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## *Cylindromonium dirinariae* sp. nov. (Ascomycota, Hypocreales), a new nectrioid lichenicolous species on *Dirinaria applanata* in Japan

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**Abstract:** A nectrioid fungus forming a pinkish colony with mainly solitary phialides producing ellipsoid, aseptate conidia in mucoid packets was isolated from *Dirinaria applanata*. Our taxonomic study based on morphology and phylogenetic analysis using ITS rDNA sequences revealed that the isolates represented a member of the genus *Cylindromonium*. Based on further morphological examination, nucleotide sequence comparison, and phylogenetic analysis based on LSU rDNA, *tef1*, and *rpb2* in addition to the phylogenetic analysis using the ITS rDNA sequences, the fungus from *Dirinaria* represents a new species, which is described here as *Cylindromonium dirinariae* sp. nov. Furthermore, inoculation experiments revealed that this species can also produce perithecia when inoculated on the host lichen in laboratory environments.

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### INTRODUCTION

Lichenicolous fungi is a term used to circumscribe fungi that grow on lichens. They can interact with their lichen hosts as saprophytes, parasites and commensalistic parasymbionts. Lichenicolous fungi usually establish a symbiotic relationship with a single species or genus of lichens, while some species have a wide host range (Diederich *et al.* 2018). Approximately 2 300 species of lichenicolous fungi have been described on lichens globally and they are classified into *ca.* 400 genera, *ca.* 100 families, 55 orders and 10 classes. Ninety-six percent of the total number of lichenicolous fungi are ascomycetes and four percent of the fungi are basidiomycetes (Diederich *et al.* 2018). About 166 lichenicolous fungi have been reported from Japan to date (Frisch *et al.* 2018, Ohmura & Kashiwadani 2018, Tadome *et al.* 2018, Zhurbenko & Ohmura 2018a, b, Zhurbenko *et al.* 2018, Zhurbenko & Ohmura 2019, 2020, Frisch *et al.* 2020, Tadome & Ohmura 2021, 2022, Tadome *et al.* 2022). In spite of these reports, there has been relatively little research conducted on this fungal group in Japan. Therefore, many species remain to be discovered and described.

The genus *Cylindromonium*, with the type species *C. eugeniicola*, was segregated from *Acremonium* based on analyses of ITS and LSU rDNA sequence data (Summerbell *et al.* 2011, Crous *et al.* 2019b). *Cylindromonium* species are known to be lichenicolous, mycophilic, or saprophytic (Gams 1971, Crous *et al.* 2019b, 2020, 2021). *Cylindromonium* was established as a

genus to accommodate acremonium-like taxa with unbranched, hyaline, phialidic conidiophores, and cylindrical 1-septate conidia (Crous *et al.* 2019b). A total of five asexual species have been assigned to *Cylindromonium*, for which no sexual morph has thus far been reported (Crous *et al.* 2019b, 2020, 2021). During our research on the diversity of lichenicolous fungi in Japan, a fungus colonising the *Physciaceae* lichen *Dirinaria applanata* was found. The purpose of this study is to describe the morphological, physiological, and ecological features of this species, clarify the link to its sexual morph, and discuss its taxonomic placement.

### MATERIALS AND METHODS

#### Collection materials

Field investigations were performed from September 2020 to March 2021 in Tsukuba city, Ibaraki prefecture, Japan. Specimens of the fungus growing on the lichen host *D. applanata* were found on the bark of *Zelkova serrata*. A voucher specimen was deposited in the National Museum of Nature and Science (TNS), Tsukuba, Japan. A living ex-type culture was deposited in the Biological Resource Center of the National Institute for Technology and Evaluation (NBRC). *Cylindromonium lichenicola* strains (CBS 188.70 and CBS 415.70A) were also examined for comparison purposes.

## Morphological observations

Samples were observed using a dissecting microscope [M165 C (Leica, Wetzlar, Germany)] and a differential interference contrast compound microscope [BX53 (Olympus, Tokyo, Japan)]. Anatomical examination was performed using hand-cut sections mounted in a drop of water or clear lactophenol. Photographs were taken using a microscope digital camera [Flexacam C3 (Leica, Wetzlar, Germany) or DP23 (Olympus, Tokyo, Japan)]. Dimensions of ascospores, conidia, conidial mass, phialide and hyphal width are given as (minimum–) range of mean  $\pm$  standard deviation (–maximum) ( $n$  = number of measurements). Chemical reactions of the perithecia were observed by using 10 % KOH. To determine if there is a significant difference between each dimension of the present fungus and *C. lichenicola*, the t-test was performed using Microsoft Excel.

## Isolation of fungal cultures

Fungal cultures were isolated from freshly collected material. Mycelium or single ascospores were picked up using a flamed needle and plated on 1 % malt extract agar (MEA). To confirm differences in colony characteristics on each agar medium, mycelial plugs were subcultured on 1 % MEA, potato dextrose agar (PDA) (Nissui Pharmaceutical, Tokyo, Japan), oatmeal agar (OA) (Becton Dickinson and Co, New Jersey, USA), Sabouraud maltose agar (SMA) (Thermo Fisher Scientific, Massachusetts, USA), malt yeast extract agar (MYA) (Ahmadjian 1961) and Sabouraud glucose agar (SGA) (Stocker-Wörgötter 2002), confirming the recipes of these media according to Crous *et al.* (2019a). Colour of colonies were determined based on Kornerup & Wanscher (1978).

## DNA extraction, PCR amplification and sequencing

Perithecia were sampled from specimen TNS-L-131533, mycelium from specimen TNS-L-131534, and mycelia from a culture derived from specimen TNS-L-131535. For DNA extraction, fungal tissues were suspended in 20  $\mu$ L of DNA extraction buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01 % sodium dodecyl sulfate (SDS), 0.01 % Proteinase K], incubated at 37 °C for 60 min, and denatured 90 °C for 10 min; 30  $\mu$ L of sterile distilled water (SDW) added to the tubes, and stored in a freezer at -20 °C.

Partial sequences of the nuc rDNA ITS1-5.8S-ITS2 (ITS), large subunit (LSU) nuc rDNA regions, elongation factor 1-alpha (*tef1*), and RNA polymerase II second largest subunit (*rpb2*) were amplified as these regions are frequently used for phylogenetic analyses of *Nectriaceae* (Lombard *et al.* 2015, Crous *et al.* 2019b, 2020, 2021). The ITS region was amplified using the primers ITS5 and ITS4 (White *et al.* 1990), LSU rDNA using primers LIC24 (Miadlikowska & Lutzoni 2000) and LR7 (Vilgalys & Hester 1990) or LROR (Rehner & Samuels 1994) and LR6 (Vilgalys & Hester 1990), *tef1* using EF1-983F and EF1-1567R (Rehner & Buckley 2005), and *rpb2* using RPB2-5F2 and RPB2-7cR (O'Donnell *et al.* 2007). PCR was performed in a 15  $\mu$ L reaction volume containing 1  $\mu$ L DNA template, 7.5  $\mu$ L GenRED PCR Mix Plus (Nippon Gene, Tokyo, Japan), 1.5  $\mu$ L each primer (2 pmol/ $\mu$ L), and 3.5  $\mu$ L distilled water. The PCR was performed in a TaKaRa PCR Thermal Cycler Dice® Touch (TaKaRa, Shiga, Japan) as follows for the ITS region; 5 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 53 °C, 1 min at 72 °C, and a final step of 8 min at 72 °C. PCR conditions

for LSU, *tef1* and *rpb2* were set according to Frisch *et al.* (2020), Rehner & Buckley (2005) and O'Donnell *et al.* (2007), respectively.

PCR products were checked by electrophoresis on a 1.5 % agarose gel stained with Midori Green Direct DNA Stain (Nippon Genetics, Tokyo, Japan) and visualised using WSE-5200 Printgraph 2 M (ATTO, Tokyo, Japan). The PCR products were purified using a FastGene™ Gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan) and ExoSAP-IT™ (Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer's instructions.

Sequences were obtained via a DNA sequencing service using Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific, Massachusetts, USA) (Eurofins Genomics, Tokyo, Japan). Data and accession number of the voucher specimen, and the obtained sequences from the International Nucleotide Sequence Database (INSD) are shown in Table 1.

## Phylogenetic analysis and comparison of sequence data

Both newly generated ITS sequences and reference sequences were used in the phylogenetic analysis. Sequence data of loci other than ITS do not exist for some isolates of the genus *Cylindromonium*. Therefore, only ITS, LSU and *rpb2* of four *Cylindromonium* species were used for the phylogenetic analysis (Table 1). ITS sequence data of species with relatively high identity (> 85 %) in the BLAST search and other hypocrealean fungi in the *Nectriaceae* were included in the analysis to infer the taxonomic position of the targeted fungi (Table 1). In these analyses, *Stachybotrys chartarum* (KM231858) was chosen as the outgroup (Lombard *et al.* 2015). Sequences of each locus (ITS, LSU, *tef1* and *rpb2*) were compared with those of *C. lichenicola*. All sequences analysed in this study were deposited in the DNA Data Bank of Japan (DDBJ), a member of International Nucleotide Sequence Database Collaboration (INSDC).

Assembling forward and reverse strands of the sequenced loci were carried out with MUSCLE v. 3.6 (Edgar 2004) in MEGA v. 7 (Kumar *et al.* 2016) to obtain consensus sequences. DNA sequences were aligned using the online version MAFFT v. 7 (Katoh *et al.* 2019) (<https://mafft.cbrc.jp/alignment/server/>) with default settings. MEGA v. 7 (Kumar *et al.* 2016) was used to truncate sequences up to the determined edge of the dataset.

Phylogenetic analyses were performed with Maximum likelihood (ML) using an online version W-IQ-Tree v. 1.6.12 (Trifinopoulos *et al.* 2016) (<http://iqtree.cibiv.univie.ac.at/>). All characters were equally weighted, and gaps were treated as missing data. The ML analysis for the ITS region alignment using the TIM2+F+I+G4 model and for a combined alignment of the three loci, ITS, LSU, *rpb2* using the TN93+G (for ITS and LSU) and TN93+I (for *rpb2*) were performed with 1 000 bootstrap replicates. FigTree v. 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>) and MEGA X were used for plotting the phylogenetic trees. Sequence alignments were deposited in TreeBASE (<http://purl.org/phylo/treebase/phylovs/study/TB2:S30024>).

## Inoculation experiments

Symptomless thalli of *D. applanata*, the host lichen of *C. dirinariae*, and those of the non-host lichen *Parmotrema tinctorum* were collected in Tsukuba city, Ibaraki prefecture, Japan and confirmed as non-infected via microscopy. Following this step, the lichen surface was cleaned using an ultrasonic cleaner with 0.005 % Aerosol® OT (a surface-active agent) for 1

**Table 1.** Sources of DNA sequence data used in phylogenetic analyses and comparison of sequence data.

Species	Strain numbers	Host/Substrate	Collection sites	ITS Accession No.	LSU Accession No.	tef1 Accession No.	rpb2 Accession No.	References
<i>Calostilbe striispora</i>	CBS 133491	<i>Erythrina glauca</i>	Trinidad and Tobago	KM231789	–	–	–	Lombard <i>et al.</i> (2015)
<i>Ciliopodium brevipes</i>	CBS 691.83	<i>Fagus sylvatica</i>	Netherlands	KM231856	–	–	–	Lombard <i>et al.</i> (2015)
<i>Ciliopodium hyalinum</i>	CBS 106.13	Soil	Switzerland	KM231857	–	–	–	Lombard <i>et al.</i> (2015)
<i>Cylindrium aeruginosum</i>	CBS 693.83	<i>Fagus sylvatica</i>	Netherlands	KM231854	–	–	–	Lombard <i>et al.</i> (2015)
<i>Cylindrium elongatum</i>	CBS 685.83A	<i>Fagus</i> sp.	Netherlands	KM231852	–	–	–	Lombard <i>et al.</i> (2015)
<i>Cylindromonium alloxyl</i>	CPC 38159	<i>Meliola</i> on leaves of <i>Alloxylon pinnatum</i>	Austria	MW175339	MW175379	–	MW173114	Crous <i>et al.</i> (2020)
<i>Cylindromonium eugeniicola</i>	CPC 37170	Leaf litter of <i>Eugenia capensis</i>	South Africa	NR166338	–	–	–	Crous <i>et al.</i> (2019b)
<i>Cylindromonium everniae</i>	CBS 148255	<i>Evernia prunasti</i>	Netherlands	NR175231	OK664736	–	NR175231	Crous <i>et al.</i> (2021)
<i>Cylindromonium dirinariae</i> sp. nov.	TNS–L–131533	<i>Dirinaria appplanata</i>	Japan	LC731273	LC731274	LC731275	LC744391	This study
	TNS–L–131534	<i>Dirinaria appplanata</i>	Japan	LC731276	LC744402	LC744396	LC744390	This study
	TNS–L–131535	<i>Dirinaria appplanata</i>	Japan	LC731277	LC744401	LC744395	LC744392	This study
<i>Cylindromonium lichenicola</i>	CBS 415.70A	Aerial algae	Netherlands	MH859774	LC744400	LC744397	LC744394	Vu <i>et al.</i> (2019); This study
	CBS 303.70	<i>Alnus</i> sp.	Germany	MH859675	–	–	–	Vu <i>et al.</i> (2019)
<i>Cylindromonium rhabdosporum</i>	CBS 188.70	Apothecia of lichen	Germany	MH859549	LC744399	LC744398	LC744393	Vu <i>et al.</i> (2019); This study
<i>Falcocladium multivesiculatum</i>	CBS 438.66	<i>Cladonia furcata</i>	Austria	MH858850	–	–	–	Vu <i>et al.</i> (2019)
	CBS 120386	Leaf litter	Brazil	JF831936	–	–	–	Rungjindamai <i>et al.</i> (unpublished)
<i>Falcocladium sphaeropedunculatum</i>	CBS 111292	Leaf litter	Brazil	JF831938	–	–	–	Rungjindamai <i>et al.</i> (unpublished)
<i>Falcocladium thailandicum</i>	CBS 121717	<i>Eucalyptus camaldulensis</i>	Thailand	JF831939	–	–	–	Rungjindamai <i>et al.</i> (unpublished)
<i>Hyaloseta nolinae</i>	CBS 109837	<i>Nolina micrantha</i>	USA	KM231846	–	–	–	Lombard <i>et al.</i> (2015)
<i>Lectera colletotrichoides</i>	CBS 109728	<i>Medicago sativa</i>	Turkey	KM231851	–	–	–	Lombard <i>et al.</i> (2015)
<i>Nectria balansae</i>	CBS 123351	<i>Coronilla</i> sp.	France	HM484552	–	–	–	Hirooka <i>et al.</i> (2011)
<i>Nectria cinnabarina</i>	CBS 125165	<i>Aesculus</i> sp.	France	HM484548	–	–	–	Hirooka <i>et al.</i> (2011)
<i>Nectria dacryocarpa</i>	CBS 121.87	Tree fern	Sulawesi	KM231850	–	–	–	Lombard <i>et al.</i> (2015)
<i>Nectria mariae</i>	CBS 125294	<i>Buxus sempervirens</i>	France	JF832629	–	–	–	Hirooka <i>et al.</i> (2012)
<i>Nectria nigrescens</i>	CBS 125148	Wood	USA	HM484707	–	–	–	Hirooka <i>et al.</i> (2011)
<i>Phialoseptomonium eucalypti</i>	CBS 145542	Leaves of <i>Eucalyptus</i>	Australia	MK876402	–	–	–	Crous <i>et al.</i> (2020)
<i>Pochonia</i> sp.	CBS 634.75	<i>Arcyria</i> sp.	Netherlands	KM231845	–	–	–	Lombard <i>et al.</i> (2015)
	CBS 892.7	Myxomycete	Netherlands	KM231844	–	–	–	Lombard <i>et al.</i> (2015)
	CBS 401.70	Myxomycete	Netherlands	KM231843	–	–	–	Lombard <i>et al.</i> (2015)
<i>Rodentomyces reticulatus</i>	CBS 128675	Rodent dung	Italy	JF832659	–	–	–	Hirooka <i>et al.</i> (2012)

Table 1. (Continued).

Species	Strain numbers	Host/Substrate	Collection sites	ITS Accession No.	LSU Accession No.	tef1 Accession No.	rpb2 Accession No.	References
<i>Sarocladium kiliense</i>	CBS 400.52	<i>Ficus carica</i>	UK	KM231849	-	-	-	Lombard et al. (2015)
<i>Septofusidium berolinense</i>	CBS 731.70	-	Germany	KM231841	-	-	-	Lombard et al. (2015)
<i>Stachybotrys chartarum</i>	CBS 129.13	-	Unknown	KM231858	MH866145	-	KM232434	Lombard et al. (2015)
<i>Thyronectria lamyi</i>	CBS 417.89	<i>Berberis vulgaris</i>	Germany	KM231837	-	-	-	Lombard et al. (2015)
<i>Thyronectria pyrrochlora</i>	CBS 125131	<i>Acer campestre</i>	Austria	HM484545	-	-	-	Hirooka et al. (2011)
<i>Thyronectria quercicola</i>	CBS 128976	<i>Quercus ilex</i>	Spain	JF832624	-	-	-	Hirooka et al. (2012)
<i>Thyronectria sinopica</i>	CBS 462.83	<i>Hedera helix</i>	Netherlands	HM484542	-	-	-	Hirooka et al. (2011)
<i>Tilachlidium brachiatum</i>	CBS 505.67	<i>Hypoholoma fasciculare</i>	Poland	KM231839	-	-	-	Lombard et al. (2015)
<i>Trichonectria rectipila</i>	CBS 132.87	-	USA	MH862058	-	-	-	Vu et al. (2019)
<i>Trichonectria setadpressa</i>	J.E.20-13	<i>Lobariella pallida</i>	France	MT153969	-	-	-	Flakus et al. (2019)

CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands; CPC: Culture collection of Pedro Crous, housed at the Westerdijk Fungal Biodiversity Institute; J.E.: The private herbarium of Javier Etayo, Pamplona.

min. This step was repeated five times. After this process, samples were rinsed with distilled water. Agar pieces including hyphae of isolates of *C. dirinariae* and *C. lichenicola* were inoculated onto the lichen thalli and maintained in 90-mm-diam glass Petri dishes with a sheet of wet filter paper to retain a high humidity at room temperature. Lichens inoculated were misted from above and moistened when they began to dry, every 2 to 3 d. In addition, some lichens were not inoculated and maintained under the above conditions as a control.

## RESULTS

### Taxonomy

*Classification:* Nectriaceae, Hypocreales, Sordariomycetes.

*Cylindromonium dirinariae* Ohmaki & Okane, *sp. nov.* MycoBank MB 846061. Fig. 1.

*Etymology:* Name refers to the host genus *Dirinaria* from which it was isolated.

*Diagnosis:* Ascomata perithecial, globose, orange; phialides short; conidia aggregated in mucoid packets in the apex of phialides, ellipsoid, aseptate; the species differs from all other *Cylindromonium* species by its characteristic DNA sequences (ITS, LSU, *tef1*, *rpb2*) and from its closest relative *C. lichenicola* also by its shorter phialides.

*Description:* Ascomata occur on the upper surface or soralia of the host lichen thallus; perithecial, scattered, globose, 80–100 µm diam, poculiform when dry, pale orange when young but later becoming dark, KOH negative, ascomatal wall layers of *textura globosa*, 14–18 µm thick. *Asci* broadly cylindrical to clavate, non-stipitate, (21.2–)24.5 ± 2.3(–29.1) × (3.9–)5.0 ± 0.8(–6.5) µm (n = 12), unitunicate, apex simple, 8-spored. *Ascospores* biseriolate, ellipsoid, hyaline, smooth, medially 1-septate, (5.0–)7.8 ± 1.1(–10.1) × (1.8–)2.6 ± 0.4(–3.7) µm, length/breadth (l/b) = (2.1–)2.6 ± 0.4(–3.9) (n = 40). *Mycelium* consisting of hyaline, smooth, septate, branched, 2 µm diam hyphae. *Conidiogenous cells* arising directly from aerial hyphae, hyaline, smooth, subcylindrical, 20–35 µm tall, 2 µm wide at the base, tapering to 1 µm at the apex, phialidic, with non-flared collarete. *Conidia* solitary, adhering in a slimy mass, hyaline, smooth, aseptate, ellipsoid with obtuse ends, (4.1–)5.9 ± 0.9(–10.3) × (1.5–)2.4 ± 0.4(–3.5) µm, l/b = (1.5–)2.55 ± 0.5(–3.9) (n = 75).

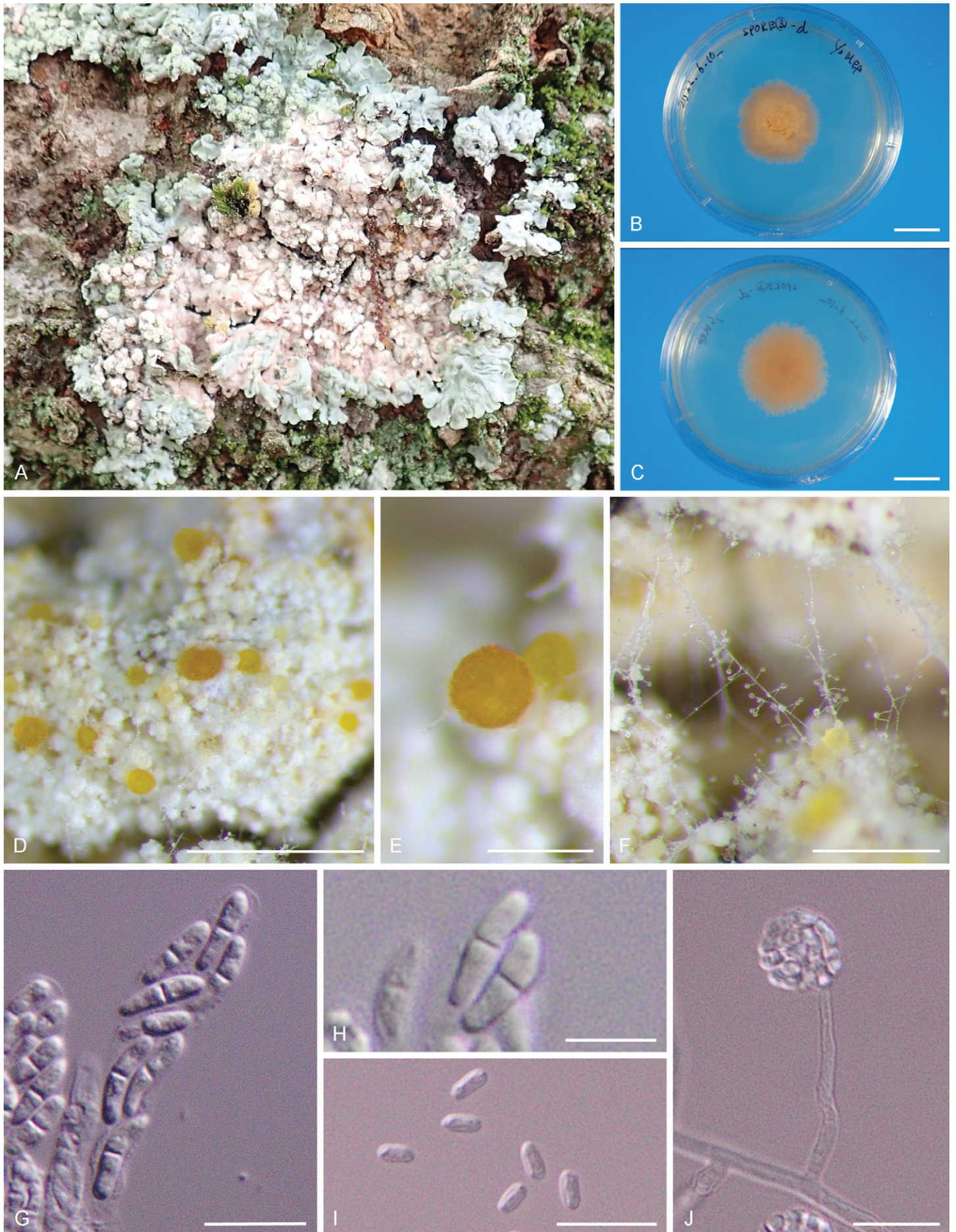
*Culture characteristics:* Colonies flat, circular or irregular, with moderate aerial mycelium and smooth, lobate margin, reaching 20 mm diam on MEA, PDA, OA, SMA and MYA, 30 mm diam on SGA after 3 wk at 23 °C in darkness. On MEA, PDA, SMA, SGA and MYA surface and reverse strong brownish orange; on OA surface grayish orange.

*Host:* *Dirinaria applanata*.

*Distribution:* Japan.

*Typus:* Japan, Ibaraki, Tsukuba, Tennodai, Univ. of Tsukuba, 36°06'08"N, 140°06'24"E, lichenicolous on *Dirinaria applanata* on bark of *Zelkova serrata*, 1 Aug. 2020, A. Ohmaki, I. Okane, K. Ohmachi, K. Miyazawa & K. Gibu, FAO 005 (holotype TNS-L-131533, culture ex-type NBRC 115852); DDBJ: ITS = LC731273; LSU = LC731274; *tef1* = LC731275; *rpb2* = LC744391.





**Fig. 1.** *Cylindromonium dirinariae*. **A.** *C. dirinariae* colonizing on *Dirinaria applanata*. **B, C.** Colonies on MEA. **B.** Surface. **C.** Reverse. **D.** Growth habit on *Dirinaria applanata*. **E.** Perithecium. **F.** Conidiogenous cells and conidia. **G.** Asci. **H.** Ascospores. **I.** Conidia. **J.** Phialide and conidial mass. Scale bars: **B, C** = 3 cm; **D** = 0.5 mm; **E, F** = 0.25 mm; **G, I, J** = 10  $\mu$ m; **H** = 5  $\mu$ m.



**Additional materials examined:** **Japan**, Ibaraki, Tsukuba, Tennodai, Univ. of Tsukuba, 36°06'08"N, 140°06'24"E, from *Dirinaria applanata* on the bark of *Zelkova serrata*, 1 Aug. 2020, A. Ohmaki, I. Okane, K. Ohmachi, K. Miyazawa & K. Gibu, NBRC 115851 = TNS-L-131534 (FAO 004), DDBJ: ITS = LC731276; LSU = LC744402; *tef1* = LC744396; *rpb2* = LC744390; 6 Nov. 2020, A. Ohmaki, TNS-L-131536 (FAO 090), in an inoculation experiment with isolates on *D. applanata* (Ibaraki: Tsukuba, 1 Aug. 2020, A. Ohmaki, I. Okane, K. Ohmachi, K. Miyazawa & K. Gibu, TNS-L-131533); Tsukuba, Tennodai, Univ. of Tsukuba, 36°06'38"N, 140°06'16"E, from *Dirinaria applanata* on the bark of *Zelkova serrata*, 15 Mar. 2021, A. Ohmaki, NBRC 115853 = TNS-L-131535 (FAO 006), DDBJ: ITS = LC731277; LSU = LC744401; *tef1* = LC744395; *rpb2* = LC744392.

**Notes:** The ex-type strain of *C. lichenicola* (CBS 425.66) was not available for study, so we conducted morphological observations on other available strains of *C. lichenicola* (CBS 188.70 and CBS 415.70A) that are similar in terms of their collection sites and hosts. In addition, these strains were also studied when the genus *Cylindromonium* was established in Crous et al. (2019b).

Morphological features of the *C. lichenicola* strains (CBS 188.70 and CBS 415.70A) shown in Table 2 correlated well with the original description provided by Gams (1971) [conidia size 5.5–9.8 × 1.5–2.5 µm (l/b = 3.0–4.4), phialide length 30–60 µm, phialide width base 2.0–3.0 µm, apex 0.7–1.5 µm].

As a result of the t-test, there were significant differences between *C. dirinariae* and *C. lichenicola* in conidia, phialide length and width (Table 2). As for phialide length, phialides of *C. dirinariae* were about half as long as those of *C. lichenicola*.

**Materials examined of *Cylindromonium lichenicola*:** **Germany**, Probststeierhagen, Schüttbrehm, from unnamed apothecia of lichen on tree bark, Oct. 1996 (living strain CBS 188.70), GenBank: LSU = LC744399; *tef1* = LC744398; *rpb2* = LC744393. **Netherlands**, Utrecht, Amelisweerd, from aerial algae on tree bark, Oct. 1968 (living strain CBS 415.70A), GenBank: LSU = LC744400; *tef1* = LC744397; *rpb2* = LC744394.

## Phylogeny

The ITS sequences derived from DNA extracted from a perithecium (specimen TNS-L-131533) and that from mycelia from another specimen (TNS-L-131534) of *C. dirinariae* were identical. The Phylogenetic analysis based on the ITS region revealed that all three sequences, adding that from mycelia (TNS-L-131535) to the above two, were grouped in a fully supported clade (Fig. 2). The clade was sister to *C. lichenicola* and linked with five *Cylindromonium* species including the type species of the genus *Cylindromonium*, *C. everniae*. The clade including the *Cylindromonium* species also included species of *Trichonectria* (*Bionectriaceae*) and *Phialoseptomonium* (*Nectriaceae*).

Sequences of LSU, *tef1*, and *rpb2* derived from specimen TNS-L-131533, TNS-L-131534 and TNS-L-131535 of *C. dirinariae* were identical. The ITS sequence from TNS-L-131535 differed

from the other two sequences from TNS-L-131533 and TNS-L-131534 in one site. While the sequences of ITS, LSU, and *tef1* of *C. lichenicola* strains CBS 188.70 and CBS 415.70A were identical, those of *rpb2* of the two stains were different in 18 sites. Comparison of *C. dirinariae* with *C. lichenicola* (CBS 188.70 and CBS 415.70A) showed that there were 19 gap sites (96.7 % in nucleotide identity) in ITS, 11 gap sites including 2 bp deletions (98.8 %) in LSU, 26 gap sites in *tef1* (93.7 %), 153 gap sites (CBS 415.70A vs. *C. dirinariae*) or 155 gap sites (CBS 188.70 vs. *C. dirinariae*) including 44 bp insertions in *rpb2* (84.9–85.1 %). Phylogenetic analysis based on the concatenated sequences of ITS, LSU and *rpb2* showed that three sequences of *C. dirinariae* grouped together with full bootstrap support and clearly segregated from *C. lichenicola* and other species (Fig. 3).

## Inoculation experiments

The two *Cylindromonium* spp. studied were able to colonise the inoculated lichens, except for failures due to contamination, extreme dryness or moisture (Fig. 4; Table 3).

*Cylindromonium dirinariae* colonised and produced perithecia on the thalli of *D. applanata*. Colonies reached 5 mm diam about 1 wk post inoculation. Pinkish discolouration was observed in the part of lichen's thalli covered with hyphae of *C. dirinariae*. The asexual morph also developed on inoculated thalli. Perithecia developed 2 wk post inoculation. Morphological features of *C. dirinariae* on the inoculated lichens coincided well with those of *C. dirinariae* observed in the field.

On the other hand, although *C. lichenicola* also colonised and produced the asexual morph on *D. applanata*, no perithecia were produced.

*Cylindromonium dirinariae* and *C. lichenicola* colonised and sporulated asexually, but no perithecia were produced on *P. tinctorum*. The lichen's thalli were covered with hyphae of the lichenicolous fungi, and discoloured brownish around the point of inoculation. Colonies reached 5 mm diam after 1 wk and 1.5–2 cm diam after 2 wk post inoculation. Lichens used for controls remained healthy. In addition, three single ascospore cultures were obtained of *C. dirinariae*, and each culture was inoculated onto thalli of *D. applanata*. As a result, they colonised the lichen and produced perithecia.

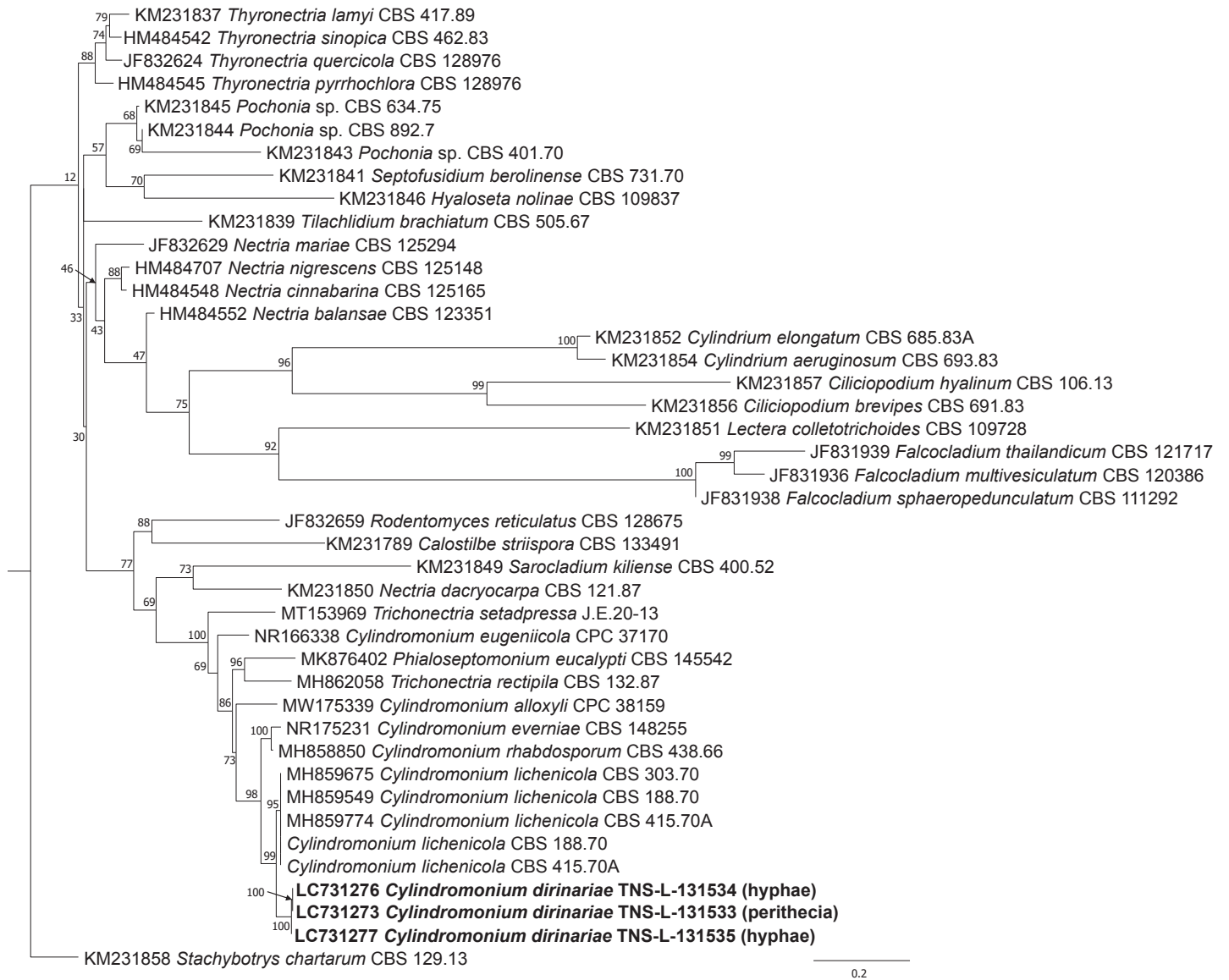
## DISCUSSION

In the molecular phylogenetic analysis based on ITS sequence data and concatenated sequences of three loci, the three sequences of *C. dirinariae* clustered in a single clade and positioned as sister to *C. lichenicola*, which is the most closely related species in morphology and DNA phylogeny (identity = 96.7 %), supported by high bootstrap values. Furthermore, *C. dirinariae* was related to the group consisting of *C. everniae* and

**Table 2.** Comparison of dimensions (µm) between *Cylindromonium dirinariae* and *C. lichenicola*.

	Conidia*	Phialide length*	Phialide width (Base)*	Phialide width (Apex)*	Hyphae width
<i>C. dirinariae</i>	(4.1–)5.9 ± 0.9(–10.3) × (1.5–)2.4 ± 0.4(–3.5)	(14.7–)27.4 ± 8.1(– 56.7)	(1.7–)2.3 ± 0.3(–3.1)	(1.0–)1.4 ± 0.2(–2.0)	(1.2–)2.0 ± 0.3(–2.6)
<i>C. lichenicola</i>	(4.1–)6.8 ± 1.1(–10.0) × (1.5–)2.4 ± 0.5(–3.3)	(33.9–)51.7 ± 7.6(– 69.9)	(2.0–)3.2 ± 0.5(–4.0)	(1.1–)1.6 ± 0.3(–2.5)	(1.5–)2.3 ± 0.5(–3.3)

\*Significantly different.



**Fig. 2.** A phylogenetic tree of *Cylindromonium dirinariae* and other *Nectriaceae* and hypocrealean fungi constructed from a maximum-likelihood (ML) analysis based on the ITS sequences. The outgroup is *Stachybotrys chartarum*. Number at the nodes represents the bootstrap value.

*C. rhabdosporum*, supported by high bootstrap values (99 %) (Fig. 2). Comparison of other loci sequences also showed that similarity between *C. dirinariae* and *C. lichenicola* were 96.7 % in ITS, 98.8 % in LSU, 93.7 % in *tef1*, 84.9–85.1 % in *rpb2*. In addition to phylogeny, *C. dirinariae* is also morphologically distinct. In our morphological observations and the inoculation experiments, *C. dirinariae* had 20–35 µm tall phialides and produced perithecia on *D. applanata*, while *C. lichenicola* had longer phialides (45–60 µm tall), and failed to produce perithecia on *D. applanata* and *P. tinctorum*. Hence, we concluded that *C. dirinariae* represents

a new species. In addition, this is the first report of the sexual morph for the genus *Cylindromonium*.

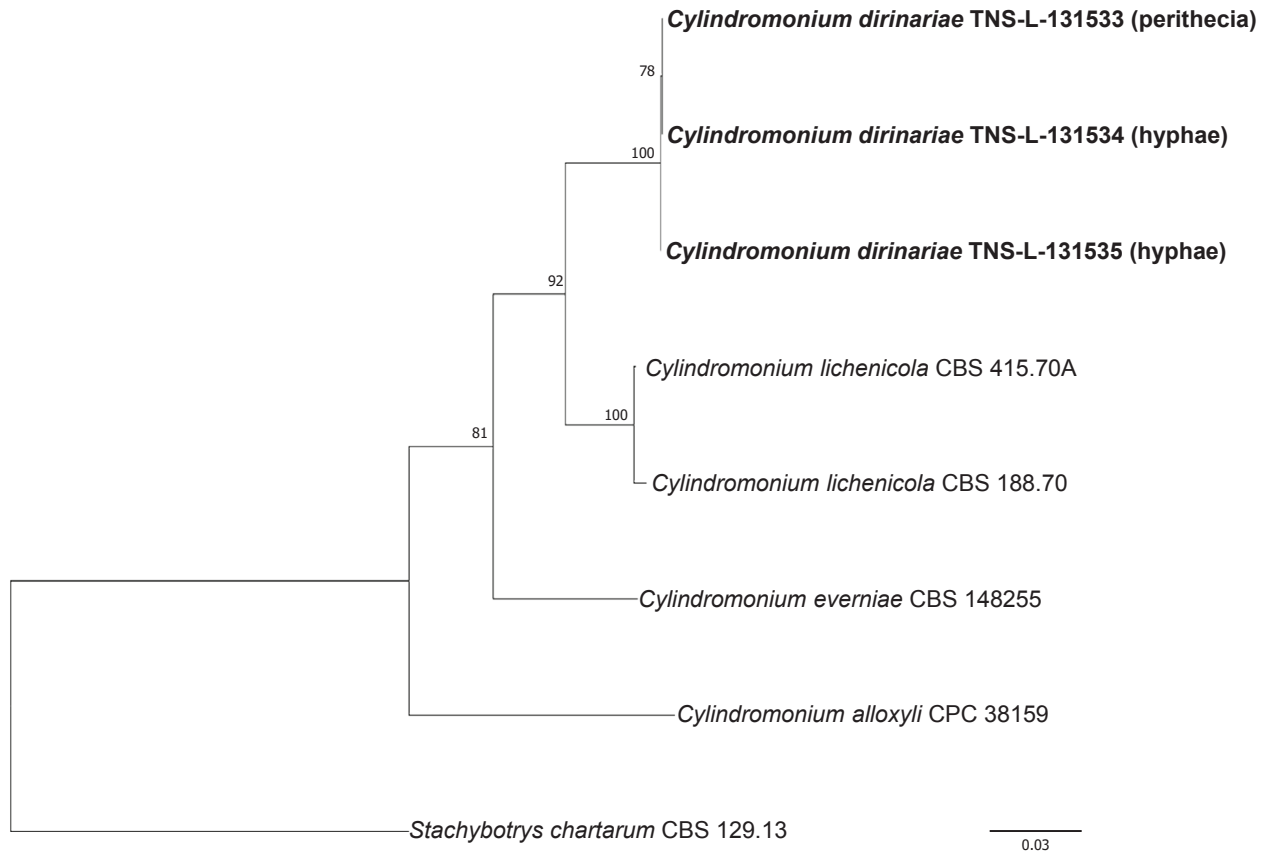
The genus *Cylindromonium* has been reported from Belgium (Gams 1971, Diederich 1989), Germany (Gams 1971, Brackel 2010), Great Britain (Hitch 1995), France (Roux 2012), Luxembourg (Diederich 1989), the Netherlands (Brand *et al.* 2013, Crous *et al.* 2021), Czech Republic (Kocourková 2009), India (Joshi *et al.* 2016), Ukraine (Khodosovtsev *et al.* 2018), and Australia (Gams 1971, Crous *et al.* 2020). This is the first report of the genus *Cylindromonium* from Japan.

**Table 3.** Colonization rate in inoculation experiments.

Lichen species inoculated	Inoculum		
	<i>Cylindromonium dirinariae</i>	<i>Cylindromonium lichenicola</i>	Control
<i>Dirinaria applanata</i>	16/20* (80 %)**	13/15 (87 %)	3/12 (25 %)
<i>Parmotrema tinctorum</i>	4/4 (100 %)	2/2 (100 %)	0/5 (0 %)

\*Number of colonised lichenicolous fungi/the number of inoculated lichens.

\*\*Perithecia produced.



**Fig. 3.** A phylogenetic tree for *Cyindromonium dirinariae* and other *Cyindromonium* spp. constructed from a maximum-likelihood (ML) analysis based on concatenated sequence dataset of ITS, LSU rDNA and *rpb2*. The outgroup is *Stachybotrys chartarum*. Number above a branch represents the bootstrap value.

Inoculation experiments using single ascospore cultures revealed that this fungus is homothallic. The newly described *C. dirinariae* was isolated from *D. applanata*, while the hosts of *C. lichenicola* are diverse, including the lichens *Cladonia*, *Hypogymnia*, *Parmelia saxatilis*, *Tremella cladoniae*, lichens overgrowing *Stereum* species, fungi; *Bulgaria inquinans*, algal-covered bark, *Alnus* bark and *Betula* litter (Gams 1971, Diederich 1989, Hitch 1995, Brackel 2010, Roux 2012, Brand et al. 2013, Tsurykau et al. 2016). Results of our inoculation experiments are suggestive of host specificity; i.e., *C. dirinariae* was able to form perithecia on its original host lichen, *D. applanata*, but not on *P. tinctorum*. *Cyindromonium dirinariae* could therefore be considered host specific.

Glenn et al. (1997) found that *Cyindromonium rhabdosporum* (as *Acremonium rhabdosporum*), occurred on healthy-looking thalli in the field, and perithecia of *Nectriopsis rubefaciens* (as *Nectria rubefaciens*) appeared on the same thalli in the closed plates. Although they mentioned the relationship between *C. rhabdosporum* and *N. rubefaciens*, they did not confirm that they belong to the same holomorph. Further study is therefore needed to determine the homogeneity between *C. rhabdosporum* and *N. rubefaciens*.

Presently there are only a few reports of inoculation experiments of lichenicolous fungi, and they were conducted in the field (Fatma et al. 2019). Glenn et al. (1997) found that continuously moist conditions probably play a pivotal role for lichenicolous fungi to produce perithecia. Inoculation experiments in the moist condition in Petri dishes using axenic cultures of lichenicolous fungi may therefore be a useful technique for

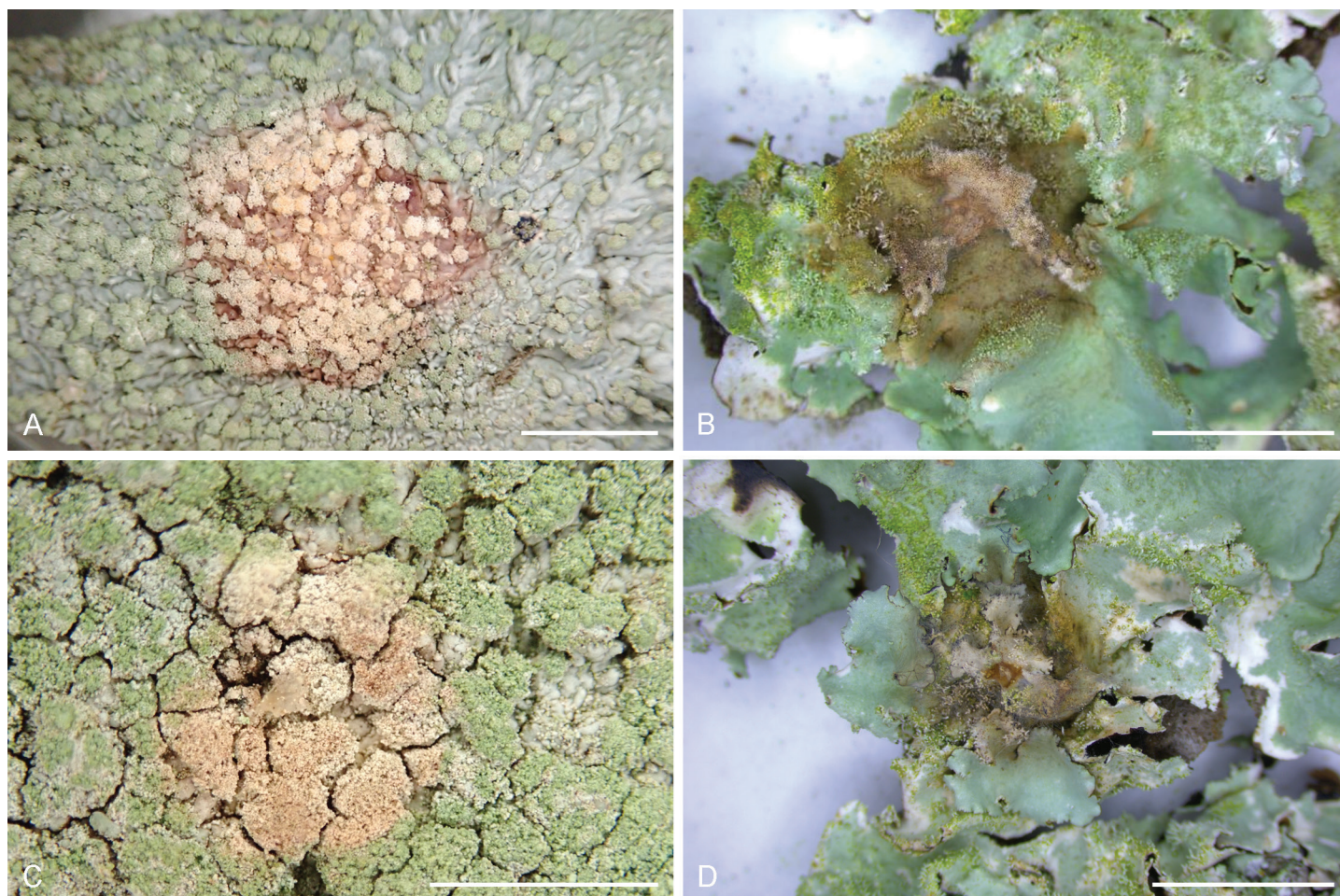
studying morphological, physiological and ecological features of lichenicolous fungi. This work has demonstrated the potential of inoculation experiments to investigate the morphological feature of perithecia and host specificity.

Presently the ecology of *C. dirinariae* remains unclear. Other species of *Cyindromonium* were reported to be mycophilic or saprophytic (Crous et al. 2019b, 2020). We expect that inoculation experiments will reveal the interaction between lichenicolous fungi and their host lichens which could help us to better understand the ecological role of lichenicolous fungi.

The ascomycete family *Nectriaceae* includes numerous important plant and human pathogens as well as several facultatively fungicolous or insecticolous species (Rossman 1996, Lombard et al. 2015). Members of *Nectriaceae* are characterised by uniloculate ascocata that are white, yellow, orange-red or purple, unitunicate asci and phialidic asexual morphs (Rossman et al. 1999, Lombard et al. 2015). In many cases ascocata show a change of colour when mounted in KOH (Lombard et al. 2015). In our study, perithecia of the *C. dirinariae* were orange in colour and did not react in KOH.

About 400 genera and 2 300 species of lichenicolous fungi are known from lichens, but the actual number of lichenicolous fungal species could be much higher (Diederich et al. 2018). Diederich et al. (2018) estimated 3 000–5 000 lichenicolous fungal species will eventually be described based on Hawksworth's global estimates of fungal diversity (Hawksworth 1991, 2001) and the total number of lichen species (Lücking et al. 2017a, 2017b). Lichenicolous fungi are assumed to be an important source of new species in many groups of fungi, including *Nectriaceae*.





**Fig. 4.** Lesions on the lichen thalli after inoculation. **A.** Colony of *C. dirinariae* on *D. applanata*. **B.** Colony of *C. dirinariae* on *P. tinctorum*. **C.** Colony of *C. lichenicola* on *D. applanata*. **D.** Colony of *C. lichenicola* on *P. tinctorum*. Scale bars = 5 mm.

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