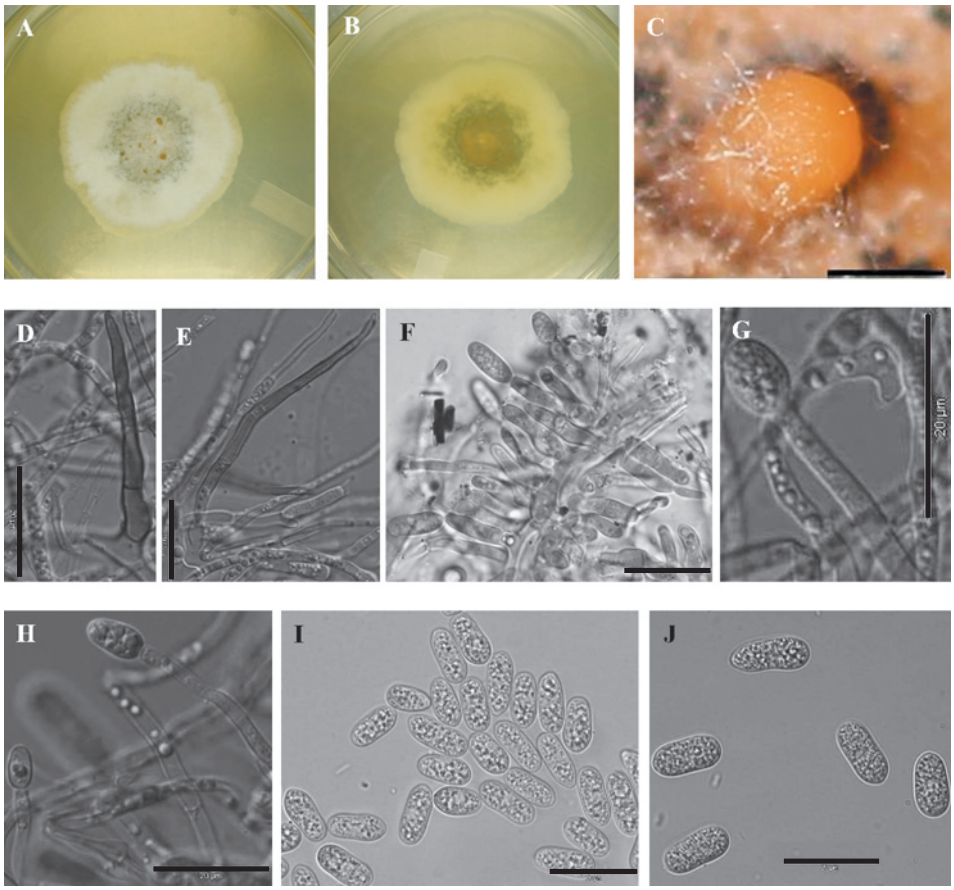

Fig. 2. *Colletotrichum phyllanthi* (MTCC 11391) **A**. Colony morphology on PDA after 7 days (front), **B**. Colony morphology on PDA after 7 days (reverse), **C**. Conidioma, **D–E**. Setae on PDA after 7 days, **F–H**. Conidiogenous cells on PDA after 7 days, **I**. Conidia on PCA after 7 days, **J**. Conidia on PDA after 7 days (scale bar of C = 100 µm, D–J = 20 µm). Photos by Gunjan Sharma.

variegata and *Bougainvillea* sp., respectively, at IMTECH, Chandigarh, India. Both isolates sporulated well in PDA, PCA as well as SNA media.

Colletotrichum phyllanthi, anamorph on PDA. Colonies on PDA are initially white, turning cream to orange with orange conidial mass, the reverse is cream to slightly pink and the growth rate is 5.9 mm per day at 20 °C, attaining 4.1 ± 0.5 cm in 7 days, n = 10 (Fig. 2). Vegetative hyphae hyaline, septate and branched, 1.2–5.5 µm in diam. Appressoria not observed. Setae medium to dark brown, basal cell lighter in colour, 1–2-septate, 32.4–64.8 µm long and 4.5–7.6 µm in diam., tapered to the apices, base slightly bulbous, tip slightly acute. Conidiomata acervular. Conidiophores hyaline to brown, septate, branched at base, smooth. Conidiogenous cells

enteroblastic, phialidic, hyaline and smooth, 12.8–18.5 µm long and 3.2–4.5 µm in diam. Conidia hyaline, smooth and cylindrical, with granular contents, 10.3–17.3 µm × 5.0–6.9 µm (n = 100, mean 14.3 ± 0.4 × 6.0 ± 0.2 µm). The teleomorph was not observed in culture.

DISCUSSION

In this study, we performed multi-gene sequence-based species identification of six isolates belonging to the *C. boninense* species complex: *C. phyllanthi* (MTCC 11391 and MTCC 11394), *C. cymbidiicola* (MTCC 11393) and *C. karstii* (MTCC 11392, MTCC 6948 and NFCCI 1611). *Colletotrichum* isolates MTCC 11391 and MTCC 11394 were isolated as leaf endophytes from *Bauhinia variegata* and *Bougainvillea* sp., respectively, in Chandigarh. This is the first report of *C. phyllanthi* associated with *Bauhinia variegata* and *Bougainvillea* sp. and also hints at a wider host range and versatile survival strategies of *C. phyllanthi*.

Colletotrichum cymbidiicola is a presumably host-specific and phytopathogenic species, only reported from *Cymbidium* sp. (*Orchidaceae*) (Damm et al. 2012a). The *Colletotrichum* isolate MTCC 11393 was isolated from *Cymbidium* sp. in Sikkim, India, thus further supporting the host specificity of this species. MTCC 11393 was originally deposited in ITCC and identified as "*C. gloeosporioides*" (ITCC 6328). In the present study, the fungus is shown to belong to *C. cymbidiicola*. This is the first time *C. cymbidiicola* has been reported from India.

Colletotrichum karstii is known to have a wide host range. In the original paper, Yang et al. (2011) reported it to be pathogenic to *Vanda* sp. and other hosts. In this study, three isolates, MTCC 11392, MTCC 6948 and NFCCI 1611, were found to be members of *C. karstii*. The association of *C. karstii* with *Olea dioica* (Rose sandalwood) has been reported for the first time in this paper. It is possible that *C. karstii*, in its present circumscription (Damm et al. 2012a), could represent a species complex, as many subclades within the *C. karstii* clade were found to have good bootstrap support. Nevertheless, further analyses with more isolates are required to validate this.

It is increasingly becoming clear that morphology alone cannot solve the problems associated with identification and phylogenetic classification of *Colletotrichum* species (Shenoy et al. 2007, Cai et al. 2009). Recent revisions of the *C. boninense* species complex (Damm et al. 2012a), *C. gloeosporioides* species complex (Weir et al. 2012) and *C. acutatum* species complex (Damm et al. 2012b) have clarified many issues associated with species boundaries and DNA sequence-based identification of *Colletotrichum* species. Further phylotaxonomic studies from poorly sampled regions such as India are needed to provide interesting insights into evolution, species boundaries, host range and pathogenic poten-

tials of *Colletotrichum* species. Importantly, in the light of cryptic speciation and recent major revisions in *Colletotrichum* taxonomy, microbial culture collection centres of India need to revisit their *Colletotrichum* collections and confirm their identity based on sequence data.

ACKNOWLEDGEMENTS

We thank Dr. D. Ananthapadmanaban for his guidance in morphological characterisation. We also thank Deepak Bhatt, Amandeep Kaur, Navinder Kumar, and Yamini Agrawal of CSIR-IMTECH, Chandigarh for their encouragement. Drs. Kevin D. Hyde, Lei Cai, Bevan Weir, and Roger Shivas are thanked for their inspiration and useful discussions on *Colletotrichum* taxonomy. We would like to thank the reviewers for their excellent comments and helping us in improving this manuscript. This work was supported by CSIR-IMTECH-OLP0071 and OLP0022/GAP0010 projects and CSIR-SRF fellowship awarded to GS.

REFERENCES

CAI L., HYDE K.D., TAYLOR P.W.J., WEIR B.S., WALLER J., ABANG M.M., ZHANG J.Z., YANG Y.L., PHOULIVONG S., LIU Z.Y., PRIHASTUTI H., SHIVAS R.G., MCKENZIE E.H.C., JOHNSTON P.R. (2009): A polyphasic approach for studying *Colletotrichum*. – Fungal Divers. 39: 183–204.

CANNON P.F., DAMM U., JOHNSTON P.R., WEIR B.S. (2012): *Colletotrichum* – current status and future directions. – Stud. Mycol. 73: 181–213.

DAMM U., WOUDENBERG J.H.C., CANNON P.F., CROUS P.W. (2009): *Colletotrichum* species with curved conidia from herbaceous hosts. – Fungal Divers. 39: 45–87.

DAMM U., CANNON P.F., WOUDENBERG J.H.C., JOHNSTON P.R., WEIR B.S., TAN Y.P., SHIVAS R.G., CROUS P.W. (2012a): The *Colletotrichum boninense* species complex. – Stud. Mycol. 73: 1–36.

DAMM U., CANNON P.F., WOUDENBERG J.H.C., CROUS P.W. (2012b): The *Colletotrichum acutatum* species complex. – Stud. Mycol. 73: 37–113.

DEAN R., VAN KAN J.A.L., PRETORIUS Z.A., HAMMOND-KOSACK K.E., DI PIETRO A., PIETRO D.S., RUDD J.J., DICKMAN M., KAHMANN R., ELLIS J., FOSTER G.D. (2012): The Top 10 fungal pathogens in molecular plant pathology. – Mol. Plant Pathol. 13: 414–430.

FARR D.F., AIME M.C., ROSSMAN A.Y., PALM M.E. (2006): Species of *Colletotrichum* on *Agavaceae*. – Mycol. Res. 110: 1395–1408.

FARR D.F., ROSSMAN A.Y. (2012): Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. – http://nt.ars-grin.gov/fungaldatabases/. [Accessed September 27, 2012]

FELSENSTEIN J. (1985): Confidence limits on phylogenies: an approach using the bootstrap. – Evol. 39: 783–791.

KISHINO H., HASEWAGA M. (1989): Evaluation of maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of *Hominoidea*. – J. Mol. Evol. 29: 170–179.

LUBBE C.M., DENMAN S., CANNON P.F., GROENEWALD J.Z., LAMPRECHT S.C., CROUS P.W. (2004): Characterization of *Colletotrichum* species associated with diseases of *Proteaceae*. – Mycologia 96: 1268–1279.

MORIWAKI J., SATO T., TSUKIBOSHI T. (2003): Morphological and molecular characterization of *Colletotrichum boninense* sp. nov. from Japan. – Mycoscience 44: 47–53.

NIRENBERG H.I. (1976): Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. – Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem 169: 1–117.

PAGE R.D.M. (1996): TREEVIEW: An application of display phylogenetic trees on personal computers. – Comput. Appl. Biosci. 12: 357–358.

PAI H.S. (1966): A new anthracnose disease of *Phyllanthus acidus* from India. – Plant Dis. Rep. 50: 313–314.

PAI H.S. (1970): Life cycle of *Colletotrichum heveae* inciting spot anthracnose of *Phyllanthus acidus*. – Mycopathol. Mycol. Applic. 42: 65–72.

PRIHASTUTI H., CAI L., CHAN H., MCKENZIE E.H.C., HYDE K.D. (2009): Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand. – Fungal Divers. 39: 89–109.

SHENOY B.D., JEEWON R., HYDE K.D. (2007): Impact of DNA sequence-data on the taxonomy of anamorphic fungi. – Fungal Divers. 26: 1–54.

SWOFFORD D.L. (2003): PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. – Sinauer Associates, Sunderland, Massachusetts.

TAMURA K., PETERSON D., PETERSON N., STECHER G., NEI M., KUMAR S. (2011): MEGA5: Molecular evolutionary genetics analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony methods. – Mol. Biol. Evol. 28: 2731–2739.

VAIDYA G., LOHMAN D.J., MEIER R. (2011): SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. – Cladistics 27: 171–180.

WEIR B.S., JOHNSTON P.R., DAMM U. (2012): The *Colletotrichum gloeosporioides* species complex. – Stud Mycol. 73: 115–180.

YANG Y.L., LIU Z.Y., CAI L., HYDE K.D., YU Z.N., MCKENZIE E.H.C. (2009): *Colletotrichum* anthracnose of *Amaryllidaceae*. – Fungal Divers. 39: 123–146.

YANG Y.L., CAI L., LIU Z.Y., HYDE K.D., YU Z.N. (2011): *Colletotrichum* species on *Orchidaceae* in southwest China. – Cryptogamie Mycol. 32: 229–253.