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Clonostachys indicus sp. nov. from India

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ABSTRACT

***Clonostachys indicus* sp. nov. is described and illustrated from pure culture, obtained from bark of twigs of** *Ficus virens* (Miq.) Domin, collected **from Chandigarh, India. The new species was disguised by morphological and *in vitro* cultural characters from its related species. Additionally, internal transcribed spacer (ITS) rDNA sequence analysis support this species as distinct within *Clonostachys*. A comprehensive table of *Clonostachys* species possessing dimorphic conidiophores has been provided.**

KEY WORDS: *Hyphomycete, conidia, conidiophores, systematic, ITS*

INTRODUCTION

The north western India is rich in mycobiota and many new species of hyphomycetes have been reported from this region (Adamčik et al., 2015; Prasher and Singh, 2015a, b; Prasher and Verma, 2014, 2015a, b, c). As a part of ongoing efforts of exploring and documenting fungal diversity an interesting species of *Clonostachys* Corda was isolated from dead branches of *Ficus virens* (Miq.) Domin collected in Chandigarh. A critical morphological and molecular examination of the specimens revealed it to be an undescribed species of *Clonostachys*.

MATERIALS AND METHODS

Clonostachys indicus was isolated from the bark of dead twigs of *Ficus virens*, collected from Department of Botany, Panjab University, Chandigarh, India. Microscopic descriptions were made from 10 days old culture grown on MEA at 24°C. Mycelia were stained in 1% Congo red and structures were studied microscopically and photographed using a Matrix Transmission Microscope (VRS-2f). All the measurements were made with the help of Pro MED software. The line drawings were prepared with the help of Olympus 2 li microscope with a drawing tube attached to it. Measurements are given as n_1n_2, (n_1) $n_4n_3n_5$ (n_2), where n_1 = minimum value observed; n_2 = maximum value observed; n_3 = arithmetical means; n_4 = first quartile; n_5 = third quartile (Schroers, 2001). Ex-type culture is deposited in the herbarium of Botany Department, Panjab University, Chandigarh (PAN).

Isolation: Pure culture was isolated on Malt Extract Agar (MEA) medium (Malt Extract 20g, Agar agar 20g, distilled water to make 1000 ml) by hyphal tipping method after seven days of incubation at 24°C in Petri-plate (**Fig 1**). Two sets of pure cultures were prepared and maintained on the MEA plates at ±4°C with periodic transfer.

DNA extraction and amplification: Fungal strain was maintained on PDA slant. DNA was extracted from cultures grown on PDA plates for two weeks at 24°C using HiPurA™ SP Fungal DNA mini kit (HiMedia) by following the manufacturer's instructions. Fragments containing the ITS region was amplified using primer pair ITS1/ITS4 (White *et al*., 1990).

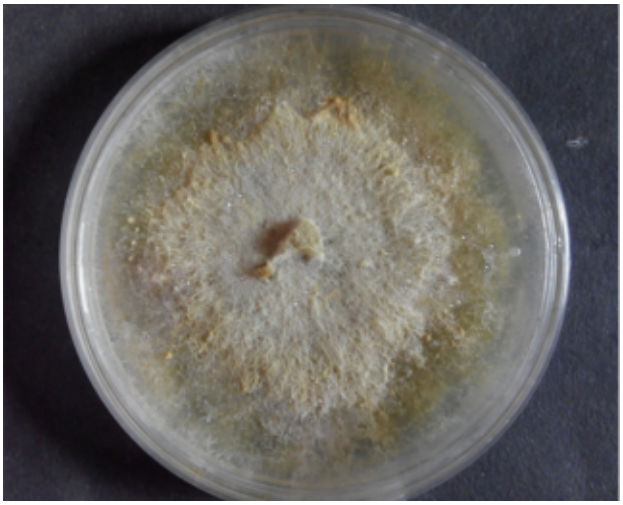

Fig. 1 Colony of *Clonostachys indicus* on Malt Extract Agar after 10 days.

DNA amplification was performed in a 25 µl reaction using 2 µl of template DNA (25 ng), 1U of Taq DNA polymerase i.e. 0.5 µl (Finnzyme Phusion™ High-Fidelity DNA Polymerase-F-530S), 5 µl of 5x Phusion HF Buffer, 0.5 µl of 10 mM of each dNTPs (Genei, Bangalore, India), 1 µl of 10 pmol primer, 15 µl H_2O (Sterile Ultra Pure Water, Sigma) to make up 25 µl. Amplification in an Eppendorf Mastercycler Gradient 5331 AG used the following parameters: 5 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C; and final 7 min extension step at 72°C for ITS region amplification. The PCR products were purified with an Axygen PCR cleanup kit (Axygen Scientific, CA, USA) and sequenced with the same primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were run on an ABI 3100 automated DNA sequence (Applied Biosystems, USA).

TAXONOMY

Clonostachys indicus Prasher I. B. and Chauhan R. sp. nov.

Figs. 2-4

MycoBank MB 821402

DIAGNOSIS: Conidial masses slimy dome-shaped.

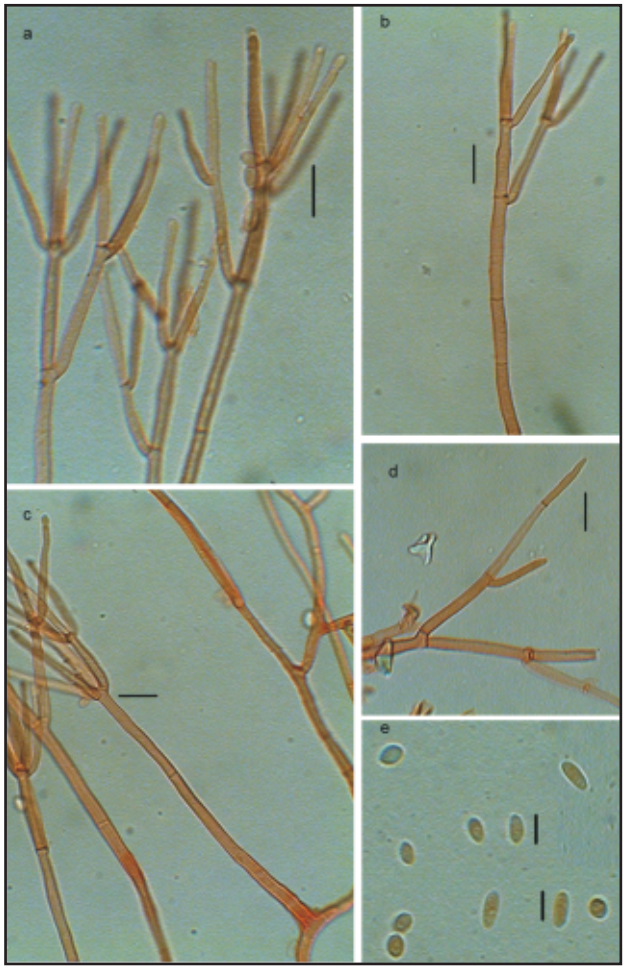

Fig. 2 *Clonostachys indicus* (a,b) Primary conidiophores with phialides (c,d) Stipe of conidiophore arising from vegetative hyphae (e) Conidia from primary conidiophores. Scale bars: ad = 10 µm, e = 5 µm.

Conidiophores dimorphic. Primary conidiophores verticillium like, mononematous. Secondary conidiophores bi- to quarter verticillate. Conidia hyaline, minutely curved, distally broadly rounded with a laterally displaced hilum, ovoid to subglobose.

Etymology: The specific epithet 'indicus' has been named after the country of origin i.e. India.

Conidial masses white, pale yellow or pale orange, normally as watery to slimy dome-shaped masses, less frequently in imbricate chains. **Conidiophores** dimorphic. Primary conidiophores verticillium-like, formed throughout the colony, dominating towards the margin, mononematous, arising from the agar surface; stipe longer than penicillus, 30-78 µm long, 2.1-3.6 µm wide at base; penicillus 43.6-65.7(77.7) µm high; phialides cylindrical but slightly tapering towards the tips, (18 20.9-23.7-25.9 (35.9) µm long, (2.1) 2.4-2.8-3.2 (3.9) µm wide at base, longer than in secondary conidiophores; each producing a small, hyaline drop of conidia that frequently collapses to form a single head on several phialides of the same metula or of several side-branches; collarette lacking. Secondary conidiophores bi- to

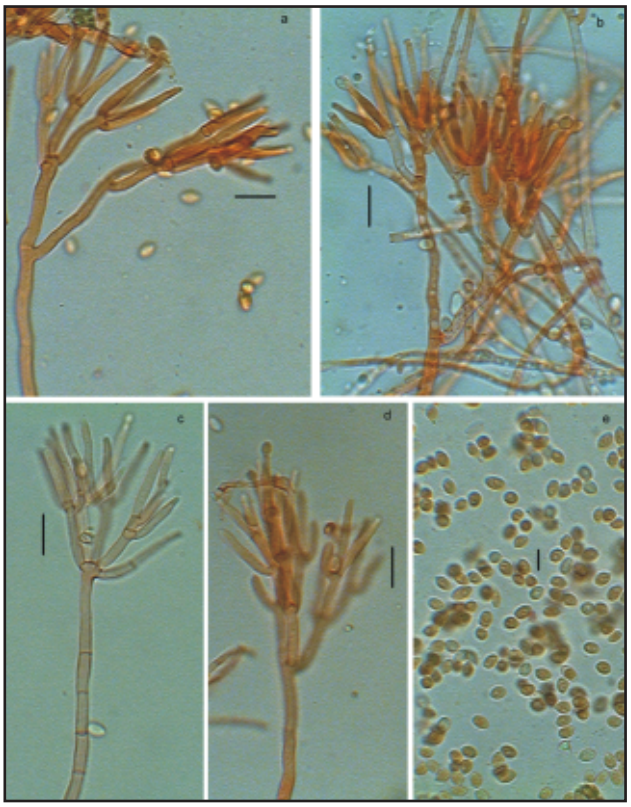

Fig. 3 *Clonostachys indicus* (a, b, d) Secondary conidiophores with phialides diverging at acute angles (c) Typical conidiophore lacking branches and with divergent phialides (e) Conidia from secondary conidiophores. Scale bars: ad = 10 µm, e = 5 µm.

quarter-verticillate, densely aggregated, formed from strands of aerial mycelium; branches either divergent at acute angles, adpressed or short when arising laterally from the hyphal

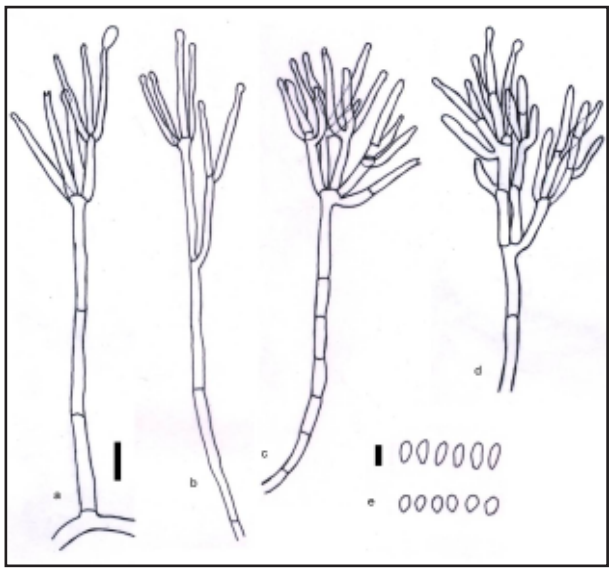

Fig. 4 *Clonostachys indicus* (a,b) Primary conidiophores with phialides (c,d) Secondary conidiophores with phialides diverging at acute angles (e) Conidia from primary and secondary conidiophores. Scale bars: ad = 10 µm, e = 5 µm.

strands; stipe up to 78.5 µm, 3.2 µm wide at base; penicillus (37.3) 39.9-75.8 µm high; metullae 10.5-14.9 (24.4) µm long, 23 (3.8) µm wide at base; phialides in loose whorls of 35, (10) 14.7-16.4-17.8 (24.8) µm long, (1.9) 2.2-2.4-2.5 (3.3) µm wide at base, straight to slightly curved, narrowly flask-shaped, tapering in the upper part, without a visible collarette; intercalary phialides not observed. **Conidia** hyaline, minutely curved, distally broadly rounded, with a laterally displaced hilum, ovoid to subglobose, conidia from primary conidiophores slightly curved to almost ellipsoidal, (4.2) 5.0-5.6-6.3 (7.4) × (2.1) 2.3-2.7-2.9 (3.6) µm from primary conidiophore, (3.9) 4.2-4.6-5.0 (5.6) × (2) 2.9-3-3.2 (3.7) µm from secondary conidiophore. **Perithecia** not observed.

***Ex-type culture examined**:* INDIA. CHANDIGARH: Panjab University Campus 30º45′N, 76º46′E, 355m. isolated from dead twigs of *Ficus virens*, I. B. Prasher and Radha Chauhan, 08. 09. 2011. (PAN 34502).

Phylogenetic analysis: ITS sequence from *Clonostachys indicus* was manually edited using Chromas Lite software (www.technelysium.com.au). Sequence derived in this study was deposited in GenBank (ITS: KT291441). The newly generated ITS sequence of *Clonostachys indicus* and those downloaded from GenBank based on the top-scoring match (**Table 1**) were added to a dataset that was used for the construction of phylogeny. For phylogenetic analysis, the sequences were aligned using Clustal W together with the homologous region of ITS of closely related genera and species. For construction of phylogenetic tree (**Fig. 5**), the matrix was analyzed using Neighbor-Joining method of Molecular Evolutionary Genetics Analysis (MEGA) software v6.0. (Tamura *et al*., 2013). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). The ITS gene dataset comprised of 17 taxa. The alignment comprised 443 characters, gaps and missing data were eliminated.

Table 1 The sequence of *Clonostachys indicus* and other related taxa used in phylogenetic analysis.

Taxon name	Origin	Substratum	Strain	Sequence Accession No. of ITS
Bionectria apocyni	USA, New York	dead stem of *Apocynum cannabinum*	CBS 130.87	AF210688
Bionectria aureofulvella	New Zealand	root of tree	CBS 195.93	AF358226
Bionectria byssicola	Uganda	*Alchorea* branches	CBS 914.97	AF358252
Bionectria capitata	Japan	bark	CBS 218.93	AF358240
Bionectria oblongispora	Japan	bark of dying tree of *Orixa japonica*	CBS 100285	AF358248
Bionectria pseudochroleuca	French Guiana	bark	CBS 192.94	AF358238
Bionectria pseudostriata	Indonesia	bark	CBS 119.87	AF358251
Bionectria sporodochialis	U.S.A., Puerto Rico	bark	CBS 101921	AF210685
Bionectria zelandiaenovae	New Zealand	bark of ?*Agathis australis*	CBS 100979	AF358229
Clonostachys agrawalii	India	decomposing buffalo horn from animal house floor sweepings	CBS 533.81	AF358241
Clonostachys rhizophaga	Switzerland	culture contaminant	CBS 361.77	AF358228
Clonostachys rogersoniana	Brazil	soil	CBS 582.89	AF210691
Clonostachys rosea	-	-	BAFC1646	KF765504
Clonostachys rosea f. catenulata	-	-	-	HQ596905
Clonostachys rosea f. rosea	-	-	T64D	HM052816
Clonostachys solani f. *solani*	Netherlands	tuber of *Solanum tuberosum*	CBS 228.74	AF358243
Clonostachys indicus	India	twig of *Ficus virens*	**IBP 2**	**KT291441**

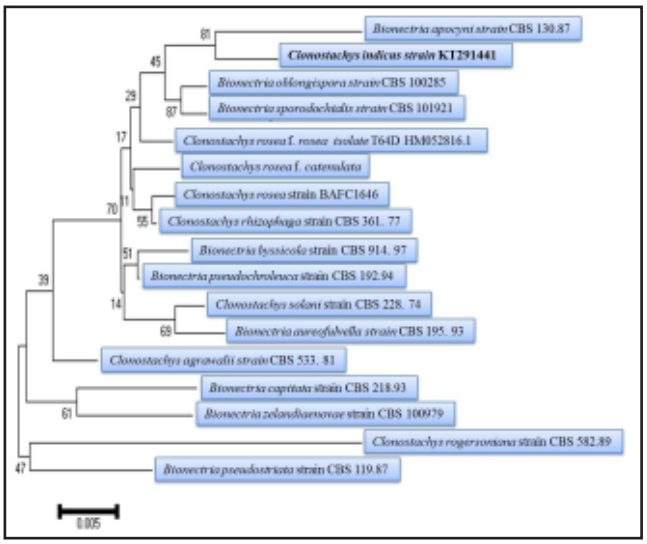

Fig. 5 Neighbour-Joining tree of *Clonostachys indicus* based on ITS sequence.

DISCUSSION

The genus *Clonostachys* was established by Corda (1839) with type species *C. araucaria*. The genus is characterized by penicillate condiophores and imbricate conidia. Later on several species have been described and transferred from other genera like *Gliocladium, Penicillium* and *Verticillium* into *Clonostachys* by different authors (Link, 1816; Hawksworth and Punithalingam 1975; Domsch *et al*., 1980; Gams, 1984; Samuels, 1988; Schroers *et al*., 1999). The anamorphs of *Bionectria* are classified into the genus *Clonostachys* (Schroers, 2001), which are characterized by penicillate and dimorphic conidiophores in many cases. The primary conidiophores are mononematous: verticillium like or penicillate like forming mucoid conidial masses (Bainer, 1905, 1907). The secondary conidiophores generally form imbricate conidia collapsing to form slimy conidial masses (mainly in sporodochia). Domsch *et al*. (1980) observed various colours of conidial masses such as white, pale orange or green. *Clonostachys indicus* is morphologically more similar to *C. byssicola* in having similar primary and secondary conidiophore but differs from it in having smaller primary phialides [(18) 20.9-23.7-25.9 (35.9) µm long, (2.1) 2.4-2.8-3.2 (3.9) µm wide at base in *C. indicus* and (12.4) 22.6-26.2-29.2(48) µm long, (1.4) 1.8-2-2.2 (2.8) µm wide at base in *C. byssicola*] and longer secondary phialides [(10) 14.7-16.4-17.8 (24.8) µm long, (1.9) 2.2-2.4-2.5 (3.3) µm wide at base in *C. indicus* and (7.6) 10.8-13.8-15.4 (27.8) µm long, (1.4) 1.8-2-2.2 (2.8) µm wide at base in *C. byssicola*]. Moreover, the primary conidia are larger i.e. (4.2) 5-5.6-6.3 (7.4) × (2.1) 2.3-2.7-2.9 (3.6) µm in *C. indicus* as compared to *C. byssicola* i.e. (3.2) 4.4-5.2-5.8 (10.8) × (1.8) 2.4-2.6-2.8 (4) µm *Clonostachys indicus* unlike *C. byssicola* does not form sporodochia in culture conditions. It differs significantly from anamorphs of subgenus *Astromata, Epiphloea* and *Myrothecium* in possessing dimorphic conidiophores (monomorphic conidiophores in later anamorphs) and from anamorphs of subgenus *Zebrinella* in lacking setae (prominent in anamorphs of subgenus *Zebrinella*). It differs significantly from species under subgenus *Bionectria* with respect to morphological details given in **Table 2** (Schroers, 2001).

Table 2 Comparative account of *Clonostachys* spp. (after Schroers, 2001).

Species	Primary Conidiophore			Secondary Conidiophore			Conidia (µm)
	Stipe (µm)	Penicillus (µm)	Phialide (µm)	Stipe (µm)	Penicillus (µm)	Phialide (µm)	
C. agrawalii	10–60 long or more, 4 wide at the base	-	18.8–32–42 long, 2.2–2.6–3.0 wide at base	-	-	7–18.2 long, 2.2–3 wide at base	(3.8–) 4.2–4.6–4.8 (–5.8) × (2.2–) 2.4–2.6–2.9 (–3)
C. aureofulvella	30–190 long, 3–5 wide at the base	30–100 high	(10.6–) 16.4–20–22.4 (–33.4) long, (1.2–) 1.8–2–2.2 (–3.2) wide at base	-	-	(8.4–) 11.4–13.4–15.2 (–21.2) long, (1.6–) 2–2.4–2.6 (–3) wide at base	(3.6–) 4.8–5.8–7 (–9) × (1.8–) 2.4–2.8–3.2 (–3.8)
C. byssicola	10–100 long, 3–5 wide at base	-	(12.4–) 22.6–26.2–29.2 (–48) long, (1.4–) 1.8–2–2.2 (–2.8) wide at base	-	-	(7.6–) 10.8–13.8–15.4 (–27.8) long, (1.4–) 1.8–2–2.2 (–2.8) wide at base	(3.2–) 4.4–5.2–5.8 (–10.8)× (1.8–) 2.4–2.6–2.8 (–4); from sporodochia (3.2–4.8×2.8–3.8)
C. capitata	100 long, 3.5 wide at base	-	(18.6–) 28.4–33.8–40.2 (–46.6) long, (1.4–) 2–2.2–2.4 (–2.6) wide at base	-	-	(8.8–) 11.6–13.6–15 (–24) long, (1.6–) 2–2.4–2.6 (–3) wide at base	(4.6–) 6–6.8–7.2 (–12.4) × (2.2–) 2.8–3.2–3.4 (–4.2)
C. compactiuscula	40–250 long, 2.5–4.5 wide at base	40–130 high	(17–) 22.2–28–31.2 (–56) long, (1.8–) 2–2.4–2.6 (–3) wide at base	100 long, to 7 wide at base	60 high	(5.2–) 8.4–8.8–11 (–17.2) long, (1.2–) 1.8–2–2.2 (–3) wide at base	(3.9–) 5.4–6.6–7.5 (–12.4) × (1.5–) 1.9–2.2–2.5 (–3.2)
C. kowhaii	-	-	-	40–100 long, to 5 wide at base	-	(13.6–) 21.8–26.2–28.8 (–42) long, (2–) 2.2–2.6–2.8 (–3.2) wide at base	(4.4–) 7.6–10.6–13.2(–18.2) × (2.8–) 3.4–4–4.6 (–5.8)
C. macrospora	inconspicuous, sometimes absent	-	-	-	-	(7–) 11.2–15.4–18.6 (–24.0) long, (2–) 2.2–2.4–2.6 (–3.2) wide at base	(6.0–) 11.2–13–15 (–20.2) × (3.2–) 4.6–5–5.4 (–7)
C. oblongispora	-	-	-	-	-	(11.8–) 13.2–18.2–20.4 (–38), (2.6–) 2.6–3–3.2 (–3.8) wide at base	(9–) 2.6–13.6–14 (–19.8) × (2.6–) 3.2–3.6–3.8 (–4.2)
C. pseudochroleuca	50–180 long, 2–5.5 wide at the base	40–90 high	(13.6–) 17.4–20.8–22.8 (–34) long, (1.3–) 1.6–1.8–2 (–2.4) wide at base	-	-	(6.2–) 10.2–11.4–12.6 (–17.6) long, (1.3–) 1.8–1.9–2 (–2.2) wide at base	(3.2–) 4–4.4–4.6 (–6.4) × (1.2–) 2.2–2.2–2.4 (–3)
C. pseudostriata	50–300 long, 3.5–6 wide at base	25–80 high	(11.6–) 16.2–22.8–26.8 (–44.2) long, (1.6–)1.8–2.2–2.4 (–3.2) wide at base	-	-	(7–) 12.2–15.8–18.8 (–30.4) long,(1.8–) 2.6–2.8–3 (–3.2) wide at base	(3.6–) 5–5.6–6.2 (–8) × (2–) 2.6–3–3.2 (–3.8); from primary conidiophores 13 × 4
C. rhizophaga	(10–) 40–100 long, 2.5–5 wide at base	30–100 high	(15.6–) 22–28.4–34.2 (–48.2) long, (2.2–) 2.6–2.6–2.6 (–3.2) wide at base	-	-	(5.8–) 12.4–14.6–17.2 (–25.2) long, (2.2–) 2.6–2.6–2.6 (–3.2) wide at base	(4.8–) 5.8–6.4–7 (–9) × (2.4–) 2.6–3–3.2 (–4.2)
C. rogersoniana	60–200 long, 3–5 wide at base	40–150 high	(14.9–) 20.8–23.6–26.8 (–38) long, (2.2–) 2.4–2.6–2.6 (–3.2) wide at base	70–160 long, 5–7.5 wide at base	50–100 and higher, 100 diameter at widest point	(10.2–) 11.8–12.4–13.4 (–16.6) long, (1.6–) 2.4–2.6–2.8(–3.2) wide at base	(4.8–) 5.8–6.6–7.2 (–9.6) × (2.2–) 3–3.2–3.8 (–4.2)
C. rosea f. *catenulata*	-	-	(25–) 29–31–37 (–45) long, (1.6-) 2–2.2–2.4 (–3) wide at base	-	-	(8–) 10.4–12–14 (–18) long, (2–) 2.2–2.2–2.4 (–2.8) wide at base	(4–) 4.8–5–5.4 (–6) × (2.2–) 2.4–2.6–2.8 (–3)
C. rosea f. rosea	(25–)70–200 long, 3.5–5.5 wide at base	-	(16.6–) 22.8–27.8–31.2 (–46.6) long, (1.6–) 2.2–2.4–2.6 (–3.4) wide at base	60–110 long, 3.5–6.5 wide at base	30–60 high, 16–50 diameter at widest point	(5.6–) 10.6–12.4–14.4 (–19.6) long, (1.2–) 2.0–2.4–2.6 (–3.2) wide at base	(5.2–) 7.6–8.2–9.0 (–15.4) × (2.2–) 2.8–3.2–3.4 (–4.8); from secondary conidiophore (4.2–) 4.8–5.2–5.6 (–6.6) ×(2–) 2.4–2.8–3 (–3.4)
C. solani f. solani	60–240 long, 3–6.5 wide at the base	20–100 high	(11.2–) 15.6–19–20.6 (–38.6) long, (1.6–) 2–2.2–2.4 (–3) wide at base	-	50–100 high, 70–150 wide	(9–) 11.6–14–15.2 (–25) long, (1.8–) 2.2–2.4–2.6 (–3.4) wide at base	(5–) 6.4–7–7.4 (–10) long; from secondary conidiophores (3.8–) 4.4–4.8–5 (–6.8) × (2–) 2.4–2.6–2.8 (–3.8)
C. solani f. nigrovirens	-	-	(11–) 14–16.6–18.6(–22.8) long, (1.6–) 2–2.2–2.4 (–3) wide at base			(8–) 11.2–13.4–14.4 (–23.4) long, (2.4–) 2.6–2.8–3 (–3.2) wide	4.2–9 × 2.1–3.4; from secondary conidiophores (3.4–) 4.2–4.6–4.8 (–6.4) × (2.2–) 2.6–2.8–3 (–3.8)
C. sporodochialis	-	-	-	-	50–120 high, 50–100 wide	(13.4–) 18.8–21.6–24.6 (–35.4) long, (1.6–) 1.8–2–2.2 (–2.6) wide at base	(3.2–) 4.4–4.8–5.4 (–6.8) × (1.6–) 2.0– 2.2–2.2 (–2.6)
C. verrucispora	50 long, 3 wide at base	-	(15.6–) 19–26.2–33(–41.6) long, (1.8–) 2–2.2–2.4 (–2.8) wide at base	-	-	(4–) 10.4–13.6–16.6 (–22.6) long, (1.4–) 2.0–2.4–2.8 (–3.2) wide at base	(5.6–) 7.4–8.4–9.2 (–15.6) × (2.2–) 3–3.2–3.6 (–4.4)
C. zelandiaenovae	50 long, 4 wide at base	-	(21.2–) 27–32.6–34 (–46.6) long, (2.2–) 2.6–2.8–3.2 (–3.2) wide at base	-	-	(4.8–) 11.4–13.6–16 (–20.6) long, (1.6–) 2.2–2.4–2.6 (–3.4) wide at base	(4–) 5.2–6–6.4 (–13.2) × (2.4–) 2.8–3–3.2 (–4.2)
C. indicus	30.0–78.0 long, 2.1–3.6 wide at base	43.6-65.7 (–77.7) high	(18.0–) 20.9–23.7–25.9 (–35.9) long, (2.1–) 2.4–2.8–3.2 (–3.9) wide at base	Up to 78.5, 3.2 wide at base	(37.3–) 39.9–75.8 high	(10.0–) 14.7–16.4–17.8 (–24.8) long, (1.9–) 2.2–2.4–2.5 (–3.3) wide at base	(4.2–) 5.0–5.6–6.3 (–7.4) × (2.1–) 2.3–2.7–2.9 (–3.6) from primary conidiophore; (3.9–) 4.2–4.6–5.0 (–5.6) × (2.0–) 2.9–3.0–3.2 (–3.7) from secondary conidiophore

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