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Hymenoscyphus albidus is not associated with an anamorphic stage and displays slower growth than *Hymenoscyphus pseudoalbidus* on agar media

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Summary

Examination of isolates of *Hymenoscyphus albidus* from France revealed that this fungus does not form an anamorphic stage in culture. The lack of an asexual stage in this fungus is a conspicuous morphological difference to the ash dieback pathogen *Hymenoscyphus pseudoalbidus*, which is associated with its *Chalara fraxinea* anamorphic state. In growth studies on malt extract agar (MEA) and MEA amended with ash leaflets (ash leaf malt extract agar, AMEA) at 20°C, isolates of *H. albidus* grew slower than those of *H. pseudoalbidus*. On AMEA, the growth of cultures of both species was greatly enhanced.

1 Introduction

The ascomycete fungi *Hymenoscyphus albidus* (**Roberge ex Desm.**) W. Phillips, a saprotrophic or endophytic species native to Europe, and the ash dieback pathogen *Hymenoscyphus pseudoalbidus* V. Queloz, C. R. Grünig, R. Berndt, T. Kowalski, T. N. Sieber & O. Holdenrieder, native to Asia and an alien invasive species in Europe, form black pseudosclerotial layers on leaf petioles and rachises of common ash (*Fraxinus excelsior* L.) in the leaf litter, on which apothecia develop during the vegetation period (Queloz et al. 2011; Solheim et al. 2011; Zhao et al. 2012). Teleomorph characteristics of the two *Hymenoscyphus* species are very similar. Ascospores of *H. pseudoalbidus* are on an average longer and its ascocarps tend to be larger than those of *H. albidus* (Queloz et al. 2011; Solheim et al. 2011). Zhao et al. (2012) reported the presence of croziers at the base of asci in *H. pseudoalbidus* and their absence in *H. albidus* as consistent morphological difference between the two taxa. *H. pseudoalbidus* is associated with an asexual stage, which was discovered prior to the teleomorph and described as *Chalara fraxinea* T. Kowalski (Kowalski 2006). So far, there has been no report as to whether or not *H. albidus* is also associated with an anamorph. Likewise, the colony morphologies and growth rates of the two species in culture have not been compared.

2 Materials and methods

In late July 2012, common ash leaf rachises with pseudosclerotial plates and ascocarps of *H. albidus* were collected from the leaf litter beneath about five *F. excelsior* trees at a riparian woodland along the creek Le Gouyandeur (47°37′25.6″ N, 03°04′20.6″ W), near Carnac in southern Brittany (France), where ash dieback has at that time not yet been occurring. For fungal isolation, about 3-5-cm-long segments containing pseudosclerotia were cut from the rachises, surface sterilized (1 min in 96% ethanol, 3 min in 4% NaOCl, 30 s in 96% ethanol) and dried shortly until the ethanol had evaporated. Thereafter, pseudosclerotial layers were carefully scrapped off with a scalpel and small tissue samples were removed and placed onto malt extract agar [MEA; 20 g DiaMalt malt extract, Hefe Schweiz AG, Stettfurt, Switzerland; 16 g Becoagar agar, W. Behrens & Co, Hamburg, Germany; 1000 ml tap water; 100 mg streptomycin sulphate (added after autoclaving)]. Alternatively, rachis segments were sprayed with 96% ethanol and allowed to dry shortly. Pseudosclerotial layers were carefully peeled off and small tissue samples beneath these layers were removed and placed onto MEA. To obtain pure cultures, suspected *H. albidus* mycelia growing from the rachis samples were transferred to fresh plates containing MEA or ash leaf malt extract agar (AMEA; as MEA but amended with 50 g fresh or frozen *F. excelsior* leaflets which were removed after autoclaving, during the course of pouring the medium into Petri dishes).

Seventeen strains of *H. albidus*, each from the tissue of a different rachis, were isolated from the French collection. The identity of four representative isolates as *H. albidus* (Table 1) was confirmed by DNA sequence comparisons. DNA extraction was done from cultures grown on MEA or AMEA using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The ITS region and 5.8S gene of the rDNA were amplified by PCR with primers ITS1 and ITS4 (White et al. 1990). PCR products were sent to a commercial company (Eurofins MWG Operon, Ebersberg, Germany) for sequencing. The obtained DNA sequences (see GenBank accession numbers in Table 1) were analysed using the BLAST (Basic Local Alignment Search Tool) option of the GenBank, maintained by the National Center for Biotechnology Information (NCBL), Bethesda, USA (Altschul et al. 1997).

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[Correction added on 30 May 2013, after first online publication. The words in 'bold' were corrected after online publication]

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Table 1. Collection data, rDNA ITS GenBank accession numbers and growth rates of isolates of *Hymenoscyphus albidus* and *Hymenoscyphus pseudoalbidus* on malt extract agar (MEA) and ash leaf malt extract agar (AMEA).

				Radial growth rate (mm/day)			
				On MEA		On AMEA	
Hymenoscyphus spp.	Origin and year	IFFF code[1]	rDNA ITS GenBank accession no.	Mean[2]	SD[3]	Mean[2]	SD[3]
H. albidus	France, Brittany, Carnac, 2012	Car1	KC495075	0.08 a A	0.08	1.28 a B	0.12
H. albidus	France, Brittany, Carnac, 2012	Car5	KC509944	0.18 a A	0.01	2.03 b B	0.15
H. albidus	France, Brittany, Carnac, 2012	Car6	KC509945	0.21 a A	0.04	1.56 a B	0.06
H. albidus	France, Brittany, Carnac, 2012	Car7	KC509946	0.09 a A	0.07	1.33 a B	0.20
H. pseudoalbidus	Austria, Lower Austria, Tulln, 2008	TU/3/2/1	KC529350	0.28 ab A	0.10	2.59 b B	0.26
H. pseudoalbidus	Austria, Styria, St. Lorenzen ob Murau, 2010	STL/1/6	KC529349	0.82 d A	0.06	2.12 b B	0.18
H. pseudoalbidus	Austria, Vienna, Neuwaldegg, 2008	NWE/1/2/H1	KC529351	0.39 bc A	0.03	2.32 b B	0.12
H. pseudoalbidus	Austria, Styria, Altaussee, 2007	N/5/4/A	KC529352	0.59 c A	0.08	2.36 b B	0.20
All four isolates of *H. albidus*				0.14 a A	0.07	1.56 a B	0.34
All four isolates of *H. pseudoalbidus*				0.52 b A	0.24	2.34 b B	0.19

[1]Accession numbers of the culture collection of IFFF-BOKU.
[2]Means in the same column followed by different lower case letters and means in the same row followed by different uppercase letters were significantly different ($p < 0.05$) according to one-way analyses of variance (ANOVA) followed by the Games–Howell test (comparisons of isolates) or Duncan's multiple range test (comparison of *H. albidus* and *H. pseudoalbidus*). Separate analyses were done for comparing the different isolates and the two *Hymenoscyphus* species (using mean values of the four isolates per species), respectively.
[3]Standard deviation.

All 17 *H. albidus* isolates and 20 isolates of *H. pseudoalbidus* from Austria and St. Petersburg (Russia), maintained in the culture collection of IFFF-BOKU, were transferred to MEA and AMEA in 5.2-cm-diameter plastic Petri dishes. The plates were incubated at 20°C in the dark or at room temperature in diffuse daylight. After establishment on MEA and AMEA (i. e. after 2–3 weeks growth), some cultures were subsequently incubated at low temperatures (about 4–8°C). During a period of 4 months, the isolates were examined for the occurrence of asexual structures at irregular intervals.

The four isolates of *H. albidus* from France, whose identity was confirmed by DNA sequencing and four *H. pseudoalbidus* strains from Austria, isolated from symptomatic *F. excelsior* trees as described previously (Kirisits et al. 2009), were used in a growth test on MEA and AMEA (Table 1). The identity of the isolates as *H. pseudoalbidus* was confirmed by DNA sequencing as described above. AMEA plugs bearing mycelium from actively growing cultures were punched out with a sterile, 5-mm-diameter cork borer and transferred, with the mycelium side orientated upwards, to the centre of 8.7-cm-diameter plastic Petri dishes containing MEA or AMEA. Five replicates of each of the eight isolates on each medium were incubated at 20°C in the dark. After 4–20 days, depending on isolate and medium, the margins of the colonies were delineated on the plates. This procedure was repeated 12–89 days after the start of the experiment. Thereafter, a line was drawn through the centre of the inoculum at the maximum diameter of the colony and a second one at right angle. With a ruler, growth increment of the cultures between the first and the final marking was measured twice along each line, to an accuracy of 1 mm, and a mean value per plate was calculated from the four measurements. By dividing this value by the number of days between the first and the final marking, ranging from 6 to 69 days depending on isolate and medium, the radial growth rate of each isolate was calculated in mm/day for each replicate plate.

Statistical analyses of radial growth rates were done with the program IBM SPSS Statistics, version 20. A two-way analysis of variance (ANOVA) was conducted to test the effect of isolate and medium on the growth rates. Thereafter, differences in growth rate between all isolate and medium combinations were analysed with a one-way ANOVA, followed by the Games–Howell test. In another set of analyses, isolate means were used and the influence of *Hymenoscyphus* species and medium on growth rate was tested with a two-way ANOVA. Subsequently, differences in growth rate between all species and medium combinations were analysed with a one-way ANOVA, followed by Duncan's multiple range test.

3 Results and discussion

No variation in the ITS sequences of the four putative *H. albidus* isolates from France (see GenBank accession numbers in Table 1) was detected, and they matched perfectly with sequences of *H. albidus* accessions in GenBank (e. g. GU586877, on *F. excelsior* in Switzerland, Queloz et al. 2011). Likewise, the ITS sequences of the four *H. pseudoalbidus* isolates from Austria used in the growth studies (see GenBank accession numbers in Table 1) were identical and showed 100% similarity with *H. pseudoalbidus* accessions in GenBank (e. g. GU586904, from the type specimen of *H. pseudoalbidus* on *F. excelsior* in Switzerland, Queloz et al. 2011). Designation of the strains as *H. albidus* and *H. pseudoalbidus*, respectively, was thus confirmed. As the colony morphologies of the other 13 strains from Brittany corresponded to those of the sequenced isolates, it is safe to conclude that they also belong to *H. albidus*.

Isolates of *H. albidus* and *H. pseudoalbidus* generally showed slow growth on the agar media, especially on MEA, as reported for *H. pseudoalbidus* by Kowalski and Bartnik (2010). On MEA, radial growth rates of *H. albidus* strains ranged from 0.08 to 0.21 mm/day (mean for all isolates 0.14), while those of *H. pseudoalbidus* isolates varied between 0.28 and 0.82 mm/day (mean 0.52) (Table 1). On AMEA, radial growth rates of *H. albidus* were within the range of 1.28 and 2.03 mm/day (mean 1.56), whereas *H. pseudoalbidus* isolates had growth rates between 2.12 and 2.59 mm/day (mean 2.34) (Table 1). A two-way ANOVA revealed a statistically significant effect of the factors 'medium' ($F = 3142.84$, $p < 0.001$) and 'isolate' ($F = 69.78$, $p < 0.001$) as well as of their interaction ($F = 24.59$, $p < 0.001$) on radial growth rates. All *H. albidus* and *H. pseudoalbidus* isolates grew much faster on AMEA than on MEA, and differences were statistically significant for all isolates (Table 1). On both media, all *H. pseudoalbidus* isolates grew faster than the *H. albidus* strains (Table 1). However, on MEA, the radial growth rate of one *H. pseudoalbidus* isolate (TU/3/2/1) did not differ significantly from the growth rates of the *H. albidus* strains, and on AMEA, the radial growth rate of one *H. albidus* isolate (Car5) did not differ significantly from those of the *H. pseudoalbidus* strains (Table 1). When mean radial growth rates of the isolates were subjected to a two-way ANOVA, 'species' ($F = 25.31$, $p < 0.001$) and 'medium' ($F = 192.59$, $p < 0.001$) were statistically significant factors, while the interaction 'medium' × 'species' ($F = 3.18$, $p = 0.10$) was not. A one-way ANOVA followed by Duncan's multiple range test revealed statistically significant differences between all species and medium combinations: *H. pseudoalbidus* on AMEA grew fastest, followed by *H. albidus* on AMEA, *H. pseudoalbidus* on MEA and *H. albidus* on MEA (Table 1).

Colony morphologies of isolates of *H. albidus* and *H. pseudoalbidus* varied considerably, as it has been described for *H. pseudoalbidus* by Kowalski and Bartnik (2010). On MEA, *H. albidus* most frequently formed brown, grey or black, compact pseudoparenchymatous structures, while the margins of cultures remained light-coloured or a white to light brown aerial mycelium developed (Fig. 1a,b). In faster growing cultures, the mycelium often remained mostly white (Fig. 1c); light brown or orange-brown colours also occurred, and pseudoparenchymatous structures were restricted to the centre, that is, the oldest parts, of the colonies (Fig. 1c). In other cultures, the mycelium at the margin of the colonies became submerged and no or only sparse aerial mycelium developed. On AMEA, dark pseudoparenchymatous structures were regularly formed

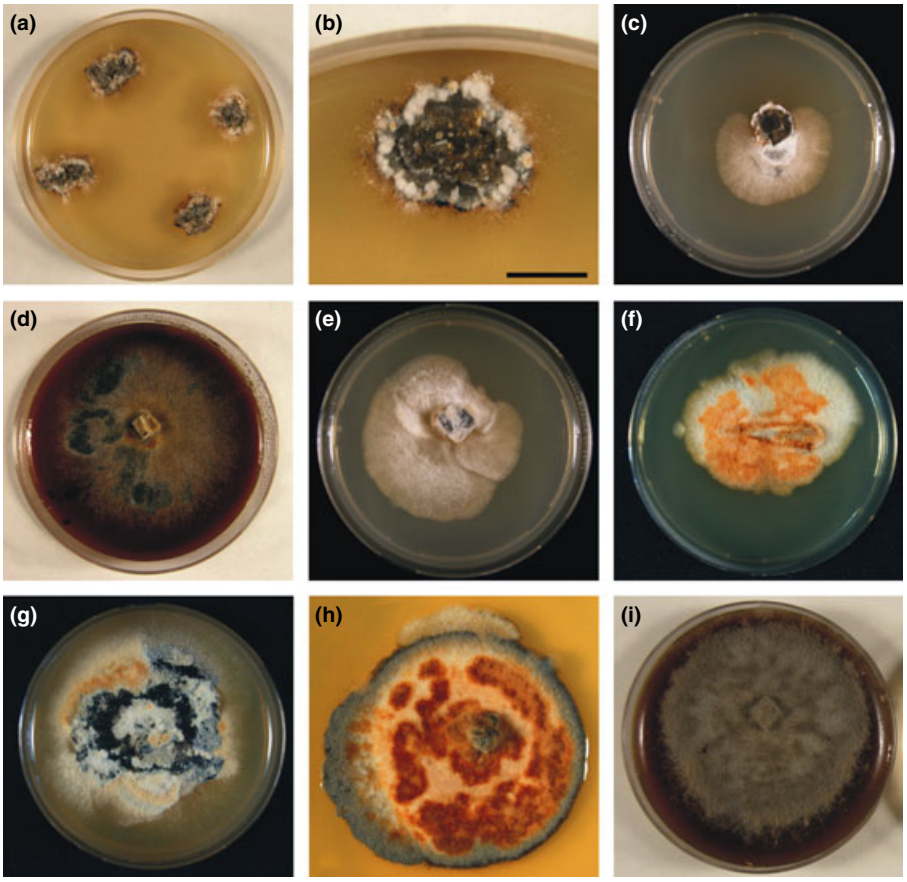

Fig. 1. Cultures of *Hymenoscyphus albidus* and *Hymenoscyphus pseudoalbidus* on malt extract agar (MEA) or ash leaf malt extract agar (AMEA) in 5.2-cm-diameter Petri dishes. (a) to (d) *Hymenoscyphus albidus*: (a) 90-day-old primary isolations (onto MEA) from an ash leaf rachis in the leaf litter; (b) Detailed view of a 80-day-old primary isolation (onto MEA), bar = 5 mm; (c) and (d) 43-day-old cultures on MEA (c) and AMEA (d). (e) to (i) *Hymenoscyphus pseudoalbidus*: 43-day-old (e), 23-day-old (f) and 69-day-old (g) cultures on MEA; (h) Culture on MEA first incubated at room temperature (3 weeks) and then at 4°C (6 weeks) showing intensive sporulation of the *Chalara fraxinea* anamorph at its colony margin (grey areas), colony diameter = 25 mm; (i) 43-day-old culture on AMEA with intensive phialophore formation of the *C. fraxinea* anamorph.

after prolonged incubation, and frequently they covered considerable portions of the cultures (Fig. 1d). Development of aerial mycelium was more intensive than on MEA and the cultures showed, outside the pseudoparenchymatous zones, white, light brown and/or orange-brown colours (Fig. 1d).

The variation of the cultural characteristics of the *H. pseudoalbidus* isolates on MEA was similar to that described for this species by Kowalski and Bartnik (2010). In brief, colonies varied from white, light brown, orange-brown, reddish-brown to grey, and the cultures often developed sectors varying in colour (Fig. 1e–h). Dark, compact pseudoparenchymatous structures were also formed in cultures of *H. pseudoalbidus* (Fig. 1g) but less consistently than in those of *H. albidus*. When cultures that had first grown at 20°C or at room temperature were incubated at low temperatures, newly formed parts of the colonies were grey, due to the abundant formation of phialophores and phialospores of the *C. fraxinea* anamorph (Fig. 1h). On AMEA, cultures of the two *Hymenoscyphus* species looked similar, but those of *H. albidus* showed a tendency to develop pseudoparenchymatous structures more frequently (Fig. 1d and i).

The most conspicuous difference between *H. albidus* and *H. pseudoalbidus* cultures concerned the formation of anamorph structures. Cultures of *H. pseudoalbidus*, which were incubated under the same conditions as the *H. albidus* strains, regularly developed phialophores of *C. fraxinea*. While phialophore formation was sparse at 20°C and room temperature and mostly occurred in old cultures, asexual sporulation was greatly enhanced by low temperatures (Fig. 1h), as described previously (Kirisits et al. 2009; Kowalski and Bartnik 2010). Similarly, AMEA stimulated phialophore formation (Fig. 1i). In contrast, no asexual structures were observed in cultures of any of the *H. albidus* isolates from France, neither on MEA nor on AMEA and regardless the conditions under which the cultures had been incubated. We therefore conclude that *H. albidus* is not associated with an anamorph. The absence of an asexual stage in *H. albidus* and its presence in *H. pseudoalbidus* is a conspicuous morphological feature distinguishing the two species.

In previous studies, the spores of the *C. fraxinea* stage of *H. pseudoalbidus* did not germinate on various agar media and on detached *F. excelsior* leaflets, and inoculation of *F. excelsior* shoots and leaves with asexual spores did not result in ash dieback symptoms (Kirisits et al. 2009; Zhao et al. 2012). It was therefore suggested that the *C. fraxinea* spores are not infectious but are spermatia (Kirisits et al. 2009; Zhao et al. 2012). Gross et al. (2012) clarified the reproductive modes of *H. albidus* and *H. pseudoalbidus*: *H. pseudoalbidus* is heterothallic, and the *C. fraxinea* spores were indeed proposed to act as fertilizing spermatia in the course of sexual reproduction. In contrast, *H. albidus* has a homothallic reproductive system (Gross et al. 2012). Under the premise that the *C. fraxinea* spores of *H. pseudoalbidus* are spermatia, the absence of an asexual stage in *H. albidus* corresponds well with its homothallism, where crossing of opposite mating types is not necessary for ascocarp formation to occur.

The AMEA medium used in the present growth studies is useful for laboratory and field work with cultures of *H. albidus* and *H. pseudoalbidus*. As it enhances the growth of both *Hymenoscyphus* species, we generally use it to grow isolates for subsequent DNA extraction and to prepare inoculum for wound-inoculation experiments. AMEA may also facilitate the identification of *H. pseudoalbidus* based on morphological characteristics because it stimulates phialophore formation of the ash dieback pathogen. Although we did not test this thoroughly, AMEA could also be useful for isolating *H. pseudoalbidus* from symptomatic ash trees: it may increase the frequency of positive isolations compared with MEA and other standard laboratory media. Similarly, the stimulation of phialophore formation of *H. pseudoalbidus* by low temperatures can be utilized for morphology-based species identification and for confirming the ash dieback pathogen on and isolating it from diseased ash trees.

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References

Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J., 1997: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389–3402.

Gross, A.; Zaffarano, P. L.; Duo, A.; Grünig, C. R., 2012: Reproductive mode and life cycle of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. Fungal Genet. Biol. **49**, 977–986.

Kirisits, T.; Matlakova, M.; Mottinger-Kroupa, S.; Cech, T. L.; Halmschlager, E., 2009: The current situation of ash dieback caused by *Chalara fraxinea* in Austria. In: Proceedings of the Conference of IUFRO Working Party 7.02.02, Eğirdir, Turkey, 11–16 May 2009. Ed. by Doğmuş-Lehtijärvi, H. T. Isparta: SDU Faculty of Forestry Journal, ISSN: 1302-7085, Serial: A, Special Issue: pp. 97–119.

Kowalski, T., 2006: *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. For. Pathol. **36**, 264–270.

Kowalski, T.; Bartnik, C., 2010: Morphological variation in colonies of *Chalara fraxinea* isolated from ash (*Fraxinus excelsior* L.) stems with symptoms of dieback and effects of temperature on colony growth and structure. Acta Agrobot. **63**, 99–106.

Queloz, V.; Grünig, C. R.; Berndt, R.; Kowalski, T.; Sieber, T. N.; Holdenrieder, O., 2011: Cryptic speciation in *Hymenoscyphus albidus*. For. Pathol. **41**, 133–142.

Solheim, H.; Timmermann, V.; Børja, I.; Hietala, A. M., 2011: A small ascomycete, *Hymenoscyphus pseudoalbidus*, is a threat to common ash in Europe. Agarica **30**, 82–88 (in Norwegian).

White, T. J.; Bruns, T. D.; Lee, S.; Taylor, J., 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: A guide to methods and applications. Ed. by Innis, M. A.; Gelfand, D. H.; Sninsky, J. J.; White, T. J. San Diego: Academic Press, pp. 315–322.

Zhao, Y.-J.; Hosoya, T.; Baral, H.-O.; Hosaka, K.; Kakishima, M., 2012: *Hymenoscyphus pseudoalbidus*, the correct name for *Lambertella albida* reported from Japan. Mycotaxon **122**, 25–41.