Morpho-molecular characterisation and epitypification of *Colletotrichum capsici* (*Glomerellaceae*, *Sordariomycetes*), the causative agent of anthracnose in chilli

Belle Damodara Shenoy[1*], Rajesh Jeewon[2], Wing Hon Lam[2], Darbhe Jayarama Bhat[3], Po Po Than[4], Paul W.J. Taylor[5] and Kevin D. Hyde[2*]

[1]Biodiversity (Mycology and Botany), Eastern Cereal and Oilseed Research Centre, 960 Carling Avenue, Ottawa, Ontario, K1A 0C6, Canada
[2]Centre for Research in Fungal Diversity, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, PR China
[3]Department of Botany, Goa University, Goa - 403 206, India
[4]The Mushroom Research Centre**,** 128 Moo3, Bahn Pha Deng, T. Papae, A. Mae Taeng, Chiang Mai, 50150, Thailand
[5]BioMarka, School of Agriculture and Food Systems, Faculty of Land and Food Resources, Corner of Royal Parade and Tin Alley, University of Melbourne, 3010, Victoria, Australia

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Colletotrichum capsici is an economically important anamorphic taxon that causes anthracnose in chilli (*Capsicum annuum*, *C. frutescens*). *Vermicularia capsici* (= *Colletotrichum capsici*) deposited by H. Sydow in S was not designated as a holotype in the protologue and is, therefore, designated as the lectotype in this paper. The specimen is in relatively good condition, but could not provide viable cultures necessary to obtain DNA sequence data. Fresh specimens of this species were, therefore, collected from chilli fruit (*Capsicum frutescens*) in the proximity of the original location. Morphological characters of the fresh specimens are compared with those of the lectotype. The taxon is redescribed and illustrated. An epitype for *Colletotrichum capsici* with living cultures is designated in order to stabilise the application of the species name. Phylogenies based on the combined partial β-tubulin gene and ITS nu-rDNA sequence-data suggest a close phylogenetic relationship of the epitype with *C. capsici* strains from Thailand. The use of named strains of *Colletotrichum* from culture collections is advised against.

Key words: holotype, molecular phylogeny, pathogen, systematics, *Vermicularia capsici*

[*]Corresponding author: B.D. Shenoy, e-mail: belleshenoy@gmail.com; K.D. Hyde, e-mail: kdhyde1@gmail.com

Introduction

Colletotrichum capsici (Syd.) E.J. Butler & Bisby was originally described as *Vermicularia capsici* Syd. (Sydow, 1913) in India. Although described from chilli (*Capsicum frutescens*, *Solanaceae*), it has also been putatively reported from 121 host-genera in 45 plant families (Farr *et al*., 2007) from different parts of the world. It is widely known to be responsible for causing anthracnose in chilli (Bailey and Jeger, 1992; Latunde-Dada, 2001; Than *et al*., 2007). Additionally, it is putatively reported to be associated with symptoms such as leaf tip die-back, stem die-back, foliar blight, leaf spot, leaf lesions and boll rot of various plants (Farr *et al*., 2007).

Given the economic, nomenclatural and taxonomic importance of *Colletotrichum capsici*, the dried type specimen of *C. capsici* (= *Vermicularia capsici*) from the Swedish Museum of Natural History (S199) was procured and re-examined. The type specimen is in relatively good condition but could not provide viable cultures necessary to obtain DNA sequence or other molecular data. Fresh specimens of this species, therefore, were collected from chilli fruit (*Capsicum frutescens*) in the proximity of the type locality (Coimbatore, India). This publicationhas the following objectives: 1) to characterise and epitypify *Colletotrichum capsici* with morpho-molecular characters and living cultures in order to stabilise the application of the species name, and 2) to investigate the phylogenetic placement of the *C. capsici* epitype with known species in the genus based on the combined partial β-tubulin gene and internal transcribed spacer (ITS) regions of nuclear rDNA sequence data.

Materials and methods

Morphological examination of Colletotrichum capsici *specimens*

Fresh chilli fruits (*Capsicum frutescens*) infected with *Colletotrichum capsici* were collected from the proximity of the type locality as stated in the protologue, i.e. *Maskalipalayam*, Coimbatore in India. The original type locality is now a township and chilli is not cultivated there. Therefore the sample was collected from close proximity to *Maskalipalayam*, i.e. from the outskirts of Coimbatore, India. Pure cultures of *C. capsici* were obtained by single spore isolation as described in Choi *et al.* (1999). The cultures were maintained on potato dextrose agar (PDA), subjected to natural illumination at room temperature for 7 days before observation of cultural and morphological characters.

Squash preparations of the conidiomata of the herbarium and fresh specimens was mounted in distilled water and morphological characters were recorded. Ten replicates of the sizes (length and width) of setae, 20 replicates of sizes (length and width) of conidiophores, and 50 replicates of sizes (length and width) of conidia were recorded. The mean and standard deviation of the measurements were calculated. Colony characteristics derived from single spore isolates from the fresh specimens were also examined. In particular, colour, shape of the colony, and the nature of mycelia were recorded. The growth rate was measured for 10-day-old colonies on PDA initiated from 5 mm diam plugs. Mycelial plugs were excised from the margins of actively growing PDA cultures. For appressoria morphology of fresh specimens, slide cultures were prepared according the method of Sutton (1992). Fifty replicates of appressoria were used to record shapes and sizes (length and width). The epitype is deposited at the University of Hong Kong Mycological Herbarium (HKU (M)). Cultures ex-types were deposited in Centraalbureau voor Schimmelcultures culture collection (CBS) and Hong Kong University Culture Collection (HKUCC).

DNA extraction, PCR and sequencing

Isolates were grown on PDA for 7 days before total genomic DNA was extracted from mycelia following the protocols as outlined by Jeewon *et al*. (2003) and Kodsueb *et al*. (2006a,b). DNA amplification was performed by polymerase chain reaction (PCR). The Bt2A–Bt2B (Glass and Donaldson, 1995) and ITS4–ITS5 (White *et al*., 1990) primer-pairs were used to amplify partial β-tubulin (tub2) gene and complete ITS/5.8 nu-rDNA, respectively. Amplification reactions were performed in a 50 μL reaction volume as outlined by Jeewon *et al*. (2002) and Shenoy *et al*. (2006). The PCR thermal cycle was as follows: 95°C for 3 min, followed by 34 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 30 s and elongation at 72°C for 1 min, with a final extension step of 72°C for 10 min. The PCR products, spanning approximately 500 bp (tub2) and 600 bp (ITS/5.8) were visualised on 1% agarose electrophoresis gels stained with ethidium bromide. The PCR products were then purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amersham Biosciences, Buckingamshire, UK; product code – 27-09602-01). DNA sequencing was performed using the above-mentioned primers in an Applied Biosystem 3730 DNA analyzer at the Genome Research Centre, The University of Hong Kong.

Table 1. Taxa used, strain number, and GenBank accession numbers.

Taxon	Strain No.	Host	Location	β-tubulin	ITS nu-rDNA
Colletotrichum boninense	STE-U 194	*Eucalyptus* sp.	South Africa	AY376569	AY376521
	STE-U 3000	*Leucospermum* sp.	Australia	AY376570	AY376522
C. capsici	HKUCC 10928; CBS 120709**	*Capsicum frutescens*	Coimbatore, India	EF683602	EF683603
	HKUCC 10859 ("Ccmj10")	*Capsicum annuum*	Chiang Mai, Thailand	DQ454054	DQ453990
	HKUCC 10875 ("Ccmj2")			DQ454045	DQ453987
	HKUCC 10857 ("Ccmj3")			DQ454047	DQ453988
	HKUCC 10870 ("R11")			DQ454049	DQ454016
	HKUCC 10880 ("R12")			DQ454057	DQ454017
	HKUCC 10868 ("R4")			DQ454046	DQ454013
	HKUCC 10852 ("R5")			DQ454056	DQ454014
	HKUCC 10869 ("R7")			DQ454053	DQ454015
	HKUCC ("Skp16")			DQ454055	DQ454025
	HKUCC 10855 ("Skp4")			DQ454052	DQ454024
	STE-U 5304	*Arachis hypogea*	Tanzania	AY376574	AY376526
	HKUCC 10876 ("U10")	*Capsicum annuum*	Ubonrachathani, Thailand	DQ454050	DQ454027
	HKUCC 10867 ("U12")			DQ454051	DQ454028
C. caudatum	STE-U 5300	*Cymbopogon martinii*	India	AY376575	AY376527
C. crassipes	STE-U 4445	*Peperomia* sp.	Unknown	AY376578	AY376530
	STE-U 5302	*Dryas octopetala*	Switzerland	AY376577	AY376529
C. dematium	STE-U 5299	*Dryandra* sp.	Madeira	AY376579	AY376531
C. musae	HKUCC ("B15")	*Musa* sp.	Thailand	DQ454038	DQ453986
	HKUCC ("B4")			DQ454037	DQ453983

* Abbreviations: HKUCC, Hong Kong University Culture Collections; STE-U, The culture collection of the Department of Plant Pathology at the University of Stellenbosch, South Africa. ** Isolate designated as epitype in this study. CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Sequence alignment and phylogenetic analyses

DNA sequences obtained were aligned using Clustal X (Thomson *et al*., 1997). Three datasets were prepared and analysed: dataset based on ITS nu-rDNA (dataset I), dataset based on partial β-tubulin sequence-data (dataset II), and dataset based on combined ITS nu-rDNA and partial β-tubulin sequence-data (dataset III). Additional sequences were retrieved from GenBank and their accession numbers are listed in Table 1. Alignments were manually adjusted to allow maximum alignment and minimum gaps in a manual sequence alignment editor, Se-Al v2.0a11 (Rambaut, 1996).

Phylogenetic analyses were conducted in MEGA4 (Kumar *et al*., 2004). Phylogenetic relationships of *Colletotrichum capsici* epitype with closely related taxa were analysed based on Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method, and all positions containing gaps and missing data were eliminated from the datasets. Clade stability was assessed in bootstrap analyses with 1000 replicates. Other details are outlined in Kodsueb *et al.* (2007) and Promputtha *et al.* (2007). Trees were figured and edited in MEGA4 and Dataset III has been submitted to TreeBASE (www.treebase.org).

Results

Taxonomy

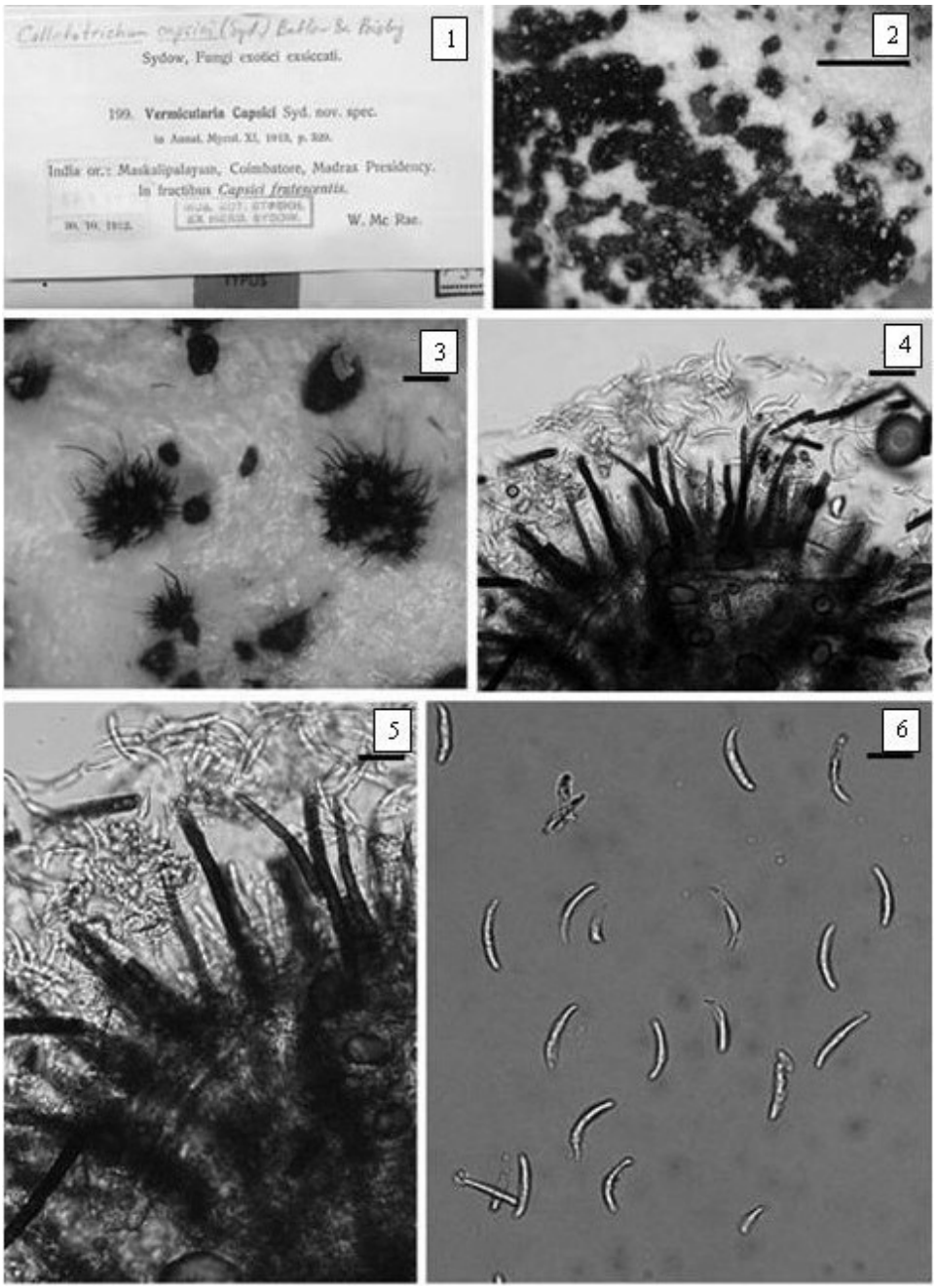
Colletotrichum capsici (Syd.) E.J. Butler & Bisby, *Fungi of India*, *Imperial Council of Agricultural Research Scientific Monograph* 1: 152 (1931)

≡ *Steirochaete capsici* (Syd.) Sacc., *Philippine Journal of Science* 18: 605 (1921)

≡ *Vermicularia capsici* Syd., *Annales Mycologici* 11: 329 (1913)

Description from lectotype (Figs 1-6)

Fruit lesions elliptical to circular. *Acervuli* on chilli fruit, 85-245 µm in diam. ($\bar{x}$ = 172±70.8 µm, n = 10), arranged concentrically on lesion, sub-epidermal, disrupting outer epidermal cell wall of host, densely setose, with grey spore masses (Figs 2-3). *Setae* 70-135 µm long ($\bar{x}$ = 98.8±20.8, n = 10) × 5 µm wide at base ($\bar{x}$ = 5 ± 0, n = 10), abundant, dark brown, rigid, smooth-walled, 1-5-septate, slightly swollen at base, tapered to the paler acute apex (Figs 4-5). *Conidia* 17-26 µm long ($\bar{x}$ = 19.7±3.31, n = 50) × 3.75 µm wide ($\bar{x}$ = 3.75±0, n = 50), one-celled, smooth walled, hyaline, falcate, sometimes fusiform, tapered towards both ends, acute at the apex (Fig. 6).



Figs 1-6. Photomicrographs of *Coletotrichum capsici* from lectotype (S199). **1.** Label of the herbarium packet. **2.** Congregation of acervuli on chilli lesion surface. **3.** Individual acervuli with setae. **4-5.** Setae and conidia. **6.** Conidia. Scale Bars: 2 = 200 µm, 3 = 100 µm, 4 = 50 µm, 5-6 = 15 µm.

Description from epitype (Figs 7-20)

Fruit lesions elliptical to circular, 750-1500 µm (Figs 7-8). *Acervuli* on fruit surface: 85-200 µm in diameter ($\bar{x}$ = 122.7 ± 33.4 µm, n = 10), brown to black, arranged concentrically on lesions, sub-epidermal, disrupting the outer epidermal cell wall of the host, densely setose, with grey spore masses (Figs 9-10). *Setae* 90-140 µm long × 4-7 µm wide ($\bar{x}$ = 109±14.39 × 5.2±1 µm, n = 10), abundant, brown, 1-5-septate, smooth-walled, rigid, hardly swollen at the base, slightly tapered to the paler acute apex (Figs 10-11). *Conidiophores* 16-26 µm long × 3-4 µm wide ($\bar{x}$ = 19±2.6 × 3.8±0.38 µm, n = 20), unicellular, hyaline to faintly brown, cylindrical, phialidic, sometimes branched, tapered towards the apex (Figs 12-13). *Conidia* 16-25 µm × 3-4 µm ($\bar{x}$ = 21.5±2.4 × 3.74±0.45 µm, n = 50), one-celled, smooth walled, hyaline, falcate, tapering at towards each end with acute apex and truncate base (Figs 14-15).

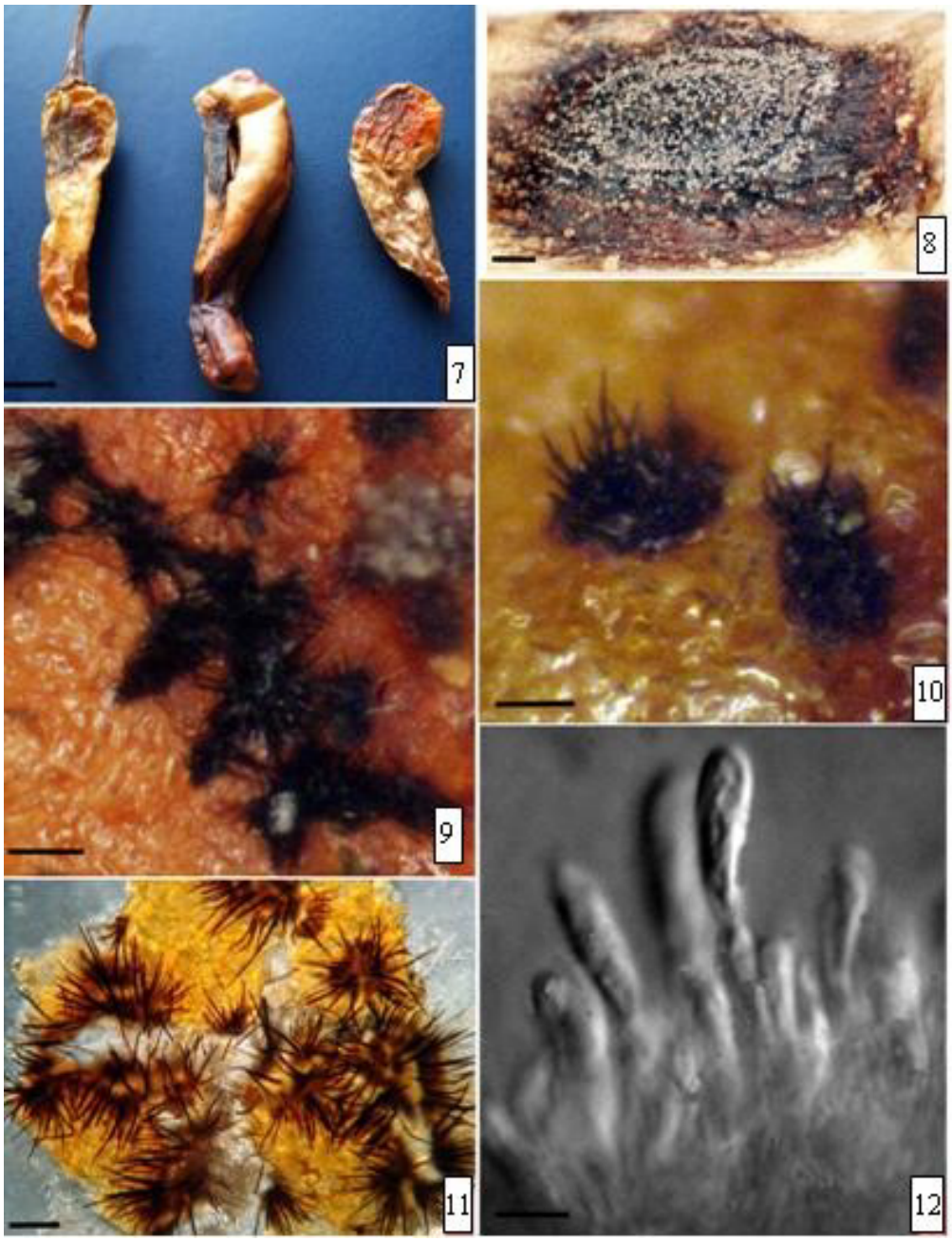
Colonies on PDA at first white and becoming greyish-green with age, reverse greyish-green, attaining 85 mm radial diam. in 10 days (Figs 16-17). Aerial mycelia white to grey. *Acervuli* dark brown to black, conspicuous for their dark setae. *Conidiophores* unicellular, hyaline, cylindrical, phialidic, septate, sometimes branched, tapered towards the apex, 20 µm long × 3 µm wide (Fig. 20). *Conidia* formed in white masses, one-celled, smooth walled, hyaline, falcate, tapering towards each end with acute apex and truncate base, 16-22 µm × 4 µm ($\bar{x}$ = 18.29±1.17 × 4 µm, n = 50) (Figs 18-20). *Appressoria* in slide culture dark brown, spherical, ovate or obclavate, smooth walled, 8-30 µm × 5-10 µm ($\bar{x}$ = 18.2±7.03 × 7.66±1.85 µm, n = 50), supporting hyphae hyaline, branched, septate, sometimes develop into complex chlamydosporic structures.

Teleomorph: unknown.

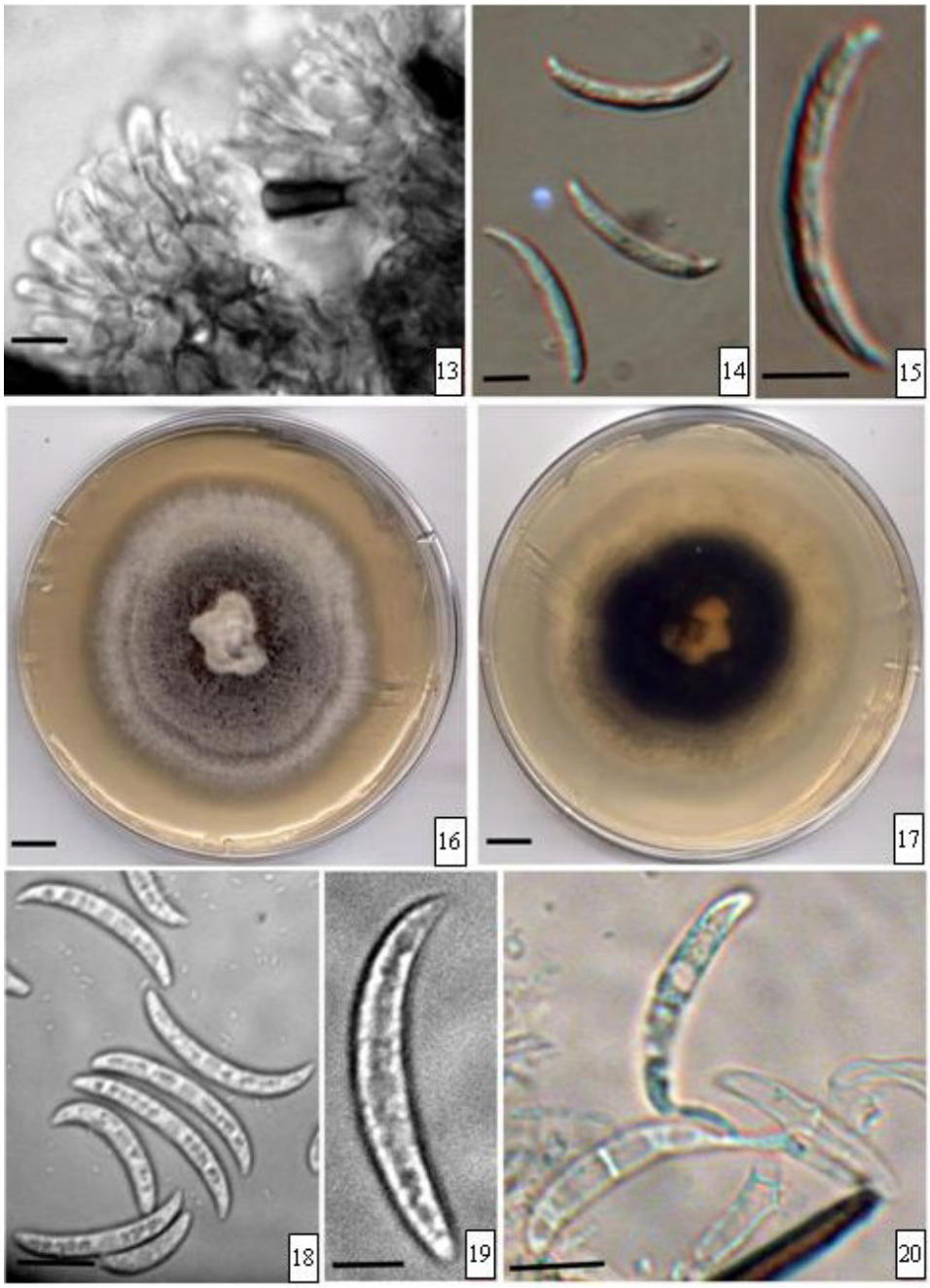
Materials examined: INDIA, Coimbatore, on fruits of *Capsicum frutescens*, October 1912, W. McRae (S199; **lectotype** designated here); INDIA, Coimbatore, on fruits of *Capsicum frutescens*, April 2006, D.J. Bhat (HKU (M) 17516; **epitype** designated here; ex-type living cultures deposited in HKUCC 10928, CBS 120709).

Habitat: *Colletotrichum capsici* has been reported on a wide range of hosts in many families. For example in IMI herbarium, there are 473 collections from 176 different host genera (Sutton, 1980). Farr *et al*. (2007) list 121 host genera in 45 host families for *Colletotrichum capsici*.

Known distribution (putative): Australia, Bangladesh, Brazil, Borneo, Brunei, Cambodia, China, Cuba, Denmark, Egypt, Ethiopia, Fiji, Gambia, Hong Kong, India, Indonesia, Japan, Kenya, Korea, Malawi, Malaysia, Myanmar, Nepal, New Zealand, Nigeria, Pakistan, Papua New Guinea, Poland, Sierra Leone, Solomon Island, Sri Lanka, Sudan, Taiwan, Tanzania, Thailand, Tonga, Turkey, Trinidad-Tobago, UK, USA, Venezuela, Vietnam, Zambia,



Figs 7-12. *Colletotrichum capsici* (epitype). **7.** Chilli fruits (*Capsicum frutescens*) infected with *C. capsici*. **8.** Individual infection on chilli fruit. **9-10.** Acervuli on fruit surface. **11.** Squash preparation of acervuli. **12.** Conidiogenous cells (from the specimen). Scale Bars: 7 = 750 µm; 8 = 150 µm; 9= 100 µm; 10 = 75 µm; 11 = 100 µm; 12 = 5 µm.



Figs 13-20. *Colletotrichum capsici* (epitype). **13.** Conidiogenous cells (from the specimen). **14-15.** Conidia from the specimen. **16-17.** Colony morphology on PDA. **18-19.** Conidia (from culture). **20.**Conidiogenous cells (from culture). Scale Bars: 13 = 5 µm; 14 -15 = 5 µm; 16-17 = 9 mm; 18 = 10 µm; 19-20 = 5 µm.

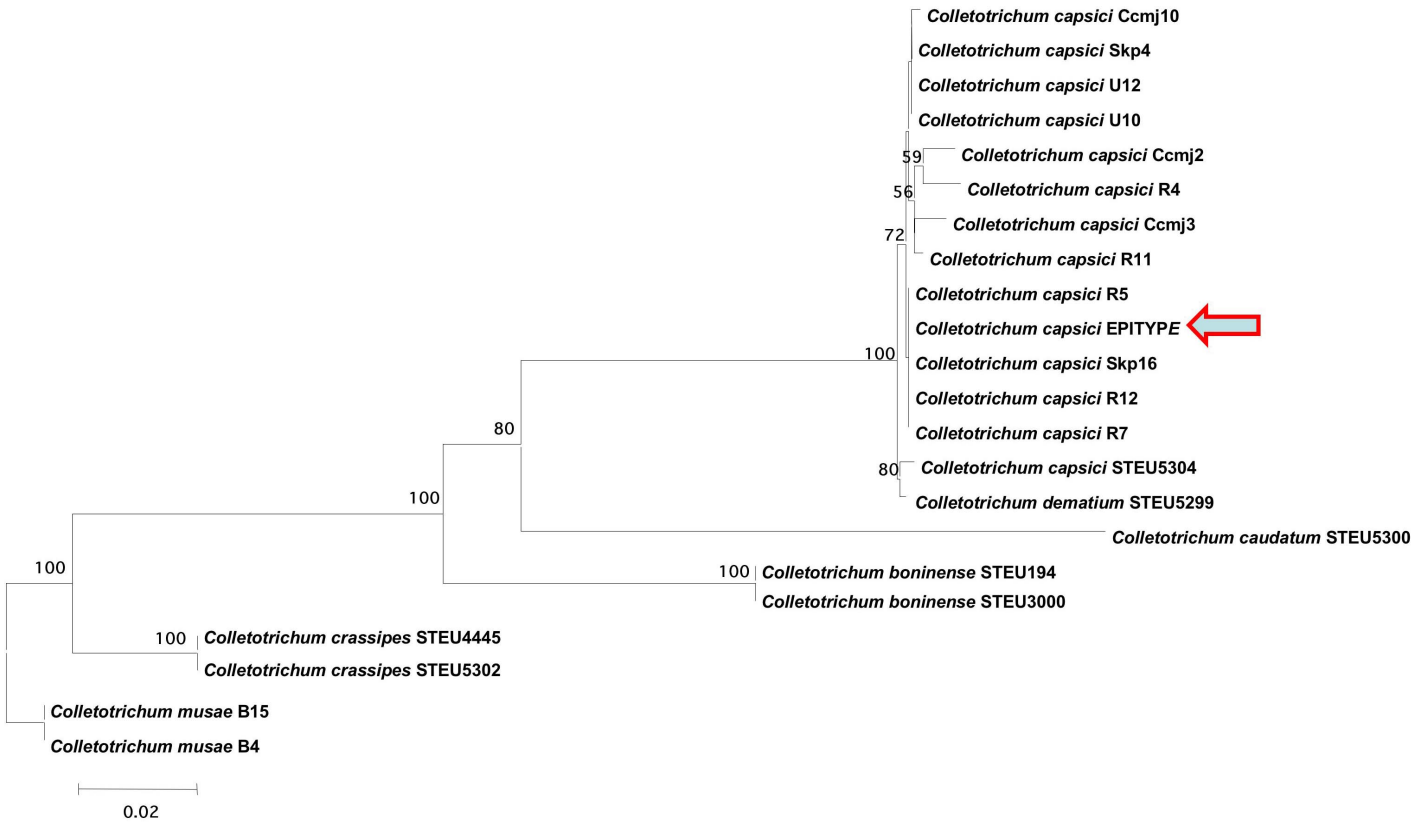


Fig. 21. Neighbor joining tree based on the combined ITS nu-rDNA and partial β-tubulin sequence-data of *Colletotrichum capsici* epitype and 21 strains of *Colletotrichum* species. The optimal tree with the sum of branch length = 0.35123274 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances. *Colletotrichum musae* is the designated outgroup.

Zimbabwe (CBS Filamentous Fungi database; DSMZ; IMI Herbarium database; NBRC Culture Catalogue; Farr *et al*., 2007).

Phylogenetic placement of Colletotrichum capsici

The trees generated from individual datasets could not provide any clarity with respect to the placement of the epitype (data not shown). The neighbor-joining analysis of the combined dataset (Dataset III) (Fig. 21) showed a close phylogenetic relationship of the epitype with *C. capsici* strains from Thailand. Two other taxa, *Colletotrichum capsici* (STE-U5304) and *C. dematium* (STE-U5299) were found to be basal to the above-mentioned clade with 100% bootstrap and Bayesian support (Fig. 21).

Discussion

There have been relatively few phylogenetic studies on the genus *Colletotrichum* in the past five years despite its importance as a plant pathogen and in post harvest disease (Lubbe *et al*., 2004; Photita *et al*., 2005; Farr *et al*., 2006; Than *et al*., 2007). These studies incorporated strains of type species that were not corroborated against herbarium specimens and thus may well have been wrongly named. With the availability of DNA sequences from Genbank and a need to publish phylogenetic data, many mycologists have failed to verify the reliability of those sequences deposited by others and assume they reflect the taxa to which they have been assigned. Scientifically, this can be highly erroneous as in most cases taxonomists have not double checked and compared their identity (nomenclature) with available herbarium specimens. In recent publications on *Colletotrichum*, we (Photita *et al*., 2005; Than *et al*., 2007) and others (e.g Lubbe *et al*., 2004) have isolated *Colletotrichum* taxa from various hosts and given them names based on available morphological descriptions. Phylogenetic data from DNA sequence analyses failed to provide clear cut taxonomic resolution and there did not seem to be any correlation between morphology and phylogeny. While DNA sequences can be authentic (i.e. reliable), we suspect that 1) many species deposited in culture collections have not been identified correctly (thus have erroneous names), while many researchers assume they are accurate, and 2) the "taxon names" associated with sequences in Genbank need revision or should be interpreted with caution in any phylogenetic framework.

The importance of correct taxonomic identification is illustrated in the paper of Than *et al*. (2007). The latter showed that *Colletotrichum acutatum*, *C. capsici* and *C. gloeosporioides* isolated from chilli in Thailand were the causal

agents of chilli anthracnose of a susceptible Thai elite cultivar of *Capsicum annuum* (Bang Chang). Furthermore, only the *C. acutatum* strain caused anthracnose on a resistant Thai elite cultivar of *Capsicum chinense* (PBC-932). This has very important implications for breeding cultivars of chilli, since new varieties must be developed that are resistant to *C. acutatum*.

The identification of *Colletotrichum* species are difficult and their relationship to hosts and distribution are confused. Most *Colletotrichum* names are ancient and type material are in poor condition and will not provide DNA sequence data or cultures for comparative molecular work. There is an urgent need to epitypify the important species of *Colletotrichum* so that living specimens are available for future researchers. This has taken place in other important plant pathogenic genera e.g. *Botryosphaeria* (Phillips *et al*., 2006), *Diplodia* (Alves *et al*., 2006), *Mycosphaerella* (Verkley *et al*., 2004) and it is important that this also occurs in *Colletotrichum*.

Examination of the type specimen of *Colletotrichum capsici* from S revealed characters consistent with the protologue (Sydow, 1913). In the original publication, the holotype was not designated and is designated here as lectotype according to Article 9.2 of the International Code of Botanical Nomenclature (ICBN) (Greuter *et al*., 2000; McNeill *et al*., 2006). The microscopic features available in the original specimen are, however, limited to the conidia and setae morphology and could not provide DNA sequence-data to establish its phylogenetic placement. This collection of *C. capsici* from Coimbatore in India fits well within the morphological circumscription of *C. capsici* (Table 2) and provided a living culture. In such a scenario, Article 9.7 of the Botanical Code allows for designation of an epitype, which we have nominated in this paper, to serve as an interpretative type of the lectotype (Greuter *et al*., 2000; McNeill *et al*., 2006).

The phylogenetic placement of the epitype of *Colletotrichum capsici* in the genus is inconclusive possibly because of 1) inadequate taxon sampling (especially in the combined dataset), 2) weak phylogenetic signal from the gene sequences used, and 3) misidentification of *Colletotrichum* strains sequenced and deposited in GenBank. Although the genes we used provided some resolution of the species under study, we are not confident that ITS nu-rDNA and β-tubulin gene are entirely suitable for species resolution in *Colletotrichum*. Therefore other genes, such as mtDNA, CO1 or elongation factor should be tested in the future. However until we can be confident of the accuracy of the names placed on the strains we sequence, we will never be able to resolve species, no matter how many genes or strains we use.

The epitype (isolated from India) and *C. capsici* strains isolated from *Capsicum annuum* (*Solanaceae*) in Thailand (Than *et al*., 2007) are

Table 2. Comparison of the lectotype and the epitype of *Colletotrichum capsici*

Feature	Lectotype (S199)	Epitype
Herbarium Number	Swedish Museum of Natural History (S199)	The University of Hong Kong Mycological Herbarium: HKU (M) 17516
Collection Date	October, 1912	April, 2006
Collected By	"W. Mc Rae"	Darbhe Jayarama Bhat
Collected from	*Maskalipalayam*, Coimbatore in India	The outskirts of Coimbatore in India
Host /substrate	*Capsicum frutescens* / living fruits	*Capsicum frutescens*/ living fruits
Living culture	Not available	HKUCC 10928 and CBS 120709
Acervuli on fruit surface	85-230 µm in diam. ($\overline{x}$ = 172± 70.8 µm, n = 10),	85-200 µm in diam. ($\overline{x}$ = 122.7 ±33.4 µm, n = 10),
Setae on acervuli (on the specimen)	70-135 µm long × 5 µm wide at base ($\overline{x}$ = 98.8±20.8 × 5±0, n = 10)	92-140 µm long × 4-7 µm wide ($\overline{x}$ = 109±14.39 × 5.2±1 µm, n = 10)
Conidiophores on the fruit surface	Size not available	16-26 µm long × 3-4 µm wide ($\overline{x}$ = 19±2.6 × 3.78±0.38 µm, n = 20)
Conidia from the fruit surface	17-26 µm long × 3.75 µm wide ($\overline{x}$ = 19.7±3.31 × 3.75±0, n = 50)	16-25 µm × 3-4 µm ($\overline{x}$ = 21.5± 2.4 × 3.74±0.45 µm, n = 50)
Growth date on PDA	No culture available	8.5 mm per day
Conidiophores on PDA	No culture available	20 µm long × 3 µm wide
Conidia on PDA	No culture available	16-22 µm × 4 µm ($\overline{x}$ = 18.29± 1.17 × 4 µm, n = 50)
Appressoria in slide culture	No culture available	8-30 µm × 5-10 µm ($\overline{x}$ = 18.2± 7.03 × 7.66±1.85 µm, n = 50)

phylogenetically closely related, probably because they were isolated from the same host genus. One particular strain of *Colletotrichum capsici* STE-U 5304, isolated from *Arachis hypogea* (*Fabaceae*) in Tanzania, clusters with *C. dematium* strain STE-U 5299 (Lubbe *et al*., 2004) and these two taxa constituted a monophyletic lineage basal to the main *C. capsici* clade. *Colletotrichum capsici* was regarded as a synonym of *C. dematium* by Arx (1957). *Colletotrichum capsici* possesses wider conidia (3-4 µm vs. 2-3 µm) (Sutton, 1980). Mordue (1971) considered *C. dematium* as a saprobic taxon, while *C. capsici* was pathogenic. Mordue (1971) also noted that *C. capsici* showed exceptionally complex development of appressoria in slide cultures. It is, however, possible that *Colletotrichum dematium* is synonymous to *C. capsici*. Further studies, therefore, are needed to test monophyly of *Colletotrichum capsici* and to resolve the inter-specific relationships in *Colletotrichum*.

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