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A new species of *Infundichalara* from pine litter

Ondřej Koukol

Department of Botany, Charles University in Prague, Benátská 2, CZ-128 01 Prague, Czech Republic

Correspondence to: *ondrej.koukol@natur.cuni.cz*

Abstract — A new species of *Infundichalara* (anamorphic *Helotiales*), a hitherto monotypic genus, is described from pine needle litter. *Infundichalara minuta* sp. nov. forms two conidiophore types. Erect penicillate conidiophores producing catenulate non-septate hyaline conidia form more frequently than *Chalara*-like conidiophores with funnel-shaped collarettes produce wedge-shaped phialoconidia. Although its morphological characters correspond more with *Xenopolyscytalum*, a three-region DNA analyses (ITS, 28S rDNA and EF-1α) of *Infundichalara*, *Xenopolyscytalum,* and related *Chalara* species in the *Helotiales* confirm the phylogenetic position of the new species within *Infundichalara*. It differs from *I. microchona* by smaller phialides and wedge-shaped conidia.

Key words — hyphomycete, dimorphic conidiophores, litter saprotroph

Introduction

Scots pine (*Pinus sylvestris* L.) needles in litter host several microfungal species producing straight erect penicillate conidiophores with catenulate hyaline conidia. *Polyscytalum fecundissimum* Riess and *P. pini* P.M. Kirk & Minter produce uniseptate conidia from denticulate conidiogenous cells growing on a straight, erect conidiophore (Kirk 1983). *Hormiactella asetosa* Hol.-Jech., differing from *Polyscytalum* by conidia with rounded ends and dark grey colonies (Holubová-Jechová 1978), predominantly colonizes bark but may also be found on needles in the litter (Koukol 2007). A monotypic genus *Xenopolyscytalum* Crous was described for *X. pinea* Crous to accommodate two strains isolated from pine needles (Crous & Groenewald 2010). Unlike *Polyscytalum* and *Hormiactella* representatives, *X. pinea* forms white tufts on needles and is characterized by aseptate conidia with somewhat darkened hila and a *Chalara*-like synanamorph. The synanamorph produces phialides with a flaring collarette that differs from the tubular collarette found in *Chalara* (Corda)

Rabenh. (Nag Raj & Kendrick 1975). The only *Chalara* species characterized by a funnel-shaped collarette, *C. microchona* W. Gams, was recently placed into a new genus, *Infundichalara* Réblová & W. Gams (Réblová et al. 2011).

I have isolated one fungus with dimorphic (penicillate/*Chalara*-like) conidiophores from needles of several pine species from various parts of Europe. These isolates are morphologically similar to both *Xenopolyscytalum* (abundant production of penicillate conidiophores) and *Infundichalara* (*Chalara*-like conidiophores with funnel-shaped collarettes) but obviously represent a distinct species. Both morphological and molecular criteria were considered to accommodate the species in a genus reflecting evolutionary relationships.

Materials & methods

Collection and isolation

Cultures were isolated from needles of Scots pine (*Pinus sylvestris*), Swiss stone pine (*P. cembra* L.), Siberian pine (*P. sibirica* Du Tour), and Siberian dwarf pine (*P. pumila* (Pallas) Regel) in the litter. Needles were sampled in pure or mixed pine forests in the NP Bohemian Switzerland (Czech Republic), along the rivers Timpton (Republica Sakha) and Vyerchni Sakujan (Zabaykalskaya Oblast) in Russia, and in the Windachtal Valley, Tyrol (Austria). Surface sterilisation of needles and cultivation conditions were identical to those described in Koukol (2010). Culture characteristics were observed on 2% malt agar (2MA, final sucrose content 2% w/v, 18 g agar, 1 l distilled water) prepared from brewer's wort (Staropramen Brewery, Prague, Czech Republic), potato carrot agar (PCA), oatmeal agar (OA), and potato-dextrose agar (PDA), all prepared from fresh ingredients according to Fassatiová (1986). Agar plates were maintained at laboratory temperature (22–25°C). For microscopy and measurements, conidiophores with conidia were mounted in Melzer's reagent and examined with phase or differential interference contrast (using an Olympus BX51 microscope with digital camera, measured using Quick Photo software). Pictures were further edited in Adobe Photoshop®. Microscopic measurements are reported as the mean ± standard deviation of 30–50 measurements with the extremes given in parentheses.

The holotype specimen is deposited in the PRM (National Museum, Prague, Czech Republic) and living cultures are maintained in the CCF (Culture Collection of Fungi, Prague, Czech Republic).

DNA extraction, amplification and sequencing

DNA was isolated from cultures grown on 2MA using the ZR Fungal/Bacterial DNA kit (Zymo Research, Orange, CA, USA). DNA analyses were made with sequences from the regions ITS1+5.8S+ITS2 (referred to it as the ITS rDNA) and 28S rDNA together with the gene coding for the translation elongation factor 1 α (EF-1α). Amplification and sequencing were performed according to Koukol (2011) with the exception that only the shorter part of EF-1α was amplified with primer pairs 983F a 1567R (Rehner & Buckley 2005). New sequences generated in this study from newly isolated strains are listed in Table 1. Sequences of ITS and 28S rDNA of *X. pinea* were obtained from

Table 1. Accession numbers of new sequences generated in this study.

Species / culture	ITS	28S rDNA	EF-1α
Infundichalara minuta			
CCF4156, ex-type	HE603986	HE603981	HE603978
CCF4157	HE603987	HE603982	HE603977
CCF4158	HE603988	HE603983	HE603976
CCF4159	HE603989	HE603984	—
CCF4160	HE603990	HE603985	—
Xenopolyscytalum pinea			
CPC14225, ex-type	—	—	HE603980
CPC14234	—	—	HE603979

GenBank. The remaining sequences of *Chalara* spp., *Cistella acuum* (Alb. & Schwein.) Svrček, and *Infundichalara microchona* (W. Gams) Réblová & W. Gams originated in Koukol (2011).

Phylogenetic analyses

All markers were aligned using MUSCLE (Edgar 2004) and adjusted manually in BioEdit, v. 4.7.1 (Hall 1999) to maximize similarities. The dataset consisted of 32 sequences and comprised 1322 characters with 272 parsimony-informative sites. Phylogenetic analyses were conducted using MEGA v. 5 (Tamura et al. 2011), PhyML v. 3.0 (Guindon et al. 2010) and MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003). For the Bayesian analyses, General Time Reversible model with invariant sites and a gamma rate distribution (GTR+I+G) was used due to its maximum complexity. The dataset was divided to three partitions (ITS, 28S rDNA and EF-1α). The proportion of variable sites, rate frequencies and the gamma shape were estimated separately for the three partitions. Markov chains were initiated from a random tree and were run for 6,000,000 generations; samples were taken every 100th generation. Posterior probabilities (PP) were used as a Bayesian branch support on the consensus trees. In addition, bootstrap branch support (BS) values were estimated using the Maximum-Likelihood (ML) analyzes with 10,000 bootstrap replicates (MEGA 5.0, PhyML v. 3.0). The tree was rooted with *Tryblidiopsis pinastri* (Pers.) P. Karst. (AFTOL-ID 1319, sequences of 28S rDNA DQ470983, EF-1α DQ471106).

Results

Five strains isolated during this study (CCF4156–4160) formed a strongly supported group sister to *I. microchona* (CBS175.74 and CBS889.73). The isolate B2S2 obtained from pine needles in Tyrol (Austria) clustered with *X. pinea* (Fig. 1). It most probably belonged to *X. pinea*, but was not morphologically observed, because it was lost due to contamination after DNA extraction. *Xenopolyscytalum pinea* (CPC14225 and CPC14234) clustered with low support with *Cistella acuum* (CCF3970).

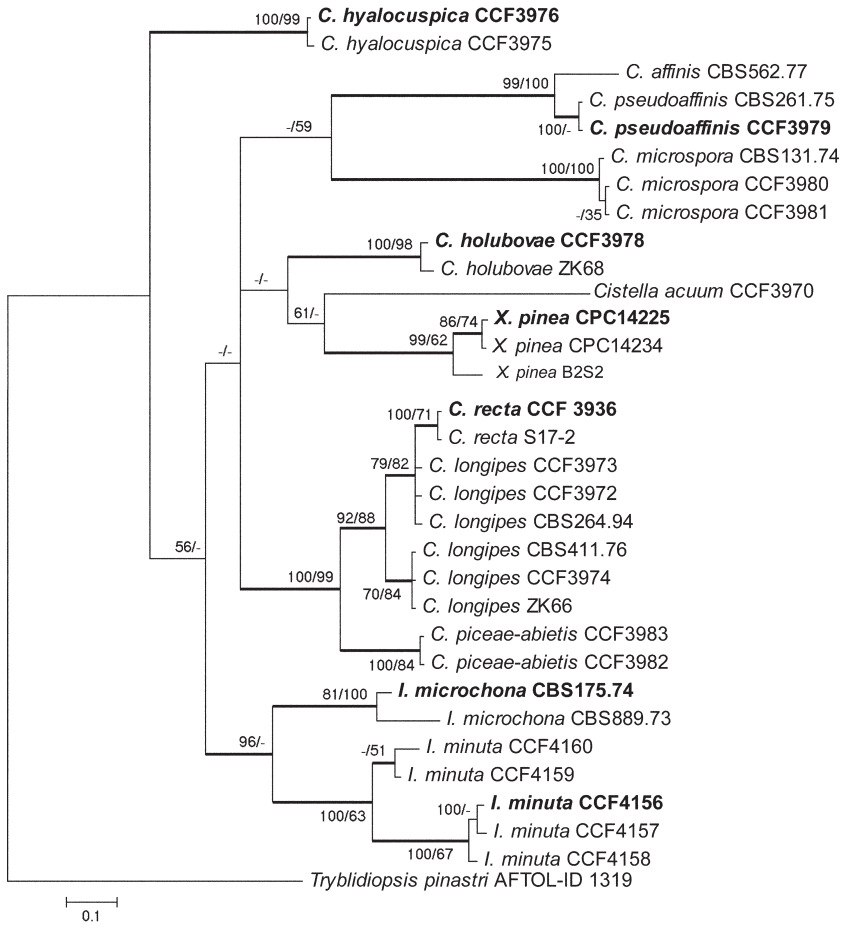

Fig. 1. Phylogenetic relationships of *Infundichalara minuta* and selected members of *Helotiales* derived from ITS, 28S rDNA and EF-1α gene regions using ML and BA analyses. Genera are abbreviated as *C.* = *Chalara*, *X.* = *Xenopolyscytalum*, and *I.* = *Infundichalara*. Ex-type sequences are in bold. Bootstrap values > 50% are indicated along nodes. Thick lines show PP > 0.95. The tree was rooted with *Tryblidiopsis pinastri*.

Taxonomy

Infundichalara minuta Koukol, **sp. nov.** Figs 2, 3 a,b

MycoBank MB 563538

Differs from *Infundichalara microchona* by its smaller *Chalara*-like phialides producing wedge-shaped conidia with truncate base and synanamorph with penicillate conidiophores, ramoconidia, and subcylindrical conidia.

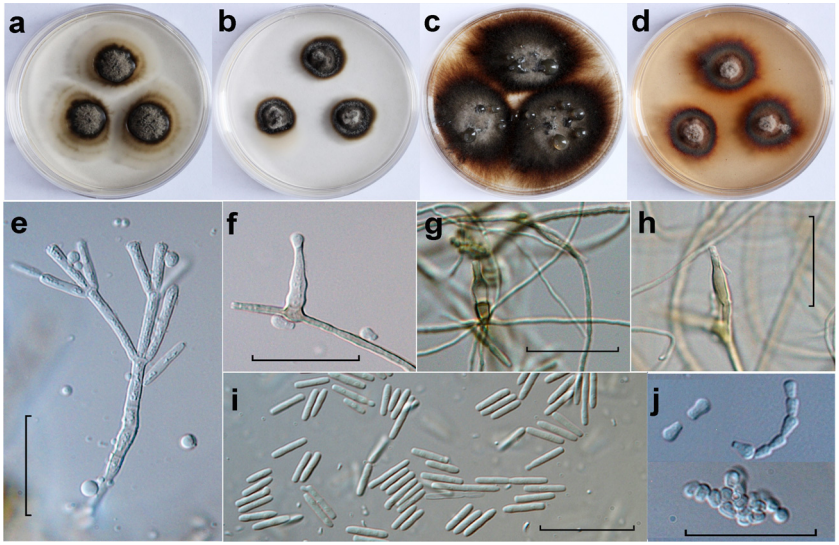

Fig. 2. ***Infundichalara minuta:*** colonial and microscopic morphology. a–d, Morphological variation on 2MA after 30 d incubation at 22–25°C; e, penicillate conidiophore with ramoconidia. f–h, *Chalara*-like conidiophores (f, intercalary; h, terminal); i, conidia produced on penicillate conidiophores; j, conidia produced from *Chalara*-like conidiophores—single, short chain, and cluster. (a,e,g,i,j = ex-type strain CCF4156; b = CCF4160; c,f,h = CCF4158; d = CCF4159. Bars = 20 µm.)

Type: Czech Republic, Bohemian Switzerland National Park, Doubice, Tokáň, forest of *P. sylvestris* on sandstone rocks, 50°53.3′N 14°24.8′E, 450 m asl., on needles in the litter of *P. sylvestris*, 11 Dec 2006, leg. O. Koukol (**Holotype**, PRM899344; ex-type strain CCF4156).

Etymology: *minutus* = referring to the minute *Chalara*-like conidiophores.

Vegetative mycelium consisting of smooth or warted, branched, septate, hyaline to pale brown hyphae 1.5–3 µm diam. Conidiophores dimorphic. Penicillate conidiophores erect, producing branched, catenulate conidia visible as white tufts on needles and in culture, pale brown, 3–5-septate, ≤ 40 µm tall, 3–3.5 µm wide. conidiogenous cells apical, hyaline to pale brown, smooth, 9.5–13.5(–15.5) × 1.5–3.5 µm, proliferating sympodially, usually giving rise to 3 ramoconidia. Ramoconidia hyaline, smooth, aseptate, cylindrical to subcylindrical, (8–)9–13(–16) × 1.5–3 µm. Conidia subcylindrical, hyaline, smooth, aseptate, occurring in unbranched dispersible chains, ends with a flattened, slightly protruding scar, 0.8 µm wide, (5–)7–9.5(–11.5) × 1.5–2.5 µm. Chalara-like conidiophores produced less frequently, formed either by conidiogenous cell on the mycelium or by conidiogenous cell on 1–4 basal cells,

that are erect, cylindrical, unbranched, pale brown, smooth, 4–10 µm tall (rarely ≤ 50 µm), 2.5–4 µm wide. Conidiogenous cells phialidic, hyaline to slightly pigmented, mostly formed terminally or intercalary directly on mycelium or on one supporting cell, subconical to lageniform, (8–)9.5–13.5(–20) × 3–4.5 µm, collarette mostly funnel-shaped, rarely cylindrical, 2.5–5 × 1.5–2.5 µm. Conidia wedge-shaped with truncate base, hyaline, smooth, 3–4(–5) × 2–3 µm, forming either short chains or clusters on top of the phialide.

Colonies variable, different strains produced different morphologies in the dark, after 1 mo at 22–25°C (Fig. 2a–d). Colonies on 2MA erumpent in the centre, with dense aerial mycelium, surface dark gray to brown with pale margin; reverse pale gray to dark brown, reaching 8–28 mm diam. On PDA aerial mycelium developed, colonies gray to pale brown, protuberant in the centre, reaching 10–26 mm diam. On OA dendritic, slimy, aerial mycelium only at margins, surface dark gray to brown, reaching 17–22 mm diam. On PCA gray to pale brown, immersed, with protuberant centre, reaching 8–33 mm diam. The strain CCF4157 produced pale white immersed mycelium on OA, 2MA and PCA and diffused violet pigment into agar on PDA and reached ≤ 40 mm diam. on each agar medium tested. Most intensive sporulation of all strains was observed on 2MA.

Ecology & Distribution — colonizes needles in the litter and humus of *P. sylvestris*, *P. cembra*, *P. sibirica,* and *P. pumila,* in Czech Republic, Austria, Russia (this study) and Sweden (concluded from Lindahl et al. 2010).

Additional specimens examined: **AUSTRIA**, **Tyrol**, Windachtal Valley, sparse *P. cembra* forest above river, 46°57.57′N 11°02.61′E, 1828 m asl., on *P. cembra* needles in litter, 25 Jun 2010, leg. O. Koukol (PRM899346, living culture CCF4160). **RUSSIA**, **Republica Sakha**, sparse growth of *P. sibirica* within *Larix gmelinii* (Rupr.) Rupr. on the right bank of river Timpton near the branching with Ojumrak, 57°11.3′N 126°6.6′E, 550 m asl., on *P. sibirica* needles in litter, 4 Aug 2010, leg. D. Svoboda (living culture CCF4157); sparse growth of *P. sibirica* within *L. gmelinii* on the right bank of river Timpton near the branching with Bolshoy Ulimakh 58°37.6′N 127°1.1′E, 270 m asl., *P. sibirica* needles in litter, 10 Aug 2010, leg. D. Svoboda (PRM899345, living culture CCF4158); **Zabaykalskaya Oblast**, *P. pumila* forest on the right bank of river Vyerchni Sakujan, 56°48.5′N 118°6.9′E, 766 m asl., on *P. pumila* needles in litter, 12 Aug 2010, leg. D. Svoboda (living culture CCF4159).

Discussion

Infundichalara minuta shares morphological characteristics with both *Infundichalara* and *Xenopolyscytalum*. It produces hyaline to slightly pigmented phialides with funnel shaped collarettes and conidia that are truncate on the base and rounded at the apex (Fig. 2f–h,j), which is consistent with *Infundichalara* (Réblová et al. 2011). On the other hand, *Chalara*-like phialides and a *Polyscytalum*-like synanamorph with penicillate conidiophores (Fig. 2e,i) forming white tufts on the substrate are typical for *Xenopolyscytalum*

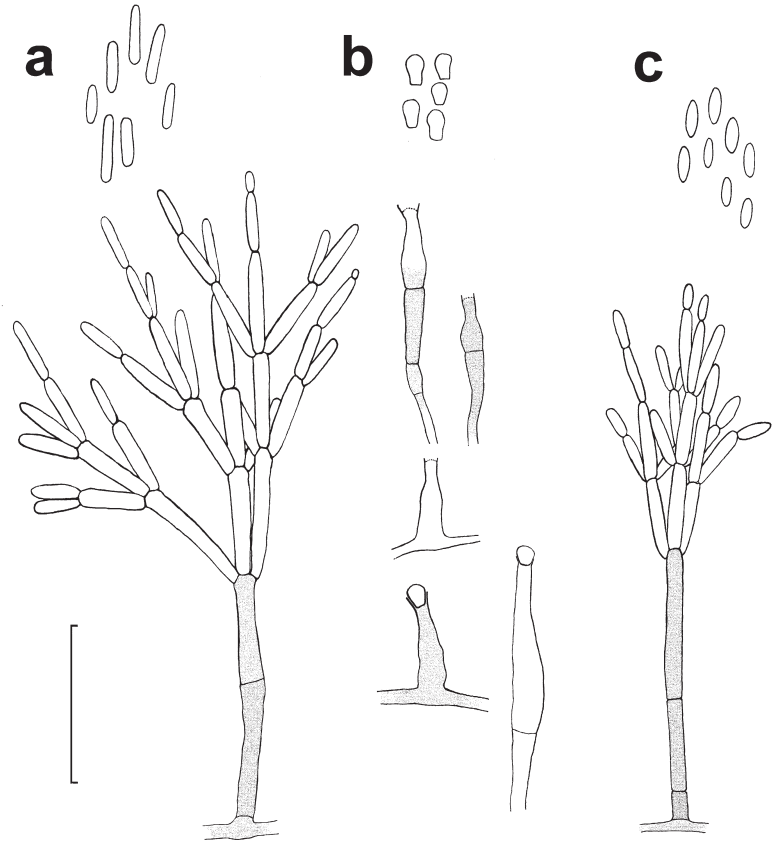

Fig. 3. ***Infundichalara minuta*** (ex-type strain CCF4156): a, Penicillate conidiophore and conidia; b, *Chalara*-like conidiophores and conidia. ***Xenopolyscytalum pinea*** (ex-type strain CPC14225): c, penicillate conidiophore and conidia. (Bar = 20 µm).

(Crous & Groenewald 2010). Molecular data confirmed placement of the new species in *Infundichalara,* despite the closer morphological similarity with *Xenopolyscytalum*. Obviously, the presence of a *Polyscytalum*-like synanamorph is not phylogenetically significant at the genus level but may be used for recognizing the species (e.g., *C. holubovae* Koukol) that form typical *Chalara* phialides and produce a synanamorph with fusiform conidia (Koukol 2011).

The previously monotypic *Infundichalara* and *Xenopolyscytalum* represented by *I. microchona* and *X. pinea* respectively may be distinguished from *I. minuta*

based on morphology. *Infundichalara microchona* produces larger phialides (18–35 × 2.5–4.0 µm) but with shorter collarettes. The similarly sized phialoconidia of *I. minuta* and *I. microchona* differ in shape — wedge-shaped in *I. minuta* but clavate in *I. microchona* (Gams & Holubová-Jechová 1976). *Infundichalara minuta* phialides with the rather narrow collarette typical of *Chalara* s.str. are considered exceptional. Such variation has been noted as well for *I. microchona* (strain CBS125.74) with slightly longer collarettes (to 4 µm deep; Gams & Holubová-Jechová 1976). Vice versa, molecular data support *C. longipes* (strain CBS867.73), which produces phialides with both types of collarettes, in *Chalara* (Koukol 2011). *Xenopolyscytalum pinea* is distinguished by shorter conidia formed on penicillate conidiophores (3–4(–7) × 1.5(–2) µm (Fig. 3c), larger *Chalara*-like phialides (15–25 × 2–3 µm), and cylindrical phialoconidia. Culture characteristics also differ, for *X. pinea* grew more quickly on all tested media (Crous & Groenewald 2010).

In this study, only freshly isolated cultures of *I. minuta* were surveyed. Pure cultures were obtained by picking conidia from penicillate conidiophores (white tufts on the surface-sterilised needles) in primary isolations after several weeks of cultivation. In pure cultures, this synanamorph also dominated and *Chalara*-like phialides were formed relatively rarely. Penicillate conidiophores and phialides were never observed growing from the same hyphae, but potential contamination of surveyed strains may be disproved as individual isolates were obtained independently from three distant localities.

It remains unknown whether *I. minuta* forms predominantly penicillate synanamorph also on field substrates. Similarly, Crous & Groenewald (2010) do not mention the prevalence of either form of *X. pinea* on natural substrates. The assumption that penicillate conidiophores may dominate on the substrate as well is indicated by the white colonies formed by an unknown *Polyscytalum*-like fungus that have been repeatedly found on Scots pine needles sampled close to the type locality of *I. minuta* and cultivated in damp chambers (Koukol 2007). Herbarium specimen "*Polyscytalum* sp. X07" (private herbarium of the author) sampled in September 2000 and surveyed in this study contained a fungus producing relatively dense growth of *Polyscytalum*-like conidiophores (12–18 × 2 µm) with chains of cylindrical, 0–1-septate, hyaline conidia (5–13 × 1.5 µm). Except for the septate conidia, its description matches *I. minuta,* but a fresh collection must first be isolated into pure culture and molecularly analyzed before it can be regarded as conspecific with *I. minuta*.

All *I. minuta* strains were obtained from pine litter, but the species also colonizes Scots pine humus according to 99% similarity (505/507 bp) of the ITS rDNA region with sequence GU559086 obtained in the study of Lindahl et al. (2010). *Infundichalara minuta* seems to have a negligible effect on degradation of structural polysaccharides in the litter. In a parallel study, strain CCF4156,

analyzed for the production of ten hydrolytic enzymes during submersed cultivation in a low-nitrogen medium, expressed only very low amounts of cellobiohydrolase and β-glucosidase (Koukol & Baldrian 2012).

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