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Xenochalara, a new genus of dematiaceous hyphomycetes for chalara-like fungi with apical wall building conidial development

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Ceratocystis autographa Bakshi is unusual amongst the ophiostomatoid fungi in that it produces two distinct anamorphs, typifying different teleomorph genera. A chalara-like anamorph characteristic of the genus *Ceratocystis sensu stricto* Ellis & Halst. and a *Sporothrix* Hektoen & C.F. Perkins anamorph typical of the genus *Ophiostoma* Syd. & P. Syd., have been described for the fungus. In a recent study, an isolate resembling *C. autographa*, that was collected from *Juniperus* L., was shown to have a different type of conidiogenesis to that of typical species of *Chalara* (Corda) Rabenh. Furthermore, cycloheximide tolerance and the presence of rhamnose in the cell walls of this isolate also suggested that it was more closely related to *Ophiostoma* than to *Ceratocystis s. str.* Parsimony analysis of ribosomal DNA sequence data in this study showed that this isolate formed a monophyletic group, distinct from *Ceratocystis* isolates and from *Ophiostoma ulmi* (Buisman) Nannf. The chalara-like anamorph from *Juniperus* is also distinct from typical *Chalara* anamorphs of *Ceratocystis s. str.* in that it produces conidia from phialides via apical wall building, in contrast to the ring wall building typical of *Chalara* species. We, therefore, propose the establishment of a new genus, *Xenochalara* gen. nov., for this and other chalara-like species producing conidia by apical wall building.

Keywords *Ceratocystis sensu stricto*, phylogeny, *Ophiostoma*, rDNA, *Xenochalara*.

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Introduction

The ophiostomatoid fungi include three genera namely, *Ophiostoma* Syd. & P. Syd., *Ceratocystis sensu stricto* Ellis & Halst. and *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. (De Hoog & Scheffer 1984). Anamorphs of *Ceratocystis s. str.* are restricted to the genus *Chalara* (Corda.) Rabenh., which produce conidia through ring wall-building (Minter *et al.* 1982), contain no cellulose (Rosinski & Campana 1964) or rhamnose (Spencer & Gorin 1971) in their cell walls, and are sensitive to the antibiotic, cycloheximide (Harrington 1981). In contrast, species in *Ophiostoma* and *Ceratocystiopsis* form conidia through apical wall building and the anamorphs are in genera other than *Chalara* (Upadhyay & Kendrick 1975; De Hoog & Scheffer 1984; Mouton *et al.* 1994). These species also contain cellulose and rhamnose in their cell walls (Rosinski & Campana 1964; Spencer & Gorin 1971) and are tolerant to high concentrations of cycloheximide (Harrington 1981).

Ceratocystis autographa Bakshi is unusual amongst the ophiostomatoid fungi in that it is reported to produce two anamorph forms with distinct modes of conidial development. Bakshi (1951) first isolated *C. autographa* from the galleries of the bark beetles, *Dryocoetes autographus* and *Hylurgops palliatus* infesting *Larix leptolepis* (Japanese larch) in Scotland. Bakshi described *C. autographa* as producing two distinct anamorphs (Bakshi 1951). In the one, the hyaline, round to ovoid conidia are produced exogenously and are carried singly or in clusters. The other anamorph produces conidia endogenously in flask-shaped conidiophores. The conidia formed by the latter anamorph are hyaline, barrel-shaped, one-celled and are formed in chains (Bakshi 1951).

Subsequent to the initial description of *C. autographa* (Bakshi 1951), much controversy has arisen concerning the taxonomy of the chalara-like anamorph. Hunt (1956) confirmed the description of Bakshi, and further observed that the conidia were cylindrical with truncate ends. Nag Raj and Kendrick (1975) and Gams and Holubová-Jechová (1976) also described the barrel-shaped endoconidia as short, clavate, rounded at the apex with truncate bases. Nag Raj and Kendrick (1975), furthermore, described the endoconidiophores as phialidic, lageniform with a distinct constriction at the base of the collarette and identified this anamorph as a species of *Chalara*. Unfortunately, no culture of the type *C. autographa* was preserved. In a subsequent study, Gams and Holubová-Jechová (1976) collected an isolate from *Juniperus* that they identified as *C. autographa* (CBS 670.75). Wingfield *et al.* (1995) re-examined the *Juniperus* isolate and observed that the chalara-like anamorph was unusual. Although the conidia were formed in chains, they had single attachment points that are typical of apical wall building. This was in contrast to ring wall-building conidial development, typical of *Chalara*, where conidia that occur in chains have two attachment points (Minter *et al.* 1982).

Wingfield *et al.* (1995) showed that the *Juniperus* isolate had a high degree of tolerance to cycloheximide. This suggested a closer relationship to the genus *Ophiostoma* than to *Ceratocystis s. str.* Although a *Sporothrix* anamorph was commonly observed on the type (IMI 20162) of *C. autographa*, Wingfield *et al.* (1995) found that the *Juniperus* isolates very rarely formed a *Sporothrix* synanamorph, and that the latter was usually inconspicuous when present. Furthermore, in a comparison of sequence data from the SSU and LSU rDNA genes, Hausner *et al.* (1993) suggested that this isolate (CBS 670.75) could have been misidentified as it grouped separately from both

Ceratocystis s. str and *Ophiostoma* species in their analyses. Based on its unique mode of conidiogenesis, Wingfield *et al.* (1995) suggested that this fungus belonged to a genus other than *Chalara,* and that its morphological similarity to *Chalara* is probably a result of convergent evolution.

The aim of this study was to consider the placement of isolate CBS 670.75, previously labelled as *C. autographa,* within *Ceratocystis sensu lato.* We made use of the variable ITS 1 and ITS 2 regions, as well as the conserved 5.8S gene, of the rDNA operon in an attempt to resolve a long-standing taxonomic enigma surrounding this culture. Previously published sequence data of isolates known to reside in the genera *Ceratocystis s. str.* and *Ophiostoma.* were chosen for comparative purposes (Witthuhn *et al.* 1998, 1999). *Ophiostoma ulmi* (Buisman) Nannf. was chosen as a representative of the genus *Ophiostoma.* Sequence data from isolates of *Ceratocystis fimbriata* Ellis & Halst. and *Ceratocystis douglasii* (R.W. Davidson) M.J. Wingf. & T.C. Harr. (Witthuhn *et al.* 1998, 1999), were chosen to represent *Ceratocystis s. str.* In addition, sequence data of *Chalara australis* Walker and *Chalara neocaledoniae* Kiffer & Delon (Witthuhn *et al.* 1998) were also included in this study to provide further comparison with well-defined species of *Chalara.*

Materials and Methods

Morphology

The isolate of the fungus from *Juniperus* in the Netherlands (CBS 670.75) and single conidial transfers (CMW 1099, CMW 1901 and CMW 2547) are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria, South Africa. These isolates were grown on 2% MEA (20 g Malt Extract, 20 g Biolab Agar/1000 ml H_20) in Petri dishes and incubated at 25°C under near-ultraviolet light. The holotype specimen of *C. autographa* (IMI 20162) was also examined and compared with the cultures used in this study. Scanning and transmission electron micrographs from the study of Wingfield *et al.* (1995) were also used in our interpretation of the conidium development of the isolate CBS 670.75.

Molecular studies

Single conidial transfers (CMW 1099, CMW 1901 and CMW 2547) were grown on cellophane discs placed on MEA and incubated at 25°C until a mycelial mat had formed. The discs were transferred to sterile Petri dishes, lyophilised and stored at -20°C. Nucleic acid was extracted from the lyophilised material following a modification of the procedure described by Raeder and Broda (1985). The freeze-dried mycelium was transferred to sterile Eppendorf tubes and 500 µl Extraction Buffer (200 mM Tris-HCl, pH 8.5; 250 mM NaCl; 25 mM EDTA, 0.5% SDS) added. The mixture was immersed in liquid nitrogen and ground with a plastic pestle in an Eppendorf tube. Phenol (350 µl) was added after grinding and the solution homogenised. Chloroform (150 µl) was added and the suspension was rapidly mixed, after which it was centrifuged for 1 hr at 10 000 rpm and 4°C. The aqueous phase was immediately transferred to sterile Eppendorf tubes and 1 volume of chloroform was added. The upper phase was transferred to sterile Eppendorf tubes and the DNA precipitated by the addition of 0.54 vol. isopropanol and 0.1 vol. 3 M NaAc. After incubation for 60 min at -20°C, the mixture was centrifuged for 30 min at 8 000 g at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in 100 µl sterile H_20. The DNA yield was assessed by agarose gel electrophoresis.

A region within the ribosomal DNA operon, which includes the 5.8S gene and the internal transcribed spacer regions (ITS 1 and ITS 2), was amplified using the Polymerase Chain Reaction (PCR) (Saiki *et al.* 1988). Primers ITS1 (5'-TCCGTAGGTGAACCT-GCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (10 OD) were used for amplification (White *et al.* 1990). Reactions were performed in a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, U.K.) for 35 cycles using *Taq* DNA Polymerase (Promega Corporation. Madison, U.S.A.) and using conditions described by Visser *et al.* (1995). The final reaction conditions were 6.25 mM $MgCl_2$, 1.6 mM dNTPs and 50 pmol of each primer in a 100 µl reaction volume.

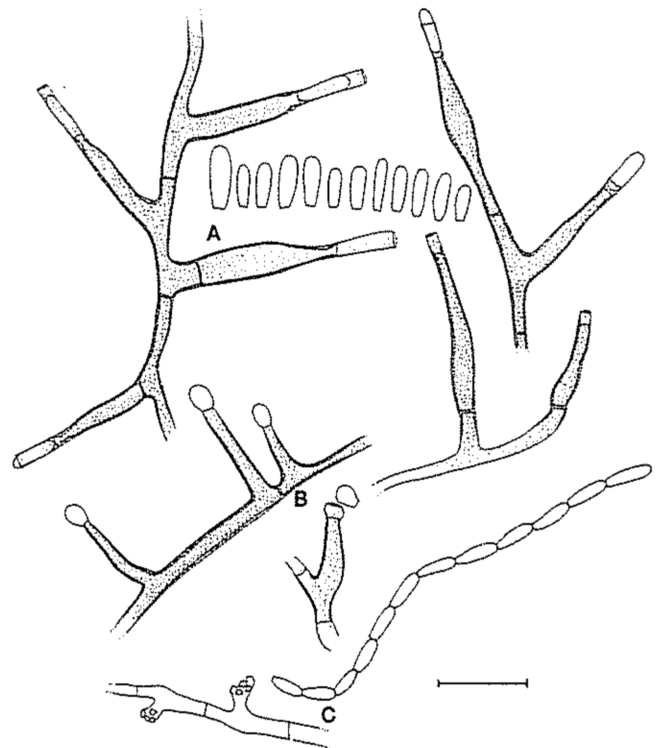

Figure 1 *Xenochalara juniperi* and its *Sporothrix* synanamorph sporulating on malt extract agar. **A**, Conidiophores, lageniform conidiogenous cells and short-clavate, catenulate conidia. **B**, Phialophora-like synanamorph. **C**, *Sporothrix* synanamorph with catenulate conidia (bar = 10 µm).

The amplified DNA fragments were visualised on a 1% (w/v) agarose gel to assess the size and quality of amplification products. The products were purified using the Magic PCR Preps (Promega Corporation. Madison, USA) and the resulting fragments were sequenced using the Sequenase PCR Product Sequencing Kit from Amersham (USB, Cleveland, Ohio, USA). The ITS1, ITS4, CS2 (5'CAATGTGCGTTCAAAGATTCG3') and CS3 (5'CGAATCTTTGAACGCACATTG3') primers (White *et al.* 1990; Visser *et al.* 1995) were used to determine the DNA sequence of both strands. The sequence data were visually aligned and the phylogenetic relationships determined using PAUP version 4.0b2a (Phylogenetic Analysis Using Parsimony) (Swofford 1999). The branch and bound options, as well as the heuristic options were used in the analysis. Confidence in the clustering and tree topology was determined using Bootstrap analysis and the gl statistic. Sequence data were deposited in GenBank (NF184887–184889).

Results

Morphology

Morphological characteristics observed for the chalara-like state of the *Juniperus* isolate and the holotype of *C. autographa* were similar. Furthermore, single attachment points were observed on the conidia, which is consistent with the detailed observations of Wingfield *et al.* (1995). This is indicative of apical wall building conidium development (Minter *et al.* 1983) and atypical of species of *Ceratocystis s. str.*, that produce conidia through ring wall building conidium development (Minter *et al.* 1982). However, the *Juniperus* and *Larix* isolates were distinct in that the latter had a prominent and well-developed *Sporothrix* state, and

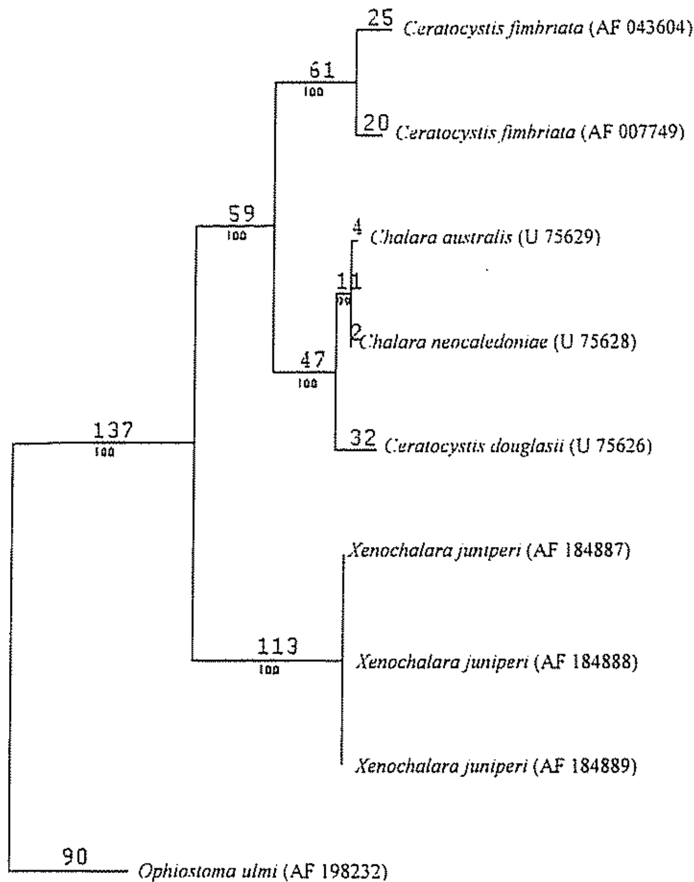

Figure 2 Phylogram generated from PAUP analysis depicting the phylogenetic relationships between *Xenochalara juniperi* (CMW 1099, CMW 1901, CMW 2547) and other ophiostomatoid fungi. Tree length = 601. The number of base substitutions is indicated above the branches and the bootstrap percentages (100 bootstrap replicates) are indicated below the branches.

formed sclerotia in culture (Bakshi 1951). In contrast, the *Juniperus* isolate very rarely formed an inconspicuous sporothrix-like synanamorph and formed no chlamydospores or sclerotia in culture (Figure 1).

Molecular studies

Amplification of the DNA in each of the three isolates yielded a single DNA fragment of approximately 550 base pairs (bp). Approximately 500 bp were sequenced, visually aligned and edited using MacClade 3.0 (Maddison & Maddison 1993). The exhaustive and random trees options in PAUP were used to determine the *gl* statistic for the obtained trees (Hillis & Huelsenbeck 1992; Swofford 1999).

The same tree configuration was observed using the consensus tree options with the branch and bound and heuristic options (Figure 2). Bootstrap analysis revealed high confidence intervals for each cluster branch (Figure 2). The minimum tree length obtained was 601 steps, with a Consistency Index (CI) of 0.900, a Homoplasy Index (HI) of 0.100 and a Retention Index (RI) of 0.894. The *gl* value obtained from the exhaustive search and the random trees option for this study is -1.00, indicating a strong support for the parsimony analysis and the resulting tree topology. The *gl* statistics discriminate phylogenetic signals from random noise in systematic data sets (Huelsenbeck 1991; Hillis & Huelsenbeck 1992). Sequence data for *C. fimbriata* and the two *Chalara* spp. formed a group distinct from the *Juniperus* species. However, the *Ceratocystis*, *Chalara* and *Juniperus* isolates comprised a larger group that was very distinct from the isolate of *O. ulmi*. This clustering confirmed the previous findings (Hausner *et al.* 1993; Wingfield *et al.* 1995) that the chalara-like anamorph of the *Juniperus* species (incorrectly referred to as the *Chalara* anamorph of *C. autographa*) is not a typical species of *Ceratocystis s. str.*

Discussion

One question that needs to be resolved is whether *C. autographa* (IMI 20162), which was collected from Larch in the U.K. is the same as the isolate (CBS 670.75) collected from *Juniperus* in the Netherlands. Although Bakshi (1951) described the *Chalara* conidia of *C. autographa* to be barrel-shaped, a re-examination of the type showed them to be similar to that of the *Juniperus* isolate. Although the *Chalara* states are similar, the *Sporothrix* state is much more common and prominent in the Larch specimen than in the one from *Juniperus*. Furthermore, no teleomorph was observed in the *Juniperus* isolate, nor any sclerotia which Bakshi (1951) reported as commonly occurring in *C. autographa*. Because of these uncertainties we prefer to treat the isolate from *Juniperus* as a separate taxon. The presence of a *Chalara*, as well as a *Sporothrix* anamorph, in a species of *Ceratocystis sensu lato* is contradictory to the current accepted generic concepts applied to the group (Weijman & De Hoog 1975; De Hoog & Scheffer 1984). The taxonomic placement of *C. autographa* is thus clearly in dispute and is in need of further investigation.

In 1995, Wingfield and co-workers conducted a study on the culture deposited in CBS as *C. autographa* (CBS 670.75). These authors observed many unusual features for the chalara-like anamorph of *C. autographa*. Species of *Chalara* are characterised by ring wall building conidiogenesis resulting in chains of conidia with two attachment points (Nag Raj & Kendrick 1975; Minter *et al.* 1982). However, the chalara-like anamorphs observed for the type specimen of *C. autographa* (IMI 20162) and also for the isolate from *Juniperus* (CBS 670.75) represent two similar species that have conidia with single attachment points, typical of apical wall building conidiogenesis (Minter *et al.* 1982). Wingfield *et al.* (1995), therefore, suggested that further studies were needed to determine the correct classification of the chalara-like anamorph. In a comparison of sequence data, Hausner *et al.* (1993) also suggested that the isolate CBS 670.75 probably did not represent the fungus originally described from Larch by Bakshi (1951).

Comparisons of ribosomal DNA sequence data obtained in the current study, clearly showed that the *Juniperus* isolate had no affinity with either *Ceratocystis s. str*, *Ophiostoma* or typical *Chalara* species. This confirms the observations of Hausner *et al.* (1993) and Wingfield *et al.* (1995), although not their interpretation of the problem. In our view, the chalara-like anamorph of *C. autographa* (IMI 20162) and the chalara-like species from *Juniperus* (CBS 670.75) probably do not represent the same fungus, for reasons discussed above. Furthermore, based on the molecular and morphological data, we conclude that the chalara-like anamorph of the fungus isolated from *Juniperus* represents a genus other than *Chalara*. We, therefore, describe a new genus, characterised by chalara-like conidiophores and conidia with single attachment points that arise through apical wall building conidium development, to represent this fungus.

Xenochalara M.J. Wingf. & Crous, gen. nov.

Species typica *Xenochalara juniperi* M.J. Wingfield *et* Crous

Hyphomycetosum genus morphologia simile Chalarae, sed distinctum quia conidia apicali pariete construendo producit quodque 'cycloheximide' tolerat. Mycelium constans ex hyphis

ramosis septatis; hyphae hyalinae et leves, brunnescentes asperascentesque, repertae singulatim vel in filis. Chlamydosporae absentes. Conidiophora micronematosa, exorientia ex aerio mycelio vel ex submersis hyphis, erecta, simplicia, saepe ad cellas conidiogenas reducta, vel 1-septata, subcylindrica, recta vel exigue curvata, pallide brunnea, leves. Conidiogenae cellae phialidicae, terminales, subcylindricae vel lageniformes, leves, subbrunneae vel pallide brunneae, venter conicus vel ellipsoideus, collariculum anguste obconicum vel subcylindricum. Conidia hyalina, levia, aseptata, reperta in falsis catenis usque 55; breviclavata, apice rotundato, basi truncata.

A hyphomycete genus morphologically similar to *Chalara*, but distinct in producing conidia through apical wall building, and being tolerant of cycloheximide. Mycelium consisting of branched, septate hyphae; hyphae hyaline and smooth, becoming brown and rough, occurring singly or in strands. Chlamydospores absent. Conidiophores micronematous, arising from aerial mycelium or submerged hyphae, erect, simple, frequently reduced to conidiogenous cells, or 1-septate, subcylindrical, straight to slightly curved, light brown, smooth. Conidiogenous cells phialidic, terminal, subcylindrical or lageniform, smooth, pale to light brown, venter conical to ellipsoid, collarette narrowly obconical to subcylindrical. Conidia hyaline, smooth, aseptate, occurring in false chains of up to 55; short clavate, apex rounded, base truncate.

Xenochalara juniperi M.J. Wingf *et* Crous, sp. nov.

Synanamorphs: *Sporothrix* and phialophora-like spp.

Teleomorph: unknown.

Mycelium constans ex ramosis septatis hyphis; hyphae hyalinae et leves, brunnescentes et asperascentes, repertae singulatim vel in filis usque 10, 1.5–2.5 µm diametro. Chlamydosporae absentes. Conidiophora micronematosa, exorientia ex mycetio aerio vel hyphis submersis, erecta, simplicia, saepe redacta ad cellulas conidiogenas, vel 1-septata, subcylindrica, recta vel exigue curvata, pallide brunnea, levia, 13–35 µm longa, 2.5–4 µm lata. Conidiogenae cellulae phialidicae, terminales, subcylindricae vel lageniformes, leves, subbrunneae vel pallide brunneae, 13–20 µm longae, 2.5–3(–4) µm latae; venter conicus vel ellipsoideus, 10–15 × 2.5–3(–4) µm; collariculum anguste obconicum vel subcylindricum, paries parum obscurior et crassior quam paries ventris, 5–8 × 1.5–2 µm; transitio a ventre ad collariculum vel abrupte vel paulatim fit, saepe quoque constricta ad basilare conidiophori septum. Conidia hyalina, levia, aseptata, evenientia in falsis usque ad 55 catenis; brevi-clavata, apice rotundato, basi truncata, (3–)3.5–4(–5) × 1–1.5 µm.

Holotype: The Netherlands, Llheedorp, on decaying needles of *Juniperus communis*, Nov. 1975, W. Gams, dried specimen deposited as PREM 56210; culture ex-type, CBS 670.75.

Mycelium consisting of branched, septate hyphae; hyphae hyaline and smooth, becoming brown and rough, occurring singly or in strands of up to 10, 1.5–2.5 µm diam. Chlamydospores absent. Conidiophores micronematous, arising from aerial mycelium or submerged hyphae, erect, simple, frequently reduced to conidiogenous cells, or 1-septate, subcylindrical, straight to slightly curved, light brown, smooth, 13–35 µm long, 2.5–4 µm wide. Conidiogenous cells phialidic, terminal, subcylindrical or lageniform, smooth, pale to light brown, 13–20 µm long, 2.5–3(–4) µm wide; venter conical to ellipsoid, 10–15 × 2.5–3(–4) µm; collarette narrowly obconical to subcylindrical, wall slightly darker and thicker than that of venter, 5–8 × 1.5–2 µm; transition from venter to collarette abrupt to gradual, also frequently constricted at basal conidiophore septum. Conidia hyaline, smooth, aseptate, occurring in false chains of up to 55; short-clavate, apex rounded, base truncate, (3–)3.5–4(–5) × 1–1.5 µm.

No other examples are known of *Ceratocystis* species having both a *Sporothrix* and a *Chalara* anamorph. It is possible, if not probable, that the *Sporothrix* sp. present on the type specimen (IMI 20162) is the anamorph linked to the teleomorph structures described as *C. autographa* by Bakshi (1951). If this is true, then the fungus would best reside in *Ophiostoma*. The chalara-like state, however, would be better accommodated in *Xenochalara*. Our re-examination of the dried type studied by Bakshi showed that it had indeed been contaminated with mites, which could have contaminated the plates with different fungi, as is evident from the *Penicillium* sp. sporulating on one. However, given the absence of cultures, this matter cannot be resolved further. Fresh collections from Larch in Scotland infested with *D. autographus* and *H. palliatus* would be required to finally resolve the identity of *C. autographa*. For the present we consider it a species of dubious status.

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