# Neotypification of *Colletotrichum falcatum*, the causative agent of red-rot disease in sugarcane

Haryudian Prihastuti[1,2,3,4], Lei Cai[1], Jo Anne Crouch[5], Sitthisack Phoulivong[2,6], Mohamed A. Moslem[7], Eric H.C. McKenzie[8] and Kevin D. Hyde[2,4,7]

[1] Key Laboratory of Systematic Mycology & Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100190, P.R. China.
[2] School of Science, Mae Fah Luang University, Thasud, Chiang Rai 57100, Thailand.
[3] Faculty of Agriculture, Brawijaya University, Malang 65145, Indonesia.
[4] International Fungal Research & Development Centre, The Research Institute of Resource Insects, Chinese Academy of Forestry, Bailongsi, Kunming 650224, PR China.
[5] Cereal Disease Laboratory, U.S. Department of Agriculture, Agricultural Research Service, 1551 Lindig Street, St. Paul, MN 55108, USA.
[6] Department of Plant Science, Faculty of Agriculture, National University of Laos, Vientiane, 7322, Lao PDR.
[7] Botany and Microbiology Department, College of Science, King Saud University, P.O. Box: 2455, Riyadh 1145, Saudi Arabia.
[8] Landcare Research, Private Bag 92170, Auckland, New Zealand.

Prihastuti H., Cai L., Crouch J. A., Phoulivong S., Moslem, M. A., McKenzie, E. H. C. & Hyde K. D. (2010) Neotypification of *Colletotrichum falcatum*, the causative agent of red-rot disease in sugarcane. – Sydowia 62 (2): 283–293.

*Colletotrichum falcatum* is an important pathogen of sugarcane causing red rot disease. The type specimen of this species could not be located in any herbaria. A fresh specimen was therefore collected and isolated from the location as stated in the protologue. A neotype of *C. falcatum* with a living culture is designated in order to stabilize the application of the species name. The morphology of conidia and appressoria, and cultural characters of the ex-neotype culture is provided and its pathogenicity on sugarcane (*Saccharum officinarum*) is confirmed by re-inoculation. Phylogenetic analysis showed that *C. falcatum* clusters in a distinct lineage in the curved-spored *Colletotrichum* species.

Key words: disease, plant pathogen, *Saccharum officinarum*, taxonomy

*Colletotrichum* species are important pathogens infecting a wide range of plants, especially in the tropics (Hyde *et al.* 2009a, b, Prihastuti *et al.* 2009). *Colletotrichum falcatum* is a destructive pathogen of sugarcane (*Saccharum officinarum* L.) that causes red-rot, a disease responsible for substantial losses in crop yield and quality in many

* Correspondence author: Lei Cai, mrcailei@gmail.com

parts of the world, particularly in India, Pakistan, Bangladesh and Taiwan (Crouch & Beirn 2009). Went (1893) described the disease from Java (Indonesia) where it was known as "*Sereh*" and was threatening yield in the sugar industry. He collected and isolated the fungus in 1892 at the Tjomal Estate in Java and published his work in 1893, providing a brief protologue (Went 1893). The fungus was subsequently identified from diseased sugarcane in India and the USA (Barber 1901, Edgerton 1910) and the teleomorph *Glomerella tucumanensis* was described in 1954 (Arx & Muller 1954).

Recent molecular studies have shown that *Colletotrichum* isolates from sugarcane are composed of at least two morphologically similar but phylogenetically divergent species (Crouch *et al*. 2009a,c). The morphology of both taxa is consistent with descriptions of *C. falcatum* (Went 1893, Sutton 1980), resulting in ambiguity as to which of the two species the name *C. falcatum* should be applied (Crouch *et al*. 2009a,c). Given the importance of this disease to the economy of several countries, resolution of this issue is of immediate practical importance.

The first step in determining the correct application of the *C. falcatum* name is to examine the type material for the species and connect it with modern collections of the fungus. Unfortunately, type material of this species is not deposited in Bogor (BO) and we could not locate it in any other mycological collection. It is therefore necessary to designate a neotype for *C. falcatum* in order to stabilize the application of the name, to facilitate subsequent taxonomic work in the genus, and to serve as a foundation for applied research of this important pathosystem (Hyde *et al*. 2009a).

The process of choosing neotype or epitype material must be conducted carefully, in order that further confusion does not ensue (Hyde & Zhang 2008, Cai *et al*. 2009). It is necessary to select a collection that conforms to the original description based on the morphology, pathogenicity and ecological characters (Cannon *et al*. 2008). Strains of *C. falcatum* are available from several international culture collections. However, none of these isolates are derived from Java. As no suitable *C. falcatum* isolates were available from extant materials, we visited the original site of the protologue at Tjomal (now Comal) in central Java (Indonesia) during March 2008 and recollected diseased sugarcane tissue, and isolated a *Colletotrichum* species that conforms to the species protologue. In this study, we characterize this new collection through morphological and molecular phylogenetic analysis, and designate this isolate as the neotype of *C. falcatum* with an ex-neotype living strain.

## Materials and methods

### Isolation, morphological examination and re-inoculation experiment

*Colletotrichum falcatum* was isolated from infected sugarcane leaves collected in Indonesia from the original type locality at Tjomal

in central Java, Indonesia. Although partially destroyed for housing, remarkably, the same sugarcane plantation sampled by Went in 1893 is still present in its original location. Single-spore isolation (Choi *et al.* 1999) was carried out from the sample with distinct red-rot disease. Morphology and cultural characteristics were examined. Appressoria were produced using a slide culture technique, in which 10 $mm^2$ squares of PDA were placed in an empty Petri dish. The edge of the agar was inoculated with spores taken from a sporulating culture and a sterile cover slip was placed over the inoculated aga. After 3–7 days, the shape and size of the appressoria formed across the underside of the cover slip were studied. The neotype is deposited in The Mycological Herbarium of Institute of Microbiology, Chinese Academy of Sciences (HMAS). The ex-neotype strain is deposited at China General Microbial Culture Collection (CGMCC).

To confirm the pathogenicity of the ex-neotype strain on sugarcane, spore suspensions were inoculated back to healthy leaves of *Saccharum officinarum* (3 replicates). Sterilized distilled water was used as control. Symptoms were examined after 14 days incubation under room temperature. Detailed protocols follow that of Cai *et al.* (2009).

### DNA extraction, PCR and sequencing

Genomic DNA was extracted from mycelia (7 days-old culture) using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) according to the instructions of the manufacturer. Primer pairs and PCR amplifications conditions were followed as previously described (Prihastuti *et al.* 2009, Crouch *et al.* 2009c). Partial actin (ACT), DNA lyase (APN2), ß-tubulin (TUB2), calmodulin (CAL), DNA lyase *Apn2* and mating type protein *Mat1*-like gene (MAT1/APN2), glyceraldehyde-3-phosphate dehydrogenase (GPDH), glutamine synthetase (GS) and the complete internal transcribed space (ITS)/5.8 rDNA (ITS) were generated for the ex-neotype strain. These gene regions were selected because they have been widely used in previous studies and have been shown to be successful in phylogenetic reconstruction (Crouch *et al.* 2009a, Prihastuti *et al.* 2009). DNA sequencing was performed at the SinoGenoMax Company Limited, Beijing.

### Sequence alignment and phylogenetic analyses

Sequences from forward and backward primers were aligned to obtain a consensus sequence. Sequence of the neotype isolate, along with reference sequences obtained from GenBank (Table 1), were aligned by Clustal X (Thompson *et al.* 1997). Alignments were optimized manually in Bioedit (Hall 1999). To compare *C. falcatum* with other graminicolous *Colletotrichum* species, a combined ITS, APN2 and MAT1/APN2 sequences dataset was used for phylogenetic reconstruction.

**Table 1.** Sequences used in the phylogenetic analysis.

| ***Colletotrichum*** **Species** | **Culture Collection** | **GenBank Accession Number** | | |
|---|---|---|---|---|
| | | **ITS** | **Apn2** | **Apn2/Mat1** |
| *C. axonopodi* | IMI279189 * | EU554086 | EU364993 | FJ377907 |
| *C. cereale* | MAFF305076AS | EU554090 | EU364997 | FJ377911 |
| *C. falcatum* | CGMCC 3.14187* | **HM171677** | **HM569770** | **HM569769** |
| *C. hanaui* | MAFF305404DC* | EU554101 | EU365008 | FJ377922 |
| *C. hanaui* | MAFF511014DC | EU554124 | EU365031 | FJ377944 |
| *C. jacksonii* | MAFF511344EE | EU554133 | EU365040 | FJ377952 |
| *C. jacksonii* | MAFF511152EE | EU554130 | EU365037 | FJ377950 |
| *C. jacksonii* | MAFF305460EE* | EU554108 | --- | --- |
| *C. miscanthi* | MAFF510857 * | EU554121 | EU365028 | --- |
| *C. navitas* | 9032d | GQ919068 | GQ919070 | GQ919072 |
| *C. navitas* | CBS125086 * | GQ919067 | GQ919069 | GQ919071 |
| *C. nicholsonii* | MAFF511115PD * | EU554126 | EU365033 | FJ377946 |
| *C. nicholsonii* | MAFF510916PD | EU554122 | EU365029 | FJ377942 |
| *C. paspali* | MAFF305403 PN* | EU554100 | EU365007 | FJ377921 |
| *C. sublineolum* | S3001 * | DQ003114 | EU365121 | FJ378029 |
| *C. sublineolum* | MAFF510020SB | EU554116 | EU365024 | FJ377937 |

* Indicate the ex-type cultures. The newly generated sequences in this study are shown in bold. CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CGMCC: China General Microbial Culture Collection. IMI: CABI Europe – UK, Bakeham Lane, Egham, Surrey, UK; MAFF: Ministry of Agriculture, Forestry and Fisheries of Japan. Other gene regions sequenced but not used in the analysis include: ACT (HM171665), CAL (HM171668), GPDH (HM171671), GS (HM171674), TUB2 (HM171680).

Phylogenetic analyses were performed by using PAUP* 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded from all analyses. Unweighted parsimony (UP) analysis was performed. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics such as tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], homoplasy index [HI], and log likelihood [-ln L] (HKY model) were calculated for trees generated under different optimality criteria. Clade stability was assessed in a bootstrap analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa. Kishino-Hasegawa tests (Kishino & Hasegawa 1989) were performed in order to determine whether trees were significantly different. Trees were figured in Treeview (Page 1996).

Model of evolution was estimated by using Mrmodeltest 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001), using the estimated model of evolution. Six simultaneous Markov chains were run for 1 000 000 generations and trees were sampled every 100$^{th}$ generation, resulting in 10 000 total trees. The first 2 000 trees, which represented the burn-in phase of the analyses, were discarded and the remaining 8 000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree.

## Results

A pure culture of *C. falcatum* was obtained by single spore isolation. The cultural characteristics, conidial and appressorial morphology are illustrated in Fig. 1. Three sugarcane leaves re-inoculated with spore suspensions all developed typical red-rot disease (Fig. 2). Eight sequences of the ex-neotype of *C. falcatum* (CGMCC3.14187) are deposited in GenBank (accession numbers: HM171665, HM171668, HM171671,HM171674,HM171677,HM171680,HM569769,HM569770). Phylogenetic relationships were inferred using combined ITS, *Mat1/Apn2* and *Apn2* sequences. Other gene regions were not used in the analysis because most of the falcate-spored graminicolous *Colletotrichum* species were only sequenced with ITS, *Mat1/Apn2* and *Apn2* regions in previous studies (Crouch *et al.* 2009a,c). Maximum parsimony analysis generated only one tree (TL=1119, CI=0.819, RI=0.850, RC=0.697, HI=0.181) as shown in Fig. 3. Multilocus sequence analysis shows that *C. falcatum* appears as a distinct lineage among the graminicolous species of *Colletotrichum*. A close phylogenetic relationship between *C. falcatum* and *C. sublineolum* is supported by this analysis, in agreement with that of Crouch *et al.* (2009a,c).

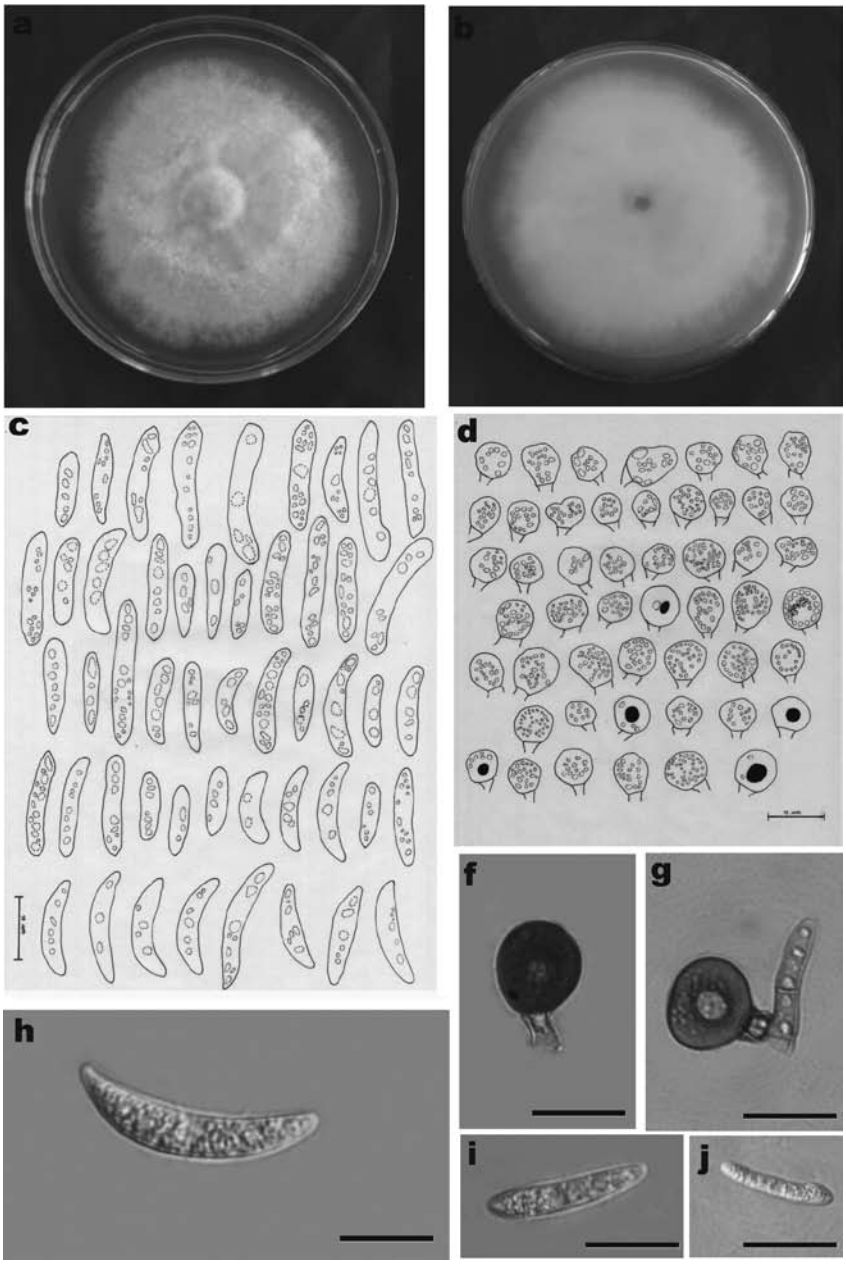


**Fig. 1. –** *Colletotrichum falcatum* (from CGMCC 3.14187). **a, b.** Upper and reverse view of culture on PDA 7 days after inoculation; **c, h, i & j.** Short falcate conidia. **d, f & g.** Rounded appressoria; (bar: c–d = 15 µm; f–j = 10 µm).

## Taxonomy

***Colletotrichum falcatum*** Went, Arch. Java Suikerindustrie 1, 265 (1893). – Figure 1.
MycoBank no.: MB157602

Colonies on PDA circular, raised, at first white and becoming grey with age, aerial mycelia white, dense, cottony without visible conidial masses, reverse white to pale yellowish-green, attaining a diameter of 58.5-81.5 mm ($\bar{x}$ = 76.35 ± 6.770, n = 10) in 7 days. Acervuli absent. Sclerotia absent. Setae absent. Conidia 16-35 µm long ($\bar{x}$ = 23.33 ± 4.892, n = 50), 4–5 µm wide ($\bar{x}$ = 4.65 ± 0.443, n = 50), one celled, smooth-walled, hyaline, falcate, sometimes fusiform. Appresoria in slide cultures 7–14.5 µm long ($\bar{x}$ = 9.44 ± 1.541, n = 50), 6.5–11.5 µm wide ($\bar{x}$ = 8.99 ± 1.268, n = 50), abundant, medium to dark brown, clavate or circular, with edge entire.

Neotype designated here: Indonesia, Central Java, Comal, Gedheg Village on leaves of *Saccharum officinarum*, 23 March 2008, N.F. Wulandari, HMAS 240681; ex-neotype culture deposited in CGMGC 3.14187, with a duplicate strain deposited in CBS.

## Discussion

Arx (1957) included most of the described *Colletotrichum* species on *Gramineae* as synonyms of *C. graminicola* (Ces.) Wils. However, host-specificity of *C. falcatum* towards *Saccharum* species is well documented (Mordue 1967). Additionally, Sutton (1968) demonstrated a

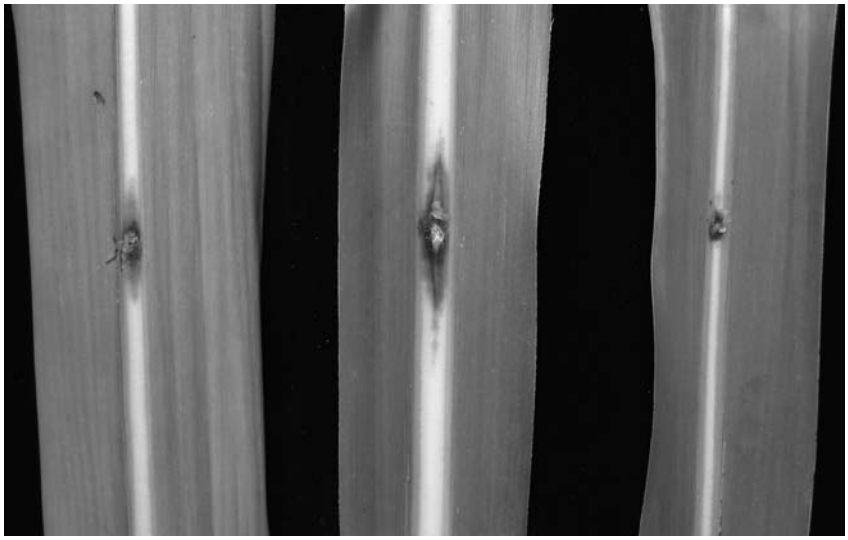

**Fig. 2. –** Results of re-inoculation experiment, anthracnose symptoms on 3 sugarcane leaves after 14 days incubation.

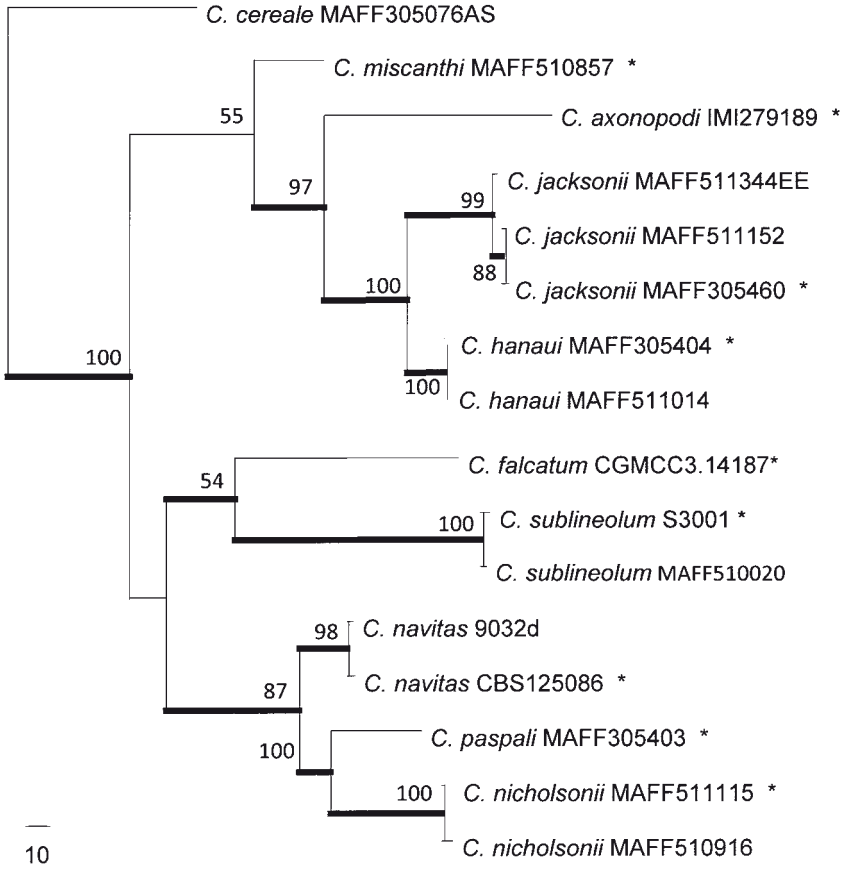


**Fig 3.** – Phylogram of tree generated maximum parsimony analysis based on combined ITS, *Mat1/Apn2* and *Apn2* sequences. Values above the branches are parsimony bootstrap (≥ 50%). Thickened branches indicate posterior probability values ≥ 95 %. The tree is rooted with *Colletotrichum cereale*. * Indicate the ex-type strains.

morphological difference in appressoria between *C. falcatum* and taxa described from *Zea* (*C. graminicola)* and *Sorghum* (*C. sublineola*). The appressoria of *C. falcatum* are broadly clavate, with entire edges and are rarely irregularly lobed, while those of *C. graminicola* are obovate, pyriform or elliptical and frequently with irregular edges (Sutton 1968). The appressorial morphology of the neotype agrees with that described in Sutton (1968). Sutton (1968), however, also showed slight differences in conidial size between graminicolous taxa and a less obvious distinction in conidial shape; these vague differences were unsatisfactory for species delimitation, an observation that has been repeatedly confirmed in subsequent work (Baxter *et al.* 1983, Crouch *et al.* 2009a, Du *et al.* 2005, Sutton 1980, 1992).

The objective in the present study is to designate a suitable neotype specimen for *C. falcatum*, a fungus that is responsible for one of the most important diseases of sugarcane in many countries worldwide. Comparison of ITS sequence data from the neotype against sequences deposited in GenBank (accessed on 6 April 2010) show that only one ITS sequence (AB462376, generated from strain MAFF306170) is identical to the neotype (100 % similarity), and only four of the 23 *C. falcatum* sequences cluster in the same lineage in the parsimonious tree (result not shown). This diversity of sequences found in the GenBank database raises the possibility that undescribed species of *Colletotrichum* occur on sugarcane and await description, consistent with findings from multi-locus phylogenetic analyses of the grass-associated *Colletotrichum* group (Crouch *et al.* 2009a,c). A similar situation has been described for many *Colletotrichum* species, including *C. acutatum* J.H. Simmonds, *C. gloeosporioides* (Penz.) Penz & Sacc. and *C. graminicola* (Ces.) G.W. Wilson where it has been determined that sequences and names in GenBank cannot be relied upon by researchers unless they are linked to type strains (Cai *et al.* 2009, Crouch *et al.* 2009b, Shivas & Tan 2009).

By formally establishing a neotype and ex-type culture for *C. falcatum* that is consistent with the original type with respect to morphology, host and geographic derivation, and generating a multilocus sequence dataset for this strain, we have taken the first and vital step towards informative and meaningful studies of the taxa responsible for this important disease of sugarcane.

## Acknowledgements

This study was supported by research grants 51101010029 and 52101010002 awarded by Mae Fah Luang University Chiang Rai, Thailand. This research was also funded by China NSFC 31070020 and the Knowledge Innovation Program of the Chinese Academy of Sciences, No. KSCX2-YW-Z-1026. Fang Liu and Yuanying Su are thanked for technical assistance.

## References

Arx J. A. von (1957) Die Arten der Gattung *Colletotrichum* Cda. *Phytopathologische Zeitschrift* **29**: 413–468.

Arx J. A. von, Muller E. (1954) *Beiträge zur Kryptogamenflora der Schweiz* **11**: 195.

Barber C. A. (1901) Sugarcane diseases in Godawari and Ganjam Districts. *Madras Department of Land Records and Agricultural Bulletin* **512**: 181–194.

Baxter A. P., van der Westhuizen G. C. A., Eicker A. (1983) Morphology and taxonomy of South African isolates of Colletotrichum. *South African Journal Botany* 2: 259–270.

Cannon P. F., Buddie A. G., Bridge P.D. (2008) The typification of Colletotrichum gloeosporioides. *Mycotaxon* **104**: 189–204.

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. and Johnston P. R. (2009) A polyphasic approach for studying Colletotrichum. *Fungal Diversity* **39**: 183–204.

Choi Y. W., Hyde K. D., Ho W. H. (1999) Single spore isolation of fungi. *Fungal Diversity* **3**: 29–38.

Crouch J. A., Beirn L. A. (2009) Anthracnose of cereals and grasses. *Fungal Diversity* **39**: 19-44.

Crouch J. A., Clarke B. B., White J. F., Hillman B. I. (2009a) Systematic analysis of the falcate-spored graminicolous Colletotrichum and a description of six new species of the fungus from warm season grasses. *Mycologia* **101**: 717–732.

Crouch J. A., Clarke B. B., Hillman B. I. (2009b) What is the value of ITS sequence data in Colletotrichum systematics and species diagnosis? A case study using the falcate-spored, graminicolous Colletotrichum group. *Mycologia* **101**: 648–656.

Crouch J. A., Tredway L. P., Clarke B. B., Hillman B. I. (2009c) Phylogenetic and population genetic divergence correspond with habitat for the pathogen Colletotrichum cereale and allied taxa across diverse grass communities. *Molecular Ecology* **18**: 123–135.

Du M., Schardl C. L., Nuckles E. M., Vaillancourt L. (2005) Using mating-type gene sequences for improved phylogenetic resolution of Colletotrichum species complexes. *Mycologia* **97**: 641–658.

Edgerton C. W. (1910) Colletotrichum falcatum in the United States. Science **31**: 717–718.

Hall T. A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic Acids Symposium Series*. Vol. **41**: 95–98.

Huelsenbeck J. P., Ronquist F. R. (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Biometrics* **17**: 754–755.

Hyde K. D., Cai L., Cannon P. F., Crouch J. A., Crous P. W., Damm U., Goodwin P. H., Chen H., Johnston P. R., Jones E. B. G., Liu Z. Y., McKenzie E. H. C., Moriwaki J., Noireung P., Pennycook S. R., Pfenning L. H., Prihastuti H., Sato T., Shivas R. G., Tan Y. P., Taylor P. W. J., Weir B. S., Yang Y. L., Zhang J. Z. (2009a) Colletotrichum – names in current use. *Fungal Diversity* **39**: 147–182.

Hyde K. D., Cai L., McKenzie E. H. C., Yang Y. L., Zhang J. Z., Prihastuti H. (2009b) Colletotrichum: a catalogue of confusion. *Fungal Diversity* **39**: 1–17.

Hyde K. D., Zhang Y. (2008) Epitypification: should we epitypify? Journal of Zhejiang University, *Science* **B 9**: 842–846.

Kishino H., Hasegawa M. (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of Hominoidea. *Journal of Molecular Evolution* **29**: 170-179.

Mordue J. E. M. (1967) Colletotrichum graminicola. C.M.I. *Descriptions of Pathogenic Fungi and Bacteria*, No. **132**. Commonwealth Mycological Institute, Kew, England.

Nylander, J. A. A. (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University

Page R. D. M. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. Computer *Applications in the Biosciences* **12**: 357-358.

Prihastuti H., Cai L., Chen H., McKenzie E. H. C., Hyde K. D. (2009) Characterization of Colletotrichum species associated with coffee berries in Chiang Mai, Thailand. *Fungal Diversity* **39**: 89–109.

Rannala B. and Yang Z. (1996) Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. *Journal of Molecular Evolution* **43:** 304–311.

Shivas R. G., Tan Y. P. (2009) A taxonomic re-assessment of Colletotrichum acutatum, introducing C. fioriniae comb. et stat. nov. and C. simmondsii sp. nov. *Fungal Diversity* **39**: 111–122.
Sutton B. C. (1968) The appressoria of Colletotrichum graminicola and C. falcatum. *Canadian Journal of Botany* **46**: 873–876.
Sutton B. C. (1980) *The coelomycetes: Fungi imperfecti with pycnidia, acervuli, and stromata*. Commonwealth Mycological Institute, Kew, UK.
Sutton B. C. (1992) The genus Glomerella and its anamorph Colletotrichum. Pages 1-26 in: *Colletotrichum: Biology, Pathology and Control*. JA Bailey and MJ Jeger, eds. CAB International, Wallingford, UK.
Swofford D. L. (2000) PAUP* 4.0: phylogenetic analysis using parsimony (* and other methods). Sinauer Associates, Sunderland, MA.
Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876– 4882.
Went F. A. F. C. (1893) Het rood snot. *Archief voor de Java-suiker-industrie* **1**: 265–282.
Zhaxybayeva O., Gogarten J. P. (2002) Bootstrap, Bayesian probability and maximum likelihood mapping: exploring new tools for comparative genome analyses. *Genomics* **3**: 1–15.

*(Manuscript accepted 25 October 2010; Corresponding Editor: R. Pöder)*