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Chalara fraxinea sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland

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Summary

A new species of the hyphomycete genus *Chalara* is described. It has been frequently isolated from stems and branches and sometimes also from roots of wilting and dying *Fraxinus excelsior* in Poland. It differs from previously described species of *Chalara* by its small, short cylindrical phialoconidia extruded in chains or in slimy droplets, morphological features of the phialophores and by colony characteristics. It is non-tolerant to cycloheximide.

1 Introduction

In Poland, intensive dieback of ash (*Fraxinus excelsior* L.) has been observed during the last 10 years. Initially, the dieback was observed only in the north-western part of Poland but currently it occurs all over the country. Trees are dying in all age classes, irrespective of site conditions and regeneration methods. Initially, small necrotic spots, without slime flux, occur on stems and branches. Later, they increase in size, resulting in wilting of leaves, top-dieback of branches and dying of whole trees (Przybył 2002; Kowalski and Łukomska 2005) (Fig. 1). A fungus from the genus *Chalara*, has been isolated from up to 70% of shoots at the beginning of the pathological process. It has also been found in dead roots of alive ash trees (Kowalski 2001; Kowalski and Łukomska 2005). Current studies confirmed that it is importantly involved in the dying of *F. excelsior* in Poland (T. Kowalski, unpublished results).

This fungus could not be assigned to any previously described species of *Chalara* (Nag Raj and Kendrick 1975; Gams and Holubová-Jechová 1976; Holubová-Jechová 1984; Kowalski and Halmschlager 1996; McKenzie et al. 2002). It is, therefore, proposed in this paper as a new species.

2 Materials and methods

Investigations were carried out on six strains isolated from ash main shoots and twigs. After surface sterilization (1 min ethanol 96%, 5 min NaOCl 4%, 30 s ethanol 96%) and removing surface bark, pieces of shoots $5 \times 2 \times 2$ mm were removed and placed in Petri dishes on the surface of 2% malt extract agar (MEA; 20 g l^{-1} malt extract Difco, Sparks, MD, USA, 15 g l^{-1}agar Difco supplemented with 100 mg l^{-1} streptomycin sulphate). The cultures were then incubated at room temperature in the dark. Growing mycelium was transferred to new MEA plates and incubated at 20°C in the dark for at least 6 weeks. Dimensions of phialophores ($n = 30$) and phialoconidia ($n = 30$) were measured for each strain in distilled water. Photomicrographs were taken using a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) in interference contrast.

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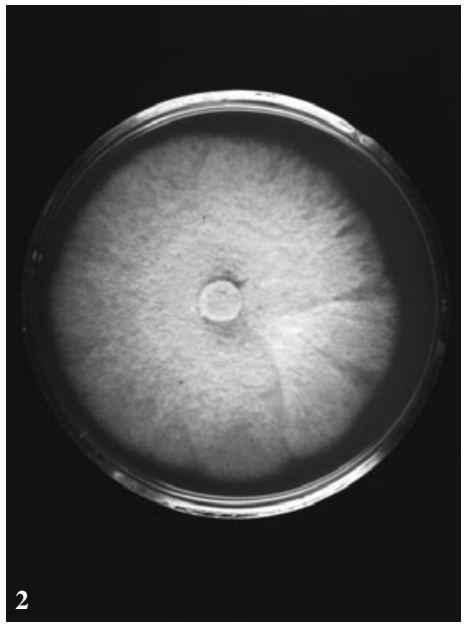


Fig. 1. Wilting of a *Fraxinus excelsior* shoot from which *Chalara fraxinea* was isolated

Fig. 2. A colony of *Chalara fraxinea* (malt extract agar, 20°C, 21 days in the dark)

A possible relationship of the isolated fungus with *Ceratocystis* s. str. was investigated by exposure to cycloheximide (HARRINGTON 1981). Six randomly selected strains of the fungus were inoculated onto MEA amended with 0.1 g cycloheximide l^{-1}. For comparison the following strains were used: *Ceratocystis polonica* (Siemaszko) C. Moreau (two strains), *Chalara ovoidea* Nag Raj & Kendrick (two strains) and *Ophiostoma piceae* (Muench) Syd. & P. Syd. (one strain). Two Petri dishes per strain were used. The experiment was conducted at 20°C in the dark. Extensions of the colonies were measured after 12, 18 or 30 days, depending on their rate of growth (Table 1).

3 Results

Chalara fraxinea T. Kowalski sp. nov.

Etym.: derived from the Latin name of the host genus – *Fraxinus*

Coloniae in agaro maltoso effusae, lanosae, infumigata alba ad ferrugineam fuscam, cum localibus maculis ravis ad obscure ravas, 9–28 mm diametrum post 21 dies in 20°C in tenebris. Hyphae vegetativae translucidae ad oleaginas fuscas, 1.2–3.0 μm latae, cum raris incrassationibus ad 4.2 μm, tenuibus parietibus, glabrae, septatae. Chlamydospora vacant. In nonnullis coloniis veterioribus quam duorum hebdomadum placentata aut in linearum forma stroma pseudoparenchymatica oritur. In agaro maltoso, 0.1 g cycloheximidi 1^{-1} addito, incrementum fungi non est observatum.

Phialophora directe in hyphis aut in stroma oriuntur; singula, saepe ad phialidas imminuta, aut cylindrica ad obclavata, cum 1 ad 3 septis in parte inferiore, oleagina brunnea, recta aut modice incurvata, cum glabris parietibus, sine constrictione ad septa, 24–37 μm longa, in phialide terminata.

Table 1. **Colony diameter (mm) of *Chalara fraxinea* and other fungi on malt extract agar (MEA) and MEA amended with 0.1 g cycloheximide l^{-1}**

Fungi	Incubation period[1] (days)	Strain no.	MEA	MEA with cycloheximide
Chalara fraxinea	30	HMIPC 17040	44	0
		HMIPC 17097	45	0
		HMIPC 18364	32	0
		HMIPC 18370	27	0
		HMIPC 18372	28	0
		HMIPC 18373	18	0
Chalara ovoidea	30	HMIPC 16664	50	0
		CBS 136.88	49	0
Ceratocystis polonica	12	HMIPC 17046	96	0
		HMIPC 17047	93	0
Ophiostoma piceae	18	HMIPC 18367	84	84

[1]Dependent on rate of growth.

In paucorum hebdomadum coloniis phialophora ad 96 μm longa et 3.0–4.2 μm circa basim lata, singula seu cum 1–5 ramificationibus. Phialides etiam in cacumine indistinctarum hypharum vegetativarum apparent.

Phialides subcylindraceae ad obclavatas, nonnumquam in forma lagoenae, 16–24 μm longae, venter brevicylindraceus ad ellipticum, 11–15 × 4–5 μm; collum cylindraceum 5–7/9/2.2–2.7 μm; ratio longitudinis colli et ventris = 0.6 : 1; transitio ex ventre ad collum gradatim, nonnumquam abrupte.

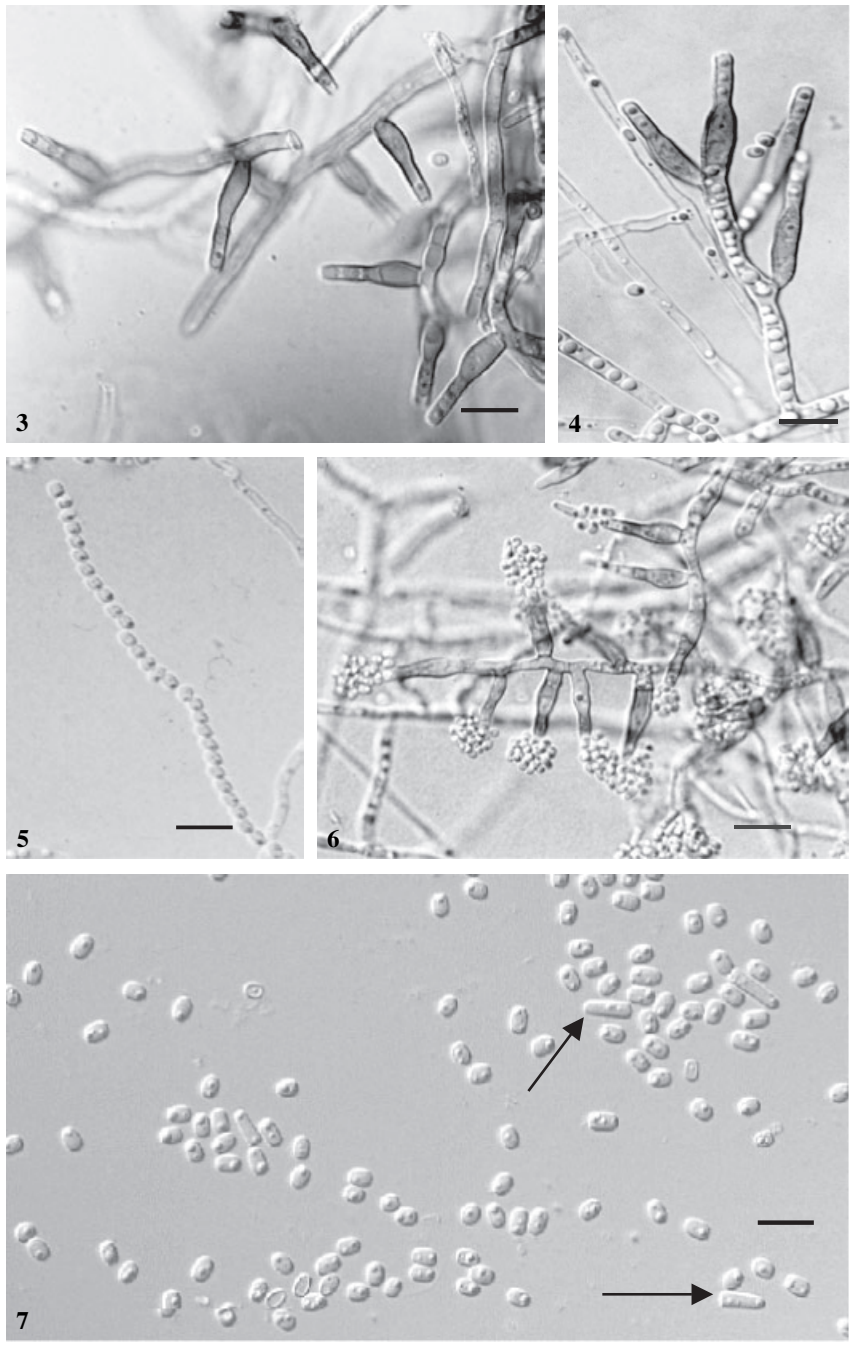
Phialoconidia in catenas breves extrusa, vel multo frequentius in mucinosis guttis, brevicylindracea, apice rotundato vel obtusato, interdum basi truncata cum parvis fimbriis marginalibus, 0–septata, hyalina ad subhyalina, cum duabus oleosis guttis, laevia, 3.2–4.0 × 2.0–2.5 μm; ratio conidii long./lat. = 1.4 : 1. Conidium primum productum breviter clavatum, 6–7 × 2.2–2.5 μm.

Holotypus: cultura desiccata ex ramo *Fraxini excelsioris*, in herbario Zuerich (Z + ZT), T. Kowalski legit, Polonia, Włoszczowa, 06.12.2000 isolata, cultura viva in CBS, Utrecht, Hollandia.

Colony on MEA effuse, cottony, dull white to fulvous brown, with some patches becoming grey to dark grey, 9–28 mm diameter after 21 days at 20°C in the dark (Fig. 2). Vegetative hyphae subhyaline to olivaceous brown, 1.2–3.0 μm wide with rare swellings up to 4.2 μm; thin-walled, smooth, septate with septa 5–21 μm apart. Chlamydospores absent. In some cultures older than 2 weeks with patchy or linear pseudoparenchymatous stroma composed of cells with thickened, dark-brown walls. No growth was observed onto MEA amended with 0.1 g cycloheximide l^{-1} (Table 1).

Phialophores arising directly on the superficial or slightly immersed vegetative hyphae or on pseudoparenchymatous stroma are solitary and scattered, often reduced to phialides, or cylindrical to obclavate up to three septate in the basal part, olivaceous brown, erect, straight or slightly bent, smooth-walled, unconstricted at the septa, mainly 24–37 μm long, terminating in a phialide (Fig. 3). In a few-week-old colonies, phialophores up to 96 μm long and 3.0–4.2 μm wide at the base, simple or with one to five branches (Fig. 4). Phialides occur also terminally on undifferentiated hyphae.

Phialides subcylindrical to obclavate, occasionally lageniform, 16–24 μm long. Venter short-cylindrical to ellipsoidal, 11–15 × 4–5 μm. Collarette cylindrical 5–7/9/ × 2.2–2.7 μm; ratio of mean length of collarette to venter = 0.6 : 1. Transition from venter to collarette gradual, occasionally abrupt (Figs 3 and 4).



Figs. 3–7. Chalara fraxinea. Bar, 10 μm: (3) Phialophores on vegetative hyphae, mostly reduced to phialid. (4.) Long and branched phialophore in a 4-week-old colony. (5) A chain of conidia. (6) Conidia in slimy droplets. (7) Conidia (arrows show first formed conidia)

Phialoconidia extruded in short chains (Fig. 5) or more frequently in slimy droplets (Fig. 6); short-cylindrical, ends rounded or blunt, sometimes with a truncate base bearing small marginal frills, unicellular, hyaline to subhyaline, filled with one or two oil droplets, smooth-walled, 3.2–4.0 × 2.0–2.5 μm (Fig. 7); mean conidium length/width ratio = 1.4 : 1. The first forming conidium is shortly clavate 6–7 × 2.2–2.5 μm (Fig. 7).

Among tested fungi only *Ophiostoma piceae* was not inhibited by cycloheximide (Table 1).

Additional specimen examined to type : Poland, HMIPC 17040 – forest district Włoszczowa, 06.12.2000, local necrosis on shoot; HMIPC17097 – forest district Miechów, 12.06.2004, local necrosis on shoot; HMIPC 18364 – forest district Niepołomice, 20.05.2005, dead shoot; HMIPC18370 – forest district Jędrzejów, 08.06.2005, dead shoot; HMIPC 18372 – forest district Limanowa, 21.06.2005, dead shoot; HMIPC 18373 – forest district Andrychów, 11.07.2005, dead shoot. All strains isolated by T. Kowalski from *F. excelsior* and preserved in the fungal strain collection at the Department of Forest Pathology, University of Agriculture, Kraków, Poland.

4 Discussion

Chalara is characterized by sessile or stalked phialides with basal venters and long collarettes, deep-seated conidiogenous loci and usually cylindrical, hyaline, or pale brown, unicellular or septate phialoconidia, formed singly or in basipetal chains (Nag Raj and Kendrick 1975; Holubová-Jechová 1984). It is represented by approximately 120 species, including anamorphs of some ascomycetous fungi (Nag Raj and Kendrick 1975; McKenzie et al. 2002). Over the last few years, genetic studies have shown that *Chalara* is polyphyletic (Paulin and Harrington 2000). Therefore, new taxonomic divisions have been proposed in the genus *Chalara*. For example, a new genus *Xenochalara* has been proposed for chalara-like fungi with apical wall building conidial development (Coetsee et al. 2000). Four anamorphic *Chalara* species, connected to the teleomorph genus *Ceratocystis*, which form aleuroconidia, were transferred to the genus *Thielaviopsis* (Paulin-Mahady et al. 2002). Paulin and Harrington (1999) distinguished two monophyletic groups within *Chalara*, pathogenic *Ceratocystis* anamorphs and non-plant pathogens.

The prevalent production of conidia by *C. fraxinea* in droplets rather than in chains makes it similar to the genus *Phialophora* Medlar or *Cadophora* Lagerb. & Melin. However, the morphology of conidiophores, phialides and collarettes, as well as its conidiogenesis, prevent classifying this new species in any of the mentioned genera (Cole and Kendrick 1973; Gams and Holubová-Jechová 1976; Harrington and McNew 2003). *C. fraxinea* is not the only species of the genus *Chalara*, which produces conidia in chains and also in slimy droplets. This is observed, e.g. in cultures of *Chalara brevispora* Nag Raj & Kendrick (Nag Raj and Kendrick 1975) and in the *Chalara* state of *Cryptendoxyla hypophloia* Malloch & Cain (Holubová-Jechová 1984). Also other characters of *C. fraxinea* occur in some other species of the genus *Chalara*. For example, stromatic structures are found in colonies of *Chalara agathidis* Nag Raj & Kendrick (Nag Raj and Kendrick 1975). The first produced conidium of *Chalara siamense* Pinnoi, *Chalara schoenoplecti* Wong and others is morphologically different from conidia produced later (McKenzie et al. 2002). Conidia with basal marginal frills occur in *Chalara acuaria* Cooke & Ellis, *Chalara angustata* Kowalski & Halmschlager, *Chalara breviclavata* Nag Raj & Kendrick, *Chalara germanica* Nag Raj & Kendrick and others (Nag Raj and Kendrick 1975; Kowalski and Halmschlager 1996; Coetsee et al. 2000; McKenzie et al. 2002).

Chalara fraxinea is distinctly different from all *Chalara* spp. and *Chalara* anamorphs of *Ceratocystis* described by NAG RAJ and KENDRICK (1975) and from all species described since then (MCKENZIE et al. 2002), primarily by very small and short cylindrical conidia. It is the only species of *Chalara* producing conidia with a length/width ratio of 1.4 : 1 or less. Closely related is *Chalara brevispora*, but its conidia are narrower with a mean ratio of conidium length/width of 1.5 : 1 and the phialophores and collarettes longer (41–145 μm and 9–20 μm respectively). Phialophores of *C. fraxinea* are two to three times shorter and collarettes are not longer than 9 μm. Also the appearance of the *C. fraxinea* colonies on malt agar is unique (NAG RAJ and KENDRICK 1975).

Chalara fraxinea belongs to a group of *Chalara* spp. forming short phialides, as *Chalara austriaca* (Fautr. & Lamb.) Nag Raj & Kendrick, *Chalara microspora* (Corda) Hughes, *Chalara sessilis* Nag Raj & Kendrick or *Chalara fusidioides* (Corda) Rabenh. They differ from *C. fraxinea* by the form of the conidia. *Cryptendoxyla hypophloia* also produces short but hyaline phialides, whereas phialides of *C. fraxinea* are pigmented (MCKENZIE et al. 2002).

HARRINGTON (1981) showed that *Chalara* spp. do not tolerate antibiotic cycloheximide. The lack of tolerance to cycloheximide suggests that *C. fraxinea* might be related to *Ceratocystis* s. str. So far, no teleomorph of this species has been observed in the laboratory cultures or in nature.

Résumé

Chalara fraxinea sp. nov. associé à un dépérissement du frêne (Fraxinus excelsior) en Pologne

Une nouvelle espèce est décrite dans le genre d'hyphomycètes *Chalara*. Cette espèce a été fréquemment isolée de tiges et branches, parfois de racines, de *Fraxinus excelsior* flétris ou fortement dépérissants en Pologne. Elle diffère des espèces précédemment décrites de *Chalara* par les phialoconidies petites et cylindriques, éjectées sous forme de chaînettes ou dans des gouttelettes mucilagineuses, par des caractères morphologiques des phialophores et des caractéristiques des colonies. Elle n'est pas tolérante au cycloheximide.

Zusammenfassung

Chalara fraxinea sp. nov., ein in Polen mit dem Absterben von Fraxinus excelsior assoziierter Pilz

Es wird eine neue Art aus der Hyphomycetengattung *Chalara* beschrieben, die aus dem Stamm und den Zweigen (gelegentlich auch aus den Wurzeln) von absterbenden Eschen (*Fraxinus excelsior*) mit Welkesymptomen in Polen isoliert wurde. Die Art unterscheidet sich von den bekannten *Chalara* spp. durch ihre kleinen, zylindrischen Phialokonidien, die in Ketten oder in schleimigen Tröpfchen ausgestossen werden, sowie durch die Morphologie der Phialophoren und der Kolonie. Der Pilz ist nicht tolerant gegenüber Cycloheximid.

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