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Cryptic speciation in *Hymenoscyphus albidus*

By V. Queloz[1], C. R. Grünig[1], R. Berndt[2], T. Kowalski[3], T. N. Sieber[1] and O. Holdenrieder[1,4]

[1]Forest Pathology and Dendrology, Institute of Integrative Biology (IBZ), ETH Zurich, 8092 Zurich, Switzerland; [2]Plant Ecological Genetics, Institute of Integrative Biology (IBZ), ETH Zurich, Zurich, Switzerland; [3]Department of Forest Pathology, Faculty of Forestry, Agricultural University, Kraków, Poland; [4]E-mail: ottmar.holdenrieder@env.ethz.ch (for correspondence)

Summary

Ash dieback caused by the mitosporic ascomycete *Chalara fraxinea* is a novel disease of major concern affecting *Fraxinus excelsior* and *Fraxinus angustifolia* in large parts of Europe. Recently, its teleomorph was detected and assigned to *Hymenoscyphus albidus*, which has been known from Europe since 1851. In this study, we present molecular evidence for the existence of two morphologically very similar taxa, *H. albidus*, which is lectotypified and *Hymenoscyphus pseudoalbidus* sp. nov. Differences were found between the species in the loci calmodulin, translation elongation factor 1-α and the internal transcribed spacers of the rDNA genes, and strong differentiation was obtained with ISSR markers. It is likely that *H. albidus* is a non-pathogenic species, whereas *H. pseudoalbidus* is a virulent species causing ash dieback. Genotyping herbarium specimens showed that *H. pseudoalbidus* has been present in Switzerland for at least 30 years prior to the outbreak of the epidemic.

1 Introduction

Chalara fraxinea T. Kowalski (Kowalski 2006) causes a rapidly spreading lethal disease of ash (*Fraxinus excelsior* L., *F. angustifolia* Vahl) in Europe (Bakys et al. 2009a,b; European Plant Protection Organization (EPPO) 2009; Kirisits et al. 2009a,b; Kowalski and Holdenrieder 2009a; Schumacher et al. 2009). The main symptoms are necrotic lesions in the bark and xylem leading to dieback of trees in all age classes (Kowalski and Holdenrieder 2008). Recently, *C. fraxinea* was linked to the helotialean ascomycete *Hymenoscyphus albidus* (Roberge ex Desm.) W. Phillips (Kowalski and Holdenrieder 2009b). The disease has been observed in Poland since the mid-1990s and the causal agent *C. fraxinea* was named in 2006 (Kowalski and Holdenrieder 2008). However, the discomycete *H. albidus* has long been known in Europe and the recent emergence of the disease remains enigmatic. The appearance of novel infectious diseases can be explained by the endemic pathogen hypothesis or the novel pathogen hypothesis. The former hypothesis assumes the development of pathogenicity in a harmless resident fungal symbiont and/or the predisposition of the host by environmental change, the latter the introduction of an invasive pathogen (e.g. Rachowicz et al. 2005). Alternatively, hybridization events can lead to aggressive lineages as was shown for *Ophiostoma novo-ulmi* Brasier (Brasier and Kirk 2010).

In the present study, we sampled *H. albidus* in asymptomatic ash stands and compared these collections with samples from epidemic regions. Herbarium specimens were also studied. We characterized the diversity found in *H. albidus* using morphological characters, inter-simple sequence repeat anchored PCR (ISSR-PCR) fingerprinting and three sequence loci.

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2 Material and methods

2.1 Fungal material

Regions with occurrence or absence of ash dieback were identified by the Swiss Forest Protection Service (R. Engesser, personal communication) and by personal observations. Cultures of *C. fraxinea* were isolated from necrotic stem lesions of *F. excelsior* in Switzerland, Poland and Germany (Table 1). Tissue samples (approx. $2 \times 2 \times 1$ mm) were taken aseptically from the xylem below necrotic bark lesions and incubated on malt extract agar (MEA) with 50 mg l^{-1} oxytetracycline (Jankovsky and Holdenrieder 2009). Subcultures were made on 2% (w/v) MEA at room temperature. Aerial mycelium was harvested and DNA extracted as described previously (Grünig et al. 2003).

Fruitbodies of *H. albidus* on ash rachises in the leaf litter were collected in several ash stands in Switzerland during July and August 2009 (Table 1). In addition, rachises with pseudosclerotia without apothecia were collected and incubated on moist vermiculite in a private garden in Zurich. The material was loosely covered with mosses and observed at weekly intervals for fructification. We also studied herbarium specimens (Table 1). A living culture of *H. albidus* (Leukerbad 090812.3c) has been deposited at CBS, Utrecht, Netherlands (CBS 126533).

DNA was extracted from fresh and dry apothecia, as described above, with the following modifications: a few grains of autoclaved, DNA-free silica sand were added to the apothecia to facilitate disruption and the protocol was adjusted for small sample volumes by adding only 250 μl CTAB lysis buffer and performing a single extraction step using 500 μl chloroform : isoamylalcohol (1 : 24).

2.2 ITS sequence analysis

The nucleotide sequences of the internal transcribed spacer regions of the rDNA (ITS) were amplified using primers prITS1 and prITS4 (White et al. 1990). Amplification of DNA was performed in 15 μl reaction volumes using approximately 2 ng of template DNA. After an initial denaturation step for 2 min at 94°C, 31 cycles were performed each comprising a denaturation step at 94°C for 30 s, an annealing step at 60°C for 1 min and an extension step at 72°C for 1 min followed by a final extension step for 10 min at 72°C. Prior to sequencing, target fragments were directly purified using an 'ExoSap' protocol (Werle et al. 1994) with the following modifications. Ten units of exonuclease I and one unit of shrimp alkaline phosphatase (Fermentas, Nunningen, Switzerland) were mixed with 5 μl PCR product and incubated for 15 min at 37°C followed by 15 min at 80°C. Cycle sequencing was performed with the Big-Dye v3.1 kit (Applied Biosystems, Rotkreuz, Switzerland) in a 10 μl reaction volume using 1 μl purified PCR amplification product, 0.5 μl BigDye v3.1, 1.9 μl 5× buffer, 5.6 μl ddH_2O and 1 μl of the sequencing primer (10 μM). Cycle sequencing reactions were performed on a Biometra T1 thermal cycler with the following conditions: 60 s at 96°C followed by 55 cycles of 10 s at 95°C, 5 s at 50°C and 4 min at 60°C. Dye-labelled fragments were cleaned using the BigDye Xterminator Purification kit following manufacturer's instructions (Applied Biosystems). Samples were analysed on an ABI 3130xl DNA Analyser (Applied Biosystems) at the Genetic Diversity Center (GDC, ETH Zurich, Switzerland). GeneBank accession nos are listed in Table 2.

2.3 Additional marker systems

To examine the two subgroups of *H. albidus* distinguished by the ITS sequences, two additional sequence loci and PCR fingerprinting were used. Nine strains collected from different sampling locations were included in this analysis for each of the two subgroups.

Table 1. **Strains and collections of *Hymenoscyphus* species included in the present study.**

Species[1]	Specimen[2]	Origin	Country	Location	Coordinates	Sampling year	Collector
H. albidus	22.11.094.95	F	FR	Bellême	NA	1994	Dougoud R
H. albidus	*GENT Pl. crypt. Fr. n°*2004	F	FR	NA	NA	1851	Desmazières J
H. albidus	Ber_02	F	CH	Bremgartenwald	N46°57′43.3″ E07°23′23.1″	2009	Senn-Irlet B
H. albidus	Ber_03	F	CH	Bremgartenwald	N46°57′49.5″ E07°23′19.8″	2009	Senn-Irlet B
H. albidus	090803.16	F	CH	Ennenda	N47°02′35.0″ E09°04′46.8″	2009	Holdenrieder O
H. albidus	090805.2	F	CH	Ennenda	N47°02′35.0″ E09°04′46.8″	2009	Holdenrieder O
H. albidus	*NMLU 2309-80*	F	CH	Giswil	N46°54′29.8″0 E08°10′01.6″	1980	Müller F
H. albidus	Cas_01	F	CH	Castro	N46°28′24.4″ E08°56′19.1″	2009	Queloz V
H. albidus	Cas_03	F	CH	Castro	N46°28′24.4″ E08°56′19.1″	2009	Queloz V
H. albidus	Lav_01	F	CH	Lavorgo	N46°26′15.3″ E08°50′23.7″	2009	Queloz V
H. albidus	Lav_02	F	CH	Lavorgo	N46°26′15.3″ E08°50′23.7″	2009	Queloz V
H. albidus	Lav_03	F	CH	Lavorgo	N46°26′15.3″ E08°50′23.7″	2009	Queloz V
H. albidus	Qui_01	F	CH	Quinto	N46°30′14.8″ E08°42′59.5″	2009	Queloz V
H. albidus	Qui_02	F	CH	Quinto	N46°30′14.8″ E08°42′59.5″	2009	Queloz V
H. albidus	Qui_03	F	CH	Quinto	N46°30′14.8″ E08°42′59.5″	2009	Queloz V
H. albidus	090707.1x	F	CH	Spruga	N46°11′53.2″ E08°34′09.5″	2009	Holdenrieder O
H. albidus	090802.2	F	CH	Spruga	N46°11′53.2″ E08°34′09.5″	2009	Holdenrieder O
H. albidus	090803.21	F	CH	Eggen	N46°18′42.4″ E07°52′56.6″	2009	Holdenrieder O
H. albidus	090812.1	F	CH	Leukerbad	N46°22′55.3″ E07°37′20.1″	2009	Holdenrieder O
H. albidus	090812.3a	F	CH	Leukerbad	N46°22′55.3″ E07°37′20.1″	2009	Holdenrieder O
H. albidus	090812.3c	P	CH	Leukerbad	N46°22′55.3″ E07°37′20.1″	2009	Holdenrieder O
H. albidus	090812.4	F	CH	Leukerbad	N46°22′55.3″ E07°37′20.1″	2009	Holdenrieder O
H. albidus	*NMLU 2107-07*	F	CH	Unterägeri	N47°06′20.1″ E08°34′22.6″	2007	Graf U
H. albidus	*ZT 87-273*	F	CH	Küsnacht	N47°19′08.0″ E08°35′26.6″	1987	Schneller J
H. albidus	090807.15	F	CH	Lägern	N47°28′39.1″ E08°19′32.6″	2009	Holdenrieder O
H. albidus	090807.2	F	CH	Wehrenbach	N47°21′11.8″ E08°34′58.2″	2009	Holdenrieder O
H. pseudoalbidus	090807.1	F	D	Singen	N47°46′31.0″ E08°49′33.3″	2009	Holdenrieder O
H. pseudoalbidus	*HMIPC 18866*	F	PL	Wesola	N50°11′14.6″ E20°03′54.1″	2008	Kowalski T
H. pseudoalbidus	*HMIPC 19.08*	F	PL	Wesola	N50°11′14.6″ E20°03′54.1″	2008	Kowalski T
H. pseudoalbidus	090811.1	F	CH	Magden	N47°31′32.5″ E07°48′32.7″	2009	Buser P
H. pseudoalbidus	090811.2	F	CH	Magden	N47°31′32.5″ E07°48′32.8″	2009	Buser P

Table 1. **(Continued).**

Species[1]	Specimen[2]	Origin	Country	Location	Coordinates	Sampling year	Collector
H. pseudoalbidus	Oth_01	F	CH	Othmarsingen	N47°23′56.0″ E08°13′55.0″	2009	Queloz V
H. pseudoalbidus	Bel_01	F	CH	Belp	N46°53′07.2″ E07°32′13.2″	2009	Senn-Irlet B
H. pseudoalbidus	Hin_01	F	CH	Hindelbank	N47°03′17.8″ E07°32′57.4″	2009	Senn-Irlet B
H. pseudoalbidus	Por_01a	F	CH	Porrentruy	N47°25′25.8″ E07°03′22.7″	2009	Queloz V
H. pseudoalbidus	Por_02	F	CH	Porrentruy	N47°25′25.8″ E07°03′22.7″	2009	Queloz V
H. pseudoalbidus	Por_03	F	CH	Porrentruy	N47°25′25.8″ E07°03′22.7″	2009	Queloz V
H. pseudoalbidus	*NMLU 0409-78*	F	CH	Aesch	N47°16′05.0″ E08°10′19.3″	1978	Müller F
H. pseudoalbidus	Sol_01	F	CH	Verena Schlucht	N47°13′23.8″ E07°32′07.1″	2009	Zaffarano P
H. pseudoalbidus	Sol_02	F	CH	Verena Schlucht	N47°13′23.8″ E07°32′07.1″	2009	Zaffarano P
H. pseudoalbidus	Sol_03	F	CH	Verena Schlucht	N47°13′23.8″ E07°32′07.1″	2009	Zaffarano P
H. pseudoalbidus	090807.12	F	CH	Lägern	N47°28′39.1″ E08°19′32.6″	2009	Holdenrieder O
H. pseudoalbidus	090807.13	F	CH	Lägern	N47°28′39.1″ E08°19′32.6″	2009	Holdenrieder O
H. pseudoalbidus	090807.14	F	CH	Lägern	N47°28′39.1″ E08°19′32.6″	2009	Holdenrieder O
H. pseudoalbidus	090807.16	F	CH	Lägern	N47°28′39.1″ E08°19′32.6″	2009	Holdenrieder O
H. pseudoalbidus	090807.6	F	CH	Lägern	N47°28′39.1″ E08°19′32.6″	2009	Holdenrieder O
H. pseudoalbidus	090807.7	F	CH	Lägern	N47°28′39.1″ E08°19′32.6″	2009	Holdenrieder O
H. pseudoalbidus	*ZT 87-236*	F	CH	Sihlwald	N47°15′57.7″ E08°33′38.8″	1987	Schneller J
H. pseudoalbidus	071026.1	N	CZ	Rudice	N49°19′07.0″ E16°44′35.0″	2007	Holdenrieder O
H. pseudoalbidus	*HMIPC 19206*	N	PL	Rokita	N53°45′55.4″ E14°50′23.1″	2009	Kowalski T
H. pseudoalbidus	*HMIPC 18679*	N	PL	Pogorzelica	N54°06′07.0″ E15°07′30.8″	2007	Kowalski T
H. pseudoalbidus	*HMIPC 18381*	N	PL	Mircze	N50°39′06.5″ E23°54′11.9″	2006	Kowalski T
H. pseudoalbidus	*HMIPC 18377*	N	PL	Wesola	N50°21′26.3″ E20°01′56.0″	2006	Kowalski T
H. pseudoalbidus	*HMIPC 18378*	N	PL	Wesola	N50°21′26.3″ E20°01′56.0″	2006	Kowalski T
H. pseudoalbidus	*HMIPC 18858*	N	PL	Stary Sacz	N49°33′00.7″ E20°38′02.5″	2008	Kowalski T
H. pseudoalbidus	*HMIPC 18859*	N	PL	Stary Sacz	N49°33′00.7″ E20°38′02.5″	2008	Kowalski T
H. pseudoalbidus	*HMIPC 18384*	N	PL	Kańczuga	N49°58′50.2″ E22°24′43.7″	2006	Kowalski T
H. pseudoalbidus	*C. fraxinea* **CBS 122504**	N	PL	Wloszczowa	N50°51′11.2″ E19°57′42.7″	2000	Kowalski T
H. pseudoalbidus	Sch_01	N	CH	Schafisheim	N47°22′18.3″ E08°07′51.0″	2009	Queloz V
H. pseudoalbidus	Cou_01	N	CH	Courgenay	N47°23′18.1″ E07°07′28.3″	2009	Queloz V
H. pseudoalbidus	Por_01n	N	CH	Porrentruy	N47°25′25.8″ E07°03′22.7″	2009	Queloz V
H. caudatus	1578	F	RC	Jiangxi	NA	NA	NA

Table 1. **(Continued).**

Species[1]	Specimen[2]	Origin	Country	Location	Coordinates	Sampling year	Collector
H. fructigenus	*CBS 650.92*	F	D	Hirschau	NA	1987	Weber E
H. fructigenus	F109077	F	E	Logroño	NA	NA	NA
H. scutulus	1278	F	RC	Beijing	NA	NA	NA
H. serotinus	F156526	F	E	Segovia	NA	NA	NA

GENT, Herbaria University Gent; ZT, Herbarium Zürich; NMLU, Mykologisches Herbar Natur-Museum Luzern; HMIPC, Department of Forest Pathology, University of Agriculture, Krakow, PL; CBS, Centraalbureau voor Schimmelcultures; F, fruitbody; N, culture from necrosis; P, culture from pseudosclerotium on rhachis with fruitbodies; FR, France; CH, Switzerland; D, Germany; PL, Poland; CZ, Czech Republic; RC, China; E, Spain; NA, not available.
[1]Type specimens (bold).
[2]Herbarium specimens (italic).

First, a partial fragment of the calmodulin gene (Cal) was amplified using primers Cal_228F and Cal_737R (Carbone and Kohn 1999) and a fraction of the translation elongation factor 1-α (EF1-α) was amplified using primers EF_728 and EF_alpha_R2 (Carbone and Kohn 1999; Grünig et al. 2007). PCR amplification and sequencing were performed as described above.

ISSR-PCR was performed using primers CCA and Herp_01 as described previously (Grünig et al. 2001; Schneider et al. 2009). These primers were selected as the most suitable for the assays in terms of polymorphic and scorable fingerprints, from a total of 10 tested in an initial screening (data not shown). GeneBank accession nos are listed in Table 2.

2.4 Phylogenetic analysis

Dataset 1 included the ITS sequence data of 58 specimens/strains collected during the present study and seven sequences derived from GenBank (Fig. 1). *Hymenoscyphus scutulus*, *H. fructigenus*, *H. caudatus* and *H. serotinus* were used as outgroups. Dataset 2 included sequence data of three loci (ITS, Cal, EF1-α) of nine strains of each *H. albidus* subgroup. In addition, sequence data for the three loci of *H. fructigenus* (CBS 650.92) were included and served as outgroup in the respective analysis. For dataset 2, each locus was analysed individually and the three loci were concatenated to form a super-gene alignment for the combined analysis. This approach produces more accurate results for inferring species phylogenies than the consensus phylogeny approach (Gadagkar et al. 2005). Maximum likelihood analyses were performed in paup using the substitution model identified by modeltest v3.7 (Posada and Crandall 1998). Bootstrapping to generate 100 pseudosamples was used for accuracy estimations in each analysis.

Fingerprinting data derived from ISSR-PCR were scored for the presence (1) or absence (0) of co-migrating bands and cluster analyses were performed in paup 4.0b10 (Swofford 2001) using the distance optimality criterion and the minimum evolution objective function (Swofford et al. 1996). Bootstrapping to generate 100 pseudosamples was used for accuracy estimations.

2.5 Search for diagnostic molecular characters for the two *H. albidus* subgroups

Nucleotide substitutions, which were unique for a subgroup of *H. albidus* were regarded as diagnostic characters. The dataset of each locus was collapsed to unique sequences (haplotypes) using the software collapse v1.2 (http://darwin.uvigo.es/software/collapse.html). Subsequently, fixed nucleotide changes and their positions in the alignment were recorded.

Table 2. **Gene bank accession numbers of sequences generated in the present study.**

Species[1]	Specimen[2]	ITS-sequence[3]	EF1-α-sequence	CAL-sequence
H. albidus	22.11.094.95	GU586876	NA	NA
H. albidus	*GENT Pl. crypt. Fr. n°*2004	NA	NA	NA
H. albidus	Ber_02	GU586877	GU586953	GU586934
H. albidus	Ber_03	GU586878	NA	NA
H. albidus	090803.16	GU586879	GU586954	GU586935
H. albidus	090805.2	GU586880	GU586955	GU586936
H. albidus	*NMLU 2309-80*	GU586881	NA	NA
H. albidus	Cas_01	GU586882	GU586956	GU586937
H. albidus	Cas_03	GU586883	NA	NA
H. albidus	Lav_01	GU586884	GU586957	GU586938
H. albidus	Lav_02	GU586885	NA	NA
H. albidus	Lav_03	GU586886	NA	NA
H. albidus	Qui_01	GU586887	GU586958	GU586939
H. albidus	Qui_02	GU586888	NA	NA
H. albidus	Qui_03	GU586889	NA	NA
H. albidus	090707.1x	GU586890	NA	NA
H. albidus	090802.2	GU586891	NA	NA
H. albidus	090803.21	GU586892	NA	NA
H. albidus	090812.1	GU586893	GU586959	GU586940
H. albidus	090812.3a	GU586894	NA	NA
H. albidus	090812.3c	GU586895	GU586960	GU586941
H. albidus	090812.4	GU586896	NA	NA
H. albidus	*NMLU 2107-07*	GU586897	NA	NA
H. albidus	*ZT 87-273*	GU586898	NA	NA
H. albidus	090807.15	GU586899	GU586961	GU586942
H. albidus	090807.2	GU586900	NA	NA
H. pseudoalbidus	090807.1	GU586901	NA	NA
H. pseudoalbidus	*HMIPC 18866*	FJ597976	GU586962	GU586943
H. pseudoalbidus	*HMIPC 19.08*	FJ597977	NA	NA
H. pseudoalbidus	090811.1	GU586902	NA	NA
H. pseudoalbidus	090811.2	GU586903	NA	NA
H. pseudoalbidus	Oth_01	GU586904	GU586963	GU586944
H. pseudoalbidus	Bel_01	GU586905	GU586964	GU586945
H. pseudoalbidus	Hin_01	GU586906	NA	NA
H. pseudoalbidus	Por_01a	GU586907	GU586965	GU586946
H. pseudoalbidus	Por_02	GU586908	NA	NA
H. pseudoalbidus	Por_03	GU586909	NA	NA
H. pseudoalbidus	*NMLU 0409-78*	GU586910	NA	NA
H. pseudoalbidus	Sol_01	GU586911	GU586966	GU586947
H. pseudoalbidus	Sol_02	GU586912	NA	NA
H. pseudoalbidus	Sol_03	GU586913	NA	NA
H. pseudoalbidus	090807.12	GU586914	NA	NA
H. pseudoalbidus	090807.13	GU586915	NA	NA
H. pseudoalbidus	090807.14	GU586916	NA	NA
H. pseudoalbidus	090807.16	GU586917	GU586967	GU586948
H. pseudoalbidus	090807.6	GU586918	NA	NA
H. pseudoalbidus	090807.7	GU586919	NA	NA
H. pseudoalbidus	*ZT 87-236*	GU586920	NA	NA
H. pseudoalbidus	071026.1	GU586921	GU586968	GU586949
H. pseudoalbidus	*HMIPC 19206*	GU586922	GU586969	GU586950
H. pseudoalbidus	*HMIPC 18679*	GU586923	NA	NA
H. pseudoalbidus	*HMIPC 18381*	GU586924	NA	NA
H. pseudoalbidus	*HMIPC 18377*	GU586925	NA	NA
H. pseudoalbidus	*HMIPC 18378*	GU586926	NA	NA
H. pseudoalbidus	*HMIPC 18858*	GU586927	NA	NA

Table 2. **(Continued).**

Species[1]	Specimen[2]	ITS-sequence[3]	EF1-α-sequence	CAL-sequence
H. pseudoalbidus	*HMIPC 18859*	GU586928	NA	NA
H. pseudoalbidus	*HMIPC 18384*	GU586929	NA	NA
H. pseudoalbidus	*CBS 122504*	FJ597975	GU586970	GU586951
H. pseudoalbidus	Sch_01	GU586930	NA	NA
H. pseudoalbidus	Cou_01	GU586931	NA	NA
H. pseudoalbidus	Por_01n	GU586932	NA	NA
H. caudatus	1578	AY348579	NA	NA
H. fructigenus	*CBS 650.92*	GU586933	GU586971	GU586952
H. fructigenus	F109077	DQ431169	NA	NA
H. scutulus	1278	AY348591	NA	NA
H. serotinus	F156526	DQ431178	NA	NA

GENT, Herbaria University Gent; ZT, Herbarium Zürich; NMLU, Mykologisches Herbar Natur-Museum Luzern; HMIPC, Department of Forest Pathology, University of Agriculture, Krakow, Poland; CBS, Centraalbureau voor Schimmelcultures; NA = not available.
[1]Type specimens (bold).
[2]Herbarium specimens (italic).
[3]Sequences produced for this study are in bold type.

2.6 Morphology

Dry apothecia were sectioned with a razor blade to evaluate morphological characters. Sections were mounted in lactic acid on microscopic slides and gently heated to facilitate soaking of the fungal structures. Preparations were examined with an Olympus 'BX51' compound microscope. Normally, 25–40 mature ascospores from 2 to 3 apothecia were measured for each specimen (Fig. 3).

3 Results

3.1 Field observations

In 2009, the first apothecia were found in the forest around Zürich on 27th June. During July 2009, mass fructifications of *H. albidus* were observed in several regions of Switzerland where pronounced ash dieback occurred (cantons Aargau, Luzern, Jura and Zürich). In regions where the disease was absent (cantons Ticino, Glarus, Wallis), few apothecia were found. Apothecia developed only rarely on artificially incubated ash rachises with pseudosclerotia by 20th August.

3.2 Detection of two subgroups within *H. albidus* based on ITS sequence data

Phylogenetic analysis based on sequence data of the ITS region placed all sequences of *H. albidus* in a cluster with high bootstrap support (Fig. 1). However, two subgroups were recognized, supported by moderate bootstrap support values. All strains isolated from necrotic lesions were in subgroup 2, which also included all samples from Poland and the type strain of the anamorph *C. fraxinea* (CBS 122504). In all but one location, apothecia belonged to a single subcluster. Only apothecia from Lägern Mountain (Zürich, Switzerland) fell into both subgroups.

The herbarium specimens from Switzerland represented both subgroups but each specimen belonged to only one subgroup (Table 1). From the type material of *H. albidus* (specimens of Desmazières in the Gent Herbarium, Belgium), no ITS sequences could be amplified.

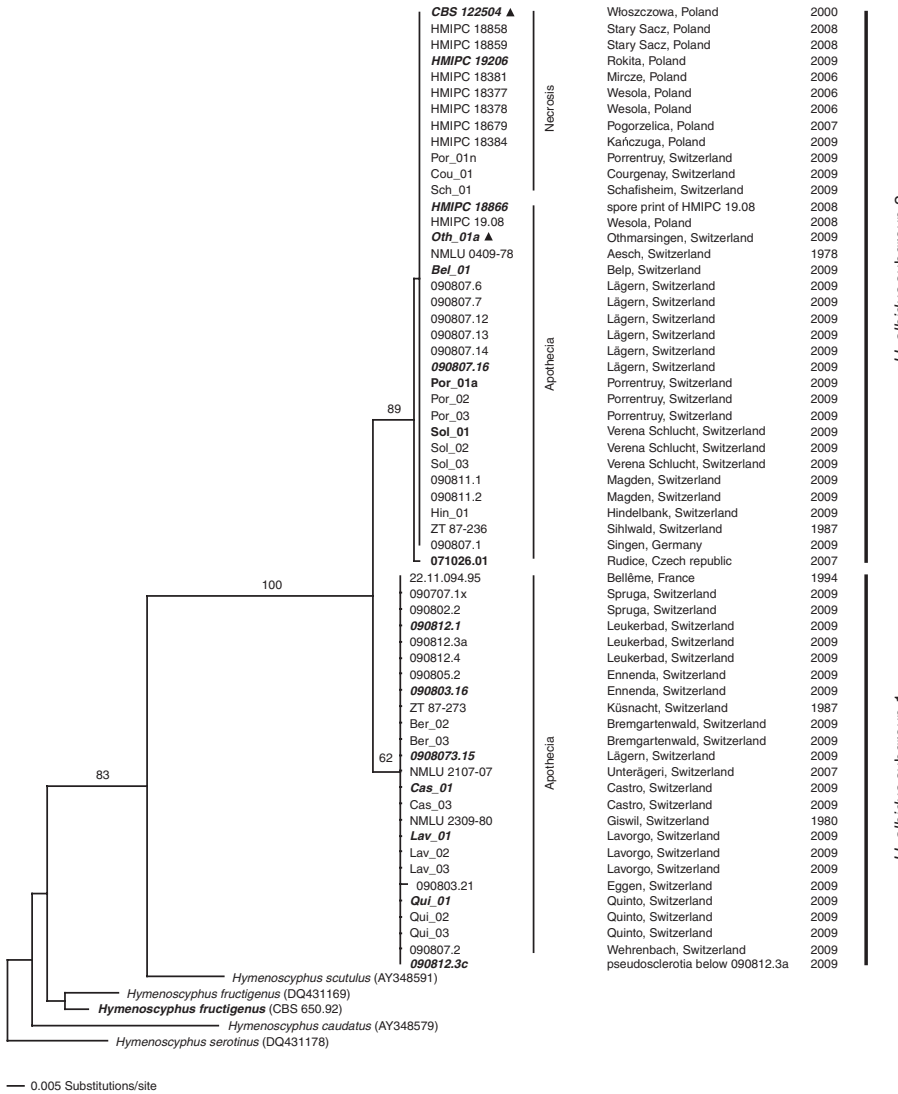


Fig. 1. Phylogenetic relationships within *Hymenoscyphus albidus* and among other *Hymenoscyphus* species based on DNA sequences of the ITS regions. Strains selected for genotyping (bold; see Fig. 2), type specimens (▲).

3.3 Confirmation of subgroups based on additional sequence markers and ISSR fingerprinting

Eighteen strains and apothecia representing both subgroups of *H. albidus* were further analysed using two additional sequence markers and ISSR-PCR. Primers Cal_228F and Cal_737R amplified a fragment with a calcium-binding motif (EF hand, cd00051) specific to the calmodulin gene. The amplified product includes three introns with a total length of 302 bp. Primers EF1-728F and EF1alpha_R1 amplified a fragment with high similarity to

other fungal EF1-α sequences and includes the EF1 alpha domain (cd01883). The EF1-α fragment includes two introns with a total length of 323 bp. Alignments for calmodulin, EF1-α, and ITS sequences for the 16 *H. albidus* strains and *H. fructigenus* (CBS 650.92) are available in TreeBase (accession no. S2661). Phylogenetic analysis of both loci confirmed the presence of two subgroups in *H. albidus.* The two subgroups were highly supported irrespective of the sequence marker applied (Fig. 2a,b) and for the concatenated dataset (Fig. 2c). Similarly, two highly supported groups were observed using PCR fingerprinting data from amplifications with primers CCA and Herp_01 (Fig. 2d).

3.4 Molecular diagnostic characters for subgroup differentiation

Twenty-one fixed nucleotide changes including one indel were found in the calmodulin fragment. Whereas 20 of the changes were found in the three introns, only one was found in the coding region. Similarly, 19 fixed nucleotide changes including one indel were found in the EF1-α fragment. All changes in the EF1-α fragment occurred in the two introns.

3.5 Morphology

The overall morphology of the apothecia of both subgroups was very similar; no distinguishing characters were found in terms of colour, size and texture. Differences were found, however, in the size of the ascospores (Fig. 3). Ascospores of subgroup 2 were significantly longer than those of subgroup 1 (ANOVA, $p = 0.038$). Nevertheless, spore measurements from both subgroups overlapped considerably making it difficult or impossible to assign a given specimen to one of the subgroups based on ascospore measurements alone.

4 Taxonomy

Based on these data, a new species is proposed for *H. albidus* subgroup 2:

Hymenoscyphus pseudoalbidus V. Queloz, C.R. Grünig, R. Berndt, T. Kowalski, T.N. Sieber & O. Holdenrieder sp. nov.

Hymenoscyphus albidus morphologia similis, sed sequentia nucleotidium valde distinctus, viz. ob 'calmodulin' positiones 37(C), 49 (T), 83(G), 124(A), 127(A), 127(A), 150(C), 163(C), 223(C), 244(C), 247(T), 256(A), 271(A), 278(A), 283(C), 284(T), 347(C), 424(G), 425(A), 439(A), 439(A); 'translation elongation factor 1-α' positiones 40(A), 67(T), 89(G), 127(C), 143(T), 148(C), 180(C), 189(T), 192(A), 199(C), 211(G), 215(T), 223(T), 230(C), 236(G), 241(G), 260(A), 453(A).

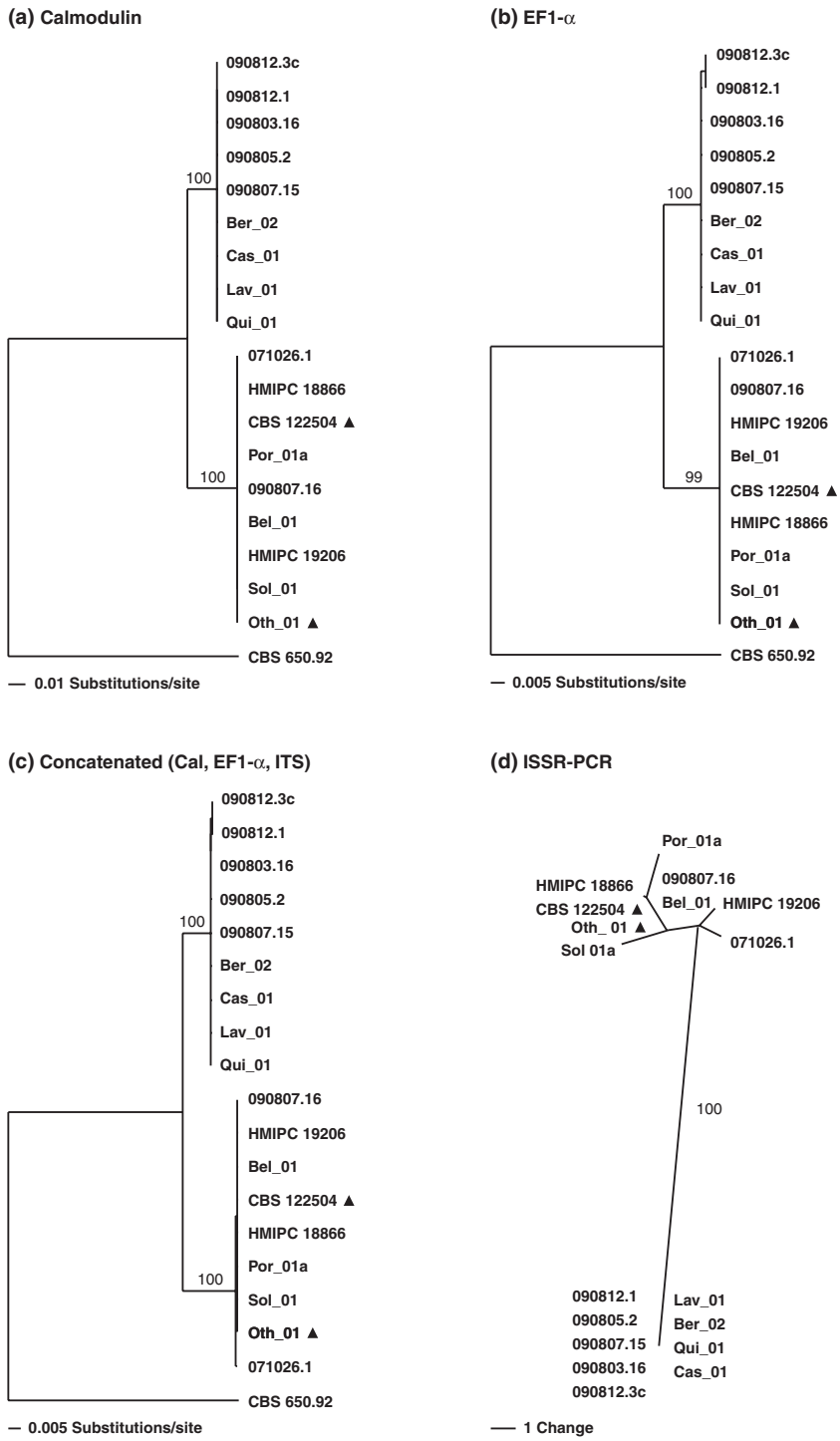
In petiolis foliorum *Fraxini excelsioris*.

HOLOTYPUS: Switzerland, Othmarsingen, forest site Gislisberg, N 47°23′56.0″/E 08°13′55.0″, on rachises of previous year's leaves of *Fraxinus excelsior* within an Aceri-Fraxinetum typicum, leg. V. Queloz 21.07.2009 (ZT Myc 2022).

Hymenoscyphus pseudoalbidus is morphologically similar to *H. albidus*, but differs in the following fixed nucleotide characters: calmodulin positions 37(C), 49(T), 83(G), 124(A), 127(A), 150(C), 163(C), 223(C), 244(C), 247(T), 256(A), 271(A), 278(A), 283(C), 284(T), 347(C), 424(G), 425(A), 439(A), 439(A); translation elongation factor 1-a positions 40(A), 67(T), 89(G), 127(C), 143(T), 148(C), 180(C), 189(T), 192(A), 199(C), 211(G), 215(T), 223(T), 230(C), 236(G), 241(G), 260(A), 453(A).

4.1 Lectotypification of *Hymenoscyphus albidus*

The original description of *H. albidus* (DESMAZIÈRES 1851) refers to material distributed by Desmazières as *Peziza albida* Rob. ex Desm. in Pl. crypt. Fr., n° 2004 (1 ed.) and n° 1604 (2


(a) Calmodulin
090812.3c
090812.1
090803.16
090805.2
100
090807.15
Ber_02
Cas_01
Lav_01
Qui_01
071026.1
HMIPC 18866
CBS 122504 ▲
Por_01a
100
090807.16
Bel_01
HMIPC 19206
Sol_01
Oth_01 ▲
CBS 650.92
0.01 Substitutions/site
(b) EF1-α
090812.3c
090812.1
090803.16
090805.2
100
090807.15
Ber_02
Cas_01
Lav_01
Qui_01
071026.1
090807.16
HMIPC 19206
Bel_01
99
CBS 122504 ▲
HMIPC 18866
Por_01a
Sol_01
Oth_01 ▲
CBS 650.92
0.005 Substitutions/site
(c) Concatenated (Cal, EF1-α, ITS)
090812.3c
090812.1
090803.16
090805.2
100
090807.15
Ber_02
Cas_01
Lav_01
Qui_01
090807.16
HMIPC 19206
Bel_01
CBS 122504 ▲
HMIPC 18866
Por_01a
100
Sol_01
Oth_01 ▲
071026.1
CBS 650.92
0.005 Substitutions/site
(d) ISSR-PCR
Por_01a
090807.16
HMIPC 18866
Bel_01
HMIPC 19206
CBS 122504 ▲
Oth_ 01 ▲
071026.1
Sol 01a
100
090812.1
Lav_01
090805.2
Ber_02
090807.15
Qui_01
090803.16
Cas_01
090812.3c
1 Change

ed.). As Desmazières did not designate a type specimen we select as lectotype the specimen of Pl. crypt. Fr., n° 2004 kept in GENT.

Hymenoscyphus albidus (Rob. ex Desm.) Phillips 1887. Lectotypus hic designatus: Pl. crypt. Fr., n° 2004 in herbario GENT.

5 Discussion

In the present study, we showed that collections assigned to *H. albidus* fall into two groups that were hardly separable by morphological characters (Fig. 3) but were clearly distinguished by the DNA sequences of ITS and at the calmodulin and translation elongation factor 1-α loci (Figs 1 and 2). No evidence for hybridization among the two species was detected (Fig. 2a,b). This finding was confirmed by ISSR-fingerprinting (Fig. 2d). It is concluded that *H. albidus*, as understood hitherto, comprises two species, *H. albidus* and *H. pseudoalbidus*. Such morphologically very similar or indistinguishable species have been termed 'cryptic species'. Recently, several ascomycete species were found to comprise cryptic species that had gone unnoticed before (e.g. Grünig et al. 2007, 2008, 2009; Carriconde et al. 2008; Giraud et al. 2008; Sato and Murakami 2008; Zaffarano et al. 2008; O'Donnell et al. 2009; Villalta et al. 2009). In the present study, a combination of two classes of markers differing in resolution was used to explore species boundaries. The combination of ISSR fingerprinting and genealogical concordance phylogenetic species recognition (Taylor et al. 2000) revealed a clear distinction of *H. albidus* into two separate taxa.

Hymenoscyphus albidus has never been reported to be pathogenic and does not seem to be the causal agent of ash dieback. This conclusion is supported by the observation that *H. albidus* was the only species present in regions without ash dieback in Switzerland. It does occur in diseased ash stands as well, however. In contrast, all strains isolated from necrotic stem lesions and the majority of apothecia collected in *F. excelsior* stands with disease symptoms were *H. pseudoalbidus*. Pathogenicity of *H. pseudoalbidus* has been convincingly demonstrated (Bakys et al. 2009a,b; Kowalski and Holdenrieder 2009a), but no experimental data are available yet for *H. albidus.*

In Switzerland, the epidemic was not evident before 2007 (Engesser et al. 2009) and the major part of the country was still considered as disease-free in autumn 2009 (R. Engesser, personal communication). It is therefore surprising that two specimens from Switzerland collected in 1978 (Aesch, NMLU 0409-78) and 1987 (Sihlwald, ZT-87-236) could be assigned to *H. pseudoalbidus*. It is possible that several introductions of *H. pseudoalbidus* occurred before 2007, but caused only very local problems on ash, which remained unnoticed and the pathogen subsequently became extinct. An alternative explanation may be a subtle climatic change, which increased host susceptibility. For example, a climatically triggered epidemic was reported for *Dothistroma septosporum* (Dorog.) Morelet needle cast on lodgepole pine in Canada (Woods et al. 2005). However, it is difficult to imagine that such an environmental change has occurred at the European scale without being noticed. Moreover, the ash dieback epidemic is associated with a high density of the pathogen and moved more or less in a front from East to West. For these reasons, we assume that *H. pseudoalbidus* (or a virulent mutant of *H. pseudoalbidus)* has been introduced to Europe from an as yet unknown region. Field observations show that *F. ornus* L. is less susceptible than the other European ash species (Kirisits et al. 2009a). *Fraxinus ornus* is more closely

Fig. 2. Phylogenetic relationships within the morphospecies *Hymenoscyphus albidus* and among other *Hymenoscyphus* species based on DNA sequences of the loci calmodulin (a) and EF1-α (b), the concatenated sequences of calmodulin, EF1-α and ITS (c) and ISSR-data (d). *Hymenoscyphus fructigenus* (CBS 650-929) was used as an outgroup. Type specimen (▲).

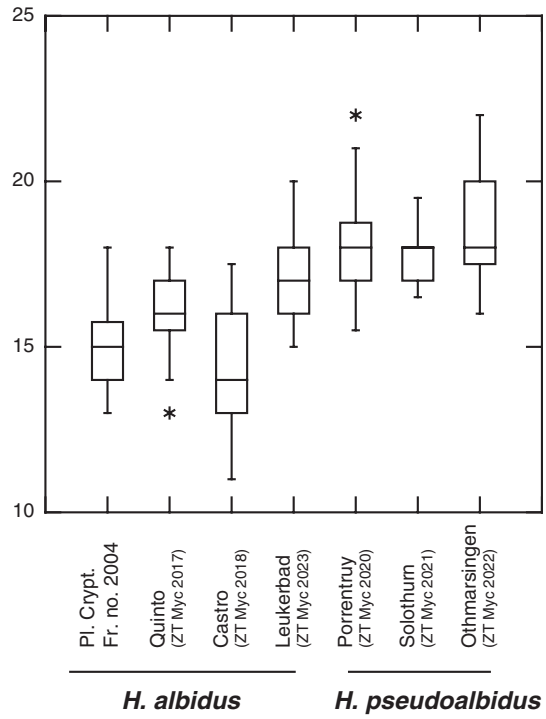


Fig. 3. Ascospore length (µm) of *Hymenoscyphus albidus* and *Hymenoscyphus pseudoalbidus* collections from different locations.

related to Asian ash species than *F. excelsior* and *F. angustifolia* (FRAXIGEN 2005, p. 89), suggesting a possible origin of the ash dieback pathogen in Asia. Population genetic studies are required to test this hypothesis.

Plant health and global change are intricately linked and emerging diseases caused by introduced pathogens provide major challenges for ecosystem management (Pautasso et al. 2010). Fungal invasions are increasingly common phenomena, which can remain unnoticed for decades, may have major implications for biosecurity and are insufficiently studied (Desprez-Loustau et al. 2007, 2010). We assume that the ash dieback pathogen is transmitted by airborne ascospores at the continental scale (Kowalski and Holdenrieder 2009b). In addition, susceptible *Fraxinus* species have a very large and continuous distribution area in Europe (FRAXIGEN 2005). Therefore, we do not believe that costly phytosanitary campaigns (e.g. see MacLeod 2007), can stop the epidemic on the European mainland. However, long distance dispersal, e.g. with infected plant material, to yet disease-free countries which are clearly separated from epidemic regions by natural barriers, might be prevented by quarantine measures.

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