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Morphology and phylogeny of *Coniothyrium chiangmaiense*: a new record from Western Ghats, India

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This paper deals with an interesting fungus isolated from a soil sample collected from a high land forest of the Mahabaleshwar region in Maharashtra state, India. Being non-sporulating, morphological and cultural studies could not reveal the identity of the said fungus. Therefore, a detailed analysis of the sequence data of combined ITS, LSU, and SSU rDNA was done, and a combined phylogenetic tree was constructed, which confirmed the identity of the fungus as *Coniothyrium chiangmaiense* (*Coniothyriaceae*) with a strong bootstrap value (99.6). To our understanding present taxon is described and illustrated for the first time from India, supported by comprehensive morphological and molecular data.

Key words: Fungi, ITS, LSU, SSU rDNA, Taxonomy

INTRODUCTION

Literature reveals that initially, *Coniothyriaceae* was synonymized with *Leptosphaeriaceae* by taxonomists (Kirk *et al*., 2008; De Gruyter *et al*. 2009; Schoch *et al*., 2009; Aveskamp *et al*., 2010), and taxonomic identity of genera like *Coniothyrium* Corda and *Paraconiothyrium* Verkley were misidentified with the genus *Phoma* Sacc. (Verkley *et al*. 2004; Damm *et al*. 2008; Woudenberg *et al*. 2009). Later on, De Gruyter and colleagues (2013) re-established the family *Coniothyriaceae* W.B. Cooke based on detailed phylogenetic analysis and further validated by several studies as a distinct family of *Pleosporales* (Hyde *et al*. 2013; Quae-dvlieg *et al*. 2013; Wijayawardene *et al*. 2016). The family *Coniothyriaceae* currently contains five genera, *Coniothyrium* Corda, *Neoconiothyrium* Crous, *Hazslinszkyomyces* Crous & R.K. Schumach, and *Ochrocladosporium* Crous & U. Braun and *Foliophoma* Crous (Crous & Groenewald 2017; Crous *et al*., 2017; Wijayawardene *et al*., 2018).

After establishing the genus *Coniothyrium* Corda, the taxonomy of its species has been in a state of flux and assumed to be the asexual state of the genera *Leptosphaeria* Ces. & De Not., *Mycosphaerella* Johanson, and *Massarina* Sac. Later on, many species of *Coniothyrium* were transferred to other genera, while some were upgraded to generic limit (Wijayawardene *et al*., 2014). *Coniothyrium* belongs to coelomycetous fungi that produce conidia from the percurrent proliferating conidiogenous cells enclosed in pycnidia. Pycnidia are unilocular, ostiolate, and immersed.

During our systematic study of fungal samples collected from a high land forest of the Mahabaleshwar region in Maharashtra state, India, we isolated the present taxon from a soil sample. Out of several isolates studied, one isolate was found to be non-sporulating, showing only vegetative structures, including chlamydospores formation. Due to limitations of morphological identity, this isolate was subjected to multigene sequencing and phylogenetic analysis. Overall, the study revealed that this taxon has a similarity (strong bootstrap value 99.6%) with *Coniothyrium chiangmaiense* Goonas., Thambugala & Hyde (2017). The same is illustrated and described here based on morphology and comprehensive molecular phylogenetic analyses, which our understanding turned out to be a new record from India.

MATERIALS AND METHODS

Collection of soil sample

The soil was sampled from the surface to 5 cm depth from Mahabaleshwar forests, Maharashtra,

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India (17.9307° N, 73.6477° E). The samples was then transferred to sterile zip-lock plastic bags and stored at 4ºC.

Isolation

Fungi from the collected soil sample were isolated using the serial dilution method (Waksman, 1916). Briefly, the stock solution was prepared by mixing 1 gm of sample in 10 ml sterile distilled water. Then dilutions from stock solution were made up to 10^{-7}. The 100 µl. of stock solution from each higher dilution (10^{-3}, 10^{-5}, 10^{-7}) was used as inoculum on three different culture media, Martin's Rose Bengal medium (MRB), Malt Extract Agar (MEA), and Potato Dextrose Agar (PDA). After 3-5 days of incubation at 25ºC, plates were observed for the appearance of fungal colonies. Colonies exhibiting different morphological characters were selected, purified, and transferred onto PDA slants for further study. Three sets of each pure culture were prepared. The One set was subjected to identifications, the second set was reserved for laboratory uses, and the third set was meant to deposit and accession in the repository. Sporulating cultures were identified based on morphology using standard literature, while non-sporulating/sterile culture was subjected to molecular identification.

Morphology of culture

The pure culture of MBS–4 was inoculated on different media such as potato dextrose agar (PDA), potato carrot agar (PCA), malt extract agar (MEA), Sabouraud dextrose agar (SDA), oatmeal agar (OMA), Czapek dox agar (CZA), cornmeal agar (CMA), and V8 juice agar to study and record morphological characters. After 14 days of incubation at 25ºC, cultural characteristics were studied. Color codes from the Methuen handbook of color were used for recording the color of the colony (Kornerup and Wanscher, 1978). Additionally, the grass leaf technique (Srinivasan *et al*. 1971) was also tried to induce the sporulation.

A pure culture of (MBS–4) was deposited in NFCCI (National Fungal Culture Collection of India), Pune (NFCCI 4773), and voucher culture was submitted in Ajrekar Mycological Herbarium (AMH), Pune (AMH 10210).

DNA extraction

Extraction of fungal genomic DNA was done as per the protocol given by Aamir *et al*. (2015). Scrapped fungal mycelia from the seven days old PDA culture plate was taken into a DNA extraction tube containing ceramic pestle and glass beads. 1 ml of lysis buffer containing 100 mM Tris–HCl of pH 8.0, 50 mM EDTA, and 3% SDS was then added into the tube and subjected to homogenization at 6 M/S for 60 seconds a FastPrep ® – 24 tissue homogenizer (MP Biomedicals, USA). The resultant lysed and homogenous mixture was subjected to centrifugation at 13,000 rpm for 10 minutes. The supernatant containing genomic DNA was collected into a new sterile microcentrifuge tube. The purification of genomic DNA was done by adding an equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) followed by centrifugation at 13,000 rpm for 10 minutes. The upper aqueous part was then collected into a 1.5 ml microcentrifuge tube, and precipitation of the DNA was done using pre-chilled Isopropanol. Pelleted DNA was then washed with 70% ethanol by centrifugation (12,000 rpm for 5 minutes). The resultant DNA pellet was air-dried and dissolved in TE buffer (1x).

Polymerase chain reaction

Internal transcribed spacer (ITS), large subunit (LSU), and small subunit (SSU) regions of ribosomal DNA (rDNA) were amplified from the genomic DNA by Polymerase Chain Reaction (PCR) using Applied Biosystems ProFlex PCR machine (Table 1). Primer pairs used to amplify ITS, LSU, and SSU rDNA include ITS4 & ITS5, LROR & LR7, and PNS–1 & NS–41, respectively (White *et al*. 1990; Vilgalys and Hester 1990; Hibbett 1996).

Using FavorPrepTM PCR Purification Kit (Favorgen Biotech Corporation, Taiwan), amplified PCR products were purified and then used for sequencing by an ABI Avant 3100 automated DNA sequencer and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Finally, obtained sequences of ITS, LSU and SSU region of rDNA were edited manually and deposited in the NCBI nucleotide sequence database (GenBank Acc. No. ITS–MZ188969, LSU– MZ189001, and SSU– MZ189085).

Sequence alignment and phylogenetic analysis

Sequence alignment of ITS, LSU, and SSU rDNA sequences of the present taxon were subjected to MegaBlast searches. A combined dataset of ITS,

LSU, and SSU rDNA sequences comprising genetically related species of the genus *Coniothyrium, Neoconiothyrium,* and *Alternaria* was prepared. A phylogram has been constructed based on ITS, LSU, and SSU rDNA sequence data of a total of 21 genetically related isolates (including the present taxon) (Table 2). *Phaeosphaeria oryzae* CBS 110110 was used as an outgroup.

With the help of the MUSCLE algorithm implemented in MEGA 7 (Kumar *et al*., 2016), multiple sequence alignment has been carried out, and ends of the aligned sequences were trimmed manually. Phylogenetic analysis was performed in IQ–TREE multicore version 1.6.11 (Nguyen *et al*., 2015) by the Maximum–Likelihood method using the best suitable model (SYM + R2 model). One–thousand bootstrap replicates were analyzed to get bootstrap values, and the values above 50% were represented on nodes in the tree.

RESULTS and DISCUSSION

Taxonomy

Coniothyrium chiangmaiense Goonas., Thambugala & K.D. Hyde, Mycosphere 8(4): 697–796 (2017) (Figs. 1–2)

Holotype: THAILAND, Chiang Mai Province, Mae Taeng, Mushroom Research Center, MFLU 16–2854, from the dead stem of grass litter collected by Ishani D. Goonasekara.

Colour codes as described in Materials and Methods were followed. It was isolated from the soil.

Asexual morph: Hyphae septate to aseptate, hyaline to olivaceous brown, 1.52–5.69 μm wide, simple to branched, thin, and smooth-walled, consisting of solitary or series of inflated cells (chlamydospores), and hyphae showed anastomosis and frequent spiral twisting. Hyphal cells, subglobose to cylindrical, 10.49–37.09 × 2.21–5.68 μm ($\overline{x}$ = 17.58 × 3.92 μm n = 30). ***Chlamydospores*** hyaline as well as light to dark olivaceous brown, frequently observed, terminal to intercalary, solitary, in branched chains and bunches, the wall thickened and darkened, variable in shape, globose to subglobose, simple to muriform, with lateral budding, 9.22–94.24 × 7.02–31.81 μm ($\overline{x}$ = 31.66 × 17.03μm, n= 30) **Sexual morph:** Undetermined.

Culture characteristics: Colonies growing on PDA reaching 44–46 mm diameter after two weeks at 25°C; colonies from above white (1A1) at the center and orange white (5A2) at the periphery, irregular, raised, with slightly cottony aerial mycelium, entire with irregular margin; colony from below, brownish-orange (5C3) at center and olive (3F8) at the periphery, sulcate, with diffusible yellow (2A4) pigment.

Colonies were growing on PCA, reaching 55–56 mm diameter after two weeks at 25°C; colonies from above greenish grey (28D2) to dull green (29E3), circular, raised, with cottony aerial mycelium, entire with regular and smooth margin; colony from below, center dark green (30F5) with greenish-yellow (1B8) peripheral margin.

Colonies on MEA reaching 50–55 mm diameter after two weeks at 25°C; colonies from above greenish grey (1B2) at center and olive (1E3) at the periphery, circular, raised, with cottony aerial mycelium, irregular with filamentous margin; colony from below, olive (3F8).

Colonies on SDA reaching 40–45 mm diameter after two weeks at 25°C; colonies from above orange white (5A2), circular, raised, with cottony aerial mycelium, irregular and smooth margin; colony from below greyish yellow (4C5), sulcate.

Colonies on OMA reaching 65 mm diameter after two weeks at 25°C; colonies from above grey (1B1), circular, raised, with cottony aerial mycelium, entire with regular and smooth margin; colony from below dark green (30F8) at the center and greyish brown (6F3) at the periphery.

Colonies on CZA reaching 55 mm diameter after two weeks at 25°C; colonies from above, yellowish-white (4A2) at the center, olive (3D3) at middle and olive-brown (4F8) at the periphery, circular, umbonate, irregular with filamentous margin; colony from below, violet grey (18E2) at the center, dark violet (18F3) at middle and olive (3F8) at the periphery.

Colonies on CMA were reaching to 64–65 mm diameter after two weeks at 25°C; colonies from above, white (1A1) at the center and greenish-grey (1B2) at the periphery, circular, umbonate, with cottony aerial mycelium, irregular with smooth margin; colony from below, greenish-grey (1B2).

Colonies on V8 juice agar reaching 46–48 mm di

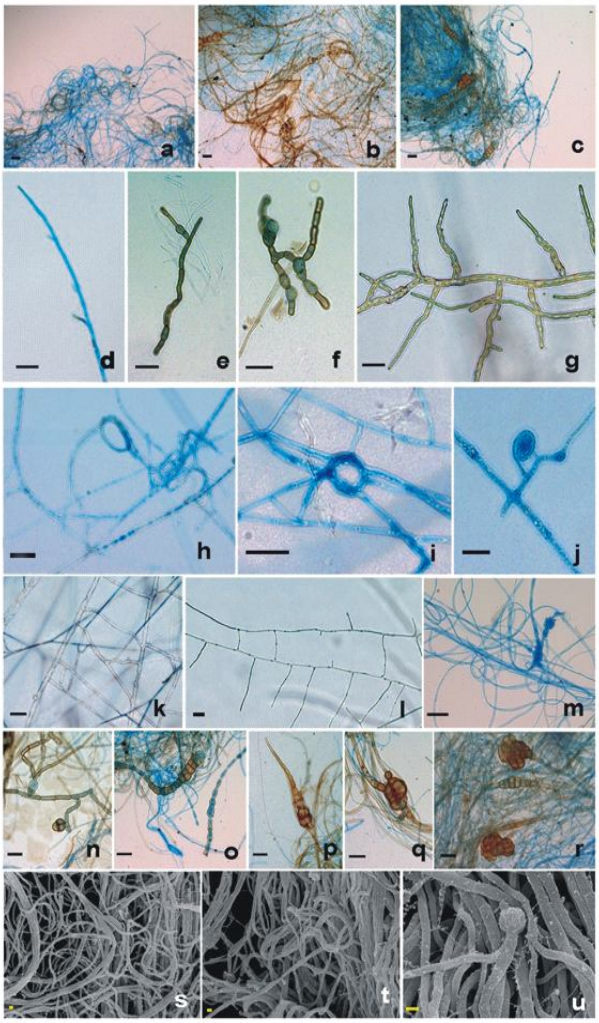

Fig.1. *Coniothyrium chiangmaiense* NFCCI 4773: **a.** Hyaline mycelia *in-vitro* fresh culture, **b–c.** Mycelia from *in-vitro* old culture, **d.** Hyaline, septate, thin-walled hypha, **e–g.** Branched, septate hyphae, **h–j.** Hyphae showing frequent spiral coiling or twisting, **k–l.** Mycelia showing frequent anastomosis, **m.** Immature hyaline, chlamydospores, **n.** Terminal and lateral solitary muriform chlamydospores **o–p.** Intercalary muriform chlamydospores in branched chains, **q.–r**. Dark olivaceous brown, muriform chlamydospores, **s–t.** SEM image of mycelia, **u.** SEM image of hyphae showing chlamydospores. Scale bars **a–r** = 20μm s-u=2 μm

ameter after two weeks at 25°C; colonies from above, white (1A1) at the center, brownish-orange (5C3) at middle and orange white (5A2) at the periphery, circular, slightly raised, with cottony aerial mycelium, regular with smooth margin; colony from below, brownish-orange (5C3) at the center and greyish orange (5C3) at the periphery.

Material examined: INDIA, Maharashtra, Mahabaleshwar (17.9307° N, 73.6477° E), from the soil, 25 November 2017, A.C. Lagashetti, living culture, NFCCI 4773, voucher specimen AMH 10210.

GenBank numbers: ITS = MZ188969, LSU = MZ189001, SSU = MZ189085

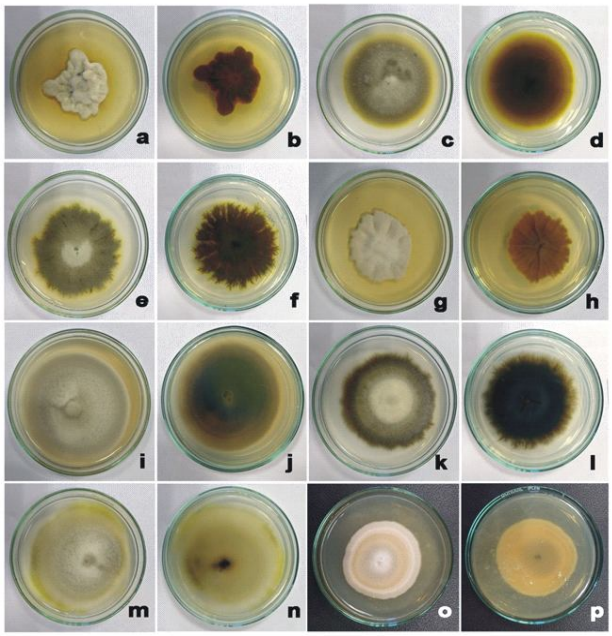

Fig.2. Colony morphology of *Coniothyrium chiangmaiense* (NFCCI 4773) after 14 days on different media: **a–b.** on PDA (front & reverse view), **c–d.** on PCA (front & reverse view), **e–f.** on MEA (front & reverse view), **g–h.** on SDA (front & reverse view), **i–j.** on OMA (front & reverse view), **k–l.** on CZA (front & reverse view), **m–n.** on CMA (front & reverse view), **o–p.** on V8–juice agar (front & reverse view).

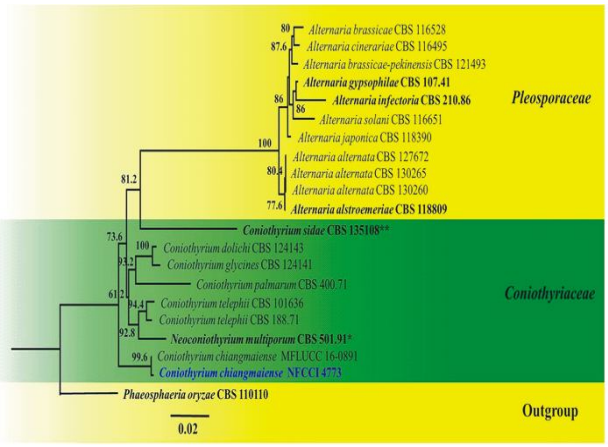

Fig.3. Phylogram generated by Maximum likelihood method for *Coniothyrium chiangmaiense* (NFCCI 4773) using combined ITS, LSU, and SSU rDNA sequence data**.** *Phaeosphaeria oryzae* CBS 110110 was used as an outgroup. The type strains are in bold and present taxon in blue.
* *Formerly* known as *Coniothyrium multiporum*
** *Formerly* known as *Setophaeosphaeria sidae*

Phylogenetic analyses

Mega blast analysis of ITS sequence of present taxon shows 99% identity (534/535) with *Coniothyrium chiangmaiense* Goonas., Thambugala & K.D. Hyde (MFLUCC 16–0891) (Thambugala

Table 1: The PCR conditions used for amplification of ITS, LSU, and SSU rDNA

PCR	PCR conditions Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles
ITS	94ºC for 5 min.	94ºC for 1 min.	48ºC for 30 sec.	72ºC for 1 min.	72ºC for 7 min.	30
LSU	94ºC for 5 min.	94ºC for 1 min.	52ºC for 50 sec.	72ºC for 1.2 min.	72ºC for 7 min.	30
SSU	94ºC for 5 min.	94ºC for 1 min.	50ºC for 30 sec.	72ºC for 1.3 min.	72ºC for 10 min.	35

Table 2: Taxa used in the phylogenetic analyses and their GenBank accession numbers

Cultures	Strain	GenBank Accession Numbers		
		ITS	LSU	SSU
Coniothyrium chiangmaiense	NFCCI 4773	MZ188969	MZ189001	MZ189085
Alternaria alstroemeriae	CBS 118809	MH863036	MH874589	NG_063029
Alternaria alternata	CBS 130265	KP124391	KP124545	KP125015
Alternaria alternata	CBS 130260	KP124387	KP124541	KP125011
Alternaria alternata	CBS 127672	KP124382	KP124536	KP125006
Alternaria brassicae	CBS 116528	KC584185	KC584258	KC584514
Alternaria brassicae–pekinensis	CBS 121493	KC584244	KC584353	KC584611
Alternaria cinerariae	CBS 116495	KC584190	KC584265	KC584521
Alternaria gypsophilae	CBS 107.41	MH856084	MH867575	NG_062884
Alternaria infectoria	CBS 210.86	NR_131263	NG_055740	NG_062886
Alternaria japonica	CBS 118390	KC584201	KC584281	KC584537
Alternaria solani	CBS 116651	KC584217	KC584306	KC584562
Coniothyrium chiangmaiense	MFLUCC 16–0891	KY568987	KY550384	KY550385
Coniothyrium dolichi	CBS 124143	JF740182	GQ387610	GQ387549
Coniothyrium glycines	CBS 124141	KF251211	GQ387598	GQ387537
Coniothyrium palmarum	CBS 400.71	AY720708	JX681084	AY642513
Coniothyrium sidae **	CBS 135108	NR_156261	KF251653	–
Coniothyrium telephii	CBS 188.71	MH860059	GQ387599	GQ387538
Coniothyrium telephii	CBS 101636	JF740190	GQ387601	GQ387540
*Neoconiothyrium multiporum**	CBS 501.91	JF740186	GU238109	GU238225
Phaeosphaeria oryzae	CBS 110110	MH862850	GQ387591	NG_061080

***** Formerly known as *Coniothyrium multiporum*
** Formerly known as *Setophaeosphaeria sidae*

et al. 2017) and similar 99% identity (773/776) for LSU analysis. Phylogram constructed based on a combined ITS, LSU, and SSU sequence dataset of 21 taxa shows that the present Indian taxon (NFCCI 4773) is clustered with species of *Coniothyrium*, i.e., *C. chiangmaiense* Goonas., Thambugala & K.D. Hyde (MFLUCC 16–0891) with a strong bootstrap value (99.6%) (Fig. 3). This has been reported as a saprophyte producing sexual morphs with ascomata and ascospores isolated from the dead stem of grass litter from Thailand. Therefore, based on similarity, Indian isolate (NFCCI 4773; AMH 10210) is assigned to *Coniothyrium chiangmaiense* Goonas., Thambugala & Hyde, being reported for the first time from India.

The present Indian isolate was isolated from the soil, and both sexual and asexual morphs were absent. The present isolate was found to be non–sporulating (absence of conidia), showing only sterile mycelia possessing terminal and intercalary chlamydospores. Attempt to induce sporulation through the Grass leaf technique (Srinivasan *et al*. 1971) was unsuccessful. Therefore, the scope for its morpho-taxonomic comparison with the allied taxa was limited. In this scenario, only genetic analy

sis of the present taxon and their comparison with related taxa in the genus would be convincing. This is probably why most recent taxonomic studies suggested applying molecular data to resolve the taxonomic ambiguity of the genus *Coniothyrium*. As per the Index Fungorum (2021) genus, *Coniothyrium* contains approximately 900 records; but the accuracy of most of the taxa is ambiguous and questioned because most of them are described based on host and lacking cultural studies and molecular data (Verkley *et al*. 2004).

Thus, for confirming the correct generic placement of Indian isolate, we have performed the phylogenetic analysis based on combined ITS, LSU, and SSU rDNA sequence data. The phylogenetic analysis has confirmed the position of the present taxon in the family *Coniothyriaceae*. A phylogenetic tree has shown that the Indian isolate was clustered with *Coniothyrium chiangmaiense* with a strongly supported bootstrap value (99.6 bootstraps). This confirms the identity of the present taxon as *C. chiangmaiense.* To our knowledge, the present Indian specimen is reported for the first time from India; therefore, it was proposed as a new record from India.

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