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Coniochaeta endophytica sp. nov., a foliar endophyte associated with healthy photosynthetic tissue of *Platycladus orientalis* (*Cupressaceae*)

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Abstract. The ecologically diverse genus *Coniochaeta* (*Coniochaetaceae*, *Ascomycota*) contains numerous endophytic strains that occur in healthy leaves and lichen thalli in temperate and boreal North America. These endophytes frequently represent undescribed species. Here we examine two endophytic isolates of *Coniochaeta* from healthy photosynthetic tissue of *Platycladus orientalis* (*Cupressaceae*), a conifer cultivated for horticultural use in Arizona, USA. On the basis of morphology, *in vitro* assays, phylogenetic analyses of two loci, and analyses of whole genome data, we designate these endophytes as a novel species, *Coniochaeta endophytica* sp. nov. Strains of *C. endophytica* are closely related to an isolate from a native lichen in North Carolina, which we also characterize here. We compare *C. endophytica* with two known species that appear to be close relatives: *C. prunicola*, associated with wood necrosis in stonefruit trees in South Africa, and *C. cephalothecoides*, isolated from soil in Asia. The new species is distinct in phylogenetic, *in vitro*, and whole-genome analyses from *C. prunicola*, and differs slightly in conidiophore morphology from that species. Although available sequence data for *C. cephalothecoides* are of uncertain relation to the type specimen for that species, our results support the distinctiveness of *C. endophytica* on the basis of morphology, perithecial formation, and phylogenetic analyses. We discuss the challenge of identifying new species in the context of fungal ecology surveys, such as those for endophytes, which often rely only on a single locus and can misidentify taxa based on their closest matches in public databases or simple comparisons of barcode sequences alone.

Key words: anamorph, bioassay, biodiversity, cryptic diversity, endolichenic, *Lecythophora*, species concept

Introduction

Endophytic fungi represent tremendous and largely undescribed diversity. In only a few cases have the fungal endophytes of woody plants been described in terms of traditional systematics and taxonomy (see Bussaban et al. 2003; Rojas et al. 2008, 2010; Gazis et al. 2011, 2012; Bills et al. 2013; Chen et al. 2015; Torres-Cruz et al. 2017). Such studies are important for linking ecological diversity with the rich history of systematics in mycology, and for establishing new phylogenetic and taxonomic links for fungi known only from ecological studies, which typically generate only environmental or barcode sequences (see Carbone et al. 2016; U´Ren et al. 2016; Torres-Cruz et al. 2017).

In temperate and boreal regions of North America, strains of *Coniochaeta* (*Coniochaetaceae*, *Coniochaetales*, *Sordariomycetes*, *Ascomycota*) often are isolated as endophytes from healthy foliage in culture-based studies (U´Ren et al. 2012; Huang et al. 2016). They typically are identified to genus or species on the basis of BLAST analyses of barcode sequence data (usually the nuclear

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ribosomal internal transcribed spacers and 5.8S gene: ITSrDNA; e.g., del Olmo-Ruiz 2012; U´Ren et al. 2012). Preliminary analyses suggest that these endophytes have the capacity to expand and reshape the generic concept for *Coniochaeta* and to revise perspectives on the ecological traits and evolution of species in this genus (del Olmo-Ruiz 2012). Such studies are of interest in part because several species of *Coniochaeta* are known as plant or human pathogens, and others produce biologically active compounds (Weber et al. 2002, Damm & Crous 2010, Khan et al. 2013, Xie et al. 2015).

As currently circumscribed, *Coniochaeta* includes species from diverse substrates, including butter, dung, wood, soil, uranium mine wastewater, and phylogenetically diverse plants (e.g., Weber 2002; Raja et al. 2012; Vázquez-Campos et al. 2014; Xie et al. 2015). With the anamorph formalized and segregated from morphologically similar genera by Gams (2000), the holomorph described in detail by Weber (2002), and the circumscription updated by García et al. (2006), *Coniochaetaceae* was segregated from *Sordariaceae* by germ-slits in the ascospores and a phialidic anamorph. The generic name *Lecythophora* Nannf. was introduced to refer to the anamorphic *C. lignicola*, which was rapidly transferred elsewhere, leading to disuse of the generic name (Melin & Nannfeldt 1934; Weber 2002). *Lecythophora* was reintroduced by Gams and McGinnis (1983) to refer to a morphologically circumscribed subset of the anamorphs of *Coniochaeta*, with this teleomorph-anamorph connection reemphasized by Weber et al. (2002). The anamorphic *Lecythophora* was synonymized with the teleomorphic *Coniochaeta* by Khan et al. (2013), with synonymization and priority of the generic name *Coniochaeta* reiterated by Réblová et al. (2016).

Given the highly plastic and taxonomically uninformative anamorph, morphological keys to species of *Coniochaeta* (e.g., Asgari et al. 2007) rely heavily on ascus and ascospore morphology (see also Gams & McGinnis 1983; Weber 2002). However, even with information from such morphological traits, species delineation in the genus remains challenging. At present the number of accepted species in *Coniochaeta* ranges from 54–100 depending on the source, reflecting a complex taxonomic history and unclear species boundaries. Among contemporary sources, Asgari et al. (2007) included 54 species in the dichotomous key to the genus, García et al. (2006) included approximately 70 species, and Kirk et al. (2008) listed 65 species. According to Species Fungorum, the genus consists of 92 species, excluding synonyms of species now placed in different genera (accessed December 2018). MycoBank suggests 119 species (accessed December 2018), reflecting some disagreement with Index Fungorum as to the higher-level classification and thus currently accepted names of a subset of the species. As of 2018, 20 described species were represented by ITSrDNA barcode sequences of type material in the GenBank RefSeq database. As of late 2018, The UNITE database (Abarenkov et al. 2010) suggests between 38 and 143 species hypotheses in the genus (including those species hypotheses labeled as *Lecythophora*) depending on the threshold of ITSrDNA divergence (3.0% to 0.5%). Most of these species hypotheses do not correspond to available type sequences from described species. The use of a polyphasic, multilocus phylogenetic species concept per Gazis et al. (2011) suggests that even the lowest ITSrDNA divergence threshold used to define this kind of species hypothesis may underestimate fungal diversity in some cases: different species can have 100% identical ITSrDNA sequences (see Lieckfeldt & Seifert 2000; Schoch et al. 2012). This is consistent with previous observations of extremely high ITSrDNA similarity between isolates from different species of *Coniochaeta* (i.e., limited ITSrDNA sequence variability in this genus; Nasr et al. 2018).

As part of a survey of endophytes associated with cultivated trees, Hoffman & Arnold (2008) isolated a strain of *Coniochaeta* as an endophyte from healthy, mature foliage of *Platycladus orientalis* (*Cupressaceae*), which was growing in an arboretum at the University of Arizona (Tucson, Arizona, USA). They did not identify the strain, designating it only by the isolation code 9094 (Hoffman & Arnold 2008). An isolate with the same ITSrDNA sequence (i.e., 100% similar) was observed later as part of a mixed culture from healthy foliage of the same *P. orientalis* individual. It was isolated in pure culture with isolation code 9055 (Hoffman & Arnold 2008). Based on phylogenetic analyses of the nuclear ribosomal large subunit (LSUrDNA) and ITSrDNA, del Olmo-Ruiz (2012) placed isolate 9094 in the anamorphic genus *Lecythophora*, but did not characterize 9094 or 9055 more finely.

Here, we use morphological data, phylogenetic analyses based on two loci, *in vitro* assays, and analyses of whole genome data to characterize endophytes 9094 and 9055 in the context of closely related strains. On the basis of these lines of evidence we describe endophyte isolates 9094 and 9055 as a new species. These isolates are closely related to an isolate from a native lichen in North Carolina (isolate NC1642), which we also characterize here. We compare 9055 and 9094 with two closely related species: *C. prunicola*, which is associated with wood necrosis in stonefruit trees in South Africa (Damm et al. 2010; see also Ivanová & Bernadovičová 2012), and *C. cephalothecoides*, originally isolated from soil in Japan (Kamiya et al. 1995; see also Han et al. 2017). Finally, we discuss the challenge of identifying new species in the context of fungal ecology surveys, which often rely only on a single locus and can misidentify taxa based on their closest matches in public databases or simple comparisons of barcode sequences.

Materials and methods

Endophytes 9094 and 9055 were isolated from healthy photosynthetic tissue of a mature individual of *P. orientalis* (L.) Franco (*Cupressaceae*) cultivated in the Campus Arboretum at the University of Arizona (Tucson, Pima County, Arizona, USA: 32.231N, 110.952W, elevation 787 m; mean annual temperature 21.6°C; mean annual precipitation 303 mm) (Hoffman & Arnold 2008). Tissues

were collected in spring 2005 and processed within 4 h of collection to isolate endophytic fungi (Hoffman & Arnold 2008). Briefly, tissues were rinsed in running tap water for 30 s and then cut into 2 mm square pieces. Tissue pieces were agitated in 95% ethanol for 30 s, 10% bleach (i.e., 0.5% NaOCl) for 2 min, and 70% ethanol for 2 min. After surface drying under sterile conditions, pieces were placed on 2% malt extract agar (MEA) and incubated at 25°C for eight weeks. All isolates that emerged were transferred to axenic culture and vouchered at the University of Arizona Robert L. Gilbertson Mycological Herbarium (ARIZ). An ITSrDNA sequence was obtained for each isolate as described previously (Hoffman & Arnold 2008), and edited sequences were identified tentatively on the basis of BLAST matches with records in GenBank (Altschul et al. 1990). Two isolates obtained in culture matched *Coniochaeta*: 9094 and 9055. These strains have identical ITSrDNA sequences. Prior to late 2017 their top identified match in GenBank BLAST searches was *C. prunicola* (the holotype, CBS 120875, sequence accession NR137037.1; Damm et al. 2010). From late 2017 forward their top identified match was *C. cephalothecoides* (accession KY064029.1; Han et al. 2017), reflecting the release of a sequence labeled *C. cephalothecoides* in GenBank in fall of that year (see Table S1). The species identity of endophytes 9094 and 9055 has not been evaluated previously, and is investigated here via morphological, phylogenetic, *in vitro*, and whole-genome analyses.

Initial taxon sampling for morphological evaluation

Initial taxon sampling for this study was guided by del Olmo-Ruiz (2012), who used LSUrDNA to infer the relationships of diverse endophytic and endolichenic strains in *Coniochaetales.* Her work placed 9094, albeit without strong support, in a clade with two publicly available isolates designated by Damm et al. (2010) as *C. prunicola*: accession GQ154603, representing strain STEU5953, reconstructed by del Olmo-Ruiz as sister to 9094; and sister to them, accession GQ154602, representing strain STEU6107. Del Olmo-Ruiz (2012) also analyzed additional loci in a subsequent analysis designed to identify the placement of unknown endophytes within *Coniochaeta*. She did not include *C. prunicola* in that analysis, but did include other known species of *Coniochaeta* that were reconstructed in the same, large, diverse clade as 9094 and other endophytic strains. These were listed under the anamorphic designations *Lecythophora mutabilis* (CBS157.44), *L. fasciculata* (CBS 205.38), *L. lignicola* (CBS267.33), *L. luteoviridis* (CBS206.38), and *L. hoffmannii* (CBS245.38). At that time, sequence data were not available for *C. cephalothecoides*, and the affinity of that species for *C. prunicola* and related strains was not yet proposed.

Based on the placement of 9094 presented by del Olmo-Ruiz (2012) we selected 16 strains for further evaluation (Table 1). We obtained reference strains of known species, listed under their teleomorph names, from the

Table 1. Metadata and GenBank accession numbers for *Coniochaeta* isolates included in this study. Asterisks: used in the final phylogenetic analyses (Fig. 1), which also included the ITSrDNA sequence for '*C. cephalothecoides*' (KY064029.1; Han et al. 2017; see Fig. S1 for the same analysis without that sequence). Daggers: isolates for which genome sequences were obtained; this group subsequently included IL0111, not listed here. Isolates from A: U´Ren et al. (2012), B: Huang et al. (2016), C: Hoffman & Arnold (2008). 1: Ex-type specimen of *C. prunicola*, 2: ex-paratype of *C. prunicola.* The same accession numbers are given for ITSrDNA and LSUrDNA in cases in which a single, concatenated sequence was accessioned in GenBank. Accession numbers in italics indicate sequence data generated for the present study.

Species	Isolate	Isolation source	Locality	Life mode	GenBank accession numbers			
					ITSrDNA	**LSUrDNA**	***RPB1***	***TEF1a***
C. hoffmanni	CBS 245.38[1]	Butter	Switzerland	Unknown	AY945807	AF353599	*MK693166*	*MK693150*
Coniochaeta sp.	dc2070	*Pinus ponderosa*	USA (Arizona)	Endophyte	*MK614055*	*MK614055*	*MK693176*	*MK693151*
C. lignicola	CBS 267.33[1]	Wood pulp	Sweden	Wood decay	NR_111520	AF353601	*MK693175*	*MK693154*
C. fasciculata	CBS 205.38[1]	Butter	Switzerland	Unknown	HE610336	AF353598	*MK693177*	*MK693152*
Coniochaeta sp.	FL0766[A]	*Selaginella arenicola*	USA (Florida)	Endophyte	JQ760426	JQ760426	*MK693178*	*MK693153*
Coniochaeta sp.	FL0232[A]	*Pinus elliottii*	USA (Florida)	Endophyte	JQ760024	JQ760024	*MK693171*	*MK693155*
Coniochaeta sp.	FL1248[A]	*Usnea subscabrosa*	USA (Florida)	Endolichenic fungus	JQ760863	JQ760863	*MK693172*	*MK693156*
Coniochaeta sp.*	FL1226[A]	*Cladonia subradiata*	USA (Florida)	Endolichenic fungus	JQ760841	JQ760841	*MK693174*	*MK693158*
Coniochaeta sp.*	FL0068[A]	*Pinus ellottii*	USA (Florida)	Endophyte	JQ759927	JQ759927	*MK693173*	*MK693157*
*C. prunicola**‡	CBS 120875[1]	*Prunus armeniaca*	South Africa	Plant pathogen	NR137037	GQ154602	*MK693170*	*MK693162*
*C. prunicola**	CBS 121445[2]	*Prunus salicina*	South Africa	Plant pathogen	GQ154541	GQ154603	*MK693168*	*MK693161*
Coniochaeta sp.*	YLH0003[B]	*Juniperus deppeana*	USA (Arizona)	Endophyte	KP991985	KP991985	*MK693169*	*MK693160*
Coniochaeta sp.* ‡	YLH0009[B]	*Juniperus deppeana*	USA (Arizona)	Endophyte	KP991991	KP991991	–	*MK693163*
Coniochaeta sp.* ‡	NC1642[A]	*Sticta beauvoisii*	USA (North Carolina)	Endolichenic fungus	JQ761997	JQ761997	–	*MK693164*
C. endophytica sp. nov.* ‡	AEA 9094[C,1]	*Platycladus orientalis*	USA (Arizona)	Endophyte	EF420005	EF420069	*MK693167*	*MK693159*
C. endophytica sp. nov.* ‡	AEA 9055[C]	*Platycladus orientalis*	USA (Arizona)	Endophyte	MK614056	EF420043	–	*MK693165*

CBS-KNAW culture collection of the Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands) (Table 1). For comparative morphology and multilocus sequencing we obtained representative strains of *Coniochaeta* from the living collection of endophytic fungi housed in ARIZ (Table 1). These strains were isolated from asymptomatic photosynthetic tissue of plants and lichen thalli as described in Hoffman and Arnold (2008), U´Ren et al. (2012), and Huang et al. (2016), and preliminary analyses placed them in the portion of *Coniochaeta* containing the reference strains in Table 1.

Morphological characterization

We examined microscopic and macroscopic characteristics of representative endophytic, endolichenic, and reference strains (Tables 2, 3, 4). Strains were first grown on 2% MEA supplemented with antibiotics to eliminate bacterial contamination (100 µg/mL ampicillin, 50 µg/mL kanamycin, 10 µg/mL tetracycline, and 40 µg/mL ciprofloxacin; see Hoffman & Arnold 2010). Hyphae were transferred to 100 mm Petri plates containing 2% MEA or 2% potato dextrose agar (PDA). Cultures were grown on a bench top at 25°C with 12 hour light/dark cycles. Colony colors were rated according to Kornerup and Wanscher (1967).

Microscopic characters were described from slide cultures (Table 2), which were prepared by rolling a cube of 2% MEA across the surface of actively growing, sporulating cultures and incubating it in a moist chamber per Riddell (1950). After 2–3 d of incubation in a moist chamber at 25°C with 12 h light/dark cycles, coverslips or slides were mounted in distilled water and imaged with a Leica DM4000B compound microscope. A minimum of 25 conidia and conidiogenous cells per isolate were measured with ImageJ and a stage micrometer.

Macroscopic characteristics on 2% MEA and 2% PDA are listed in Tables 3 and 4. In addition to waiting three months for the formation of perithecia on standard media, following Damm et al. (2010) we grew selected strains on synthetic nutrient agar (SNA) with double-autoclaved pine needles (*Pinus canariensis* C. Sm.) to encourage formation of perithecia. Structures were imaged with a Leica S8AP0 dissecting microscope. We compared all observed traits against references for *C. prunicola* (Damm et al. 2010) and *C. cephalothecoides* (Kamiya et al. 1995) as described below, after phylogenetic analyses revealed the relatedness of 9094 and 9055 to these species.

In vitro assays

We conducted two *in vitro* assays. In the first assay, we determined the ability of 9094 and the ex-type cultures of *C. prunicola* to form mature, fertile perithecia on foliage of *Prunus*, the genus from which *C. prunicola* was isolated in association with wood necroses (Damm et al. 2010). We focused on almond (*Prunus dulcis* var. *dulcis* (Mill.) D. A. Webb, 'All in one'). We used a cork borer to remove 6 mm discs from the growing edge of the mycelium on 2% MEA under sterile conditions. Each disc was transferred to a sterile 1.5 mL tube containing 1 mL of sterile water. Each agar disc was ground briefly with a sterile pestle. Healthy young leaves were harvested from greenhouse-grown trees and processed within 4 h. Leaves were rinsed in tap water and 8 mm discs were removed with a cork borer. Discs were surface sterilized (Hoffman & Arnold 2008) and allowed to surface-dry in sterile conditions. Leaf discs were plated abaxial side up on water agar in 150 mm Petri dishes. We used a pipette to place 10 µL of inoculum on each leaf disc. Negative controls were inoculated similarly with the suspension of a macerated piece of sterile 2% MEA suspended in sterile water. Five discs per treatment were placed in each dish. Each dish was wrapped three times with Parafilm and incubated at 25°C with 12 h light/dark cycles. After 28 d and 56 d we scored discs for presence of perithecia.

In the second *in vitro* assay we examined the capacity of 9055 to form fertile, mature perithecia on leaf discs of almond (as above) and peach (*Prunus persica* (L.) Batsch, 'May Pride'). In this assay we used macerated 2% MEA in water, as above, as the negative control. We also used three positive controls (two strains of *Phoma* isolated from peach in Arizona, Myco-ARIZ accessions PLFA and PLFB; one strain of *Colletotrichum* sp. isolated from southern Arizona; Arnold, unpubl.). Methods were as described above, except that six discs were used per treatment, we scored leaf discs for discoloration and fruitbody production 21 d after inoculation, and the entire experiment was repeated twice.

DNA extraction, PCR, and sequencing for phylogenetic analyses

DNA was extracted from fresh mycelium of each strain with a phenol:chloroform:isoamyl alcohol method following Arnold and Lutzoni (2007). The ITSrDNA and

Table 2. Micromorphological characteristics of known taxa and endophytes, as listed in Table 1, assessed in slide cultures after 14 d of growth on 2% MEA. FL0068 and FL1226 were selected as outgroup taxa for the final analysis (Fig. 1), but they had identical ITSrDNA and *TEF1a* sequences. Therefore we characterized only one of them (FL0068). Asterisks: characterized by del Olmo-Ruiz (2012). Data for the ex-type of *C. prunicola* were obtained from Damm et al. (2010). 1: ex-type, 2: ex-paratype. All isolates considered had intercalary and discrete phialides, and oblong conidia. No chlamydospores were observed.

Species or isolate	Conidial length (µm)	Conidial width (µm)
C. hoffmannii	(2.7) 3.7–4.1 (5.5)	(1.7) 2.0–2.2 (2.8)
dc2070*	(3.5) 4.0–4.3 (5.2)	(1.6) 2.0–2.2 (2.9)
*C. lignicola**	(2.7) 3.2–3.4 (4.1)	(1.4) 1.6–1.8 (2.1)
*C. fasciculata**	(3.1) 3.8–4.1 (4.8)	(1.4) 1.7–1.8 (2.3)
FL0766*	(3.1) 4–4.4.0 (5.2)	(1.3) 1.5–1.7 (2.1)
FL0232*	(3.8) 4.2–4.4 (4.9)	(1.2) 1.8–2.0 (2.3)
FL1248*	(3.4) 3.9–4.2 (4.9)	(1.4) 1.6–1.8 (2.5)
FL0068*	(2.6) 3.4–3.7 (4.4)	(1.2) 1.5–1.6 (1.9)
C. prunicola[1]	(2.5) 3.5–6.0 (8.0)	1.0–2.0 (3.0)
C. prunicola[2]	(2.0) 2.3–4.2 (5.0)	(0.6) 0.9–1.8 (2.0)
YLH0003	(2.2) 2.3–4.4 (4.9)	(1.2) 1.3–2.2 (2.4)
YLH0009	(1.8) 2.3–3.6 (3.7)	(0.9) 1.0–1.9 (2.3)
NC1642	(2.1) 2.4–4.5 (7.1)	(0.9) 1.1–2.0 (2.2)
C. endophytica 9094*	(2.5) 3.1–3.4 (4.4)	(1.3) 1.6–1.8 (2.4)
C. endophytica 9055	(2.1) 2.1–4.1 (4.3)	(0.9) 1.0–1.9 (2.0)

Table 3. Macroscopic characteristics of the strains after 14 d of growth on 2% MEA at 25°C with natural light/dark cycles. Color terms and codes are from Kornerup and Wanscher (1967). FL0068 and FL1226 were selected as outgroup taxa for the final analysis (Fig. 1), but they had identical ITSrDNA and *TEF1a* sequences. Therefore, we characterized only one of them (FL0068). Asterisks: characterized by del Olmo-Ruiz (2012). 1: ex-type, 2: ex-paratype.

Strain	Diameter (mm)	Color main colony	Color reverse colony	Medium color	Aerial mycelium
*C. hoffmanni**	42	Pale orange (6A2)	Pale orange (6A2)	absent	absent
dc2070*	30	Pale orange (6A2)	Pale orange (6A2)	absent	absent
*C. lignicola**	45	Black, brown-orange (7E4)	Black	absent	absent
*C. fasciculata**	48	Red-gray (7B2)	Red-gray (7B2)	absent	absent
FL0766*	32	Pale orange (6A2)	Pale orange (6A2)	absent	absent
FL0232*	35	Pale pink (7A2)	Pale pink (7A2)	absent	present
FL1248*	42	Pale orange (6A2)	Pale orange (6A2)	absent	absent
FL0068*	42	Pale orange (6A2)	Pale orange (6A2)	absent	absent
C. prunicola[1]	21	Pastel red (7A5)	Pink-orange (6A6)	absent	sparse
C. prunicola[2]	22	White; pink (7A3)	Light pink-orange, Salmon (6A4)	absent	sparse
YLH0003	36	Pale pink (7A2)	Pale pink (7A2)	absent	absent
YLH0009	39	Pastel red (7A4)	Pastel red (7A4)	absent	present
NC1642	38	Pastel red (7A4)	Pastel red (7A5)	absent	present
C. endophytica 9094*	42	Orange white (5A2)	Orange white (5A2)	absent	absent
C. endophytica 9055	25	Pink (7A3)	Pale orange (6A3)	absent	present

Table 4. Macroscopic characteristics after 14 d of growth on 2% PDA at 25°C with natural light/dark cycles. Color terms and codes are from Kornerup and Wanscher (1967). FL0068 and FL1226 were selected as outgroup taxa for the final analysis (Fig. 1), but they had identical ITSrDNA and *TEF1a* sequences. Therefore, we characterized only one of them (FL0068). Asterisks: characterized by del Olmo-Ruiz (2012). Data for the ex-type of *C. prunicola* were obtained from Damm et al. (2010). 1: ex-type, 2: ex-paratype.

Species or isolate	Diameter (mm)	Color main colony	Color reverse colony	Medium color	Aerial mycelium
*C. hoffmanni**	31	Red-brown (8D8); Pink (7A3)	Red-orange (7A8); Orange (5A6)	Orange (5A6)	present
dc2070*	29	Pale yellow (4A2)	Pale yellow (4A2)	absent	present
*C. lignicola**	33	Pastel red (7A4); Brown-gray (7E3); Light brown (6D4)	Gray-orange (5B4); Brown-gray (7E3); Light brown (6D4)	absent	present
*C. fasciculata**	42	Brown-gray (7E2);Gray (7B1)	Olive (3E4); Olive-gray (3F2); Yellow-gray (3B2)	absent	present
FL0766*	29	White; Pink (7A3)	White; Pink (7A3)	absent	absent
FL0232*	20	Light pink-orange, salmon (6A4)	Light pink-orange, Salmon (6A4)	absent	present
FL1248*	22	Orange (6A7)	Orange (6A7)	absent	absent
FL0068*	35	Red-orange (7A6)	Orange-white (6A2)	absent	sparse
C. prunicola[1]	28	Light pink-orange, salmon (6A4)	Light pink-orange, Salmon (6A4)	absent	sparse
C. prunicola[2]	21	White; Pale orange (6A2)	Light orange (5A5)	absent	present
YLH0003	31	Light pink-orange, salmon (6A4)	Orange (6A5)	absent	sparse
YLH0009	30	Pale pink (7A2)	Light pink-orange, Salmon (6A4)	absent	present
NC1642	31	Pale orange-pink (6A2)	Light orange (5A5)	absent	present
C. endophytica 9094*	16	White; Orange-white (6A2)	Orange-white (6A2)	absent	present
C. endophytica 9055	26	White; Pale pink (7A2)	Pale orange (6A3)	absent	present

a portion of the adjacent LSUrDNA were amplified as a single fragment (~1200 base pairs, bp) with primers ITS1F and LR3 (Vilgalys & Hester 1990, Gardes & Bruns 1993). The partial largest subunit of RNA polymerase II (*RPB1*, ~800 bp) was amplified with modified primers RPB1-Af (GARTGYCCDGGDCAYTTYGG) and RPB1-Crev (CCNGCDATNTCRTTRTCCATR) (Stiller & Hall 1997; Matheny et al. 2002). The partial translation elongation factor 1-alpha (*TEF1a*, ~1000 bp) was amplified with primers 983F (GCYCCYGGHCAYCGTGAYTTYAT) and 2218R (ATGACACCRACRGCRACRGTYTG) (Rehner & Buckley 2005), with thermocycler conditions following del Olmo-Ruiz (2012). Each 20 µL reaction contained 10 µL of REDTaq ReadyMix (Sigma-Aldrich, St. Louis, MO, USA), 0.8 µL of each 10 µM primer, 1.3 µL of 15 mg/mL BSA (New England BioLabs, Ipswitch, MA, USA), 3.1 µL of PCR water, and 4 µL of DNA extract. PCR amplification was confirmed with gel electrophoresis on 1% agarose gel with SYBR Green I (Molecular Probes, Invitrogen; Carlsbad, CA, USA). PCR products were cleaned by adding 1 µL ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) to each well and incubating in a thermocycler at 37°C for 60 min and then at 80°C for 15 min. Cleaned products were sequenced bidirectionally with the original primers (5 µM) on an Applied Biosystems 373*xl* (Foster City, CA, USA) at the University of Arizona Genetics Core (UAGC). Contigs were assembled and quality checked with *phred/phrap* (Ewing et al. 1998) in Mesquite v2.75 (Maddison and Maddison 2017). Base calls were manually checked by viewing the chromatograms on Sequencher v.4.5 (Gene Codes, Ann Arbor, MI, USA).

Taxon sampling for phylogenetic analyses

Our initial taxon sampling was based on analyses of LSUrDNA by Garcia et al (2006), del Olmo-Ruiz (2012), and Friebes et al. (2016), with later taxon sampling constrained to species in "clade II" *sensu* Garcia et al. (2006). Additional endophytic isolates were included following del Olmo-Ruiz (2012) (Table 1). Following preliminary analyses of our original pool of 16 strains (Table 1, Fig. S1), a subset was selected for the final phylogenetic analyses to identify the taxonomic placement of 9094 and 9055.

For the final analyses we included two additional strains not characterized above. First, in surveys during 2016, U′Ren et al. (submitted) isolated an endolichenic fungus (isolate code IL0111) with affiliation for the lineage containing 9094 and 9055. IL0111 was isolated from a healthy, mature thallus of the lichen *Flavoparmelia caperata* (L.) Hale in northern Michigan, USA (U′Ren et al., submitted). The thallus was surface-sterilized and processed as for the endophyte sampling described above (U′Ren et al. 2012). Routine BLAST analyses of the ITSrDNA sequence of IL0111 in mid-2018 revealed the release, in late 2017, of an ITSrDNA sequence for an isolate labeled '*C. cephalothecoides*', a species described originally from soil in Japan by Kamiya et al. (1995). This ITSrDNA sequence was published by Han et al. (2017) and represents a strain isolated in Tibet (isolate code L821). Although '*C. cephalothecoides*' was identified tentatively as potentially related to 9094 and 9055 on the basis of high ITSrDNA similarity, the isolate represented in GenBank (Han et al. 2017) is not from the type specimen. Thus we are cautious in our treatment of '*C. cephalothecoides*' in our analyses, as we are not able to confirm that morphological data from the original description (Kamiya et al. 1995) are consistent with the strain used to generate the available ITSrDNA sequence (Han et al. 2017). Therefore we analyzed our final data set twice: once with the ITSrDNA sequence for *C. cephalothecoides* isolate L821 (Han et al. 2017), and once without it.

Phylogenetic analyses

The first analysis focused on 16 strains for which ITSrDNA sequences were available at the start of the present study (Table 1, Fig. S1), and was designed to identify the appropriate outgroup for finer-scale analyses

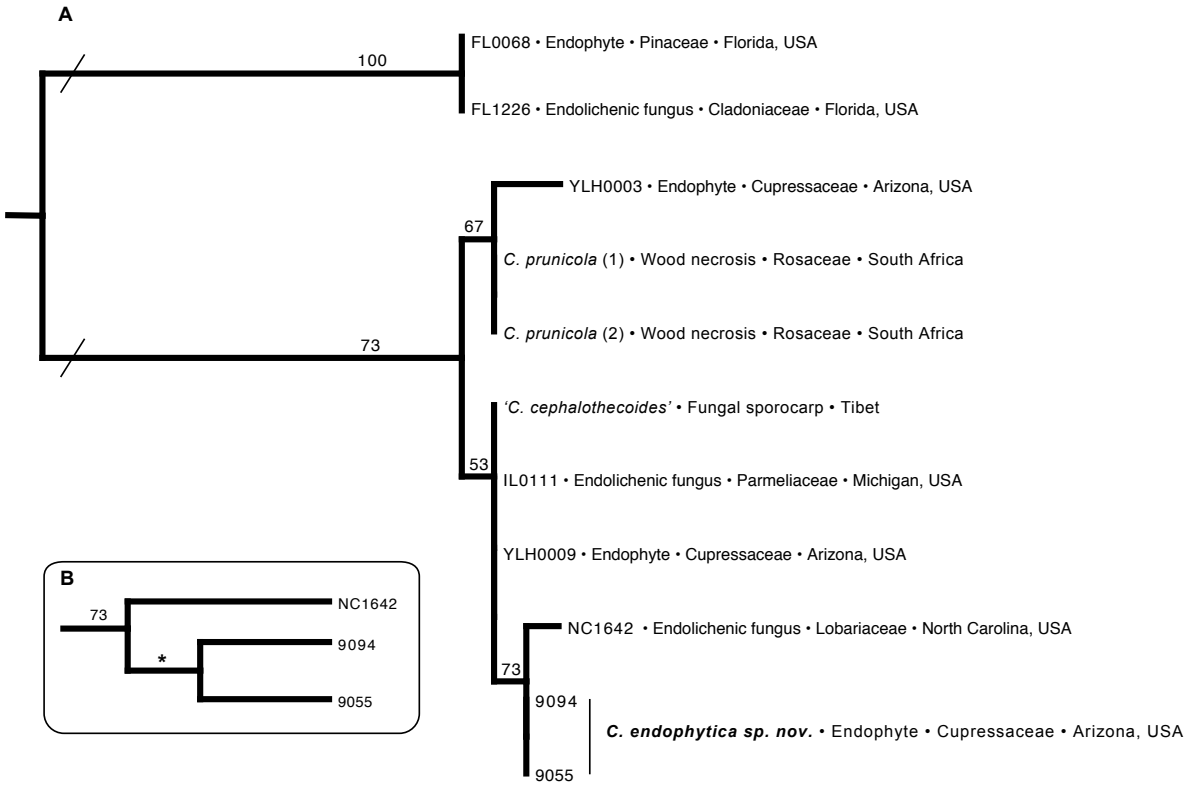

Figure 1. A, Maximum likelihood analysis based on concatenated ITSrDNA and *TEF1a* sequence data. Outgroups were chosen on the basis of analyses presented in Fig. S1. Trees for individual loci are shown in Figs S2 and S3. Values with bootstrap support ≥ 50 are shown above branches. Branch lengths are proportional to substitutions. Hash marks indicate branches shortened by 50% of their length for presentation of the tree. After trimming the final alignment consisted of 1378 characters. The sequence for '*C. cephalothecoides*' represents GenBank accession KY064029.1 (Han et al. 2017), but its relationship to the type for that species described by Kamiya et al. (1995) is unclear. If that sequence is removed, inferences regarding the placement and taxonomy of 9094 and 9055 do not change (Fig. S4). Support values for the clades containing *C. prunicola* and '*C. cephalothecoides*' are relatively low here and in Fig. S1, reflecting variable placement of YLH0009 in the single-gene analyses (see Figs S2 and S3). Exclusion of YLH0009 increases bootstrap support for the clade containing NC1642, 9094, and 9055 to 76%; groups the remaining ingroup sequences (YLH0003, '*C. cephalothecoides*', *C. prunicola* (1), *C. prunicola* (2), and IL0111) into a clade with 70% support (data not shown); and provides support of 73% for the ingroup (data not shown). For a report of similar analyses excluding both YLH0009 and '*C. cephalothecoides*', see the legend for Fig. S4. B, Zoom without branches proportional to substitutions, showing relationship of 9094 and 9055 to NC1642. Asterisk indicates that ITSrDNA and *TEF1a* are 100% identical for 9094 and 9055.

(below). We aligned ITSrDNA sequences in MUSCLE (Edgar 2004) and then trimmed the alignments manually to consistent starting- and ending points prior to analysis in GARLI (Zwickl 2006). We implemented the GTR+I+G model and evaluated topological support via 1000 bootstrap replicates.

Based on that analysis, we designated FL0068 and FL1226 as outgroup taxa for our final analysis. Taxon sampling for the final analysis included strains identified as related to 9094 and 9055 in the first analysis, with the addition per above of IL0111 (U′Ren et al., submitted) and '*C. cephalothecoides*' (Han et al. 2017) (Fig. 1). This analysis was based on concatenated sequences for ITSrDNA and *TEF1a*. Data from *RPB1* were excluded from the final analyses because they provided no resolution in the focal clade. Nucleotide sequences for *TEF1a* were translated into amino acid sequences prior to alignment via MAFFT v7.310 in Mesquite v3.2, with default settings (Katoh & Standley 2013; Maddison & Maddison 2017). The concatenated alignment was analyzed in GARLI per above (Fig. 1). Trees inferred with only ITSrDNA or *TEF1a* are shown as Figs S2 and S3, respectively. The analysis presented in Fig. 1 was repeated without the sequence for '*C. cephalothecoides*' (Han et al. 2017) because of the uncertainty regarding the taxonomic identity of that strain (see Discussion; results are presented in Fig. S4 and do not differ meaningfully from those presented in Fig. 1). All nucleotide alignments [ITSrDNA, 16 strains, Fig. S1; ITSrDNA, 11 strains, Fig. S2; *TEF1a*, eight strains, Fig. S3; the ITSrDNA+*TEF1a* concatenated alignments with '*C. cephalothecoides*' (Fig. 1); and that alignment without '*C. cephalothecoides*' (Fig. S4)] were deposited in TreeBase (S24286).

Taxon sampling for genome analyses

Based on phylogenetic analyses (Fig. 1) we selected six representative and publicly available isolates for whole-genome sequencing. These included the five isolates denoted with daggers in Table 1 (9094, 9055, NC1642, YLH0009, and the ex-type of *C. prunicola*), and the subsequently isolated endolichenic fungus IL0111.

Genome analyses

The strains selected for genome sequencing were grown in 2.4% potato dextrose broth on a rotary shaker (120 rpm) at 25°C, except for the ex-type of *C. prunicola*, which was grown in 2% malt extract broth. Mycelia were ground with a mortar and pestle or pulverized with a bead-beater following lyophilization (U′Ren & Arnold 2017). Total genomic DNA was extracted with the Qiagen DNEasy Plant Mini Kit for all isolates except the ex-type of *C. prunicola*, for which the MoBio Power Plant kit was used. DNA extractions were quantified via Qubit fluorometer (range: 16.9–72.8 ng/uL) and nanodrop (260/280 range: 1.51–1.79; 260/230 range: 0.99–1.33). Samples were normalized to 100 ng of DNA for a 350 bp insert size library. DNA was prepped with the TruSeq Nano High Throughput Library Prep kit according to manufacturer's reference guide (Illumina, Inc., San Diego, USA). DNA libraries were run on a Fragment Analyzer and combined in equimolar concentrations into two 96 sample pools. Libraries were sequenced on a HiSeq with pair-end reads (2x150 bp) at Illumina headquarters (Illumina, Inc. San Diego). Raw sequencing reads were trimmed of adapters and low-quality bases with Cutadapt v1.9.1 (Martin 2011). Reads were assembled into contigs with MEGAHIT v1.1.2 (Dinghua et al. 2016). Reads were mapped to contigs with Bowtie2 v2.3.4 (Langmead & Salzberg 2012), and contigs were assembled into scaffolds with BESST v2.2.8 (Sahlin et al. 2014). Assembly quality was evaluated with QUAST v 4.6.0 (Gurevich et al. 2013).

Gene prediction and annotation was performed with the Funannotate pipeline v1.3.0 (Palmer 2016). Before gene prediction, repetitive sequences were soft-masked with RepeatMasker v4.0.7 (Smit et al. 2013) and the fungal repeat library. Genes were predicted with AUGUSTUS v3.3 (Stanke et al. 2006), with protein evidence and a closely related fungal training set as inputs, and with GeneMark-ES v4.33 (Lomsadze et al. 2005). Protein evidence for AUGUSTUS was generated by aligning the nucleotide sequences against the SwissProt database with Diamond BlastX (Buchfink et al. 2014) and realigning hits with Exonerate v2.4.0 (Slater & Birney 2005). Predictions from AUGUSTUS and GeneMark-ES were combined with Evidence Modeler (Haas et al. 2008). Gene models less than 50 amino acids long or with overlapped repetitive sequences were removed. tRNAs were predicted with tRNAscan-SE v1.3.1 (Lowe & Eddy 1997). Genome characteristics are shown in Table 5.

To assess genome similarity using an alignment-free method we calculated K-mer genomic distances with Mash (Ondov et al. 2016), as implemented in iMicrobe with mash-all-vs-all-0.0.5u1 (www.imicrobe.us) with default parameters (sketch size of 1,000 bp) and k-mer size of 21 bp. Mash converts a collection of sequences into a MinHash sketches (Broder, 1997) and computes the pairwise similarity among samples with the Jaccard index (i.e. the fraction of shared k-mers). A matrix of pairwise distances (1 – Jaccard) was used as the input for a principal coordinate analysis (PCoA) in iMicrobe.

Results

Phylogenetic relationships based on the concatenated ITSrDNA and *TEF1a* data reveal that 9094 and 9055 are sister to one another and are part of a well-supported clade with the endolichenic strain NC1642 (Fig. 1). This distinctive clade is part of a larger group containing '*C. cephalothecoides*' L821, albeit without strong support. The two isolates of *C. prunicola* considered here are reconstructed with marginal support in a distinct clade (Fig. 1). Together these results suggest that 9094 and 9055 are distinct from *C. prunicola* and '*C. cephalothecoides*' L821 (Fig. 1). These relationships are generally consistent with the single-locus trees (Figs S1, S2, and S3), except that the position of YLH0009 differs between reconstructions based on ITSrDNA vs. *TEF1a*, and support values for topologies based on each locus alone are generally low (see Figs S2 and S3). The same core

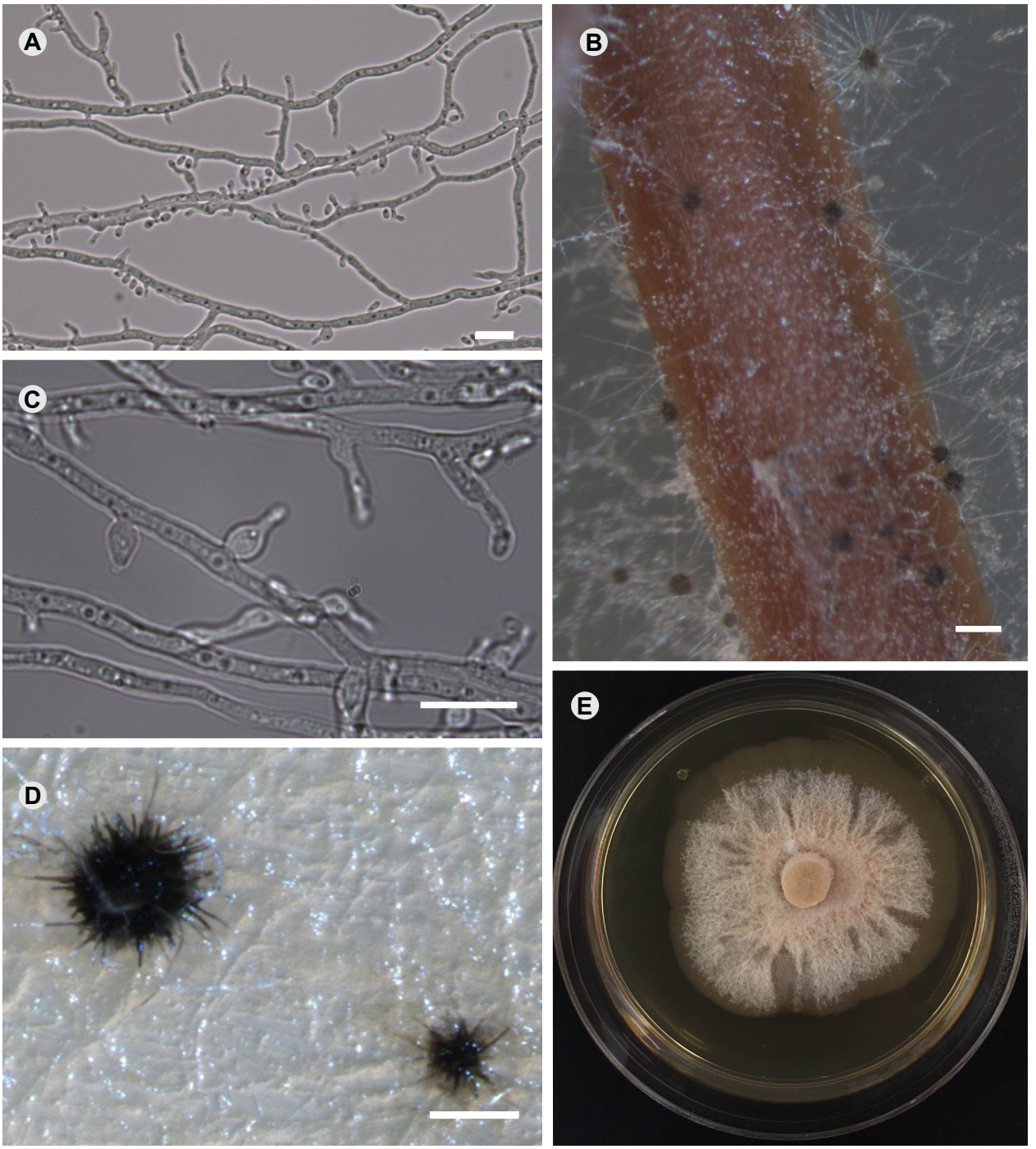

Figure 2. *Coniochaeta endophytica* sp. nov., type isolate 9094. A, C – conidiogenous cells; B – infertile perithecia on autoclaved pine needles; D – infertile perithecia on 2% MEA. E – whole-colony characteristics on 2% MEA after three weeks of growth. Materials were mounted in water and unstained. Scales: A, C = 10 µm; B = 200 µm; D = 100 µm. Compare to Fig. 8 in Damm et al. (2010) for characteristics of *C. prunicola*, and Figs. 1 and 2 in Kamiya et al. (1995) for *C. cephalothecoides.*

relationships were reconstructed when the sequence for '*C. cephalothecoides*' L821 was removed (see Discussion and Fig. S4).

We considered these results in conjunction with morphological features (Figs 2 and 3; Tables 2, 3, and 4) and whole-genome characteristics (Fig. 4 and Table 5). Together these suggest that 9094 and 9055 are distinct relative to closely related taxa that are morphologically similar. Where appropriate we focus our evaluation of morphology on comparisons with *C. prunicola* and *C. cephalothecoides*, the two described species to which 9094 and 9055 appear to be related closely. We also consider in detail NC1642, reconstructed in our analyses as a close relative of 9094 and 9055 (Fig. 1, Figs S1, S2, S3, and S4).

Morphological characterization

Morphological characteristics of 15 strains of *Coniochaeta* are shown in Tables 2, 3, and 4. The isolates considered here all feature discrete, intercalary phialides, lack chlamydospores, and have oblong conidia that vary in size and shape within and among species (Table 2).

The conidia of 9094 and 9055 are similar to, though occasionally slightly shorter in length than, those of the ex-type of *C. prunicola* (Table 2). The ex-type and ex-paratype strains of *C. prunicola* differ somewhat from one another in conidial length and width, and the values observed for 9094 and 9055 are generally within the range encompassed by the two reference strains. However, conidia of 9094 and 9055 are typically more linear and less curved than those of *C. prunicola* (Damm et al. 2010) and occasionally more spherical or ovoid, which is not recorded for *C. prunicola*. Kamiya et al. (1995) described conidia of *C. cephalothecoides* as single-celled, hyaline, and ovoid to ellipsoid or allantoid, 2.5–5.0 µm in length and 1.0–2.0 µm in width, broadly consistent with those of 9094 and 9055. Overall, standard conidial dimensions appear variable and potentially do not convey strong phylogenetic information for the strains considered here (cf. similar conidial sizes of the recognized species *C. hoffmannii*, *C. lignicola*, *C. fasciculata*, and *C. prunicola*, Table 2).

We frequently observed conidiophores in 9094 (Fig. 2) and 9055 that were more ampulliform and at times more likely to be linearly extended than those described for *C. prunicola* (Damm et al. 2010). Damm et al. (2010) also noted distinctive collarettes in *C. prunicola.* We observed only subtle-to-indistinct collarettes in 9094 and 9055. Conidiophores of 9094 (Fig. 2) differ from those depicted by Kamiya et al. (1995) for *C. cephalothecoides*, which are more cylindrical, although the authors describe them as ampulliform in the text: "conidiophores undifferentiated. Conidiogenous cells phialidic, terminal or lateral, borne on branched hyphae, hyaline, variable in shape, mostly ampulliform, sometimes ovoid or cylindrical […] with a distinct collarette" (Kamiya et al. 1995).

Whole-colony characteristics for 15 strains on 2% MEA and 2% PDA are summarized in Tables 3 and 4. In general, the strains considered here range in main colony color from white to pink, orange, red, or brown, and in reverse colony colors from orange-white and pale pink

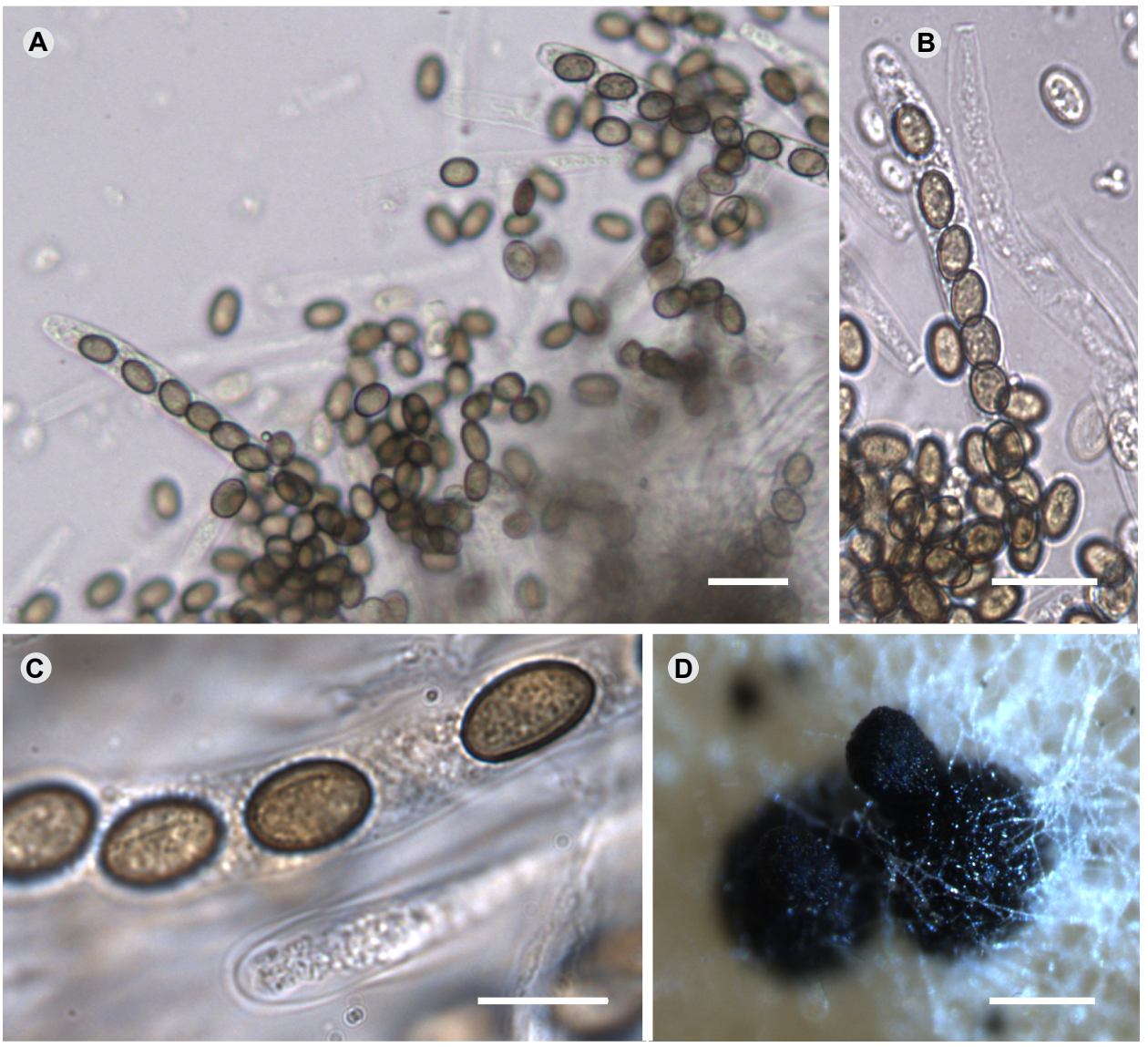

Figure 3. *Coniochaeta* sp. NC1642. A–C – ascospores in asci, with germ slits visible in C (materials were mounted in water and unstained); D – mature perithecium on 2% MEA. Perithecial and ascospore characters of NC1642, closely related to *C. endophytica* and illustrated here to confirm the distinctiveness of the clade it represents relative to characteristics of *C. prunicola* and *C. cephalothecoides.* Compare to Fig. 8 in Damm et al. (2010) and Figs. 1 and 2 in Kamiya et al. (1995), respectively. Scales: A, B = 20 µm; C, 10 = µm; D = 100 µm.

to black (Table 3). The two isolates of *C. prunicola* differ from each other in their coloration from above and below on each medium, suggesting that intraspecific variation in color may occlude interspecific comparisons.

Similarly, 9094 and 9055 differ from each other in these characteristics, and further differ from each other in their growth rates (Tables 3 and 4). Kamiya et al. (1995) reported that *C. cephalothecoides* is pale red (8A3) from above and pale orange from below (5A3), generally consistent with the range of colors observed here. All strains considered here lack secreted pigments in the growth medium on 2% MEA and may have or lack aerial mycelium, with different strains of the same species sometimes differing in the quantity of their aerial mycelium (e.g., see the ex-types of *C. prunicola* in Table 4). Thus colony color and the quantity of aerial mycelium do not appear to be phylogenetically informative.

Formation of perithecia

Endophyte 9094 formed infertile perithecia on 2% MEA and SNA with autoclaved pine needles (Fig. 2). Formation of infertile perithecia was infrequent, and we never observed maturation of those perithecia. Endophyte 9094 did not form perithecia (fertile or infertile) on almond leaf discs after 28 d or 56 d. In contrast, both isolates of *C. prunicola* formed fertile, mature perithecia on pine needles and on almond leaf discs after 28 d. Kamiya et al. (1995) noted that ascomata were produced commonly by *C. cephalothecoides* in culture, in contrast to our observations of 9094. In turn, isolate 9055 did not form perithecia (fertile or infertile) on leaves of almond or peach or on pine needles, and did not discolor leaf discs more than the negative controls (Table S2). The positive controls used in that assay (PLFA, PLFB, and *Colletotrichum* sp.) discolored leaves and frequently produced fruiting structures (Table S2, Figs S5 and S6).

Examination of NC1642

Endophytes 9094 and 9055 were reconstructed as closely allied with the endolichenic isolate NC1642. Unlike 9094 and 9055, NC1642 readily formed mature perithecia in culture and on SNA with autoclaved pine needles (Fig. 3). Perithecia of NC1642 are solitary, immersed or superficial on MEA and PDA, and dark brown (cf. black, *C. cephalothecoides*). They are comparable in diameter to those of *C. prunicola* (Damm et al. 2010), but with a shorter and broader neck. In these ways they are distinct from those of *C. prunicola* and *C. cephalothecoides* (see Damm et al. 2010 and Kamiya et al. 1995). Asci of NC1642 are generally larger than those of *C. prunicola* (Damm et al. 2010) and *C. cephalothecoides* (Kamiya et al. 1995), ranging from (81.4) 83.5–105.7 (105.9) × (5.5) 6.1–8.5 (8.9) µm. They have 8 ascospores per ascus, growing between hyaline paraphyses from the base of the perithecium. Their ascospore dimensions (7.8) 7.9–9.9 (9.9) µm × (4.9) 4.9–6.3 (6.5) µm are comparable to, but somewhat shorter and broader than, those of *C. prunicola* (Damm et al. 2010) and *C. cephalothecoides* (Kamiya et al. 1995). They are uniseriate, 1-celled, brown, broadly-ellipsoidal, smooth-walled, with granular contents and a longitudinal germ-slit. These results suggest that even though 9094 and 9055 did not form perithecia during *in vitro* assays, they are closely related to an isolate (NC1642) with distinctive perithecial and ascus morphology relative to *C. prunicola* and *C. cephalothecoides.*

Genome analyses

Genome data further distinguish 9094 and 9055 from the closely related taxa considered here (Table 5, Fig. 4). Endophytes 9094 and 9055 have smaller genomes and gene numbers than the related strains we considered here (Table 5). Alignment free k-mer analysis by Mash indicates that they, like IL0111, YLH0009, and NC1642, are distinct from *C. prunicola* (Fig. 5). In turn they are partitioned strongly by the second axis of variation from IL0111, YLH0009, and to a lesser degree, NC1642 (Fig. 4).

Taxonomy

Coniochaeta endophytica A.H. Harrington & A.E. Arnold, sp. nov. (Fig. 2)

MycoBank MB830070

Etymology: Referring to isolation from asymptomatic host plant tissue.

Type: USA: Arizona: Tucson, University of Arizona, isolated from asymptomatic photosynthetic tissue of *Platycladus orientalis* (L.) Franco, March 2005, *M. Hoffman*, (ARIZ-M-AN12490 [lyophilized material] – holotype). Reference type ITSrDNA sequence with flanking regions accessioned in GenBank as EF420005. Genome assembly accessioned in GenBank under SNVB00000000.

Ascomata infertile perithecia produced on autoclaved pine needles; solitary or rarely aggregated and superficial

Table 5. Genome characteristics for six isolates of *Coniochaeta*: sequencing coverage, genome length, GC content, estimated gene number, and accession numbers for GenBank. Compared with *C. prunicola*, strains of *C. endophytica* have smaller genomes and fewer genes. Asterisks: compared with all other strains considered here, *C. endophytica* strains have markedly smaller genome sizes (95% confidence interval [CI] for the other four strains: 32,517,397-32,780,184 nucleotides) and numbers of genes (95% CI for the other four strains: 10,740-10,836).

Strain	Fold coverage	Length (nucleotides)	% GC	Number of genes	Accession
C. prunicola [1]	44	32,578,402	54.1	10,757	SMOB00000000
IL0111	103	32,699,488	54.1	10,802	SMZL00000000
YLH0009	113	32,578,834	54.1	10,772	SMZM00000000
NC1642	171	32,738,438	54.1	10,824	SMZN00000000
C. endophytica 9094	140	32,373,870*	54.1	10,704*	SNVB00000000
C. endophytica 9055	153	32,363,791*	54.1	10,692*	SNVC00000000

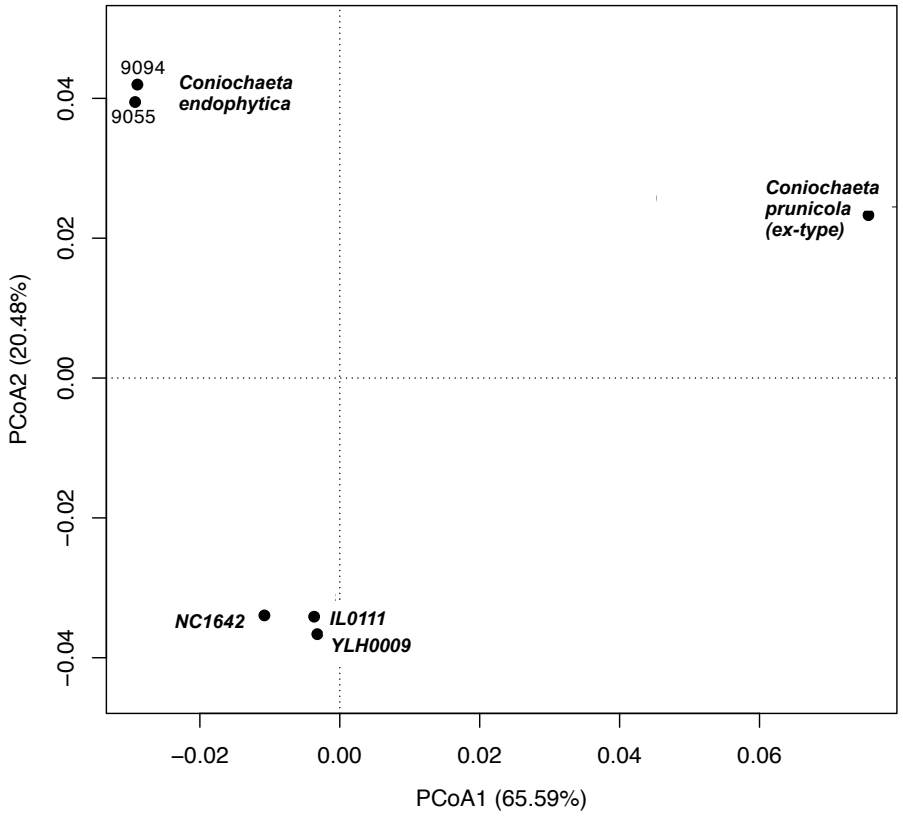

Figure 4. Mash analysis of genome data reveals distinctiveness of *C. endophytica* (9094, 9055) relative to *C. prunicola* and other isolates considered here (NC1642, YLH009, IL0111). K-mer size = 21, sketch size = 1000. As in phylogenetic analyses (Fig. 1, Figs S1–S4), NC1642 is reconstructed as similar to, but distinct from, 9094 and 9055.

or immersed on SNA and MEA; globose and lacking a neck; densely covered with dark brown unbranched setae; diameter variable from 2 to 20 µm, remaining immature with no asci or ascospores produced. *Conidiogenous cells* present either as reduced cylindrical protrusions from the hyphae or as discrete phialides; phialidic conidiogenous cells ampulliform, 5.2–10.3 × 2.3–2.9 µm at the widest part, only monophialides observed with occasional phialides of twice the length with a central constriction, interpreted as compound phialides. *Conidia* hyaline, smooth-walled, oblong and occasionally curved; abundant on all media tested. Conidia ellipsoidal to fusiform (2.5) 3.1–3.4 (4.4) µm × (1.3) 1.6–1.8 (2.4) µm. Vegetative hyphae hyaline, without chlamydospores. No microcyclic conidiation observed. Colonies after two weeks of growth on 2% MEA flat with fine felt-like aerial mycelium, orange white (9094) to pink (9055) from above, orange white (9094) or pale orange (9055) from below, diameter of 42 mm (9094) or 25 mm (9055). Sterile perithecia inconsistently formed after one month. From above, colonies on 2% PDA after two weeks of growth white, with orange-white (9094) or pale pink (9055) tones, with aerial mycelium not evenly distributed across the colony; colony undersides orange-white (9094) to pale orange (9055). Colony diameter after two weeks on PDA 16 mm (9094) and 26 mm (9055); colony surface with fine-felt like aerial mycelium, darker toward the center. No perithecia formed after three months (9055). Images are presented in Fig 2.

Specimens examined. USA, Arizona, Tucson, University of Arizona (9094), isolated from surface-sterilized, asymptomatic photosynthetic tissue of *Platycladus orientalis* (L.) Franco, in March 2005 by M. Hoffman. Voucher specimens of isolate "9094" deposited in the culture collection of the Robert L. Gilbertson Mycological Herbarium at the University of Arizona, and lyophilized type specimen accessioned under ARIZ-M-AN12490. Its ITSrDNA sequence, sequences for *RPB1* and *TEF1a*, and genome sequence are accessioned in GenBank (Table 1, Table 4).

USA, Arizona, Tucson, University of Arizona (isolate 9055) isolated from surface-sterilized, asymptomatic photosynthetic tissue of *Platycladus orientalis* (L.) Franco, in March 2005 by M. Hoffman. Voucher specimens "9055" deposited in culture collection of the RLG Mycological Herbarium (ARIZ), and lyophilized material accessioned under ARIZ-M-AN12489. Its ITSrDNA sequence, sequences for *RPB1* and *TEF1a*, and genome sequence are accessioned in GenBank (Table 1, Table 4).

Note: NC1642 is excluded from *C. endophytica* species designation due to its ready production of perithecia and its genome-scale differentiation from 9094 and 9055. It differs by a single nucleotide in the concatenated ITSrDNA-*TEF1a* data set relative to 9094 and 9055, which are identical to each other at these loci. See Discussion for a perspective on the challenges posed by such limited variation in key loci often used for fungal taxonomy. Its ITSrDNA sequence, sequences for *RPB1* and *TEF1a*, and genome sequence are accessioned in GenBank (Table 1,

Table 4). This isolate has been preserved in a lyophilized state for future examination under ARIZ-M-AN12491 at the Robert L. Gilbertson Mycological Herbarium.

Discussion

A major challenge in mycology is to link newly discovered fungi that are encountered through ecological surveys and barcode sequencing to existing species definitions, a process that is critical for advancing an integrative understanding of fungal diversity. Diverse species of *Coniochaeta* are common in asymptomatic tissues of woody plants and lichens of temperate and boreal North America (U´Ren et al. 2012). The genus is better known for saprotrophy and pathogenicity, but surveys suggest that *Coniochaeta* is rich in endophytes and endolichenic fungi as well (e.g., del Olmo-Ruiz 2012).

Here we describe a new species, *Coniochaeta endophytica*, which was isolated from healthy photosynthetic tissue of *Platycladus orientalis.* The two strains that represent this species, 9094 and 9055, were obtained as endophytes and tentatively identified on the basis of BLAST analysis of the ITSrDNA region as *C. prunicola* (prior to the release of an ITSrDNA sequence for a putative strain of *C. cephalothecoides*, which subsequently was the top taxonomic match; Table S1). Strains 9094 and 9055 have very similar ITSrDNA and *TEF1a* sequences relative to other isolates in the *C. prunicola*/'*C. cephalothecoides*' L821/*C. endophytica* clade. As a result, these three species could be treated inappropriately as one species given current approaches in fungal ecology, whereby unknown, and often sterile, fungi such as endophytes are delimited into operational taxonomic units based on ITSrDNA alone (see also challenges associated with identification based on BLAST results, below and Table S1). In this study we bring a two-locus phylogenetic analysis, morphological characterization, *in vitro* assays, and whole genome sequences to bear to distinguish *C. endophytica* from closely related species. Our results are consistent with its status as a non-pathogenic endophyte in a small clade not known for pathogenicity, which appears as a whole to be rich in endophytic and endolichenic strains from diverse biotic zones across the United States.

The polyphasic approach used here, which integrates phylogenetic, ecological, functional, genomic, and morphological data, is one of many approaches to defining species concepts used in fungi and has gained traction in the past decades. Quaedvlieg et al. (2014) describe this approach as the Consolidated Species Concept, though this general framework has been described previously in the broader taxonomic community as 'integrative taxonomy' (see Dayrat 2005; Valdecasas et al. 2007; Zamora & Calonge 2015). In the present study we used both a traditional approach and insights from genome-scale data to qualify our inferences. We did not use a phylogenomics approach because the process to discover appropriate loci for differentiating these closely related taxa is just beginning, and is a focus of future work. However, the exploration of genome-scale data presented here highlights that marked differences may be observed among strains that are identical or >99% similar at the traditional barcode locus (ITSrDNA) or loci typically used in phylogenetic analyses of fungi (*TEF1a* and *RPB1*). Phylogenome approaches are not tenable at present for most fungal taxonomy studies, in large part because of cost. They may be especially useful in the near term, however, in highlighting the sets of loci or markers that could be used selectively in phylogenetic analyses of taxa at various degrees of relatedness. Ultimately such approaches have the potential to complement and expand current approaches in fungal ecology by illuminating the dual promise and limitations of barcode-based approaches to enumerating and identifying fungal diversity.

One challenge highlighted by our study is that top BLAST matches frequently change as a function of the strains that are present in GenBank. Moreover, we face the challenge of not having ready access to the type of *C. cephalothecoides*, nor validation of that species name for the strain labeled with that name in GenBank. On the chance that the sequence available in GenBank was taxonomically misleading, we removed it from analyses (Fig. S4), but our main conclusions remained unchanged. Thus our results are consistent with (1) differentiation on the basis of morphology and perithecial formation from *C. cephalothecoides* sensu Kamiya et al. (1995), and (2) differentiation on the basis of phylogenetic analyses from '*C. cephalothecoides*' sensu Han et al. (2017).

The disconnect between the growing number of fungal barcoding sequences in public databases and the more slowly growing number of described fungal species highlights the need to increase rates of species descriptions, particularly for undescribed isolates already present in culture collections. While some authors (Hibbett et al. 2011; Kõljalg et al. 2013) point to the proliferation of sequences derived from environmental metabarcoding studies as a key opportunity and challenge in terms of undescribed diversity, culture-based studies like those that generated the isolates examined here remain important. Living cultures can link invisible diversity described by a single sequence in a public database with morphological, ecological, and multilocus phylogenetic data, facilitating phenotypic and genomic studies as well as traditional taxonomic work. Studies of *Archaeorhizomyces* and *Bifiguratus* (Rosling et al. 2011; Torres-Cruz et al. 2017), provide examples: both used environmental sequencing to guide investigators to undescribed lineages, which were then evaluated with traditional approaches. In addition to bringing light to some 'dark matter fungi' (typically defined by lack of cultures or descriptions, these strains also can fill in gaps in the tips of the fungal tree of life. In doing so, they help clarify the evolution of ecological characteristics in widespread, culturable, previously described genera, informing fungal biology more generally. Here, a small part of this undescribed diversity isolated during surveys for endophytic and endolichenic fungi is described, helping to clarify the ecological diversity and relationships in a genus of fungi otherwise known for its pathogenic, saprotrophic, and metabolically diverse species.

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Author contributions

Conceived of study, AEA. Isolated focal strains, MDO, MTH, YLH, JMU. Characterized morphology, MDO, DCS, AHH. Prepared material for genome sequencing, KG. Genome analyses, JMU, DP, NW. Sequencing and phylogenetic analyses, MDO, DCS, AHH. Wrote manuscript, AHH and AEA, with input from all authors.

Supplementary electronic material

Table S1. Focal strains whose sequences were used in this study. Download file

Table S2. Results of second *in vitro* leaf disc assay, 21 d after inoculation. Download file

Figure S1. Relationships of 16 isolates of *Coniochaeta* (Table 1), inferred from maximum likelihood analysis of ITSrDNA. Download file

Figure S2. Relationships of 11 isolates inferred from maximum likelihood analysis of ITSrDNA. Download file

Figure S3. Relationships of 8 isolates inferred from maximum likelihood analysis of *TEF1a*. Download file

Figure S4. Relationship of isolates shown in Fig. 1, inferred from maximum likelihood analysis based on concatenated ITSrDNA and *TEF1a* sequence data, without the sequence representing '*C. cephalothecoides*' (Han et al. 2017). Download file

Figure S5. Representative *in vitro* interactions between almond leaf discs and isolate 9055. Download file

Figure S6. Representative *in vitro* interactions between peach leaf discs and isolate 9055. Download file

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