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Hymenoscyphus pseudoalbidus, the correct name for *Lambertella albida* reported from Japan

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Abstract — Recent molecular analyses separate *Hymenoscyphus pseudoalbidus* (causal agent of ash dieback in Europe) from the morphologically scarcely distinguishable *H. albidus*. *Hymenoscyphus albidus* was reported (as "*Lambertella albida*") on petioles of *Fraxinus mandshurica* in Japan. Phylogenetic analysis in the present study shows Japanese "*L. albida*" to be conspecific with *H. pseudoalbidus* but with a higher genetic variability compared to European isolates. The presence of croziers at the ascus base was found to be a clear distinguishing character of *H. pseudoalbidus*. Our phylogenetic analysis of the combined ITS and LSU-D1/D2 dataset supports *Hymenoscyphus* as more appropriate than *Lambertella* for *H. pseudoalbidus*. As the *Hymenoscyphus* clade includes members with two major characters (presence of substratal stroma and brown ascospores) currently used to circumscribe *Lambertella*, the generic delimitation of *Lambertella* requires redefinition.

Key words — *Chalara fraxinea*, *Helotiaceae*, *Rutstroemiaceae*

Introduction

Ash dieback, an emerging infectious disease of common ash (*Fraxinus excelsior*), has reached epidemic levels in Central Europe during the last two decades (Bakys et al. 2009; Kowalski 2006). Since the first disease report around 1992 in Poland, ash dieback has spread into 22 European countries (Timmermann et al. 2011). The causal agent, *Chalara fraxinea* T. Kowalski was first described in Poland (Kowalski 2006). Later, its teleomorph was found and tentatively identified as *Hymenoscyphus albidus* (Gillet) W. Phillips (Kowalski & Holdenrieder 2009). Further research revealed that *H. albidus* was a complex of two species, and the ash dieback pathogen was described as a new

taxon *Hymenoscyphus pseudoalbidus* Queloz et al., separated from *H. albidus* based on differences in ITS rDNA, calmodulin gene (CAL), and translation elongation factor 1-α gene (EF1-α), as well as substantial differences in inter-simple sequence repeat anchored PCR (ISSR-PCR) fingerprinting (Queloz et al. 2011). The genetic distinction between *H. albidus* and *H. pseudoalbidus* was later confirmed by population genetic studies (Bengtsson et al. 2012, Gross et al. 2011, Husson et al. 2011). Queloz et al. (2011) indicated that the two species are indistinguishable in apothecial morphology except for a tendency toward slightly longer ascospores in *H. pseudoalbidus*. In fact, measurements in the two species overlapped, making it virtually impossible to distinguish *H. pseudoalbidus* from *H. albidus* based on ascospore size (Queloz et al. 2011).

However, recent morphological studies of European materials revealed a sharp difference in the ascus base that permits unequivocal distinction between *H. albidus* and *H. pseudoalbidus* (Baral & Bemmann, in prep.). The importance of ascal croziers in helotialean fungi was discovered by White (1942, 1943, 1944). Although many later workers neglected this discovery, a number of studies have confirmed that the presence or absence of ascal croziers is a stable characteristic that clearly distinguishes between closely allied species (e.g., Baral 1984; Huhtinen 1989).

Carpenter (1981) transferred *Hymenoscyphus albidus* to *Lanzia* based on the presence of a conspicuous substratal stroma. The transfer to *Lambertella* (Korf 1982) was based on the presence of some brown spores in the studied specimen (R.P. Korf, pers. comm.), and this recombination was confirmed when Japanese material was studied by Hosoya et al. (1993), who likewise found the ascospores to become brown prior to germination. Although these two characters were also recognized in the teleomorph classification of *Chalara fraxinea*, Kowalski & Holdenrieder (2009), who followed European usage to classify the species in *Hymenoscyphus,* did not place the species in *Lambertella.*

Lambertella albida (Gillet) Korf was reported as a new record for Japan by Hosoya et al. (1993) from petioles of *Fraxinus mandshurica*, a common ash in northeastern Asia. The asexual reproductive structure was also obtained in pure culture of Japanese material, but the authors regarded it as having a spermatial function. Since conidiophores have never been reported for *H. albidus*, and *H. pseudoalbidus* was considered morphologically indistinguishable from *H. albidus*, the taxonomic status of "*L. albida*" as reported from Japan needs to be reconsidered.

The aims of the present research were: 1) to clarify whether Japanese "*L. albida*" belongs to *H. albidus* or *H. pseudoalbidus* based on molecular phylogeny; 2) to examine if the presence of croziers is appropriate to separate the two species; 3) to clarify whether *Hymenoscyphus* or *Lambertella* is more appropriate for Japanese specimens currently known as "*L. albida*".

Materials & methods

Specimens and strains

Thirty-one specimens were analyzed, which include nine "*L. albida*" specimens from Japan, sixteen further specimens classified as *Lambertella* spp., four specimens classified as *Hymenoscyphus* spp., and two *Lachnum* species as outgroup (Table 1). Japanese isolates were obtained from single spores, using a Skerman's micromanipulator (Skerman 1968). Three *L. albida*" isolates of (FC-1445, FC-2793, FC-2799) were maintained on potato dextrose agar (PDA, Nissui, Tokyo, Japan) slants and deposited in Biological Resource Center, National Institute of Technology and Evaluation (NITE-BRC, Japan).

Observation of ascal base and anamorph

The bases of asci were examined for the presence of croziers or simple septa in Japanese specimens previously identified as "*L. albida*". Apothecial fragments were mounted in either water + 1% phloxine B solution or 5–10% KOH + Congo Red and strongly squashed.

In order to observe the anamorph, isolates were incubated on malt extract agar (MEA, 20 g malt extract, 20 g agar, 1000 ml distilled water) at room temperature up to 1 month.

DNA extraction, PCR amplification and sequencing

Isolates were cultivated in 2 ml of 2% malt extract broth (20 g malt extract, 1000 ml distilled water) for 2 weeks to obtain mycelium. Genomic DNA of isolates was obtained from about 50 mg of frozen mycelia using a DNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instruction. Genomic DNA from apothecia was extracted using the modified Cetyltrimethylammonium bromide (CTAB) extraction following glass milk purification methods as summarized by Hosaka (2009) and Hosaka & Castellano (2008). Briefly, samples were ground in liquid nitrogen using mortar and pestle, incubated in CTAB buffer (2% CTAB, 100 mM Tris pH 8.0, 20 mM EDTA, 1.4 M NaCl) at 65°C for 1 hour, and proteins were removed using the mixture of chloroform: isoamylalcohol (24: 1). The materials were further purified using 6M sodium iodine buffer (1M Tris pH 6.8, 2M Na_2SO_3) with glass milk, washed with ethanol/buffer solution (10 mM Tris pH 7.4, 1mM EDTA, 100 mM NaCl, 50% EtOH), and finally eluted in 100 µl Tris-EDTA buffer (TE, 10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The internal transcribed spacer regions (ITS-5.8S), the LSU-D1/D2 region of the large subunit rDNA gene (28S rDNA), the calmodulin gene (CAL) and translation elongation factor 1- α (EF1-α) were sequenced using primer pairs ITS1F and ITS4 or ITS1 and ITS4 (White et al. 1990), NL1 and NL4 (O'Donnell 1993), Cal-228F and Cal-737R (Carbone & Kohn 1999), EF1-728F and EF1α_R (Carbone & Kohn 1999, Grünig et al. 2007), respectively. PCR reactions were performed using 10 µl reaction volumes each containing: 0.5 µl genomic DNA, 0.25 µl of each primer (10 µM), 0.05 µl (0.25 unit) of ExTaq DNA polymerase (TaKaRa, Tokyo, Japan), 5.0 µl deoxynucleotide triphosphate (dNTP) mixture containing 2.5 mM each dNTP and ExTaq buffer containing 2 mM Mg^{2+} (adding 3.95 µl distilled water to get 10 µl reaction volumes). Amplifications were performed for ITS-5.8S and D1/D2 region with preliminary denaturation at 94°C for

3 min, 35 amplification cycles (94°C for 1 min, 52°C for 30 s and 72°C for 1 min), followed by a final extension at 72°C for 7 min. For both CAL and EF1-α regions, the annealing temperature was set at 54°C for 30s. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. PCR products were then purified using an ExoSAP-IT purification kit (USB, Cleveland, OH, USA) following the manufacturer's instruction.

Sequencing was carried out using the Big Dye Terminator Cycle Sequencing Kit on the DNA auto sequencer 3130x (Applied Biosystems Inc., Norwalk, CT, USA), following the manufacturer's instructions. Sequences were assembled and edited by SeqMan (Lasergen v6 DNAstar), and congruent sequences obtained from both strands were saved. A total of 92 sequences generated from this study were deposited in GenBank (Table 1).

Phylogenetic analyses

The obtained DNA sequences were aligned by Clustal W using the default parameters (Thompson et al. 1994) and edited manually when necessary using BioEdit ver. 7.0.5.2 (Hall 1999). Ambiguous regions having gaps were excluded from the analyses by checking the alignment in BioEdit. Each of the three datasets (ITS-5.8S, CAL and EF1-α) including all sequences of European *H. albidus* and *H. pseudoalbidus* used in the analyses of Queloz et al. (2011) and Japanese "*L. albida*" species were analyzed, rooted with other *Hymenoscyphus* species as used in the analyses of Queloz et al. (2011) (*H. scutula*, *H. fructigenus*, *H. caudatus*, *H. serotinus* for ITS-5.8S; *H. fructigenus* for CAL and EF1-α). 103 sequences downloaded from GenBank shown in Fig. 1–3 were used. A combined sequence data set (ITS-5.8S and D1/D2 region) was also analyzed, including seven "*L. albida*", 24 *Lambertella* species, nine *Hymenoscyphus* species, and rooted with *Lachnum abnorme* and *Lachnum virgineum*.

Maximum parsimony (MP) and maximum likelihood (ML) methods were used to analyze all four datasets. The combination of these two methods was suggested as a supportive way when constructing a phylogenetic tree (Kolaczkowski & Thornton 2004). The MP analyses were performed using PAUP version 4.0b10 (Swofford 2002). Heuristic searches were conducted with tree bisection–reconnection branch swapping algorithm (TBR), random sequence additions, and with Multrees option on. Bootstrap values (BP) of the most parsimonious trees were obtained from 1000 replications.

The ML analyses were conducted using GARLI version 0.951 (Zwickl 2006). The analyses were conducted using the GTR+G+I model (six general time reversible substitution rates, assuming gamma rate heterogeneity and a proportion of invariable sites), with model parameters estimated over the duration of specified runs. The tree topology with the highest likelihood was inferred from 10 independent runs from random starting trees. The "stopgen" parameters were set to 50,000,000 and other parameters were set to default values. Bootstrap analyses were done using 1000 replications with the same parameters as the initial tree search. The data matrix and trees have been deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study /TB2:S12500).

Genetic variation analysis

DnaSP Version 4.0 software (Librado & Rozas 2009) was used to investigate the genetic diversity among the European and Japanese populations of *H. pseudoalbidus*.

Table 1 Specimens, pure cultures, and sequences used in the present study.

Species	Pure culture	Specimen	Locality	Substrate	GenBank accession no.			
					ITS	D1-D2	CAL	EF1-α
Hymenoscyphus pseudoalbidus (as "*Lambertella albida*")	NA	TNS-F-52061	Yubari, Hokkaido Pref.	Petiole of *Fraxinus mandshurica*	AB705222	AB705262	—	—
	NA	TNS-F-52060	Tomakomai-shi, Hokkaido Pref.		—	—	—	—
	NA	TNS-F-52062	Ueda-shi, Nagano Pref.		—	—	—	—
	NA	TNS-F-12503	Sugadaira, Nagano Pref.		AB705221	AB705261	AB705208	AB705214
	FC-1445	TNS-F-12761	Sugadaira, Nagano Pref.		AB705218	AB705258	AB705209	AB705215
	FC-2793	TNS-F-40043	Yubari, Hokkaido Pref.		AB705219	AB705259	AB705206	AB705212
	FC-2799	TNS-F-40051	Chitose, Hokkaido Pref.		AB705220	AB705260	AB705207	AB705213
	NA	TNS-F-40074	Sugadaira, Nagano Pref.		AB705223	AB705263	AB705210	AB705216
	NA	TNS-F-17817	Sugadaira, Nagano Pref.		AB705224	AB705264	AB705211	AB705217
Hymenoscyphus fructigenus (Bull.) Gray	FC-2375	TNS-F-31299	Ushiku-shi, Ibaraki Pref.	Acorn	AB705225	AB705265	—	—
	FC-1093	TNS-F-11208	Towada-ko-machi, Aomori Pref.	Leaf of *Ginkgo biloba*	AB705226	AB705270	—	—
Hymenoscyphus ginkgonis J.G. Han & H.D. Shin	FC-1491	NA	—	—	AB705227	AB705266	—	—
	FC-1492	NA	—	—	AB705228	AB705268	—	—
	FC-1493	NA	—	—	AB705229	AB705269	—	—
	FC-1494	NA	—	—	AB705230	AB705267	—	—
Hymenoscyphus herbarum (Pers.) Dennis	NBRC-9520	NA	—	—	AB705231	AB705271	—	—
Hymenoscyphus. sp. 1	FC-2727	TNS-F-40037	Ueda-shi, Nagano Pref.	Grass stem	AB705232	AB705272	—	—
Hymenoscyphus sp. 2	FC-2725	TNS-F-40029	Ueda-shi, Nagano Pref.	Unknown leaf	AB705233	AB705273	—	—
Lachnum abnorme (Mont.) J.H. Haines & Dumont	FC-2118	TNS-F-16609	Ashigarakami-gun, Kanagawa Pref.	Wood of *Cephalotaxus harringtonia*	AB705234	AB705274	—	—
Lachnum virgineum (Batsch) P. Karst.	FC-2137	TNS-F-16583	Ashigarakami-gun, Kanagawa Pref.	Unknown	AB705235	AB705275	—	—
Lambertella advenula (W. Phillips) Hosoya & Y. Otani	FC-1007	TNS-F-11194	Agatsuma, Gunma Pref.	Needle of *Larix* sp.	AB705236	AB705276	—	—
	FC-2718	TNS-F-40015	Ueda-shi, Nagano Pref.		AB705237	AB705277	—	—
	FC-2722	TNS-F-40019	Ueda-shi, Nagano Pref.		AB705238	AB705278	—	—

Table 1 Continued

Species	Pure culture	Specimen	Locality	Substrate	GenBank accession no.			
					ITS	D1-D2	CAL	EF1-α
Lambertella cornimaris Höhn.	FC-2389	TNS-F-30402	Bunkyo-ku, Tokyo	Leaf of *Mallotus japonicus*	AB705239	AB705279	—	—
Lambertella sp. 1	NBRC- 9067	NA	—	—	AB705240	AB705280	—	—
	FC-1487	NA	—	—	AB705241	AB705281	—	—
	FC-1488	NA	—	—	AB705242	AB705282	—	—
	FC-1489	NA	—	—	AB705243	AB705283	—	—
	FC-1490	NA	—	—	AB705244	AB705284	—	—
	FC-2645	TNS-F-36994	Chiyoda-ku, Tokyo	Petiole of *Fatsia japonica*	AB705245	AB705287	—	—
	FC-1985	TNS-F-30018	Chiyoda-ku, Tokyo		AB705247	AB705286	—	—
	FC-1190	TNS-F-31157	Odawara-shi, Kanagawa Pref.		AB705246	AB705285	—	—
	NBRC- 6894	NA	-	-	AB705248	AB705288	—	—
Lambertella sp. 2	FC-1230	TNS-F-17633	Kikuchi-shi, Kumamoto Pref.	Leaf of *Aucuba japonica*	AB705249	AB705289	—	—
Lambertella sp. 3	FC-2726	TNS-F-40033	Ueda-shi, Nagano Pref.	Leaf of *Pinus* sp.	AB705250	AB705290	—	—
Lambertella sp. 4	NA	TNS-F-40036	Ueda-shi, Nagano Pref.	Unknown leaf	AB705251	AB705291	—	—
Lambertella sp. 5	NA	TNS-F-40038	Ueda-shi, Nagano Pref.	Unknown leaf	AB705252	AB705292	—	—
Lambertella sp. 6	NA	TNS-F-40026	Iriomote Isl., Okinawa Pref.	Unknown leaf	AB705253	AB705293	—	—
Lambertella sp. 7	NA	TNS-F-40027	Tsukuba-shi, Ibaraki Pref.	Leaf of *Camellia japonica*	AB705254	AB705294	—	—
Lambertella sp. 8	NA	TNS-F-40031	Ueda-shi, Nagano Pref.	Leaf of *Pinus* sp.	AB705255	AB705295	—	—
Lambertella yunnanensis (S.H. Ou) W.Y. Zhuang & Yan H. Zhang	NA	TNS-F-40035	Ueda-shi, Nagano Pref.	Branch of *Pinus* sp.	AB705256	AB705296	—	—
	NA	TNS-F-40028			AB705257	AB705297	—	—

* NA: not available. Apothecia were used for DNA extraction when isolates not present.

The alignments of the ITS region, and the portion of CAL and EF1-α regions were separately compared without deletions, and the genetic diversity parameter Pi was calculated.

Results

Taxonomic reconsideration of Japanese "*Lambertella albida*" based on three loci

The aligned ITS-5.8S rDNA dataset comprised 454 bp, and 11 ambiguously aligned sites (site nos. 100–102, 336–343) were excluded from the analyses. Three most parsimonious trees were obtained [tree length (TL): 67 steps, consistency index (CI): 0.8060, retention index (RI): 0.9615, rescaled consistency index (RC): 0.7750]; one is shown in Fig. 1. The ML analysis yielded an optimal tree with the best likelihood (log likelihood = –1051.7137), topologically congruent with this MP tree, which highly supported monophyly of the ingroup. In the ingroup, two major clades were supported with high BP (BP >80%) confirming Queloz et al. (2011); Japanese materials were included in the strongly supported (92/90% ML BP/MP BP) clade with European *H. pseudoalbidus*.

The aligned CAL dataset comprised 460 bp, and 18 ambiguously aligned sites (site nos. 38–42, 72–74, 82, 176–177, 201, 216–217, 247–250) were excluded from the analyses. One of the 10 MP trees obtained (TL: 28 steps, CI: 0.9286, RI: 0.9888, RC: 0.9182) is shown in Fig. 2. The ML tree (log likelihood = –1110.7132) showed the same topology with one MP tree. Here also two major clades were highly supported and Japanese materials were included in the European *H. pseudoalbidus* clade with high support.

The aligned EF1-α dataset comprised 553 bp with 31 ambiguously aligned sites (site nos. 35–36, 86–87, 121–127, 221–228, 233–239, 453–456) excluded from further analysis. Only one parsimonious tree (Fig. 3) was obtained (TL: 28 steps, CI: 0.8929, RI: 0.9779, RC: 0.8732). The ML tree (log likelihood = –1145.5894) did not show any topological conflict with the MP tree. Also, two major clades were obtained, one consisting of European *H. albidus* with high support and the other (including Japanese "*L. albida*" and *H. pseudoalbidus*) that was moderately supported (61/–%). In the latter clade, five out of six Japanese "*L. albida*" materials grouped with European *H. pseudoalbidus* with high support (97/97%).

Morphology of Japanese "*Lambertella albida*"

The presence of croziers at the base of asci was observed in all nine Japanese "*L. albida*" specimens (Fig. 4). White (1944) observed the absence of croziers in the type of *H. albidus;* Baral (Baral & Bemmann,in prep.) observed the presence of croziers in the type of *H. pseudoalbidus,* and he and Bemmann examined numerous European materials (including some specimens treated by Queloz et al. 2011) morphologically. The presence of basal croziers was found to be a clear distinguishing character between the two taxa (Baral & Bemmann,in prep.).

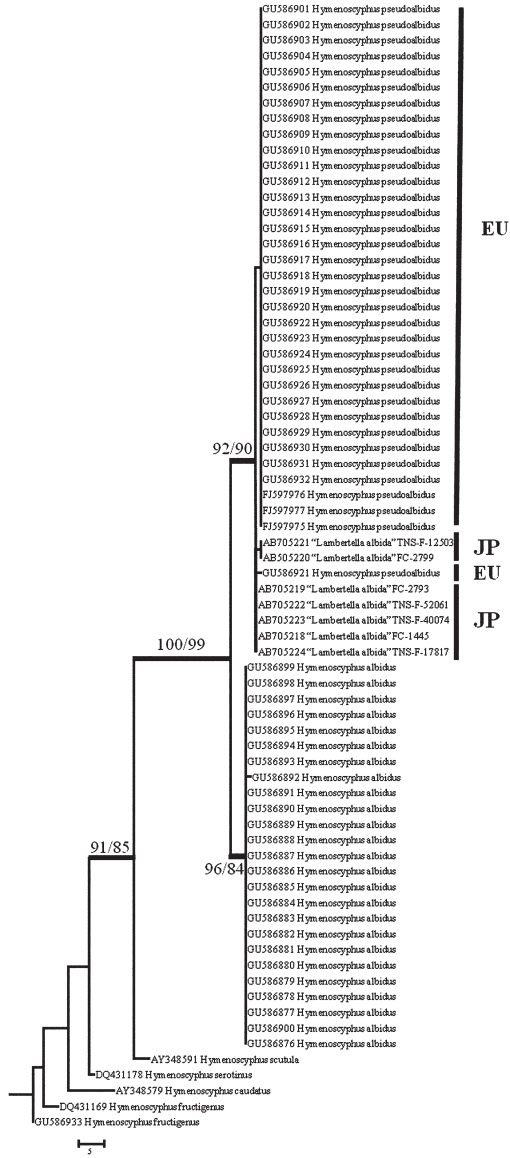

Fig. 1. One of three most parsimonious trees based on ITS rDNA sequences from Japanese "*Lambertella albida*" and European *Hymenoscyphus albidus* and *H. pseudoalbidus*. Numbers above the branches are maximum parsimony (MP) bootstrap values (BP) followed by maximum likelihood (ML) BP >50% in 1000 replications. Tree length: 67 steps; consistency index: 0.8060; retention index: 0.9615; rescaled consistency index: 0.7750. EU: European materials; JP: Japanese materials

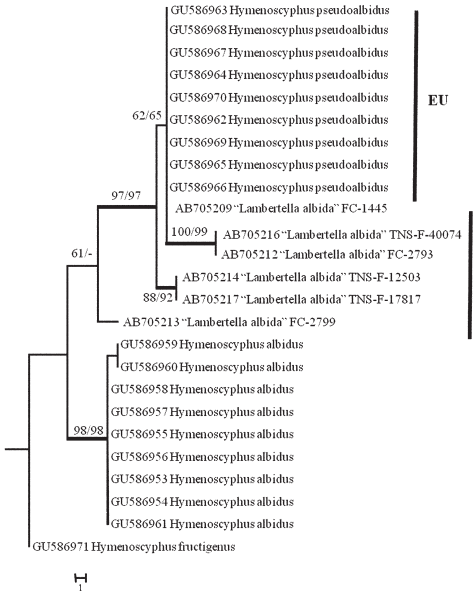

Fig. 2. One of ten most parsimonious trees based on CAL gene sequence data of Japanese "*Lambertella albida*" with European *Hymenoscyphus albidus* and *H. pseudoalbidus*. Numbers above the branches are bootstrap values (BP) in maximum parsimony analysis (MP) followed by BS from maximum likelihood (ML) >50% in 1000 replications. Tree length: 28 steps; consistency index: 0.9286; retention index: 0.9888; rescaled consistency index: 0.9182. EU: European materials; JP: Japanese materials

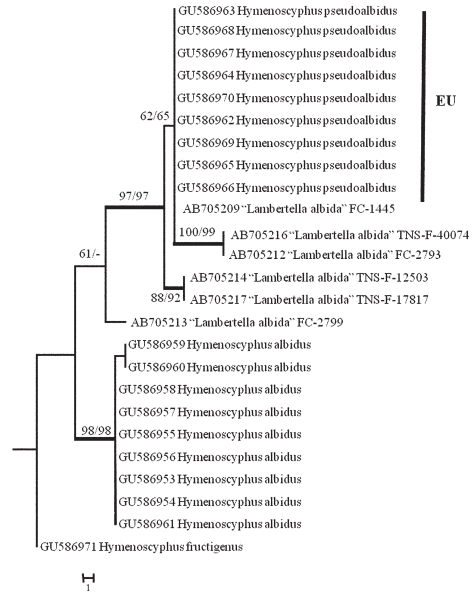

Fig. 3. Maximum parsimony tree based on EF1-α gene sequences from Japanese "*Lambertella albida*" and European *Hymenoscyphus albidus* and *H. pseudoalbidus*. Numbers above the branches are maximum parsimony (MP) bootstrap values (BP) followed by maximum likelihood (ML) BP >50% in 1000 replications. Tree length: 28 steps; consistency index: 0.8929; retention index: 0.9779; rescaled consistency index: 0.8732. "–": BP ≤50%; EU: European materials; JP: Japanese materials

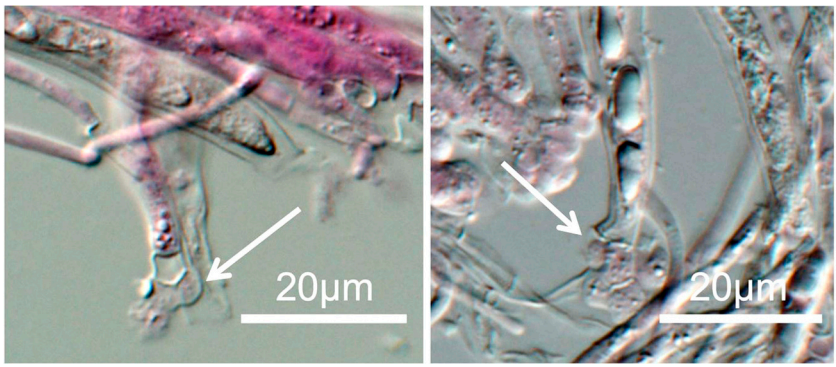

Fig. 4. Representative photos of croziers at the base of asci observed in Japanese materials of *Hymenoscyphus pseudoalbidus*. Mounted in 1% phloxine B solution (A. TNS-F-12503; B. TNS-F-52061). Scale = 20 µm

The conidia and conidiophores of *Chalara fraxinea* were obtained on MEA, but conidia did not germinate in any of the isolates during the prolonged incubation up to one month at room temperature.

Results from the above molecular, morphological, and cultural analyses all indicate that Japanese "*L. albida*" materials should be re-identified as *H. pseudoalbidus*.

Comparison of genetic variation between Japanese and European materials

Japanese *H. pseudoalbidus* showed a higher genetic variation compared with European *H. pseudoalbidus* and *H. albidus* in all the three genes, whereas no genetic difference was observed within European *H. pseudoalbidus* for CAL and EF1-α genes, and within European *H. albidus* for CAL gene. (Table 2).

Comparison of nucleotide characters between Japanese and European materials

Nucleotide differences in the EF1-α, CAL and ITS genes were found between European *H. pseudoalbidus* and *H. albidus* (Queloz et al. l.c.). Although most nucleotides from Japanese *H. pseudoalbidus* populations matched those from European *H. pseudoalbidus* populations, some positions deviated. Unexpectedly, these deviating positions consistently showed the same nucleotides as *H. albidus*. EF1-α gene nucleotides were most diverse among Japanese *H. pseudoalbidus* materials (Table 3). When comparing the 22 positions in which European *H. pseudoalbidus* differed from *H. albidus*, Japanese material showed concordance with *H. pseudoalbidus* in 7 positions, while the remaining 15 positions showed variation within Japanese materials. Thereby, each position that differed from European *H. pseudoalbidus* showed the nucleotide that characterized *H. albidus*. This phenomenon occurred mainly in positions included in the definition (protologue) of *H. pseudoalbidus* (Queloz et al. 2011), but also in 4 additional positions (232-235 in Table 3) that were not included

Table 2. Nucleotide diversity (Pi) between strains of European *H. pseudoalbidus*, Japanese *H. pseudoalbidus* ("*Lambertella albida*"), and European *H. albidus*.

Loci	Nucleotide diversity (Pi)		
	European HP	Japanese HP	European HA
ITS	0.00034 (n=35)	0.00287 (n=7)	0.00096 (n=25)
CAL	0.00000 (n=9)	0.01147 (n=6)	0.00000 (n=9)
EF1-α	0.00000 (n=9)	0.02188 (n=6)	0.00071 (n=7)

Table 3. EF1-α gene nucleotide comparisons in European *H. pseudoalbidus*, Japanese *H. pseudoalbidus* ("*Lambertella albida*"), and European *H. albidus*.

	Definition	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N
	Position	40	67	89	127	143	148	180	189	192	199	211	215	223	230	236	241	260	453	232	233	234	235
	EU HP	A	T	G	C	T	C	C	T	A	C	G	T	T	C	G	G	A	A	C	C	A	T
JP HP	FC-1445	A	T	G	C	T	C	C	T	A	C	G	T	T	C	G	G	A	A	C	C	A	T
JP HP	TNS-F-40074	A	T	G	C	T	C	C	T	A	C	G	T	T	C	G	G	A	G	C	C	A	T
JP HP	FC-2793	A	T	G	C	T	C	C	T	A	C	G	T	T	C	G	G	A	G	C	C	A	T
JP HP	TNS-F-12503	A	T	G	C	T	C	C	C	A	C	G	T	T	C	G	G	A	A	T	C	A	T
JP HP	TNS-F-17817	A	T	G	C	T	C	C	C	A	C	G	T	T	C	G	G	A	A	T	C	A	T
JP HP	FC-2799	A	T	G	C	T	C	A	C	C	T	G	C	C	A	GAP	C	G	A	T	T	GAP	GAP
	EU HA	G	C	A	G	C	G	A	C	C	T	A	C	C	A	GAP	C	G	G	T	T	GAP	GAP

* Y: Positions included in the definition of *H. pseudoalbidus* (protologue); N: Out of definition; EU: European; JP: Japanese; HP: *H. pseudoalbidus*; HA: *H. albidus*.

in the protologue. One strain (FC-2799) shared 14 nucleotides with *H. albidus* and 8 with *H. pseudoalbidus*, suggesting it is intermediate between these two species (Fig. 3, Table 3).

However, strain FC-2799 and four other Japanese materials concurred with *H. pseudoalbidus* in all 21 CAL gene positions where the European *H. pseudoalbidus* differed from *H. albidus*. Within these positions, some Japanese specimens showed variation at position 244 (specimens: TNS-F-40043, TNS-F-12503) and position 266 (specimen: TNS-F-12503) where they shared, instead, nucleotides with *H. albidus*. Further CAL region variation was noted at positions where European *H. albidus* and *H. pseudoalbidus* do not differ.

In the ITS rDNA, Japanese materials concurred with European *H. pseudoalbidus* except for two positions (site nos. 83, 124) at which all Japanese materials showed the character of *H. albidus*.

Generic taxonomy of *Hymenoscyphus pseudoalbidus*

The combined ITS-5.8S and D1/D2 dataset comprised 1183 bp (582 bp for ITS-5.8S rDNA, 601 bp for D1/D2), and 15 ambiguously aligned ITS sites (site nos. 80–81, 107–113, 127, 140–141, 206, 581–582) and 17 ambiguously aligned D1/D2 sites (site nos. 583–596, 781–782, 850) were excluded from the analysis. One of the 16406 equally parsimonious trees obtained (TL: 67 steps, CI: 0.8060, RI: 0.9615, RC: 0.7750) is shown in Fig. 5. The ML tree (log likelihood = –6682.1630) did not show any topological conflict with the MP trees. In the MP consensus tree and the best ML tree, two major clades were generated, one strongly supported (100/100%) and the other moderately supported (77/80%). The more strongly supported clade was composed solely of *Lambertella* species, including the type species *L. corni-maris*. The other clade included the *Hymenoscyphus* species and *L. yunnanensis*; *H. pseudoalbidus* grouped here with *H. fructigenus* (type species of *Hymenoscyphus*) with 99% BP support. This phylogenetic analysis shows *H. pseudoalbidus* as more distantly related to the *Lambertella* core group (including the type) and more closely related to the *Hymenoscyphus* core group (including the type).

Discussion

Based on the rapid expansion and high intensity of ash dieback in Europe, *H. pseudoalbidus* was considered as an invasive alien organism (Husson et al. 2011; Queloz et al. 2011; Timmermann et al. 2011). However, until now its

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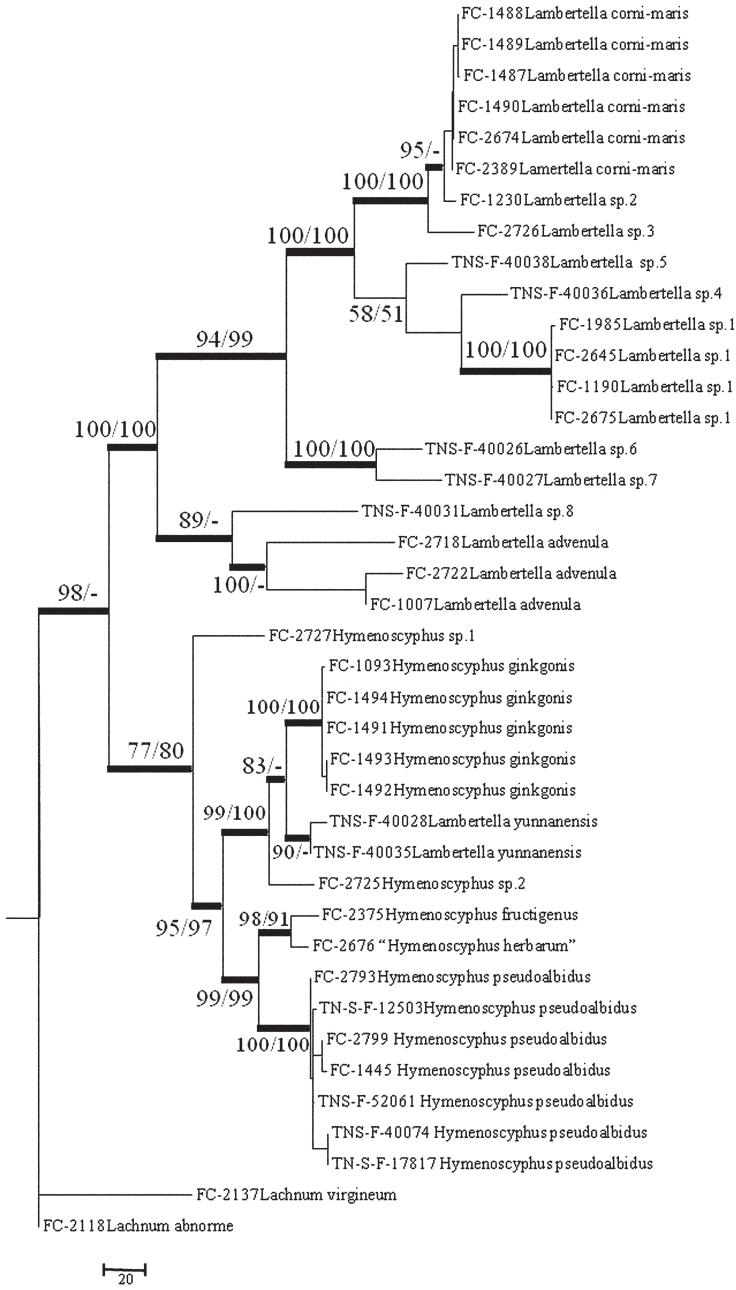
Fig. 5. One of 16406 most parsimonious trees inferred from combined dataset of ITS and D1-D2 sequences showing the relationship of Japanese *Hymenoscyphus pseudoalbidus* with other *Hymenoscyphus** and *Lambertella* species. Numbers above the branches are maximum parsimony (MP) bootstrap values (BP) followed by maximum likelihood (ML) BP >50% in 1000 replications. Tree length: 67 steps; consistency index: 0.8060; retention index: 0.9615; rescaled consistency index: 0.7750. "–": BP ≤50%.


FC-1488Lambertella corni-maris
FC-1489Lambertella corni-maris
FC-1487Lambertella corni-maris
FC-1490Lambertella corni-maris
FC-2674Lambertella corni-maris
FC-2389Lamertella corni-maris
FC-1230Lambertella sp.2
FC-2726Lambertella sp.3
TNS-F-40038Lambertella sp.5
TNS-F-40036Lambertella sp.4
FC-1985Lambertella sp.1
FC-2645Lambertella sp.1
FC-1190Lambertella sp.1
FC-2675Lambertella sp.1
TNS-F-40026Lambertella sp.6
TNS-F-40027Lambertella sp.7
TNS-F-40031Lambertella sp.8
FC-2718Lambertella advenula
FC-2722Lambertella advenula
FC-1007Lambertella advenula
FC-2727Hymenoscyphus sp.1
FC-1093Hymenoscyphus ginkgonis
FC-1494Hymenoscyphus ginkgonis
FC-1491Hymenoscyphus ginkgonis
FC-1493Hymenoscyphus ginkgonis
FC-1492Hymenoscyphus ginkgonis
TNS-F-40028Lambertella yunnanensis
TNS-F-40035Lambertella yunnanensis
FC-2725Hymenoscyphus sp.2
FC-2375Hymenoscyphus fructigenus
FC-2676 "Hymenoscyphus herbarum"
FC-2793Hymenoscyphus pseudoalbidus
TN-S-F-12503Hymenoscyphus pseudoalbidus
FC-2799 Hymenoscyphus pseudoalbidus
FC-1445 Hymenoscyphus pseudoalbidus
TNS-F-52061 Hymenoscyphus pseudoalbidus
TNS-F-40074 Hymenoscyphus pseudoalbidus
TN-S-F-17817 Hymenoscyphus pseudoalbidus
FC-2137Lachnum virgineum
FC-2118Lachnum abnorme
95/-
100/100
100/100
58/51
94/99
100/100
100/100
100/100
89/-
100/-
98/-
77/80
100/100
83/-
99/100
90/-
95/97
98/91
99/99
100/100
20

origin has remained uncertain. Field inoculations showed that *Fraxinus ornus*, closely related to many Asian ash species, is less susceptible to infection than the European common ash, *F. excelsior* (Kräutler & Kirisits 2011), leading Queloz et al. (2011) to suggest a possible Asian origin for the ash dieback pathogen. In addition, *F. mandshurica*, a northeastern Asian ash ornamental introduced in Europe, was only slightly affected by ash dieback in southeastern Estonia (Drenkhan & Hanso 2010). It must be mentioned, however, that *F. mandshurica* is not closely related to *F. ornus* but is, instead, close to *F. excelsior* (Wallander 2008). Pathogenicity of *H. pseudoalbidus* to indigenous ash has not been reported in Japan.

To clarify the origin of the pathogen, population genetic studies based on polymorphic microsatellite markers, have shown that *H. pseudoalbidus* possesses low genetic variation among and within populations (Bengtsson et al. 2012, Gross et al. 2011). The genetic homogeneity of European *H. pseudoalbidus* can be explained by the founder effect (characteristic of invasive populations established by a small number of individuals). In the present study, Japanese *H. pseudoalbidus* showed a higher genetic variation than European populations, and Japanese populations were more basal in the phylogenetic analyses. Together with a very low susceptibility of local *Fraxinus*, this does not contradict an Asian origin for *H. pseudoalbidus.* In order to unravel the origin of *H. pseudoalbidus*, however, further genetic comparisons of populations from other Asian regions are needed.

Comparison of the ITS, CAL and EF1-α gene nucleotides has shown the European *H. pseudoalbidus* and *H. albidus* to be extremely stable and with clear differences in various positions. Although Japanese *H. pseudoalbidus* shared most characters with the European *H. pseudoalbidus*, some nucleotide positions varied. Especially in the EF1-α gene, strain FC-2799 appeared intermediate between *H. pseudoalbidus* and *H. albidus*. Records of *H. albidus* in Japan are unknown up to now, and the reason for the intermediate nucleotide patterns needs further study. Possibly the mixed nucleotide positions in Japanese materials suggests a common ancestor of *H. albidus* and *H. pseudoalbidus* from eastern Asia.

Hosoya et al. (1993) was the first to report the presence of an anamorphic structure in *H. pseudoalbidus*, based on Japanese material. At the time, the authors regarded the structure as spermatia due to its minute size and the fact that conidial germination was not observed. In the present study, the absence of conidial germination was again noted (on MEA). This suggests that the asexual state of *H. pseudoalbidus* does not contribute to its long-range dispersal and that pathogen dispersal is limited to ascospores, as previously suggested (e.g. Gross et al. 2011, Timmermann et al. 2011, Gross et al. 2012).

Lambertella was chiefly characterized by the presence of a substratal stroma and ascospores that may become brown at various stages of spore development (Dumont 1971, Korf & Zhuang 1985). Our current phylogenetic analysis (Fig. 5) places *Lambertella* species in both of the two major clades, suggesting that the genus is not monophyletic. The molecular phylogeny of Holst-Jensen et al. (1997) also suggested polyphyly in *Lambertella*. Korf (1982) transferred *Hymenoscyphus albidus* to *Lambertella* because it has the major morphological characters of *Lambertella* (Hosoya et al. 1993). Both substratal stroma and brown ascospores were observed in *H. pseudoalbidus* by Kowalski & Holdenrieder (2009), but the authors retained this fungus in *Hymenoscyphus*.

Our phylogenetic analyses situate *H. pseudoalbidus* in the *Hymenoscyphus* group, suggesting that *Hymenoscyphus* is the more appropriate genus to accommodate this fungus at present. This is in concordance with morphological characteristics, such as the *Hymenoscyphus*-type of ascus apical ring and the heteropolar scutuloid ascospores in *H. pseudoalbidus* (Baral & Bemmann, in prep.). The *Hymenoscyphus* clade also contains *H. ginkgonis,* another species with a substratal stroma and ascospores becoming brown when germinated (although these characters were not mentioned by Han & Shin, 2008). In conclusion, the two major characters (presence of substratal stroma and brown ascospores) currently used to circumscribe the genus *Lambertella* seem to have resulted from convergent evolution, and the generic delimitation of *Lambertella* needs to be reconsidered.

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