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Birth of an order: Comprehensive molecular phylogenetic study excludes *Herpomyces* (Fungi, Laboulbeniomycetes) from Laboulbeniales

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ABSTRACT

The class Laboulbeniomycetes comprises biotrophic parasites associated with arthropods and fungi. Two orders are currently recognized, Pyxidiophorales and Laboulbeniales. *Herpomyces* is an isolated genus of Laboulbeniales, with species that exclusively parasitize cockroaches (Blattodea). Here, we evaluate 39 taxa of Laboulbeniomycetes with a three-locus phylogeny (nrSSU, ITS, nrLSU) and propose a new order in this class. Herpomycetales accommodates a single genus, *Herpomyces*, with currently 26 species, one of which is described here based on morphological and molecular data. *Herpomyces shelfordellae* is found on *Shelfordella lateralis* cockroaches from Hungary, Poland, and the USA. We also build on the six-locus dataset from the *Ascomycota Tree of Life* paper (Schoch and colleagues, 2009) to confirm that Laboulbeniomycetes and Sordariomycetes are sister classes, and we apply laboulbeniomyceta as a rankless taxon for the now well-resolved node that describes the most recent common ancestor of both classes.

1. Introduction

Laboulbeniomycetes is a class of perithecial fungi that are associated with arthropods as obligate biotrophs or for dispersal. The class is comprised of two orders, Laboulbeniales and Pyxidiophorales, and several unclassified organisms (e.g., *Laboulbeniopsis termitarius* Thaxt. and *Coreomycetopsis oedipus* Thaxt. on termites; Blackwell et al., 2003; Henk et al., 2003). Pyxidiophorales contains species associated with arthropods in their dispersal phase. Most species of *Pyxidiophora* Bref. & Tavel parasitize other fungi and their two-celled ascospores directly divide to develop a *Thaxteriola* Speg. asexual state (Blackwell and Malloch, 1989b; Kirschner, 2003; Weir and Blackwell, 2005), which is dependent on arthropods for dispersal. The *Thaxteriola* asexual state produces yeast-like cells that inoculate new substrates. A phylogenetic study using partial nuclear small subunit rDNA (nrSSU) sequences placed the asexual fungus *Gliocephalis hyalina* Matr. within Pyxidiophorales (Jacobs et al., 2005). Culturing of this fungus failed using standard monoxenic techniques but was successful when co-cultured with a *Fusarium* species (Barron, 1968; Jacobs et al., 2005).

Laboulbeniales are obligate biotrophs of arthropods. About 2,200 species are known to infect various groups in three subphyla – Chelicerata, Hexapoda, Myriapoda – and are known from all continents except Antarctica. Among the insects, the most basal host order (Blattodea) includes the cockroaches and termites. To date, 27 species of Laboulbeniales in three genera have been reported on cockroaches (Wang et al., 2016): *Herpomyces* Thaxt. (25 species), *Laboulbenia* Mont. & C.P. Robin (1), and *Rickia* Cavara (1). Although *Laboulbenia* and *Rickia* have a broad host range with the majority of species occurring on other host groups (e.g., Santamaria et al., 1991; Pfliegler et al., 2016), species of the genus *Herpomyces* occur only on cockroaches (Blattodea).

Herpomyces is the only genus in the family Herpomycetaceae I.I. Tav. and this is the only family in the suborder Herpomycetineae (Thaxt.) I.I. Tav. (Tavares, 1981). The genus was described by Thaxter (1902) and includes 25 species, all of which are parasites of cockroaches (Richards and Smith, 1954). *Herpomyces* is arguably the best-studied genus of the Laboulbeniales in terms of biology and thallus ontogeny. *Herpomyces*-infected cockroaches are easily reared and maintained, thus exhaustive studies have been done on the life history of these fungi, development, histopathology, and host specificity (Richards and Smith, 1954, 1955a, 1955b, 1956). In addition to the investigations by Richards and Smith cited above, Hill (1977) and Tavares (1965, 1966, 1980, 1985) contributed to our current

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knowledge of *Herpomyces* with detailed ultrastructural and developmental studies.

In his early efforts to organize the Laboulbeniales, Thaxter (1908) created two suborders based on antheridial characters, Laboulbeniineae and Ceratomycetineae, 2 families, and 22 tribes to accommodate the (at that time) 55 genera in the order. One of these tribes was Herpomyceteae, with a single genus, in the suborder Laboulbeniineae, family Laboulbeniaceae. Later, based on perithecium morphology and characters of ascus development, Tavares (1981) erected the suborder Herpomycetineae to accommodate Thaxter's tribe Herpomyceteae and to indicate the isolated position of these species. In this classification system (Tavares, 1981, 1985), *Herpomyces* species were considered sister to all other members of Laboulbeniales, which were placed in the suborder Laboulbeniineae. One important characteristic of Laboulbeniales (including *Herpomyces*) is that their perithecia have two wall layers, an outer and an inner wall. In most members of the Laboulbeniales, the perithecial wall arises from the perithecial basal cells (Tavares, 1985). In *Herpomyces* species, the inner wall cell rows start at the level of the fourth or fifth tier of the outer wall. As such, they may be comparable to periphyses, sterile elements that line the perithecial neck in many pyrenomycetes (Tavares, 1985). A further difference is marked in the number of ascospores per ascus. The asci of Laboulbeniales generally contain four two-celled ascospores. Asci of *Herpomyces* species, on the other hand, contain eight two-celled ascospores (Thaxter, 1908; Richards and Smith, 1955a; Tavares, 1985). Eight-spored asci predominate in the Ascomycota and this condition might be considered ancestral in the Laboulbeniomycetes.

Blackwell and Malloch (1989a) proposed these *Herpomyces* species as intermediate forms linking filamentous ascomycetes and other Laboulbeniales. The flask-shaped perithecia of *Herpomyces* species closely resemble those of *Pyxidiophora*. This view of the relationship and position of *Herpomyces* species was further supported by molecular phylogenetic reconstruction (Weir and Blackwell, 2001). Their phylogeny supported a sister relationship between Laboulbeniales and Pyxidiophorales, but no sequences of *Herpomyces* species were used in this study. Goldmann and Weir (2018) included a *Herpomyces* sequence in their nrSSU phylogeny of the Laboulbeniomycetes. They retrieved *H. stylopygae* Speg. in an unresolved position outside of the main Laboulbeniales clade.

Tavares (1985) suggested that the ancestral position of the genus might be related to the basal position of its hosts among the Hexapoda. Tavares (1985) presented a hypothesis that stated an origin of the group through the infection of cockroaches in the Carboniferous and a subsequent transition to Coleoptera (beetles). Laboulbeniomycetes fossils are rarely reported. Only three reports are known: *Stigmatomyces succini* W. Rossi et al. from a fly in Bitterfeld amber (35 million years old, Myo; Rossi et al., 2005), an undescribed species of *Columnomyces* from Dominican amber (16 Myo; M. Perreau and D. Haelewaters, unpubl. data), and a report we consider spurious by Poinar (2016) of an amber inclusion from Myanmar (around 100 Myo). The hypothesized evolutionary history of these fungi may be inferred from phylogenetic molecular investigations incorporating a molecular clock approach. Here we present extensive molecular phylogenetic data to resolve the position of the genus *Herpomyces* within the Laboulbeniomycetes as well as the relationships among several species in the genus.

2. Material and methods

2.1. Collection of host specimens

Cockroaches were obtained from pet supply companies and laboratory colonies. Screening for Laboulbeniales was done using a binocular microscope at 50×. Fresh specimens of *Periplaneta americana* were hand-collected in Burbank, CA; Cambridge, MA; New York City, NY; and during fieldwork in Panama (in Ancón and Gamboa). Long-term preservation was obtained by storing material in 95% ethanol at −20 °C. To present a more complete phylogeny of Laboulbeniomycetes, also other insect groups were collected and screened for the presence of fungal thalli. Hosts were collected by hand, using pyramid traps with killing agent, on an illuminated white screen at night, by fumigation, or using an entomological net. In addition, bats were captured with mist nets and their bat flies were collected using paintbrush and forceps.

2.2. Morphology

We removed entire antennae from highly infected cockroaches using forceps. These were washed 3 times in 70% ethanol and stored in 85% ethanol at −20 °C prior to identification of thalli or isolation of fungal DNA. In other cases, individual thalli were removed from the host at the point of attachment (foot or haustorium/haustoria), using Minuten Pins (BioQuip, Rancho Dominguez, CA, #1208SA) inserted onto wooden rods. Voucher slides were prepared of thalli mounted in Amann solution (Benjamin, 1971) with modifications as follows. We placed a droplet of Hoyer's medium on the microscope slide with the tip of a Minuten pin and deposited thalli in the droplet. The thalli were positioned on the slide by taking them out of the Hoyer's one by one and placing them in a single row, each thallus in a minute amount of the Hoyer's. The specimens were dried briefly, then a small droplet of Amann solution was placed on the cover glass, before lowering the latter (droplet facing down) sideways onto the Hoyer's medium. In this way, the moderately fixed thalli remained in place when the cover glass was added. To seal, the cover glass was ringed with nail polish or B-72 in acetone (Gaylord, Syracuse, NY, #AB72). We viewed mounted specimens at 400–1000× magnification. For identification, we used relevant systematic and taxonomic descriptions (listed in the References section). Most species of *Herpomyces* were identified using descriptions of Thaxter (1908, 1931) and Santamaria (2003, for *H. stylopygae*). Voucher slides are deposited at the Farlow Herbarium (FH; Harvard University, Cambridge, MA).

2.3. DNA isolation, PCR amplification, and sequencing

DNA was isolated from 1 to 18 thalli following a modified Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, St Louis, MO), a so-called "heat-extraction" protocol, and a modified REPLI-g Single Cell Kit (Qiagen, Stanford, CA). Using the Extract-N-Amp PCR Kit, 1.5 mL Eppendorf tubes were filled with 40 µL of Extraction Solution. A Minuten Pin was submerged in glycerin to allow the thalli to stick to the pin and prevent them from getting lost or flying away during transfer. Thalli were removed from the host using this Minuten Pin and placed in a droplet of glycerin on a microscopic slide. Thalli were then taken out of the droplet with the Minuten Pin and put into the Extraction Solution-filled tube. The sample was crushed with a pestle and incubated at room temperature for 10+ min and then at 95 °C for 20 min on a standard heating block (VWR Scientific, Franklin, MA, catalog no. 13259-030). Finally, 60 µL of Dilution Solution (3% BSA) was added to the tubes. DNA extractions were stored at −20 °C. Some samples in the 40 µL Extraction Solution received pre-treatment with overnight incubation in a Shake 'N Bake Hybridization Oven (Boekel Scientific, Feasterville, PA, model no. 136400-2) at 56 °C.

Samples from Hungary were subjected to a heat-extraction protocol. A portion of a heavily infected antenna, around 5 mm in length, was removed from the cockroach host, placed in a 0.2 mL PCR tube, and incubated in a microwave at 750 W for 5 min. Then 50 µL ddH_20 was added, and the submerged tissue (fungal material or section of highly infected antenna) was crushed using a sterile pipette tip under a dissecting microscope. Some loss of material occurred by capillary action, but it was minimal. PCR tubes were incubated at −20 °C for 10 min. Forceful pressure was applied to the ice inside the PCR tubes using a sterile pipette tip to further break apart thalli.

Table 1
Primer pairs used in this study, including the targeted product and reference(s).

Forward	Reverse	Product	Reference(s)	
NS1	NS4	nrSSU	White et al. (1990)	White et al. (1990)
NS1	NS2	nrSSU		White et al. (1990)
NS1	NS6	nrSSU		White et al. (1990)
NS1	R	nrSSU		Wrzosek (2000)
NSL1	NSL2	nrSSU	Haelewaters et al. (2015)	Haelewaters et al. (2015)
SL122	NSL2	nrSSU	Landvik et al. (1997)	
ITS1f	ITS4	ITS	Gardes and Bruns (1993)	White et al. (1990)
ITS1f	ITS4_kyo1	ITS		Toju et al. (2012)
ITS1f	ITS4A	ITS		Larena et al. (1999)
ITS1f	ITS-u4	ITS		Cheng et al. (2016)
ITShespL	ITShespR	ITS	Haelewaters et al. (2018a)	Haelewaters et al. (2018a)
ITS5	ITS2	ITS1 – 5.8S	White et al. (1990)	White et al. (1990)
5.8Shs2	ITS4	5.8S – ITS2	Sundberg et al. (2017)	
ITS9mun	LR3	ITS – nrLSU	Egger (1995)	Vilgalys and Hester (1990)
LabITS1	LR3	5.8S – nrLSU	This study	
LR0R	LR5	nrLSU	R. Vilgalys unpublished	Vilgalys and Hester (1990)
LIC24R	LR3	nrLSU	Miadlikowska and Lutzoni (2000)	

In addition, we developed a modified protocol for the REPLI-g Single Cell Kit (Qiagen). This protocol was used for isolation and whole-genome amplification (WGA) of DNA from single thalli. A Minuten Pin was submerged in glycerin and a single thallus was removed from the host and placed in a droplet of glycerin on a microscope slide. The thallus was then placed in a 0.2 mL PCR tube with 2 µL of phosphate-buffered saline (PBS). After adding 1.5 mL of prepared D2 buffer, the tube was incubated at 65 °C for 20 min. Subsequent steps followed the manufacturer's instructions.

Three non-protein coding DNA fragments were amplified, including nrSSU, ITS (ITS1 – 5.8S – ITS2), and nrLSU. Primer pairs used are given in Table 1. For ITS, initial attempts to amplify using previously published primers designed for fungi often resulted in weak or non-specific amplification. To improve our success rate, specific primers were designed in the course of our studies: ITShespL (5′-CTCCTGTAGAACCTA CACATC-3′) and ITShespR (5′-CAAATTTAAGCTTTTGCCGC-3′), both of which are *Hesperomyces*-specific, and the Laboulbeniomycetes-specific LabITS1 (5′-ATkGCrCTyTyTGGyAwTCC-3′). The PCR reactions were conducted on a Mastercycler ep gradient Thermal Cycler (Eppendorf, Hauppauge, NY, model no. 5341) and consisted of 13.3 µL of Extract-N-Amp PCR ReadyMix (Sigma-Aldrich), 2.5 µL of each 10 µM primer, 5.7 µL of H_2O, and 1 µL of template DNA. The amplification reactions were run under the following profiles: pre-denaturing at 94 °C for 3:00 min; 35 cycles of denaturing at 94 °C for 1:00 min, annealing at 50 °C for 0:45 min, extension at 72 °C for 1:30 min; and a final extension step of 72 °C for 10:00 min.

PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen) and sequenced. We prepared 10 µL sequencing reactions containing the same primers and 1 µL of purified PCR product. Sequencing reactions were performed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA). Generated sequences were assembled, trimmed, and edited in Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI).

2.4. Sequence alignment and phylogenetic analyses

We compiled two datasets, the ITS sequences matrix and a concatenated dataset (nrSSU, ITS, nrLSU), to investigate the phylogeny within the genus *Herpomyces* and its position among Laboulbeniomycetes. For all three available DNA regions in the combined dataset, we aligned sequences using Muscle v3.7 (Edgar, 2004) as implemented on the Cipres Science Gateway version 3.3 (Miller et al., 2010). Ambiguously aligned regions and uninformative positions were detected and removed using trimAl v1.3 (Capella-Gutiérrez et al., 2009) with 60% gap threshold and minimal coverage of 50%. The data for each region were concatenated in MEGA7 (Kumar et al., 2016) to create a super matrix of 1891 bp with phylogenetic data for 41 species.

Our ITS dataset consisting of 23 Laboulbeniomycetes sequences was complemented by four *Herpomyces* sequences that we retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and three taxa belonging to other classes: *Neurospora crassa* Shear & B.O. Dodge (Sordariomycetes, Sordariales), *Capnodium coffeae* Pat., and *C. salicinum* Mont. (Dothideomycetes, Capnodiales). All sequences were aligned using Muscle 3.7 and trimmed using trimAl v1.3 with 60% gap threshold and minimal coverage of 50%. Alignments generated during this study are available for download in NEXUS format from the figshare online repository (Haelewaters, 2018).

Phylogenetic analyses were performed using RAxML v8.2.X (Stamatakis 2014) available on the Cipres web portal (Miller et al., 2010). Maximum likelihood (ML) was inferred under a GTRCAT model, with 1000 bootstrapping replicates. Nucleotide substitution models were selected statistically with the help of jModelTest 2.1 (Darriba et al., 2012) by considering the Akaike Information Criterion (AIC). For the combined nrSSU + ITS + nrLSU dataset, the lowest -lnL value (12483.7340) was assigned to the General Time Reversible substitution model (Tavaré, 1986) with estimation of invariant sites and the assumption of a gamma distribution with six rate categories (GTR + G + I). Bayesian analyses were done with a Markov chain Monte Carlo (MCMC) coalescent approach implemented in BEAST v1.8.4 (Drummond et al., 2012), with an uncorrelated lognormal relaxed molecular clock allowing for rate variation across the tree. We selected a Speciation Yule Process tree prior with the GTR + G + I nucleotide substitution model (as selected by jModelTest 2.1). Five runs were performed from a random starting tree for 80 million generations, with a sampling frequency of 8000. All prior settings were entered in BEAUti v.1.8.4 to generate an XML file, which was run in BEAST on the Cipres web portal. The resulting log files of the five independent runs were entered in Tracer v1.6 (Rambaut et al., 2014) to check trace plots and effective sample size (ESS). Burn-in was adjusted to achieve an overall ESS of ≥200. Upon removal of a portion of each run as burn-in, log files and trees files were combined in LogCombiner v.1.8.4. TreeAnnotator v1.8.4 was used to generate consensus trees with 0% burn-in value and to infer the maximum clade credibility tree, with the highest product of individual clade posterior probabilities. BEAUti, LogCombiner, and TreeAnnotator are part of the BEAST package. Final trees with bootstrap values (BS) and posterior probabilities (pp) were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.5. Molecular clock: dataset, initial phylogenetic analyses, calibration strategies, and divergence time estimates

Data from Schoch et al. (2009a) were used as a basis for our molecular clock analysis. Their six-locus data matrix (nrSSU, nrLSU, mitSSU, RPB1, RPB2, TEF1) is available through TreeBASE under study ID #2137. Both the nrSSU and nrLSU regions were extracted from the matrix separately and sequences of Laboulbeniomycetes were added to the respective dataset. Alignment of DNA sequences was done for both loci separately using Muscle v3.7 on the Cipres Science Gateway version 3.3. The sequences of both loci were concatenated in MEGA7 and trimmed with trimAl v1.3 as implemented in the Phylemon 2.0 web resource (Sánchez et al., 2011), selecting the heuristic method 'automated1' (Capella-Gutiérrez et al., 2009). The resulting nrSSU + LSU data matrix then was complemented with Schoch et al.'s (2009a) four other loci, which were present as two-locus datasets in the downloaded NEXUS file (RPB1 + mitSSU and TEF + RPB2).

Maximum likelihood analysis of the six-locus data matrix was inferred under a GTRCAT model with 1000 BS replicates (using RAxML v8.2.X on the CIPRES web portal). In preparation for the molecular clock analysis in BEAST v1.8.4, best fitting substitution models were chosen for each locus separately and for the six-locus data matrix as a whole from 88 candidate models included in jModeltest 2.1. The Bayesian Information Criterion was employed (BIC; Schwarz, 1978). For all loci as well as the concatenated data matrix, the GTR + G + I model was selected by the Bayesian Information Criterion. Bayesian analyses were done using MCMC to check whether our selected priors were optimized for the data matrix prior to including fossil calibration points. First trials using the entire dataset with 345 taxa failed to converge, and thus we decided to continue working with a pruned data matrix (making sure that each class was represented and supported in the resulting tree). Two independent runs of 40 million generations each were made, with the following priors: GTR + G + I substitution model, uncorrelated lognormal relaxed clock, Speciation Birth-Death tree prior with incomplete sampling (Stadler, 2009), normal prior distribution on the ucld.mean hyperparameter and sampling frequency of 4000. Trace plots and ESS values were checked in Tracer v1.6, and the burn-in of each run was adjusted to achieve an ESS of ≥200 (20% for run 1, 10% for run 2). TreeAnnotator v1.8.4 was used to generate consensus trees with 0% burn-in value and to infer the maximum clade credibility tree. Final trees with bootstrap values (BS) and posterior probabilities (pp) were visualized in FigTree v1.4.3.

For fossil calibration, we used five ascomycetes fossils: *Paleopyrenomycites devonicus* Taylor et al., *Aspergillus collembolorum* Dörfelt & A.R. Schmidt, Metacapnodiaceae sp., *Parmelia ambra/P. isidiiveteris* Poinar et al., and *Stigmatomyces succini*. Ages are adopted from Beimforde et al. (2014). *Paleopyrenomycites devonicus* (Taylor et al., 2005) is from Devonian Rhynie Chert (410 Myo) and represents the oldest known ascomycete fossil. It has an uncertain position but was estimated to be best placed between Pezizomycotina divergence (=stem base) and Pezizomycotina crown (=Pezizomycetes stem base) by Lücking et al. (2009). We followed Beimforde et al.'s (2014) view and placed *Paleopyrenomycites* on the node giving rise to all Pezizomycotina, as "common ancestor of all filamentous, sporocarp-producing Ascomycota." The Baltic amber fossil *Aspergillus collembolorum* (Dörfelt and Schmidt, 2005) was used to constrain the most recent common ancestor (MRCA) of *Aspergillus protuberus*, *Penicillium freii*, *Eupenicillium limosum*, and *E. javanicum* to 50–35 Myo. The fossil Metacapnodiaceae sp. (Schmidt et al., 2014), from Early Cretaceous Charentes amber (100 Myo), was placed on the node representing the MRCA of *Scorias spongiosa*, *Capnodium coffeae*, and *C. salicinum*. The fourth calibration point is represented by two species of *Parmelia* (*P. ambra*, *P. isidiiveteris*) from Dominican amber (17 Myo). Reassessment by Beimforde et al. (2014), evaluating the use of these materials as calibration points in molecular phylogenetic models, led to the insight that both are Parmeliaceae but do not belong to *Parmelia* sensu stricto. As a result, we used them as MRCA of the family Parmeliaceae (including genera *Canoparmelia*, *Flavocetraria*, *Flavoparmelia*, *Hypogymnia*, and *Usnea*). Finally, *Stigmatomyces succini*, a member of the order Laboulbeniales, was described as an ectoparasite of a diopsid fly in Bitterfeld amber (Rossi et al., 2005). It was used to constrain the common ancestor of *Stigmatomyces gregarious*, *S. limnophorae*, *S. protrudens*, *S. rugosus*, and *S. scaptomyzae* to be 23 Myo.

Divergence times were estimated with BEAST v1.8.4 using an uncorrelated log-normally distributed clock model, allowing for rate variation across the tree. The XML input file for BEAST was constructed with BEAUti v1.8.4 (Drummond et al., 2012) by importing the NEXUS file of the concatenated, pruned data matrix. The substitution model GTR + G + I was used. Five taxon sets were created and constrained to be monophyletic in BEAUti for fossil calibration: Pezizomycotina, *Aspergillus*, Capnodiales, Parmeliaceae, and *Stigmatomyces*. Tree Prior was set to Speciation: Birth-Death Incomplete Sampling (Stadler, 2009). The prior on the ucld.mean hyperparameter was lognormally distributed. For fossil node calibrations, we used normally distributed priors (mean = 410 for Pezizomycotina; mean = 35 for *Aspergillus*; mean = 100 for Capnodiales; mean = 17 for Parmeliaceae; mean = 23 for *Stigmatomyces*; standard deviations were kept at 1). Four independent Markov chain Monte Carlo (MCMC) chains of 80 million generations and sampling frequency of 8,000 were run from random starting trees. Convergence was assessed by checking the resulting log files in Tracer v1.6 (Rambaut et al., 2014). Of each run the burn-in was adjusted such that most of the combined ESS values were ≥200. A maximum clade credibility tree with mean and 95% Highest Posterior Density (HPD) node ages and per-clade posterior probabilities was inferred using TreeAnnotator v1.8.4. All XML files generated during this study are available for download from the figshare online repository (Haelewaters, 2018).

3. Results

3.1. Nucleotide alignment datasets

The concatenated nrSSU + ITS + nrLSU dataset included 61 isolates representing 41 species and 1891 characters (GenBank accession numbers in Table 2). Of these characters, 1058 were constant and 620 were parsimony-informative. Taxonomic sampling covered 17 genera in the Laboulbeniomycetes. *Capnodium coffeae*, *C. salicinum* (Dothideomycetes, Capnodiales), and *Neurospora crassa* (Sordariomycetes, Sordariales) served as outgroup taxa. The ITS dataset included 30 isolates from 11 species and 1098 characters, of which 305 were constant and 494 were parsimony-informative. Taxonomic sampling covered 7 species in the genus *Herpomyces* in addition to *Pyxidiophora microspora* (Laboulbeniomycetes, Pyxidiophorales), *Neurospora crassa*, and as outgroup taxa *Capnodium coffeae* and *C. salicinum*. The six-locus data matrix included 345 isolates from 335 species and 16,754 characters, of which 5354 were constant and 8262 were parsimony-informative. Taxonomic sampling covered 16 classes of Ascomycota. The class Laboulbeniomycetes was represented by the genera *Arthrorhynchus* (1 isolate), *Hesperomyces* (3), *Polyandromyces* (1), and *Stigmatomyces* (5) (Laboulbeniales); *Gliocephalis* (1) and *Pyxidiophora* (4) (Pyxidiophorales); and the genus of interest, *Herpomyces* (9). The pruned six-locus data matrix included the same number of characters but only 120 taxa representing 114 species. Compared to the original dataset with 345 taxa, coverage did not change (16 classes of Ascomycota); as to the Laboulbeniomycetes, *Arthrorhynchus nycteribiae* and *Herpomyces leurolestis* were absent from the pruned data matrix (Table 2).

3.2. Phylogenetic inferences

The resulting six-locus phylogeny confirms the placement of

Table 2
Overview of all Laboulbeniomycetes sequences used in this study. All isolates for which sequences were generated are listed, with country information and GenBank accession numbers. X under ITS, 3-locus, 6-locus = sequence(s) were used in the respective dataset. (X) under 6-locus = sequences were used in the six-locus data matrix but removed from the pruned data matrix. Sequences in bold were newly generated during the course of this study.

Species	Isolate	Country	SSU	ITS	LSU	Dataset(s)		
						ITS	3-locus	6-locus
Capnodium salicinum	CBS 131.34	Indonesia	NG016491	AJ244240	DQ678050	X	X	X
Capnodium coffeae	CBS 147.52	DR Congo	DQ247808	DQ491515	NG027576	X	X	X
Neurospora crassa	FGSC 987		–	AF388914	–	X		
Neurospora crassa	OR74A		NW011929459	NW011929459	NW011929459		X	
Herpomyces chaetophilus	DH435b	USA: Massachusetts	**MG438318**	**MG438292**	–	X	X	
Herpomyces chaetophilus	DH483b	USA: Massachusetts	**MG438319**	**MG438293**	**MG438350**	X	X	X
Herpomyces chaetophilus	DH483e	USA: Massachusetts	**MG438320**	–	**MG438351**		X	
Herpomyces chaetophilus	DH602b	USA: Massachusetts	KT800023	KT800039	KT800009	X	X	
Herpomyces chaetophilus	DH1097b	Panama	**MG438321**	**MG438294**	**MG438352**	X		
Herpomyces chaetophilus	DH1097c	Panama	**MG438322**	**MG438295**	**MG438353**	X	X	X
Herpomyces ectobiae	TW793a	USA: California	–	**MG438296**	–	X	X	
Herpomyces ectobiae	MG001	Poland	KT800024	KT800040	–	X	X	
Herpomyces leurolestis	DH1417b	Hungary	–	**MG438297**	–	X		
Herpomyces leurolestis	Debr_Ppal	Hungary	**MG438323**	**MG438298**	**MG438354**	X	X	(X)
Herpomyces leurolestis	2017/0199	Hungary	–	**MG438299**	–	X		
Herpomyces paranaensis	DH1365a	Panama	–	**MG438300**	–	X		
Herpomyces paranaensis	DH1365b	Panama	–	**MG438301**	–	X		
Herpomyces periplanetae	TW437c	USA: Massachusetts	**MG438324**	**MG438302**	**MG438355**	X	X	
Herpomyces periplanetae	TW448b	USA: Massachusetts	**MG438325**	**MG438303**	**MG438356**	X	X	X
Herpomyces periplanetae	DH602a	USA: Massachusetts	**MG438326**	**MG438304**	–	X	X	
Herpomyces periplanetae	DH602c	USA: Massachusetts	KT800025	KT800041	KT800010	X	X	X
Herpomyces periplanetae	DH602d	USA: Massachusetts	**MG438327**	**MG438305**	**MG438357**	X	X	
Herpomyces periplanetae	DH620a	USA: New York	**MG438328**	**MG438306**	**MG438358**	X	X	
Herpomyces periplanetae	DH654b	Panama	**MG438329**	**MG438307**	–	X	X	
Herpomyces periplanetae	DH654c	Panama	**MG438330**	**MG438308**	**MG438308**	X		
Herpomyces periplanetae	DH1187d	USA: Massachusetts	**MG438331**	**MG438309**	**MG438359**	X		X
Herpomyces stylopygae	Bud_Bori	Hungary	**MG438332**	**MG438310**	**MG438360**	X	X	X
Herpomyces stylopygae	Bud_Bori_2	Hungary	–	**MG438311**	–	X		
Herpomyces shelfordellae	DE_HerpBL1	Hungary	KT800026	KT800042	KT800011	X	X	X
Herpomyces shelfordellae	Bud_Slat	Hungary	**MG438333**	**MG438312**	**MG438361**	X	X	X
Herpomyces shelfordellae	DH1415a	Hungary	–	**MG438313**	–	X		
Gliocephalis hyalina	DAOM 229465	Canada	AH012810	–	–		X	X
Pyxidiophora arvernensis	CBS 657.82		FJ176839	–	FJ176894		X	X
Pyxidiophora cf. *microspora*	MG200	Poland	**MG438334**	**MG438314**	**MG438362**	X		X
Pyxidiophora sp.	IMI-1989	Canada	AF313769	–	–		X	
Pyxidiophora sp. 03			AY212811	–	–		X	
Aphanandromyces audisioi	MG060	Poland	**MG438335**	–	–		X	
Arthrorhynchus nycteribiae	DH1015d	Hungary	**MG438336**	–	**MG438363**		X	(X)
Chitonomyces hyalinus		USA: New York	JN127393	JN127405	–		X	
Chitonomyces marginatus		USA: New York	JN127391	JN127404	–		X	
Corethromyces sp.	AW2001		AF431761	–	–		X	
Gloeandromyces streblae	DH1011a	Mexico	**MG438337**	–	–		X	
Gloeandromyces streblae	DH1018a	Nicaragua	**MG438338**	–	–		X	
Hesperomyces coccinelloides	AW820	USA: Louisiana	AF407575	–	–		X	
Hesperomyces coleomegillae	635A	Ecuador	KF266890	KF192897	–		X	
Hesperomyces coleomegillae	637	Ecuador	KF266893	KF192901	–		X	
Hesperomyces palustris	631K	Ecuador	KF266902	KF192902	–		X	
Hesperomyces palustris	632B	Ecuador	KF266891	KF192899	–		X	
Hesperomyces virescens	DH316a	USA: Georgia	**MG438339**	**MG438315**	**KJ842339**		X	X
Hesperomyces virescens	DH334b	Netherlands	**MG438340**	**MG438316**	**MG438364**		X	X
Hesperomyces virescens	DH646c	Germany	–	KT800045	KT800015		X	
Hesperomyces virescens	DH1188g	USA: Massachusetts	**MG438341**	**MG438317**	**MG438365**			X
Hesperomyces virescens	MT001	Poland	KT800032	KT800048	KT800018		X	
Laboulbenia calathi	DH1007a	Netherlands	**MG438342**	–	–		X	
Laboulbenia flagellata	DH1030a	USA: Massachusetts	**MG438343**	–	–		X	
Laboulbenia pheropsophi	DH1009b	Sierra Leone	**MG438344**	–	–		X	
Laboulbenia sp.	DH971a	Panama	**MG438345**	–	–		X	
Monoicomyces homalotae	DH1014c	USA: California	**MG438346**	–	–		X	
Monoicomyces invisibilis	MT004	Poland	KT800034	–	–		X	
Polyandromyces coptosomalis	DH313f	Ecuador	KT800035	–	KT800020		X	X
Polyandromyces coptosomalis	HM499a	Canary Islands	**MG438347**	–	–		X	
Prolixandromyces triandrus	HNHM1079	Hungary	LT158294	LT158296	LT158295		X	
Rhachomyces philonthinus	TM10446	Poland	KT800036	–	–		X	
Rhadinomyces pallidus			AF431763	–	–		X	
Rickia passalina			AF432129	–	–		X	
Stigmatomyces borealis	AW797	USA: Louisiana	JN835186	–	–		X	
Stigmatomyces gregarius	DH1008a	Sierra Leone	**MG438348**	–	–		X	X
Stigmatomyces limnophorae	AW785	USA: Louisiana	AF407576	–	–		X	X
Stigmatomyces protrudens			AF298232	–	AF298234		X	X
Stigmatomyces rugosus			AF431759	–	–		X	X

(*continued on next page*)

Table 2 (*continued*)

Species	Isolate	Country	SSU	ITS	LSU	Dataset(s)		
						ITS	3-locus	6-locus
Stigmatomyces scaptomyzae			AF431758	–	–		X	X
Zodiomyces vorticellarius	AW819	USA: Louisiana	AF407577	–	–		X	
Zodiomyces vorticellarius	MG003	Poland	KT800038	–	KT800022		X	

Laboulbeniomycetes as sister to the Sordariomycetes with a high level of certainty. In both the three-locus and six-locus phylogenies, support was lacking or moderate for the relationships among orders in the Laboulbeniomycetes. In all multi-locus phylogenetic analyses, the three orders are highly supported (Figs. 1 and 2). The *Herpomyces* clade and Pyxidiophorales are supported with maximum bootstrap values and posterior probabilities. Laboulbeniales is supported with BS = 87 and pp = 1.0 in the three-locus phylogeny and with BS = 94 in the six-locus phylogeny. In the pruned six-locus phylogeny, the *Herpomyces* clade (clade support: pp = 1.0) and Laboulbeniales (pp = 1.0) are sharing a most recent common ancestor, which is sister to Pxyidiophorales (pp = 1.0) (Fig. 2). The support for the sister relationship between the *Herpomyces* clade and Laboulbeniales is moderately high: BS = 68, pp = 0.8.

The ITS phylogeny supported existing species of *Herpomyces* and brought to light an undescribed species (Fig. 3). The new species is associated with *Shelfordella lateralis* (Walker, 1868) (Blattodea, Blattidae, Blattinae) from commercially available sources in Hungary, Poland, and the USA. To confirm its molecular identity, we generated ITS sequences for multiple isolates from cockroaches that were purchased in different pet stores. In addition, we applied two distinct DNA isolation techniques in two collaborating laboratories (Debrecen, Harvard). The ITS sequences match for 100% (over 721 bp), but isolates DE_HerpBL1 and DH1415a have an extra A in position 9 and an extra G in position 650 while isolate Bud_Slat presents two gaps at these positions.

Our dating estimates are shown in Fig. 4. The 95% HPD ranges for each divergence time estimate are available for download from figshare (Haelewaters, 2018). The diversification of extant Ascomycota happened in the Neoproterozoic, about 664 Mya. The subphylum Pezizomycotina split from Saccharomycotina in the early Cambrian, around 583 Mya. Within the Pezizomycotina, extant taxa within the unranked taxon sordariomyceta (=Leotiomycetes and Laboulbeniomycetes and Sordariomycetes, Schoch et al., 2009a) diversified in the Triassic (231 Mya). Laboulbeniomycetes and Sordariomycetes diverged around the Triassic-Jurassic boundary (206 Mya). Within Laboulbeniomycetes, the earliest split occurred around 160 Mya (divergence of Pyxidiophorales). Finally, the *Herpomyces* clade and Laboulbeniales diverged around 143 Mya.

4. Taxonomical section

4.1. Order Herpomycetales

To formally recognize the *Herpomyces* clade in the Laboulbeniomycetes we have proposed a new order (Haelewaters et al., 2018b). This is based on its highly supported phylogenetic placement, distinct from Laboulbeniales and Pyxidiophorales (Figs. 1, 2, and 4), in combination with evidence from developmental, morphological, and host usage data (see Discussion).

4.2. Herpomycetales Haelew. & Pfister, Index Fungorum 382:1 (2018)

Index Fungorum number IF555706.

Type family: Herpomycetaceae I.I. Tav., Mycotaxon 13:469 (1981).

Type genus: *Herpomyces* Thaxt., Proceedings of the American Academy of Arts and Sciences 38:11 (1902).

Etymology: Derived from its single genus, *Herpomyces*.

Description: Dioecious; 4-celled primary axis of thallus developing directly from ascospore; suprabasal cell in female thallus giving rise to secondary axis (or axes), producing perithecia and connecting directly with integument of the host; perithecia multi-tiered, outer wall rows consisting of many cells equal in height; ascospores 8 per ascus with median septum. On Blattodea (cockroaches).

Notes: There is a single family Herpomycetaceae with a single genus, *Herpomyces* Thaxt. (Thaxter, 1902). The type species of the genus is *Herpomyces chaetophilus* Thaxt. With the description of *H. shelfordellae* below, 26 species are now accepted in the Herpomycetales (Table 3). Acceptance is based on the combination of morphological characteristics (Thaxter, 1902, 1905, 1908, 1915, 1918, 1931; Spegazzini, 1917) and molecular data (this study).

4.3. Herpomyces shelfordellae sp. nov., Pfliegler & Haelew.

MycoBank number MB823130.

Etymology: Referring to the host genus of this species, *Shelfordella*.

Description: Male thallus hyaline, consisting of four superposed cells; second cell conspicuously flattened. Third and fourth cell each giving rise to an elongated cell at the upper-lateral corner, carrying a single slender antheridium. Fourth cell ending in a short-pointed axis, which laterally carries a minuscule blackish disc.

Female thallus hyaline. Primary axis of the receptacle four-celled, the proximal cell with a short and pointed apex. *Thalli growing on antennal setae* have a secondary axis with obliquely superposed cells. *Thalli growing on the integument surface* possess a compact secondary axis forming a single-lobed shield, 0.8–1.3× higher than wide, usually symmetrical, asymmetrical in some thalli, and usually asymmetrical in thalli possessing two perithecia, with a single lobe (rarely the basal part of the shield extends laterally, but does not form a distinct lobe with rounded apex), with broad and blunt apex; shield ornamented with concentric ridges extending between lateral edges, apical ridges slightly curved, basal ridges strongly curved to inverted U-shaped. The apex of the single lobe is broad and blunt. Perithecial basal cells flattened. Thalli usually with one perithecium, occasionally two. Perithecium slightly bent, asymmetric, fusiform; broadest in the lower third, gradually tapering upwards to a well-differentiated, bent neck; perithecial apex strongly asymmetrical, with pointed ostiole positioned sideways, distally ending in an elongated, tooth-like projection. Upper 4–5 tiers of outer wall cells conspicuously thickened, resulting in the abrupt narrowing of the inner mass at the perithecial neck.

Measurements: Male thallus 33–40 µm in length. Female thallus 214–282 µm in length. Shield: 26–56 × 28–54 µm (height × width). Perithecia: 156–224 × 33–45 µm (without basal cells). Ascospores 24–28 × 2–3 µm.

Known distribution and hosts: Only known from *Shelfordella lateralis*, with reports in Hungary, Poland, and the USA (Massachusetts).

Types: HUNGARY, Northern Great Plain Region, Hajdú-Bihar County, Debrecen, November 2014, *W.P. Pfliegler*, on antenna of *Shelfordella lateralis*, slide D. Haelew. 1414c (FH 00313669, **holotype**; Figs. 5A–B and 6A). HUNGARY, Central Hungary Region, Budapest, 10 March 2015, *W.P. Pfliegler*, on antenna of *S. lateralis*, slide D. Haelew. 1415b (FH 00313670, **paratype**; Fig. 6B). POLAND, Łódź Voivodeship, Łódź, 3 November 2017, *M. Gorczak*, on antenna of *S. lateralis*, slide MG202e (FH, **paratype**).

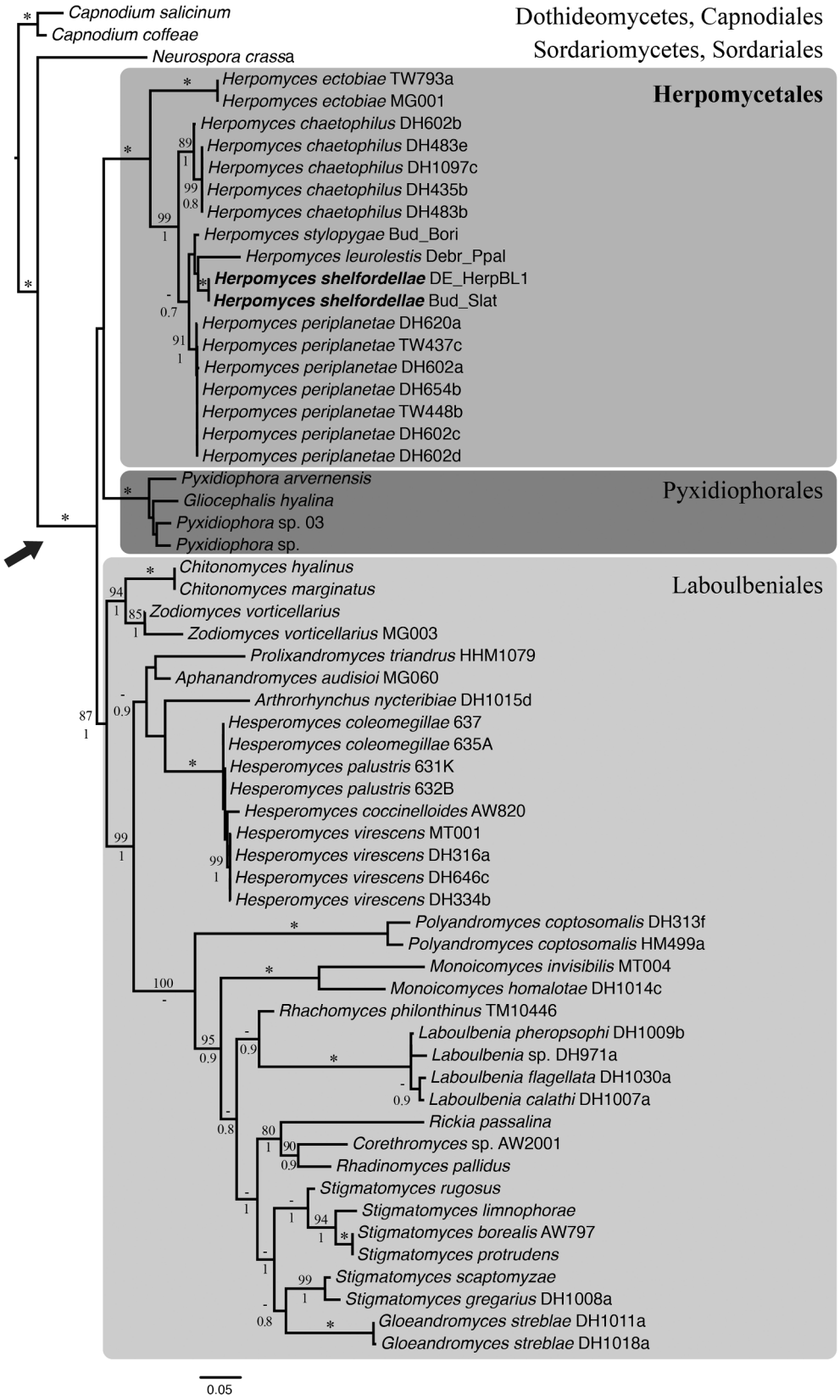

Fig. 1. Phylogeny of Laboulbeniomycetes, reconstructed from the concatenated three-locus dataset (nrSSU + ITS + nrLSU). The topology is the result of maximum likelihood inference performed with RAxML. For each node, the ML bootstraps (if > 70) and posterior probabilities (if > 0.7) are presented above/below the branch leading to that node. An asterisk (*) indicates maximum support (BS = 100, pp = 1.0). The arrow indicates the Laboulbeniomycetes class. Newly proposed taxa are highlighted in bold (Herpomycetales, *Herpomyces shelfordellae*).

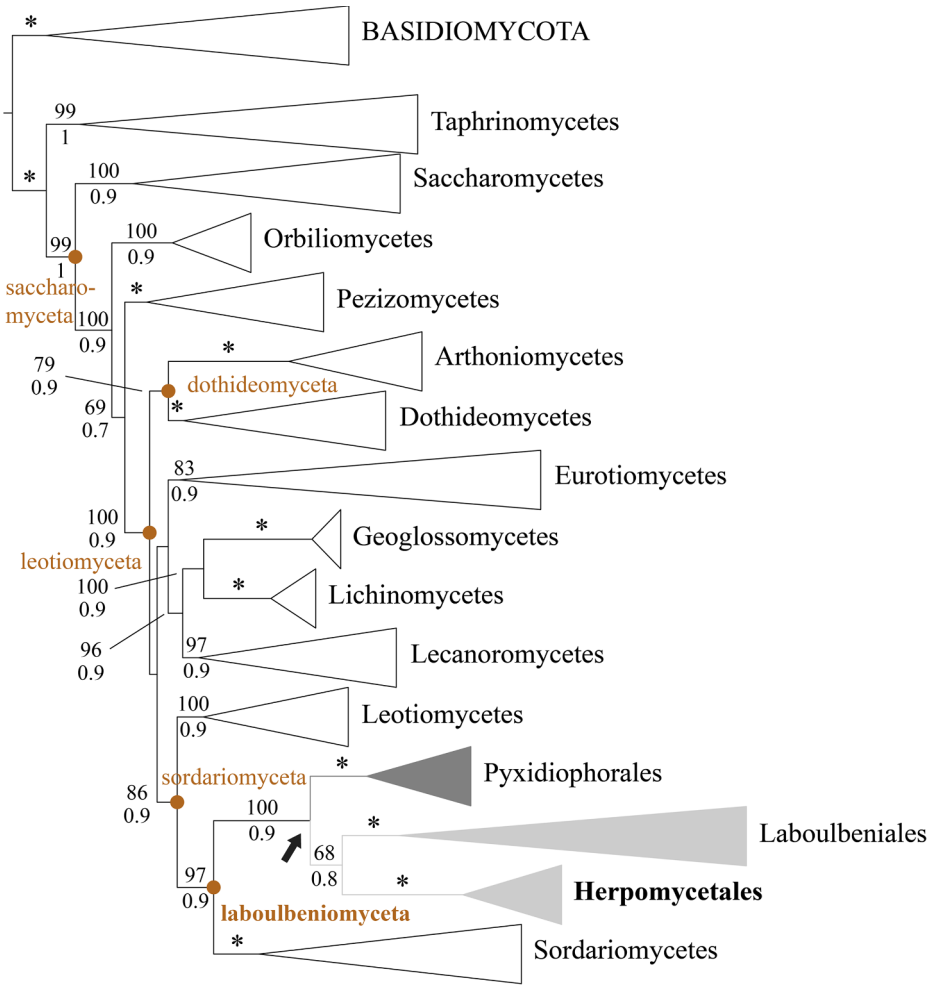

Fig. 2. Complete Ascomycota phylogeny, reconstructed from the pruned six-locus data matrix. The topology is the result of maximum likelihood inference performed with RAxML with all lineages collapsed to class level, and to order level within Laboulbeniomycetes. Rankles taxa (-myceta) are shown. The arrow indicates the Laboulbeniomycetes class. For each node, the ML bootstraps (if > 65) and posterior probabilities (if > 0.7) are presented above/below the branch leading to that node. An asterisk (*) indicates maximum support (BS = 100, pp = 1.0). Newly proposed taxa are highlighted in bold (laboulbeniomyceta, Herpomycetales).

Material sequenced: HUNGARY, Northern Great Plain Region, Hajdú-Bihar County, Debrecen, November 2014, *W.P. Pfliegler*, on antenna of *Shelfordella lateralis*, isolate DE_HerpBL1 (± 30 female thalli, SSU: KT800026, ITS: KT800042, LSU: KT800011), erroneously identified as *H. stylopygae* in Haelewaters et al. (2015). HUNGARY, Central Hungary Region, Budapest, 10 March 2015, *W.P. Pfliegler*, on antenna of *S. lateralis*, isolate Bud_Slat (20–30 female thalli, SSU: MG438333, ITS: MG438312, LSU: MG438361). Same data, isolate D. Haelew. 1415a (12 female thalli, ITS: MG438313). POLAND, Łódź Voivodeship, Łódź, 3 November 2017, *M. Gorczak*, on antenna of *S. lateralis*, isolate MG202b (ITS: MK299848). USA, Massachusetts, Barnstable County, Hyannis, 14 December 2017, *D. Haelewaters*, on right antenna of *S. lateralis*, isolate D. Haelew. 1427a (2 female thalli, ITS: MK299847).

Notes: All Hungarian host specimens were purchased from pet stores in Budapest or Debrecen and subsequently kept in escape-proof terrariums at the University of Debrecen under the following conditions: 25 ± 1 °C and 14:10 [L:D] h (Pfliegler et al., 2018). Host specimens from Poland were obtained from a private collection of *S. lateralis* cockroaches originating from an online pet store based in Warsaw. American host specimens were purchased from an online pet store based on Cape Cod (Massachusetts) and screened immediately after arrival (infection prevalence of 4.8%, $n = 62$). The American and Polish records of *H. shelfordellae* were discovered during the course of the review process of this paper. ITS sequences were generated for two isolates from this recent material (accession numbers MK299847 and MK299848). NCBI Nucleotide BLAST searches resulted in 99–100% similarity with the previously submitted ITS sequences of *H. shelfordellae*, confirming identity.

Material examined of Herpomyces stylopygae: CANADA, Québec, 20 September 1963, *A. Francoeur*, on left antenna of male *Blatta orientalis* Linnaeus, 1758, in Collection d'insectes du Québec (CIQ), slides D. Haelew. 570a (FH 00313663), 570b (FH 00313664), and 570c (FH 00313665). HUNGARY, Central Hungary Region, Budapest, May 2015, *J. Schmidt*, on antenna of *B. orientalis*, slide D. Haelew. 951a (FH 00313666). Same data, slides D. Haelew. 952a (FH 00313667; Fig. 5C) and 952b (FH 00313668). Hungarian host specimens for this species originated from a toxicological laboratory in Budapest.

5. Discussion

5.1. Placement within sordariomyceta

Schoch et al. (2009a, 2009b) used the unranked taxon sordariomyceta to circumscribe the classes Leotiomycetes, Laboulbeniomycetes, and Sordariomycetes. The distinction between Laboulbeniomycetes and Sordariomycetes was first shown by Weir and Blackwell (2001) based on nrSSU sequences. The sister relationship of these two classes was established from a six-locus Ascomycota-wide phylogeny that

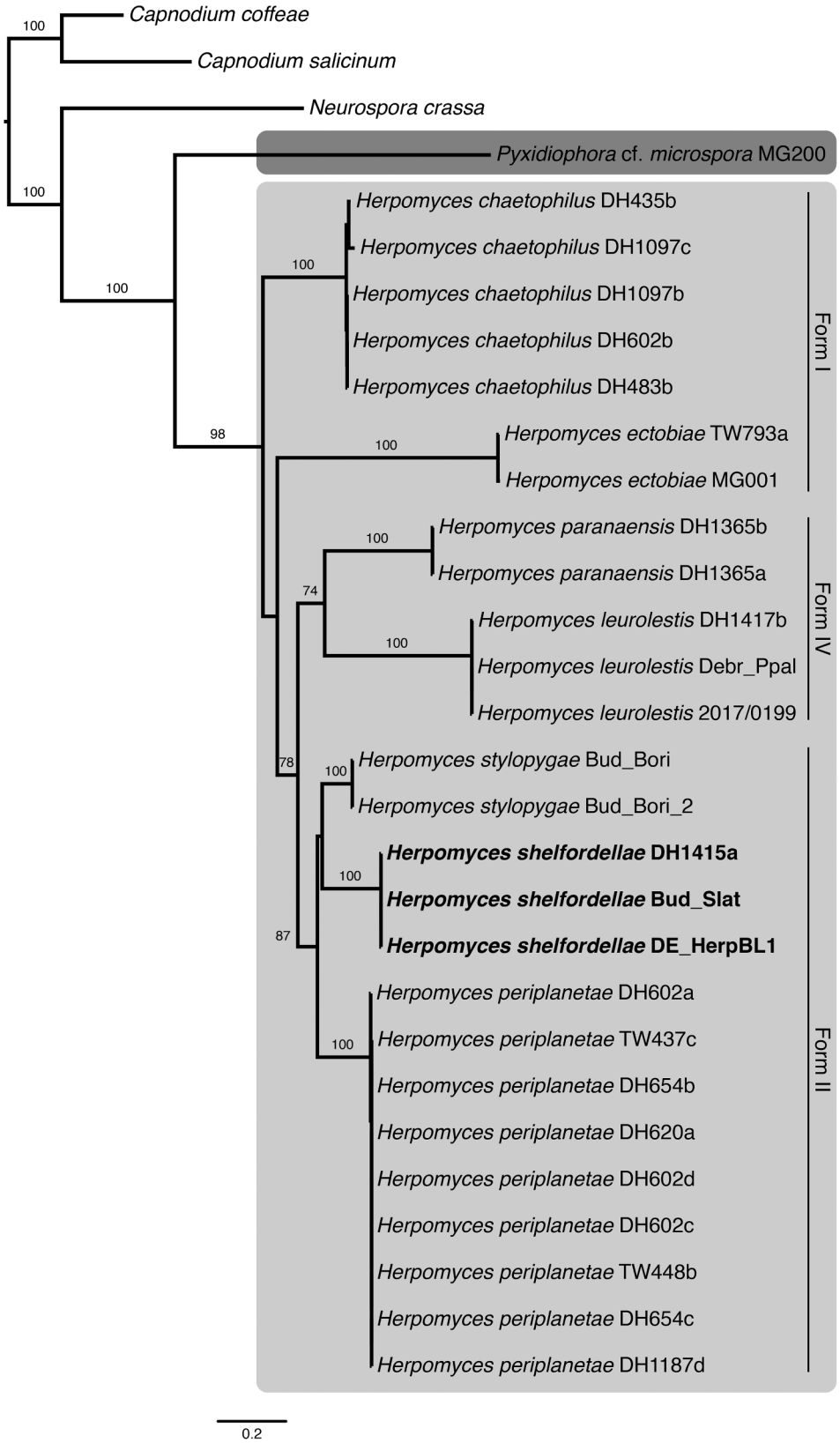

Fig. 3. Phylogeny of *Herpomyces* species, reconstructed from the ITS dataset. The topology is the result of maximum likelihood inference performed with RAxML. For each node, the ML bootstraps (if > 70) are presented next to the branch leading to that node. Newly proposed taxa are highlighted in bold (*Herpomyces shelfordellae*).

included 4 isolates of Laboulbeniomycetes (Schoch et al., 2009a). Our analyses are in agreement with Schoch et al. (2009a). Taxonomic sampling of Laboulbeniomycetes is more complete in our study, with the inclusion of 23 isolates from the three supported orders in the six-locus phylogeny. These isolates represent 17 species in Herpomycetales (5 species, 9 isolates), Laboulbeniales (8 species, 10 isolates), and Pyxidiophorales (4 species, 4 isolates).

In keeping with Schoch et al. (2009b), who applied -myceta rankless

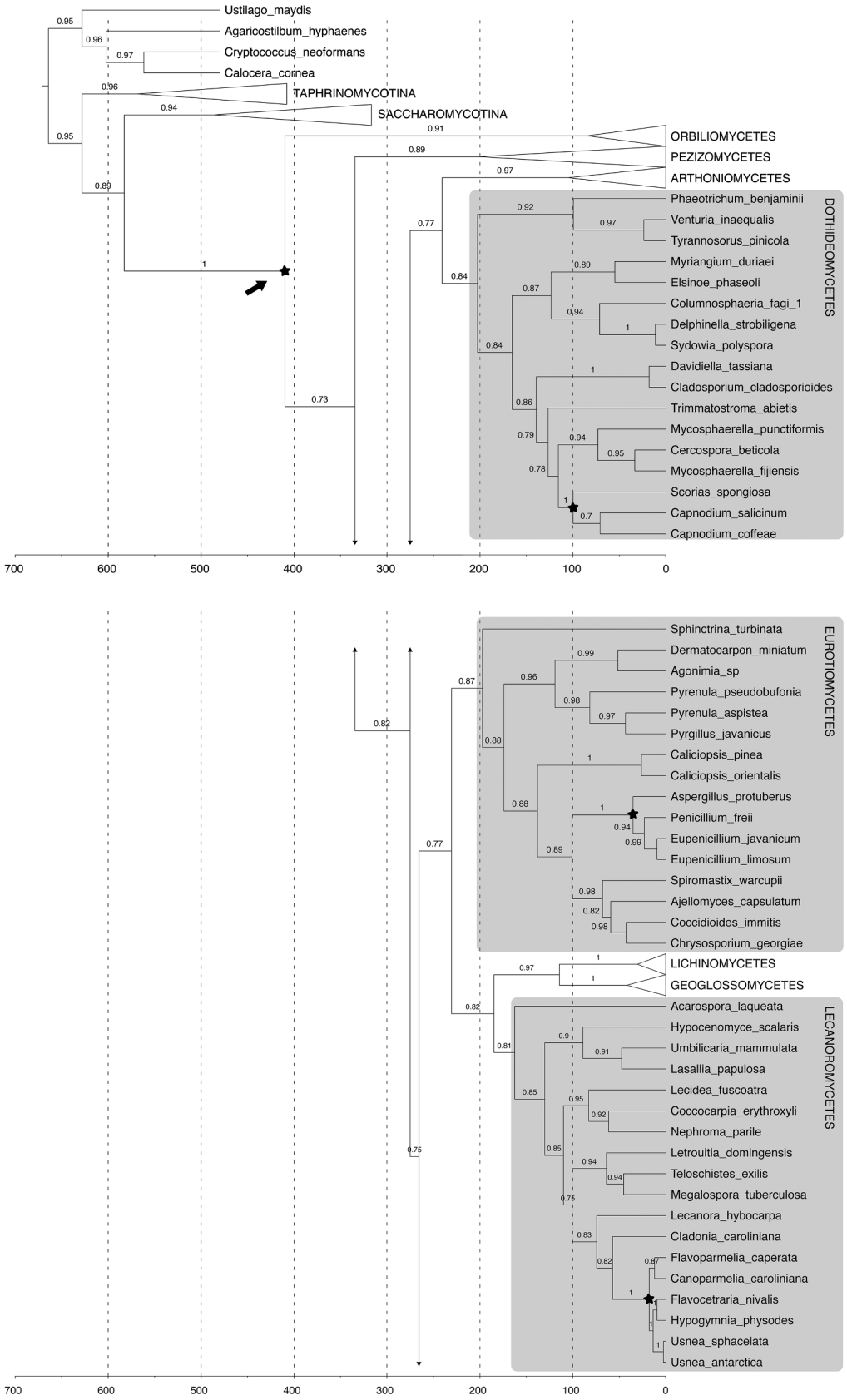

Fig. 4. Maximum clade credibility tree with divergence time estimates for main groups of Ascomycota and orders within Laboulbeniomycetes, reconstructed from the pruned six-locus data matrix. The tree is the result of a Bayesian analysis performed in BEAST, using five fossil calibration constraints. For each node, the posterior probabilities (if > 0.7) are presented next to the branch leading to that node. Assignments in the tree of the fossil calibration points are marked with black stars. The arrow indicates the Pezizomycotina subphylum. The rankles taxon sordariomyceta is shown. Fossil calibrations are *Paleopyrenomycites devonicus* (Pezizomycotina-crown, basal-most position), Metacapnodiaceae sp. (in Dothideomycetes), *Aspergillus collembolorum* (in Eurotiomycetes), *Parmelia ambra/P. isidiiveteris* (in Lecanoromycetes), and *Stigmatomyces succini* (in Laboulbeniomycetes).

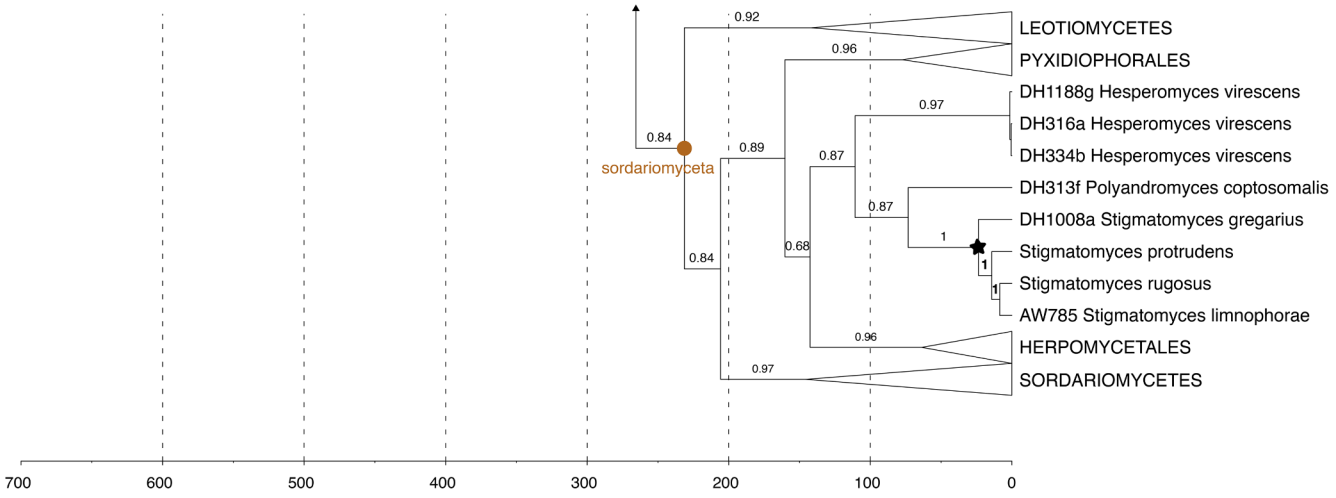

Fig. 4. (*continued*)

taxa to define well-supported clades above the class level, we apply laboulbeniomyceta as a rankless taxon to contain all the fungi with perithecial ascomata (*pyrenomycetes*). Laboulbeniomyceta excludes the earliest diverging class of sordariomyceta (the apothecial Leotiomycetes). Included in this clade are the two classes Sordariomycetes and Laboulbeniomycetes and perhaps some unclassified genera ("extralimital" pyrenomycetes; Samuels and Blackwell, 2001). It is clear from all analyses (Schoch et al., 2009a; this study) that perithecial fungi have a single origin. Within in the various groups that produce perithecia there are different developmental pathways. This is the case within the three orders of the Laboulbeniomycetes (Malloch, 1981; Parguey-Leduc and Janex-Favre, 1981; Samuels and Blackwell, 2001; Eriksson et al., 2003; Schoch et al., 2009b). Malloch (1981) and Samuels and Blackwell (2001) described multiple steps towards evolutionary simplification of taxa, such as the loss of the ostiole and loss of the arrangement of asci in a hymenium. In the case of Laboulbeniomycetes, the simplification extends to reductions in the assimilative phase and loss of asexual states in Herpomycetales and Laboulbeniales. Along with these life history simplifications, there is a reduction to the point that thallus development is restricted to a series of highly organized, determinate mitotic divisions. There are no hyphae.

5.2. Relationships within Laboulbeniomycetes

The three orders within Laboulbeniomycetes form an unresolved trichotomy. Only in the pruned six-locus phylogeny is there moderate support for the early divergence of Pyxidiophorales (BS = 68, pp = 0.8). Blackwell (1994) put forward two potential reasons to support Pyxidiophorales as the basal-most or early diverging branch of Laboulbeniomycetes. First, a switch from the two-host situation in Pyxidiophorales to a single arthropod host in Herpomycetales and Laboulbeniales is a significant simplification of life history. Second, for *Pyxidiophora* spp., successful completion of the life cycle requires an ephemeral substrate (e.g., herbivorous dung) onto which an appropriate fungus host must grow. The shift to an arthropod-only dependency has freed Herpomycetales and Laboulbeniales from this "patchiness" (Blackwell, 1994). Although arthropod hosts themselves can be considered patchy substrates, these hosts are often long-lived as adults; they have many contacts with individuals of their own species but also other species, thus providing good conditions for Herpomycetales and Laboulbeniales fungi to be transmitted, develop, and mature. These associations maintain populations. But, in time divergent isolated populations emerge, leading to microevolutionary changes and ultimately speciation. The radiation of Laboulbeniales is remarkable, given the currently 2,200 described species and estimates up to 75,000 (Weir and Hammond, 1997), but may be expected considering the high diversity of a principle host group, the beetles.

Molecular, developmental, morphological, and host usage data provide ample evidence to support formally elevating the suborder Herpomycetineae to the order level. Our molecular data consistently point to three strongly supported clades within Laboulbeniomycetes. In one analysis (the pruned six-locus phylogeny), there is support for within-class relationships. There is no doubt that more data of various types will resolve relationships. First, more taxa should be represented in the phylogenetic reconstructions of the class. Many families and genera are still highly undersampled, which we believe accounts for long branches in our phylogenetic analyses. Second, in addition to taxon sampling, effort needs to be made to develop additional markers to better resolve evolutionary relationships within Laboulbeniomycetes. Genomic studies, too, will be critical. Pyxidiophorales are separated from Herpomycetales and Laboulbeniales by their complex life cycle with hyphal growth and both asexual and sexual states. In addition, their perithecia are produced from a mycelium, are composed of single-layered cell walls, and have reduced numbers of ascospores per ascus (Blackwell and Malloch, 1989b; Kirschner, 2003; Doveri and Coué, 2006; Weir and Blackwell, 2005). Thalli of Herpomycetales and Laboulbeniales differ because they develop from an ascospore and the perithecia have two-layered walls (Weir and Blackwell, 2005). These features support the sister relationship of these two orders. The way in which these 2-layered wall cells are formed, however, differs between the two orders. In Herpomycetales, the perithecial walls develop before carpogonial upgrowth, which extends between the outer wall cells (Fig. 7). By contrast, in Laboulbeniales, the rows of outer wall cells grow upwards around and after carpogonial extension (Tavares, 1980). Ascus development differs between Herpomycetales and Laboulbeniales (Tavares, 1980, 1985): (1) The asci of Herpomycetales produce 8 ascospores; those of Laboulbeniales produce 4 ascospores. (2) In Herpomycetales, a primary septum divides the ascospore in two equal cells; in Laboulbeniales this septum is positioned near the lower end, dividing the ascospore in a smaller (directed downward) and larger cell (directed upwards). (3) Asci are produced sequentially from two series of ascogenic cells, first on one side, then the other in Herpomycetales; in Laboulbeniales the asci form in a single series. Other

Table 3
All 26 species of *Herpomyces* described thus far on cockroaches (order Blattodea) are listed. Species in bold are included in our phylogenetic analyses. Species with an asterisk (*) are only known from the type collection.

amazonicus Thaxt. 1931*	***ectobiae* Thaxt. 1902**	*nyctoborae* Thaxt. 1905*	***shelfordellae* Pfliegler & Haelew**
anaplectae Thaxt. 1905	*forficularis* Thaxt. 1902	*panchlorae* Thaxt. 1931*	***stylopygae* Speg. 1917**
appendiculatus Thaxt. 1931*	*gracilis* Thaxt. 1931*	*panesthiae* Thaxt. 1915*	*supellae* Thaxt. 1931*
arietinus Thaxt. 1902	*grenadinus* Thaxt. 1931*	***paranaensis* Thaxt. 1902**	*tricuspidatus* Thaxt. 1902
***chaetophilus* Thaxt. 1902**	***leurolestis* Thaxt. 1931**	***periplanetae* Thaxt. 1902**	*zanzibarinus* Thaxt. 1902
chilensis Thaxt. 1918*	*lobopterae* Thaxt. 1931*	*phyllodromiae* Thaxt. 1905*	
diplopterae Thaxt. 1902	*macropus* Speg. 1917	*platyzosteriae* Thaxt. 1905*	

evidence for the separation of Herpomycetales and Laboulbeniales comes from their host usage differences. All 26 species of *Herpomyces* are restricted to cockroaches. Species in the order Laboulbeniales, on the other hand, have a wide variety of hosts in three subphyla: Chelicerata (subclass Acari, mites; order Opiliones, harvestmen), Myriapoda (class Diplopoda, millipedes), and Hexapoda (class Insecta, true insects). Among the Insecta, representatives of 9 orders are hosts to Laboulbeniales: Blattodea (cockroaches and termites), Coleoptera (beetles), Dermaptera (earwigs), Diptera (flies), Hemiptera (true bugs), Hymenoptera: Formicidae (ants), Orthoptera (crickets and allies), Psocodea (lice), and Thysanoptera (thrips). This shows how widely diversified the Laboulbeniales clade is, although it is unclear why Laboulbeniales has undergone such a successful radiation whereas Herpomycetales has not.

5.3. Species delimitation and ecology within Herpomycetales

In our ITS phylogeny, seven species of *Herpomyces* are included. All species are highly supported, indicating that the internal transcribed spacer region is a good barcode marker for these fungi. A problem that we have recurrently encountered is low amplification success for this region with Laboulbeniales species (see discussion in Haelewaters et al., 2018a), but general primers work very well to amplify the ITS locus of *Herpomyces* species (Table 1). There does not seem to be any geographical signal in conspecific isolates. For example, the two isolates of *H. ectobiae* were collected in California (TW793a) and Poland (MG001). The wide distribution of the hosts is of course relatively recent and clearly associated with human activity. The taxonomic status of *H. stylopygae* as a separate species is confirmed. In the second volume of his monograph, Thaxter (1908) included this as a form on *Blatta orientalis* in his circumscription of *H. periplanetae*. It was Spegazzini (1917) who considered this form as a separate species, but Thaxter (1931) doubted its validity.

Based on our work, we not only find that *H. stylopygae* is a well-defined species, but also that it may be highly host specific. When we found *Herpomyces* thalli on *Shelfordella lateralis*, we initially had identified them as *H. stylopygae*. The host species has a complex taxonomic history, contributing to our initial confusion. It was first described by Walker (1868) as *Periplaneta lateralis* and then

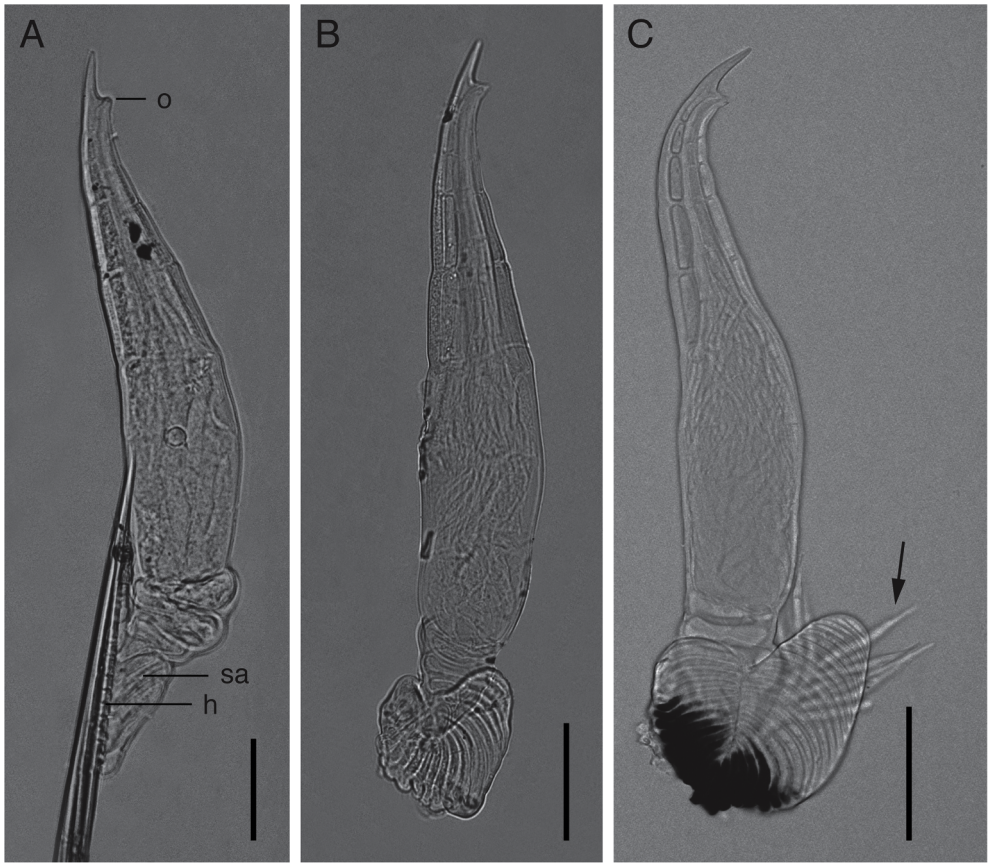

Fig. 5. *Herpomyces shelfordellae* (A–B) and *H. stylopygae* (C). A. Female thallus growing on antennal seta. Indicated are the oblique cells of the secondary axis (sa), which are attached to the host's haemocoel by haustoria (h), and the perithecial ostiole (o). B. Female thallus removed from the host's integument, with a compact secondary axis forming a single-lobed shield, ornamented with concentrical ridges. C. Female thallus of *H. stylopygae* removed from the host's integument. The most conspicuous difference from *H. shelfordellae* is its bilobed shield that is basally blackened. In addition, the male thallus of *H. stylopygae* is comparatively more developed (arrow). Scale bars = 40 μm.

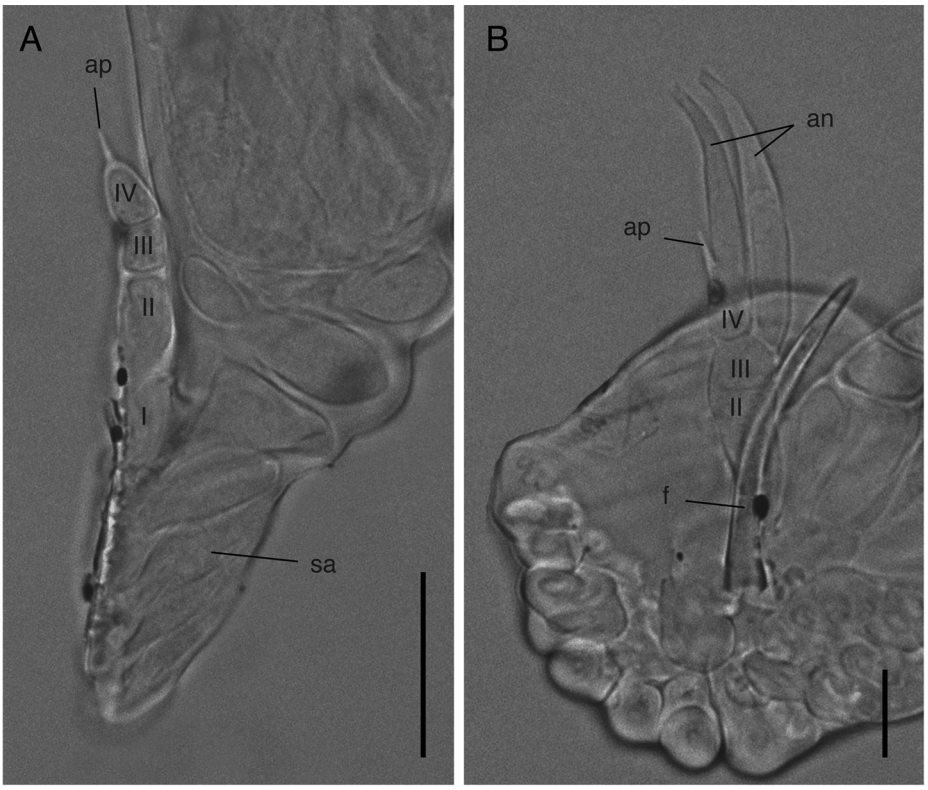

Fig. 6. *Herpomyces shelfordellae*. A. Detail of the four-celled primary axis of a female thallus, which has been removed from its host's spine. Annotated are cells I through IV, the pointed apex at the apical end of the fourth cell (ap), and the secondary axis of the receptacle (sa). B. A male thallus, attached to a spine by a small foot (f). Shown are cells II through IV, the pointed apex (ap), and two slender antheridia (an). Scale bars = 20 μm.

transferred to *Blatta* (*Shelfordella*) *lateralis* (Princis, 1966). Later, Bohn (1985) raised *Shelfordella* to genus level. To date, the phylogenetic relationships between *Blatta* and *Shelfordella* remain elusive (Djernæs et al., 2012). After careful morphological examination, it was clear that the thalli examined from this host represented an undescribed species (Pfliegler et al., 2018). This was supported by our ITS phylogeny. *Herpomyces shelfordellae* and *H. stylopygae* are retrieved as sister species, but support for this sister relationship is low (BS = 53). Both taxa could be part of a complex of species parasitic on closely related cockroach hosts. The most distinctive morphological character of *H. shelfordellae* is its secondary axis, which forms a completely hyaline shield. In comparison, the basal tip of this shield is blackened in *H. stylopygae* (Spegazzini, 1917; Thaxter, 1931). The combination of molecular data and morphology has been key to the recognition of *H. shelfordellae*. To date, blackening of cells and structures has been referred to as an unsatisfying character in Laboulbeniomycetes taxonomy, since variations of color are not uncommon (e.g., Thaxter, 1931; Rossi, 1991; Weir, 1998). We find this pigment difference to be significant in this case.

One interesting observation was that two of our sampled species, *H. chaetophilus* and *H. periplanetae*, co-occur on the same host specimens (*Periplaneta americana*). To test their status as separate taxa, we removed thalli of both species from a single host specimen, isolated and amplified their DNA, and included the ITS sequences in our phylogeny. The isolates are DH602b for *H. chaetophilus*, and DH602a, DH602c, and DH602d for *H. periplanetae*. In this case, *H. chaetophilus* thalli were removed from the left posterior leg and those of *H. periplanetae* from the antennae. Often thalli of both species occur on antennae in close proximity of each other. Wang et al. (2016) found that *H. periplanetae* almost exclusively occurs on the antennae, but *H. chaetophilus* occurs on antennae, coxae, femora, tibiae, and tarsi. What drives this strict specificity of *H. periplanetae* is unknown.

Thaxter (1931) designated four groups, that he referred to as forms, in the genus *Herpomyces*, depending on characters of the perithecial apex. Form I includes those species with a simple apex (no projections), such as *H. chaetophilus* and *H. ectobiae* in our dataset; form II circumscribes those species with an apex subtended by a single projection, such as *H. periplanetae*, *H. shelfordellae*, and *H. stylopygae*; form III is represented by *H. forficularis* (not in our dataset), in which the perithecial apex has two projections at opposite sides; and form IV includes those species with three apical projections, such as *H. leurolestis* and *H. paranaensis*. Interestingly, the species from forms II and IV form two supported clades in our ITS phylogeny. Tavares (1985) suggested that structural form I is ancestral based on three pieces of evidence: (1) Simple morphology with normally blackened foot but without shield; (2) Ectobiidae is the earliest diverging lineage of Herpomycetales-associated cockroaches (confirmed by later molecular phylogenetic studies; e.g., Legendre et al., 2015), and (3) the species of form I occur on cockroaches of different lineages. Our three-locus phylogeny shows that also *H. ectobiae* (form I), parasitic on *Blattella germanica* (Ectobiidae, Blattellinae), is the earliest diverging clade, sister to all other *Herpomyces* species in the dataset.

5.4. Origins of Blattodea and Herpomycetales

The host range of Laboulbeniomycetes species is undeniably diverse. *Herpomyces* species are parasites of cockroaches (Thaxter, 1908, 1931); *Laboulbeniopsis termitarius*, a member of the class with unconfirmed position, is associated with termites (Henk et al., 2003); most *Pyxidiophora* species are associated with various fungal hosts in decaying substrates, and beetle and phoretic mite dispersers (Blackwell et al., 1986); and the Laboulbeniales have hosts in three subphyla of Arthropoda (Weir and Hammond, 1997). Comparing the phylogeny of Laboulbeniomycetes with their arthropod hosts may enable us to speculate on the evolutionary history of these fungi. However, this

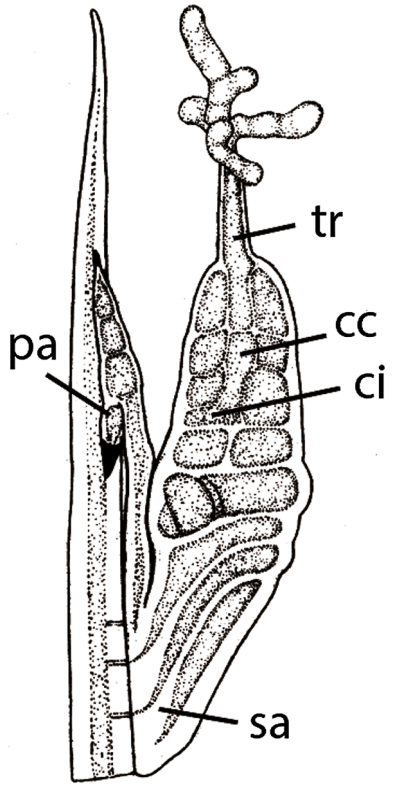

Fig. 7. *Herpomyces appendiculatus*. Developing female thallus attached to a single antennal spine of a *Platyzosteria scabra* Brunner von Wattenwyl, 1865 cockroach. This is an enlargement of Thaxter's (1931) drawing Plate XIV, Fig. 24. Indicated are: the primary axis of the receptacle (pa), secondary axis (sa) with haustoria penetrating the host's integument, the carpogonium-initiating cell (ci), the carpogenic cell (cc), and the trichogyne (tr), which receives spermatia. The carpogenic cell and trichogyne are surrounded by three tiers of perithecial outer wall cells. Image courtesy of the Archives of the Farlow Herbarium of Cryptogamic Botany.

comparison is arguably only informative when we exclude Pyxidiophorales, because these fungi are associated with organisms across multiple kingdoms. Species in Herpomycetales have cockroaches (Blattodea) as hosts. Blattodea and Mantodea (mantises) form a well-established lineage, superorder Dictyoptera, with a rich fossil record and established phylogeny (Legendre et al., 2015). Recently, termites were shown to be part of Blattodea and they should now be treated as an epifamily, Termitoidae, most closely related to the extant wood-feeding cockroach *Cryptocercus* (Inward et al., 2007; Eggleton et al., 2007; Djernæs et al., 2015; Legendre et al., 2015). Estimated dates for the split between Mantodea and Blattodea vary from 315.1 to 145.0 Mya (Table 4, Split M-C). The most recent common ancestor of the two subfamilies Blaberoidea and Blattoidea is thought to have appeared in the Late Permian—Middle Jurassic (Djernæs et al., 2015; Legendre et al., 2015; Wang et al., 2017; Table 4). The only other study that constructed a molecular clock analysis of a cockroach phylogeny estimated these dates much younger (Che et al., 2017), but their findings were based on a single mitochondrial marker and thus should be treated with more caution.

An interesting question regarding the Blattodea-associated Herpomycetales clade is whether its divergence happened simultaneously or later than that of its hosts. In our molecular clock analysis, the estimated age of sordariomyceta is 231.35 Mya (Fig. 4). Beimforde et al. (2014) did not include Laboulbeniomycetes into their analyses but dated the Leotiomycetes–Sordariomycetes split around 287 Mya (234–388) with *Paleopyrenomycites devonicus* as a single calibration, whereas with five calibration points this split was dated at 309 Mya (267–430 Mya). Further, we estimate the split between Laboulbeniomycetes and Sordariomycetes around 205.79 Mya. Surprisingly, the diversification of extant species of Laboulbeniales is much older (110.78 Mya) than for Herpomycetales (63.65 Mya). The genera *Chitonomyces* and *Zodiomyces* form a highly supported clade sister to the rest of the Laboulbeniales (Fig. 1). Species of these two genera occur on aquatic hosts (Dytiscidae and Hydrophilidae, respectively) (Tavares, 1985; Santamaria, 2004; Goldmann and Weir, 2012). It is very well possible that Laboulbeniales-like ancestors were aquatic. The Paleocene origin of the Herpomycetales clade is plausible given the divergence time estimates provided for their hosts (Djernæs et al., 2015; Legendre et al., 2015; Wang et al., 2017). This later origin compared to Laboulbeniales points to either a host shift from a laboulbenialean ancestor on a host living in close proximity to cockroaches or divergence among populations of laboulbenialean ancestors on a cockroach host.

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Table 4
Molecular phylogenetic studies including molecular dating of cockroach phylogenies based on fossil calibrations. For each reference are given: estimated dates for the split of crown-Dictyoptera into mantises and cockroaches (M-C) and the split between superfamilies Blaberoidea and Blattoidea (B-B), as well as the loci and the number of fossil calibration points used.

Reference	Split M-C	Split B-B	(Number of) loci	Fossils
Djernæs et al. (2015)	273 ± 15 Mya	~250 Mya	(6) 12S, 16S, COII, 18S, 28S, H3	3
Legendre et al. (2015)	293.7–315.1 Mya	283.2–263.6 Mya	(6) 12S, 16S, COI, COII, 18S, 28S	17
Che et al. (2017)	145.0–185.09 Mya	125–167.4 Mya	(1) COI	6
Wang et al. (2017)	204.3–289.1 Mya	173.1–229.1 Mya	(5) 12S, 16S, COII, 28S, H3	8

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Conflict of interest

None.

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