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RESEARCH ARTICLE

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Pisorisporiales, a new order of aquatic and terrestrial fungi for *Achroceratosphaeria* and *Pisorisporium* gen. nov. in the *Sordariomycetes*

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Key words

Achroceratosphaeria
freshwater
Hypocreomycetidae
Koralionastetales
Lulworthiales
multigene analysis
systematics

Abstract Four morphologically similar specimens of an unidentified perithecial ascomycete were collected on decaying wood submerged in fresh water. Phylogenetic analysis of DNA sequences from protein-coding and ribosomal nuclear loci supports the placement of the unidentified fungus together with *Achroceratosphaeria* in a strongly supported monophyletic clade. The four collections are described as two new species of the new genus *Pisorisporium* characterised by non-stromatic, black, immersed to superficial perithecial ascomata, persistent paraphyses, unitunicate, persistent asci with an amyloid apical annulus and hyaline, fusiform, cymbiform to cylindrical, transversely multiseptate ascospores with conspicuous guttules. The asexual morph is unknown and no conidia were formed in vitro or on the natural substratum. The clade containing *Achroceratosphaeria* and *Pisorisporium* is introduced as the new order *Pisorisporiales*, family *Pisorisporiaceae* in the class *Sordariomycetes*. It represents a new lineage of aquatic fungi. A sister relationship for *Pisorisporiales* with the *Lulworthiales* and *Koralionastetales* is weakly supported by Bayesian inference and maximum likelihood analyses. The systematic position of *Pisorisporium* among morphologically similar perithecial ascomycetes is discussed.

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INTRODUCTION

The genus *Achroceratosphaeria* was described for perithecial ascomycetes that are morphologically similar to *Ceratosphaeria* and *Pseudohalonectria* of the *Magnaporthaceae* (Réblová et al. 2010).

In the phylogeny inferred from sequences of the small and large subunits of nuclear ribosomal DNA (nuc18S and nuc28S rDNA) *Achroceratosphaeria* has been placed within *Sordariomycetes* incertae sedis; it was nested in a weakly supported clade as sister to the *Lulworthiales* and *Koralionastetales* containing fungi from predominantly marine habitats (Kohlmeyer 1997, Kohlmeyer et al. 2000, Campbell et al. 2005, 2008). *Achroceratosphaeria* comprises two freshwater and one terrestrial species characterised by minute, immersed, subhyaline to pale brown ascomata with a fragile, hyaline to pale brown protruding neck, tapering paraphyses, unitunicate stipitate asci with a non-amyloid apical annulus and eight hyaline, septate, ellipsoidal to fusiform ascospores. The asexual morph is unknown.

Four specimens of an unidentified fungus were collected on deciduous wood submerged in fresh water in France and Belgium during the years 2006–2014. They are characterised by non-stromatic, immersed to superficial papillate perithecial ascomata, persistent paraphyses, unitunicate asci with an amyloid apical annulus and hyaline, fusiform, cylindrical to cymbiform, transversely multiseptate ascospores with numerous guttules. No conidia were formed in vitro or on the natural substratum containing ascomata. In ascospore morphology, the unknown fungus resembles members of *Ceratosphaeria*, *Ceratosphaerella* and *Pseudohalonectria* of the *Magnaporthaceae* (Shearer 1989, Huhndorf et al. 2008).

Preliminary analysis of the three phylogenetic markers nuc18S rDNA, nuc28S rDNA and the second largest subunit of RNA polymerase II (*rpb2*) revealed that three strains of the unidentified fungus are closely related to *Achroceratosphaeria*. A sister relationship with the *Lulworthiales* and *Koralionastetales* as a basal group to the *Hypocreomycetidae* was suggested. In the *Hypocreomycetidae* four lineages contain mostly aquatic fungi. Marine fungi characterised by considerable morphological and ecological diversity are accommodated in the *Lulworthiales*/ *Koralionastetales* clade. Other marine fungi are placed in the *Halosphaeriaceae* of the *Microascales* and in four families introduced for genera of the so-called TBM clade '*Torpedospora*/ *Bertia*/ *Melanospora*' (Kohlmeyer 1972, Spatafora et al. 1998, Schoch et al. 2007, Jones et al. 2014), while *Savoryellales* comprises usually lignicolous species found in freshwater and brackish water habitats (Boonyuen et al. 2011). Other non-stromatic freshwater fungi are placed in the *Sordariomycetidae* in the *Annulatascaceae* (Wong et al. 1998, Ho & Hyde 2000, Campbell & Shearer 2004) and in other numerous small or monotypic genera of uncertain position (Hyde et al. 1997, 1999, 2000, Ho et al. 1999, Ranghoo et al. 2000, 2001, Raja et al. 2003, Vijaykrishna et al. 2005, Zelski et al. 2011a, b, Ferrer et al. 2012, Liu et al. 2012). The family *Papulosaceae* placed in the *Sordariomycetidae* originally comprised a single species growing on saltmarsh plants (Winka & Eriksson 2000). Based on molecular sequence data, the two freshwater genera *Brunneosporella* and *Fluminicola* (Wong et al. 1999, Ranghoo et al. 2001) were shown to be closely related to *Papulosa* (Réblová 2013).

This study aims to investigate and clarify the ordinal and familial relationships of *Achroceratosphaeria* and the unidentified freshwater fungus in the *Sordariomycetes* employing molecular sequence characters from protein-coding and ribosomal nuclear loci.

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Table 1 A list of fungi, isolate information and new sequences determined for this study and those retrieved from GeneBank. GenBank accession numbers in **bold** were generated for this study.

Classification	Taxon	Source	GenBank accession numbers		
			nuc28S	nuc18S	*rpb2*
Sordariomycetes					
Annulatascaceae	*Annulatascus velatisporus*	A70-18	AY316354	–	–
	Annulusmagnus triseptatus	CBS 131483, CBS 128831	GQ996540	JQ429242	JQ429258
	Ascitendus austriascus	CBS 131685	GQ996539	GQ996542	JQ429257
Boliniales	*Camaropella pugillus*	SMH 3846	EU481406	–	–
	Camarops microspora	CBS 649.92	AY083821	DQ471036	DQ470937
	Cornipulvina ellipsoides	SMH 1378	DQ231441	–	–
Calosphaeriales	*Calosphaeria pulchella*	CBS 115999	AY761075	AY761071	GU180661
	Jattaea algeriensis	STE-U 6399, CBS 120871	EU367457	EU367462	HQ878603
	Togniniella microspora	CBS 113648	AY761076	AY761073	GU180660
Chaetosphaeriales	*Chaetosphaeria ciliata*	ICMP 18253	GU180637	GU180614	GU180659
	Chaetosphaeria curvispora	ICMP 18255	GU180636	AY502933	GU180655
Coniochaetales	*Coniochaeta discoidea*	SANK 12878, CBS 158.80	AY346297	AJ875179	AY780191
	Coniochaeta ostrea	CBS 507.70	DQ470959	DQ471007	DQ470909
Coronophorales	*Bertia moriformis*	SMH 3344, SMH 4320	AY695261	–	AY780151
	Chaetosphaerella phaeostroma	SMH 4585	AY346274	–	AY780172
Diaporthales	*Diaporthe phaseolorum*	FAU 458, NRRL 13736	U47830	L36985	AY641036
	Gnomonia gnomon	CBS 199.53	AF408361	DQ471019	DQ470922
	Valsa ambiens	AR 3516	AF362564	DQ862056	DQ862025
Etheirophoraceae	*Etheirophora blepharospora*	JK 5397A	EF027723	–	EF027731
	Etheirophora unijubata	JK 5443B	EF027725	EF027718	EF027733
	Swampomyces armeniacus	JK5059C	EF027728	EF027721	–
	Swampomyces triseptatus	CY2802	AY858953	AY858942	–
Glomerellales	*Glomerella cingulata*	MCA 2498, FAU 513	DQ286199	M55640	DQ858455
	Kylindria peruamazonensis	CBS 838.91	GU180638	GU180609	GU180656
	Monilochaetes infuscans	CBS 379.77	GU180645	GU180619	GU180658
	Reticulascus clavatus	CBS 125296	GU180643	GU180622	–
Hypocreales	*Pseudonectria rousseliana*	AR 2716, CBS 114049	U17416	AF543767	DQ522459
	Trichoderma viride	GJS 89-127, IFFI 13001	AY489726	AF525230	EU252006
	Virgatospora echinofibrosa	CBS 110115	AY489724	AY489692	EF692516
Juncigenaceae	*Juncigena adarca*	JK 5235A	EF027726	EF027719	EF027734
	Fulvocentrum aegyptiacus	CY 2973	AY858950	AY858943	–
	Fulvocentrum clavatisporium	LP 83	AY858952	AY858945	–
Koralionastetales	*Koralionastes ellipticus*	JK 5769	EU863585	EU863581	–
	Pontogeneia microdictyi	JK 5748	–	EU863582	–
Lulworthiales	*Kohlmeyeriella tubulata*	PP 1105, PP 0989	AF491265	AY878997	–
	Lindra thalassiae	JK 5090A	DQ470947	DQ470994	–
	Lulwoana uniseptata	PP 7333, CBS 16760	FJ176904	AY879034	–
	Lulwoidea lignoarenaria	AFTOL 5013, IFO 32135	FJ176903	AY879010	–
	Lulworthia fucicola	PP 1235, C 21-1	AF491270	AF050481	–
	Rostrupiella danica	BBH 16759	DQ394094	–	–
	Spathulospora antarctica	JK 3530	–	AY380315	–
Magnaporthales	*Ceratosphaeria lampadophora*	CBS 117555	AY761084	AY761088	–
	Gaeumannomyces graminis	AR 3401, M 57	AF362557	JF414874	–
	Magnaporthe grisea	Ina168, 70-15	AB026819	DQ493955	–
	Muraeriata africana	GKM 1084	EU527995	–	–
Melanosporales	*Melanospora tiffanii*	ATCC 15515	AY015630	AY01561	AY015637
	Melanospora zamiae	ATCC 12340, CBS 421.87	AY046579	AY046578	DQ368634
Microascales	*Ceratocystis adiposa*	CCFC 212726, CBS 600.74	AY281101	EU984263	–
	Ceratocystis fimbriata	C89, Cef 0801, CBS 374.83	U17401	HQ908495	DQ368641
	Corollospora maritima	AFTOL 5011, JK 4834	FJ176901	U46871	DQ368632
	Custingophora olivacea	CBS 335.68	AF178566	JX070460	GU180665
	Graphium penicillioides	C 1505, CBS 506.86	AF222500	DQ471038	DQ470938
	Knoxdaviesia proteae	CMW 3936, CBS 486.88	AF221011	AY271804	–
	Lignincola laevis	AFTOL 737, A169-1D	U46890	AF050487	DQ836886
	Microascus trigonosporus	BS 218.31, ATCC52470	DQ470958	DQ471006	AF107792
	Petriella setifera	CCFC 226737, CBS 385.87, CBS 110344	AY281100	U43908	DQ368640
Ophiostomatales	*Ophiostoma piliferum*	DAOM 226737, CBS 129.32, CBS 158.74	AY281094	AJ243295	DQ470905
	Ophiostoma stenoceras	CBS 139.51	DQ836904	DQ836897	DQ836891
Papulosaceae	*Brunneosporella aquatica*	HKUCC 3708	AF132326	–	–
	Fluminicola coronata	HKUCC 3717	AF132332	–	–
	Papulosa amerospora	JK 5547F	DQ470950	DQ470998	DQ470901
Pisorisporiales	*Achroceratosphaeria potamia*	CBS 125414	GQ996538	GQ996541	**KM588908**
	Achroceratosphaeria sp.	HKU(M) 5224	AF132325	–	–
	Pisorisporium cymbiforme	CBS 138884	**KM588904**	**KM588901**	**KM588907**
	Pisorisporium cymbiforme	PRM 924378	**KM588902**	**KM588899**	**KM588905**
	Pisorisporium cymbiforme	PRM 924379	**KM588903**	**KM588900**	**KM588906**
Sordariales	*Gelasinospora tetrasperma*	CBS 178.33	DQ470980	DQ471032	DQ470932
	Lasiosphaeria ovina	SMH 1538, CBS 958.72	AF064643	AY083799	AY600292
	Sordaria fimicola	SMH 4106, MUCL 937, CBS 723.96	AY780079	X69851	DQ368647
Savoryellales	*Ascotaiwania lignicola*	NIL 00005	HQ446364	HQ446284	–
	Canalisporium exiguum	SS 00809	GQ390281	GQ390266	–
	Savoryella lignicola	NF 00204, NTOU 791	HQ446378	HQ446299	–
Torpedosporaceae	*Torpedospora ambispinosa*	CY3386	AY858946	AY858941	–
	Torpedospora radiata	JK5252C	EF027730	EF027722	EF027737
Xylariales	*Anthostomella torosa*	JK 5678E	DQ836902	DQ836895	DQ836885
	Graphostroma platystoma	CBS 270.87	DQ836906	DQ836900	DQ836893
	Xylaria hypoxylon	AFTOL 51	AY544648	AY544692	DQ470878
Leotiomycetes					
Helotiales	*Leotia lubrica*	AFTOL 1, isolate unknown for nuc18S	AY544644	L37536	DQ470876
	Microglossum rufum	AFTOL 1292	DQ470981	DQ471033	DQ470933

MATERIALS AND METHODS

Herbarium material and fungal strains

Dry ascomata were rehydrated with water; material was examined with an Olympus SZX12 dissecting microscope, and hand-sectioned centrum material (including asci, ascospores and paraphyses) was mounted in Melzer's reagent, Lugol, 90 % lactic acid, aqueous cotton-blue (1 mg/mL), and blue or black Waterman ink. Hand sections of the ascomatal wall were studied in 3 % KOH or heated chloral-lactophenol. All measurements were made in Melzer's reagent. Means ± standard deviation (SD) based on 20–25 measurements, excluding maxima and minima, are given for dimensions of asci and ascospores. Images were captured by differential interference (DIC) or phase contrast (PC) microscopy using an Olympus DP70 Camera operated by Imaging Software Cell on an Olympus BX51 compound microscope.

Multi-ascospore isolates were obtained from fresh material of three collections (PRM 924377-924379) with the aid of a spore isolator (Meopta, Prague, Czech Republic). Ascospores and asci were spread on water agar, ascospores germinated within 48 h. Germinating ascospores were transferred and isolates were grown on water agar, potato-dextrose agar (PDA, Oxoid) and potato-carrot agar (PCA, Gams et al. 1998). Colonies were examined after 7, 21 and 30 d incubation at 25 °C in the dark. The ex-type culture is maintained at CBS (CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands). Type and other herbarium material are deposited in PRM herbarium (National Museum in Prague, Czech Republic). The Online auction colour chart (2004) was used as the colour standard.

DNA extraction, amplification and sequence alignment

Cultures used for DNA isolations were grown as previously described by Réblová et al. (2011) and DNA was extracted following the protocols of Lee & Taylor (1990). Procedures for amplifying and sequencing the nuc18S, nuc28S and *rpb2* were performed as described in Réblová et al. (2011). Sequences were edited using Sequencher v. 5.0 software (Gene Codes Corp., Ann Arbor, MI, USA).

GenBank accession numbers for newly sequenced taxa and other homologous sequences of members of the *Sordariomycetes* and *Leotiomycetes* retrieved from GenBank are listed in Table 1. Sequences were manually aligned in BioEdit v. 7.0.9.0 (Hall 1999). The nuclear ribosomal loci were aligned according to the secondary structure of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen in order to improve the decisions on homologous characters and introduction of gaps (Gutell 1993, Gutell et al. 1993, www.rna.ccbb.utexas.edu). These procedures and alignment of the *rpb2* sequences were performed as described in Réblová & Réblová (2013).

The single-locus datasets (nuc28S: 1 923 characters and 77 sequences, nuc18S: 1 805 characters and 68 sequences, *rpb2* segments 5–7: 1 213 characters and 48 sequences) were examined for topological incongruence among loci. For each individual locus, 500 bootstrap replicates were generated with RAxML-HPC v. 7.0.3 (Stamatakis et al. 2005, Stamatakis 2006) and compared visually for topological conflict between supported clades in phylogenetic trees. A conflict between two loci was assumed to occur when a clade appeared monophyletic with bootstrap support of ≥ 75 % in one tree, but was supported as non-monophyletic in another (Mason-Gamer & Kellogg 1996). Individual, conflict-free alignments were concatenated to combine sequences for subsequent phylogenetic analyses. The multiple sequence alignment is deposited in TreeBASE (Study no. 16406).

Phylogenetic analysis

Phylogenetic relationships of the unidentified fungus were resolved by an analysis of nuc18S, nuc28S and *rpb2* sequences of representatives of 19 orders or individual families of the *Sordariomycetes*. We analysed the first 2/3 of the 5' half of the nuc28S, the almost entire nuc18S, and segments 5–7 of *rpb2*. Bases 1–148 of the nuc18S, 1–85 of the nuc28S, and 1–58 of the *rpb2* alignments at the 5'-end and 1 457–1 923 of the nuc28S alignment at the 3'-end were excluded from analyses because of incompleteness of the majority of the available sequences. The combined dataset was partitioned into several subsets of nucleotide sites, i.e. nuc28S, nuc18S, and first, second and third codon positions of *rpb2*. Two members of the *Leotiomycetes*, *Leotia lubrica* and *Microglossum rufum* were used to root the multilocus phylogeny.

The program MrModeltest2 v. 2.3 (Nylander 2008) was used to infer the appropriate substitution model that would best fit the model of DNA evolution for each sequence dataset and each partition of the combined datasets. Maximum likelihood (ML) and Bayesian inference (BI) analyses were used to estimate phylogenetic relationships. ML analysis was performed with RAxML-HPC v. 7.0.3 with a GTRCAT model of evolution. Nodal support was determined by non-parametric bootstrapping (BS) with 1 000 replicates.

BI analysis was performed in a likelihood framework as implemented in MrBayes v. 3.0b4 software package to reconstruct phylogenetic trees (Huelsenbeck & Ronquist 2001). For the combined nuc18S, nuc28S and *rpb2* dataset we used for each partition the GTR+I+G substitution model. Two Bayesian searches were performed using the default parameters. Analyses were run for 10 M generations, with trees sampled every 1 000 generations. Tracer v. 1.6.0. (Rambaut et al. 2013) was used to confirm convergence of trees and burn-in. The first 50 000 trees, which represented the burn-in phase of the analysis, were discarded. The remaining trees were used for calculating posterior probabilities (PP) of recovered branches (Larget & Simon 1999).

RESULTS

Phylogenetic results

In the ML analyses (conducted by RAxML) of individual nuc28S, nuc18S and *rpb2* loci, the three strains of the unidentified fungus grouped always with two species of *Achroceratosphaeria* in a strongly supported monophyletic clade distantly related to the known orders and families of the *Sordariomycetes*. The clade is introduced as the new order *Pisorisporiales*. Analyses of individual nuclear ribosomal and protein-coding loci place *Pisorisporiales* at different positions in the *Sordariomycetes*, however none of the internodes received significant statistical support. The nuc28S locus supports the placement of *Pisorisporiales* as a basal group in the *Sordariomycetes*. The phylogenies derived from individual nuc18S and *rpb2* loci consistently place *Pisorisporiales* within the *Sordariomycetes*. In the nuc18S tree, the *Pisorisporiales* are located basal to the *Hypocreomycetidae*, while in the *rpb2* locus they are at the base of the *Sordariomycetidae*. All other families and orders of the *Sordariomycetes* formed well-supported monophyletic clades in analyses of all three individual loci.

The final alignment consisted of 79 combined nuc18S, nuc28S and *rpb2* sequences of members of the *Sordariomycetes*, each with 4 941 characters after introduction of gaps. The alignment had 2 551 distinct alignment patterns (ML analysis); the ML tree is shown in Fig. 1. The *Sordariomycetes* are shown as a robust monophyletic clade (100 % ML BS / 1.0 PP) comprising

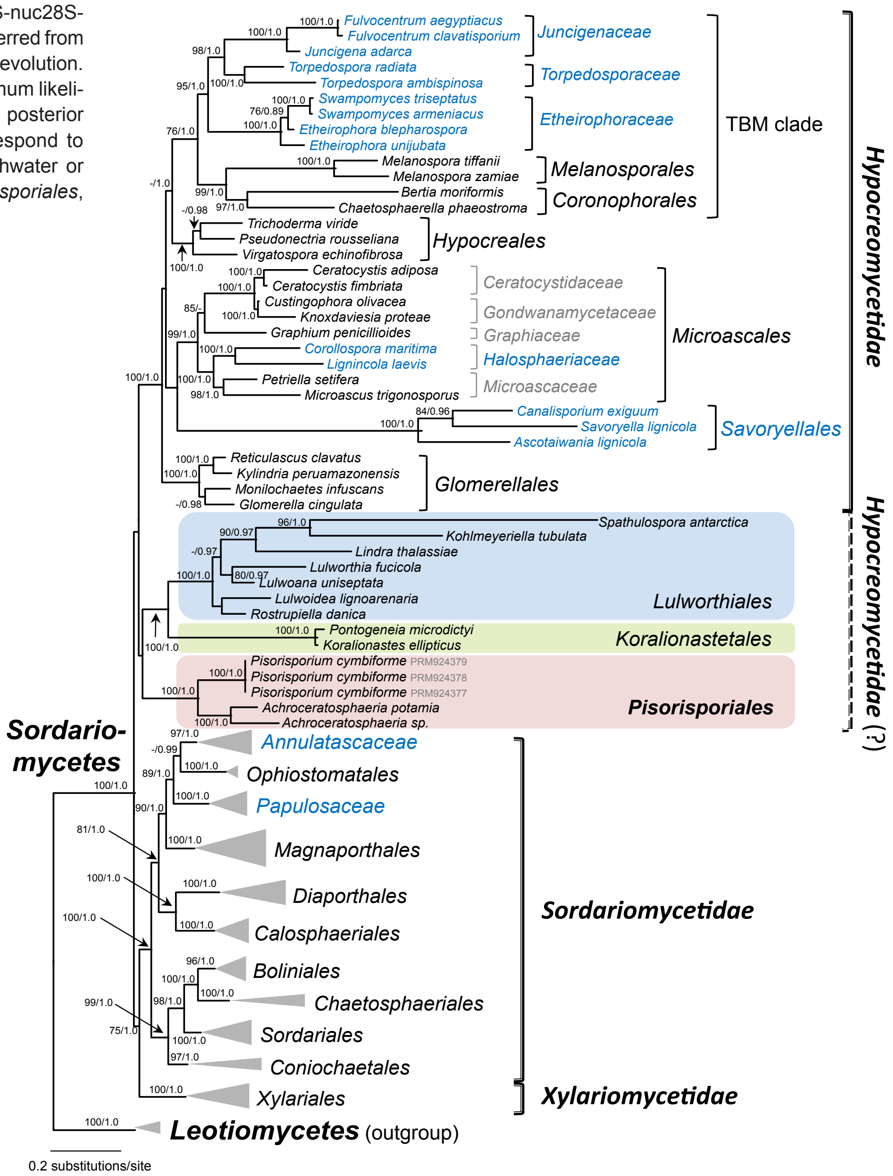

Fig. 1 Multilocus phylogenetic analysis of the nuc18S-nuc28S-*rpb2* sequences of the *Sordariomycetes*. Phylogram inferred from the ML analysis with RAxML using a GTRCAT model of evolution. Only high branch support is shown at the nodes, maximum likelihood bootstrap support (ML BS) ≥ 75 % and Bayesian posterior probability (PP) ≥ 95 %). Taxa labelled in blue correspond to groups whose members predominantly occur in freshwater or marine habitats besides the generally aquatic *Pisorisporiales*, *Lulworthiales*, and *Koralionastetales* clades.

three strongly supported lineages, the *Sordariomycetidae*, *Hypocreomycetidae* and *Xylariomycetidae*. The *Pisorisporiales* are nested in a weakly supported clade as sister to the *Lulworthiales* (100/1.0) and *Koralionastetales* (100/1.0). The whole clade is situated basal (65/0.87) to the *Hypocreomycetidae*. The other three taxonomic groups of the *Hypocreomycetidae* that contain predominantly fungi from aquatic habitats form strongly supported monophyletic clades, i.e. the *Halosphaeriaceae* (100/1.0), *Savoryellales* (100/1.0), and the complex of marine genera (95/1.0) comprising the *Etheirophoraceae* (100/1.0), *Juncigenaceae* (100/1.0) and *Torpedosporaceae* (100/1.0) of the TBM clade (76/1.0).

TAXONOMY

DNA sequences of nuclear ribosomal and protein-coding loci of specimens obtained from freshwater habitats in this study were shown to represent a new genus *Pisorisporium* and order in the *Sordariomycetes* based on phylogenetic analysis. Morphological examination showed that two species were present, described here as *P. cymbiforme* and *P. glaucum*. For the latter species DNA sequences could not be obtained but morphologically and ecologically it fits clearly within the newly described genus, while it is morphologically distinct from the first species.

Pisorisporiales Réblová & J. Fourn., *ord. nov.* — MycoBank MB810338

Type family. *Pisorisporiaceae* Réblová & J. Fourn.

Ascomata perithecial, non-stromatic. *Ostiole* periphysate. *Ascomatal wall* leathery to fragile, brown, partly carbonaceous. *Hamathecium* of true paraphyses. *Asci* unitunicate, persistent, with an amyloid or non-amyloid apical ring. *Ascospores* hyaline, transversely multiseptate. Asexual morph unknown. Saprobic on wood.

Pisorisporiaceae Réblová & J. Fourn., *fam. nov.* — MycoBank MB810339

Type genus. *Pisorisporium* Réblová & J. Fourn.

Ascomata non-stromatic, immersed to superficial, papillate or with a long neck, venter subglobose to conical, upright or lying obliquely or horizontally, neck central rarely eccentric. *Ostiole* periphysate. *Ascomatal wall* leathery to fragile, partly carbonaceous in the outer layers, pigmented dark brown, opaque to light brown to subhyaline, comprising two layers. *Paraphyses* abundant, persistent, cylindrical. *Asci* unitunicate, 8-spored, with a pronounced amyloid or non-amyloid apical annulus, cylindrical-clavate, persistently attached to the ascogenous hyphae at maturity. *Ascospores* fusiform, cylindrical to cymbi-

Fig. 2 *Pisorisporium cymbiforme*. a, b. Ascomata arranged in small groups or in rows; c, d. vertical sections of the ascomatal wall; e – g. asci; h, i. ascospores; j. paraphyses (a, b, g, i, j from PRM 924377 holotype; c, d, h from PRM 924379; e, f from PRM 924378); e – i: DIC; j: PC. — Scale bars: a, b = 200 μm; c, d = 20 μm; e – j = 10 μm.

form slightly tapering towards the ends, hyaline, transversely multiseptate, lacking a mucilaginous sheath or appendages, often with numerous guttules. Asexual morph unknown.

Pisorisporium Réblová & J. Fourn., *gen. nov*. — MycoBank MB810340

Type species. *Pisorisporium cymbiforme* Réblová & J. Fourn.

Etymology. *Pisorum* (Latin), meaning peas in a pod, referring to the numerous guttules arranged in a chain within ascospores; *spora* (Latin), referring to the ascospores.

Ascomata non-stromatic, immersed, gradually erumpent to superficial, solitary or in small groups or rows, papillate or with a short beak, glabrous, venter subglobose to broadly conical, laterally or basally flattened, upright or lying obliquely or horizontally. *Ostiole* periphysate. *Ascomatal wall* fragile, partly carbonaceous in the outer layer, 2-layered. *Paraphyses* persistent, septate, hyaline, arising from the bottom and sides in the ascomatal cavity. *Asci* unitunicate, 8-spored, cylindrical-clavate, short-stipitate, with a pronounced thimble-shaped amyloid apical annulus, persistently attached to the ascogenous hyphae at maturity. *Ascospores* fusiform, cylindrical to cymbiform, sometimes falcate, hyaline, transversely multiseptate, lacking a mucilaginous sheath or appendages, smooth-walled, with numerous guttules. Asexual morph unknown.

Notes — The paraphyses were present abundantly, they are fragile, easily broken in squash mounts, making it difficult to determine their length. They are cylindrical, arranged in parallel at the bottom of the ascomata and among the asci, tapering, sparsely branched and often intertwined in the upper half. The outer ascomatal wall is carbonaceous, grading outwards into 2–3 layers of subhyaline to pale brown, polyhedral to angular cells that probably account for the finely roughened appearance of the wall in both species.

Pisorisporium cymbiforme Réblová & J. Fourn., *sp. nov.* — MycoBank MB810341; Fig. 2

Etymology. *Cymbiform* (Latin), meaning boat-shaped (a long rowboat), referring to the shape of the ascospores.

Ascomata non-stromatic, immersed, gradually erumpent to superficial, solitary or in small groups of 2–4, or in rows, venter (240–)290–380 µm diam, 220–280 µm high, subglobose to broadly conical, dark brown to black, sometimes laterally or basally flattened, glabrous, finely roughened, upright or lying obliquely to horizontally, papillate or with a beak 30–110 µm high, conical or subcylindrical, central to lateral, opening by a rounded pore. *Ostiole* periphysate. *Ascomatal wall* fragile, carbonaceous, (12–)14–26 µm thick, becoming thicker in the neck c. 20–30 µm, 2-layered; outer layer consisting of brown, polyhedral cells of *textura prismatica* with opaque walls and lumina reduced to occluded; outwards grading into 2–3 layers of subhyaline to pale brown, polyhedral to angular cells of *textura angularis* c. 4–6 µm thick, collapsing in old ascomata and forming a persistent subhyaline amorphous coating; inwards grading into several layers of thin-walled, pale brown to hyaline, flattened cells. *Paraphyses* abundant, persistent, septate, hyaline, sparsely branched in the upper half and intertwined, c. 3.5–5.0 µm wide, tapering to c. 3.0 µm. *Asci* (180–)190–207 × 11–13(–14) µm (mean ± SD = 199.7 ± 5.4 × 12.5 ± 1.2 µm), cylindrical-clavate, obtuse to broadly rounded apically, 8-spored; apex with an amyloid apical annulus 2.7–3.2 µm wide, 1.9–2.3 µm high. *Ascospores* 40–45(–48) × (3.8–)4.3–4.8(–5.0) µm (mean ± SD = 43.7 ± 1.9 × 4.5 ± 0.3 µm), cymbiform to fusiform to cylindrical, slightly tapering towards the ends, hyaline, smooth, (8–)12–16-septate, non-constricted at the septa, each cell with a large guttule, arranged 2-seriately in the ascus.

Culture characteristics — Colonies slow growing, 18–22 mm diam on PDA after 21 d at 25 °C. Aerial mycelium beige (oac816) near the centre of the colony and on the inoculum block, white (oac909) towards the margin, felty, margin entire. Sporulation absent. Aerial mycelium composed of thin-walled, hyaline, unbranched or sparsely branched hyphae, 2.0–3.0 µm diam. Chlamydospores not observed.

Specimens examined. France, Midi-Pyrénées, Ariège, Rimont, valley of La Maille brook, c. 550 m asl, submerged decorticated wood of *Alnus glutinosa*, 9 May 2014, *J. Fournier J.F. 14046* (holotype PRM 924377, culture ex-type CBS 138884); ibid., 2 Apr. 2013, submerged decorticated wood of *Fraxinus excelsior*, *J. Fournier*, *J.F. 13067*, *J.F. 13070*, PRM 924378, PRM 924379.

Notes — The two collections PRM 924378 and PRM 924379 were acquired from the same branch submerged in water. The ascospores in PRM 924378 were slightly smaller, 34–39 × 4.0–4.7(–5.0) µm (mean ± SD = 36.9 ± 1.5 × 4.4 ± 0.4 µm), and asci slightly longer, 187–210(–230) × 11.5–13.5(–14) µm (mean ± SD = 201.4 ± 8.8 × 13.1 ± 1.3 µm) than in the type specimen. The ascospores of all three collections germinated easily on water and PDA agar within 48 h, cultures derived from PRM 924378 and PRM 924379 are no longer viable.

Pisorisporium glaucum Réblová & J. Fourn., *sp. nov.* — MycoBank MB810342; Fig. 3

Etymology. *Glaucus* (Latin), meaning blue, referring to the intense blue amyloid reaction of the apical annulus.

Ascomata non-stromatic, immersed, gradually erumpent to superficial, solitary or in small groups of 2–5, or in rows, venter 270–390 µm diam, 250–350 µm high, subglobose to broadly conical, dark brown to black sometimes laterally or basally flattened, glabrous, upright or lying obliquely to horizontally, papillate or with a beak 50–180 µm high, conical or subcylindrical, central to lateral, opening by a rounded pore. *Ostiole* periphysate. *Ascomatal wall* fragile, carbonaceous, 20–32 µm thick, becoming thicker in the neck c. 45–58 µm, 2-layered; outer layer consisting of brown, polyhedral cells of *textura prismatica* with opaque walls and lumina reduced to occluded; outwards grading into 2–3 layers of subhyaline to pale brown, polyhedral to angular cells of *textura angularis*; inwards grading into several layers of thin-walled, pale brown to hyaline, flattened cells. *Paraphyses* abundant, persistent, hyaline, septate, sparsely branched in the upper half and intertwined, c. 3.0–5.5 µm wide, tapering to 2.0–2.5 µm. *Asci* 190–245 × 12–15 µm (mean ± SD = 209.7 ± 12.8 × 12.8 ± 1.2 µm), cylindrical-clavate, obtuse to broadly rounded apically, 8-spored; apex with an amyloid thimble-shaped apical annulus 3.0–3.2 µm wide, 2.0–2.3 µm high. *Ascospores* (52–)55–67(–72) × 4.5–5.5 µm (mean ± SD = 59.5 ± 4.5 × 4.9 ± 0.3 µm), fusiform to subcylindrical, falcate, slightly tapering towards the ends, hyaline, smooth, 10–14-septate, non-constricted at the septa, each cell with a large guttule, arranged 2–3-seriately in the ascus.

Specimen examined. Belgium, Hainaut Province, Wellin, Halma, Ry des Glands brook, 26 Sept. 2006, on driftwood of *Acer pseudoplatanus*, *J. Fournier J.F. 06232* (holotype PRM *924380*).

Notes — *Pisorisporium glaucum* is easily distinguishable from *P. cymbiforme* by longer and slightly wider ascospores and longer asci. The number of septa of the ascospore is in both species comparable and varies from 10 to 16. This species has not been cultivated at the time of its collection and DNA sequences could not be obtained due to insufficient number of ascomata that would be required for successful DNA extraction. Such procedure would cause destruction of the type material.

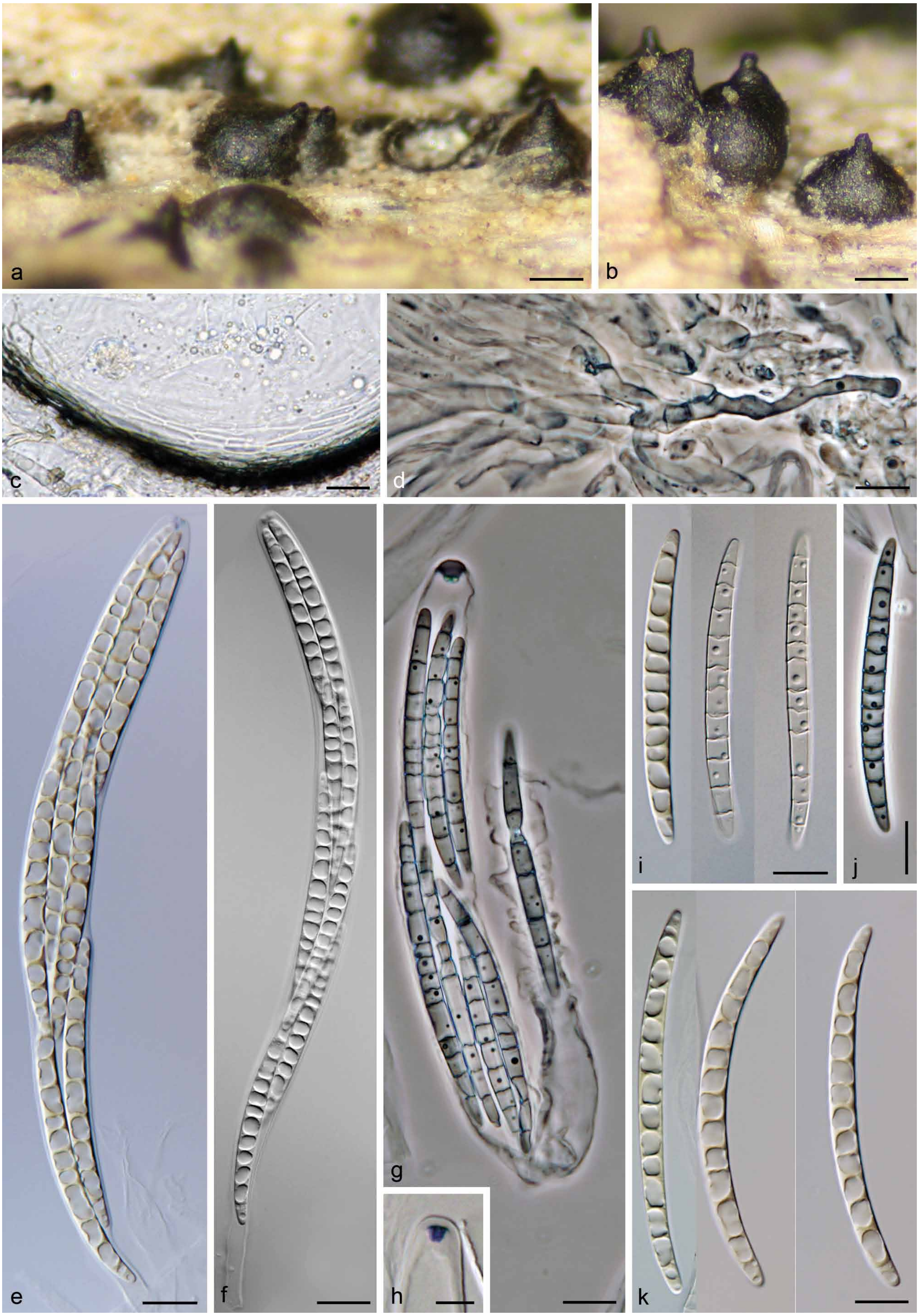

Fig. 3 *Pisorisporium glaucum*. a, b. Ascomata arranged in small groups or in rows; c. vertical section of the ascomatal wall; d. ascogenous hypha with attached bases of asci; e – g. asci; h. thimble-shaped apical annulus staining blue in Lugol; i – k. ascospores (a – k from PRM 924380 holotype); e, f, h, i, k: DIC; d, g, j: PC. — Scale bars: a, b = 200 μm; c = 20 μm; d – g, i – k = 10 μm; h = 5 μm.

DISCUSSION

The combined analysis of nuc18S-nuc28S-*rpb2* sequences (Fig. 1) led to the discovery that the three strains of *P. cymbiforme* and *Achroceratosphaeria* form a strongly supported monophyletic clade (100/1.0), which is distantly related to freshwater and marine ascomycetes of the *Annulatascaceae*, *Halosphaeriaceae*, *Papulosaceae*, *Savoryellales*, marine genera of the TBM clade, now classified as the *Etheirophoraceae*, *Juncigenaceae*, *Torpedosporaceae* and *Falcocladiaceae* (Jones et al. 2014), and other morphologically similar fungi. The newly recognised clade containing *Achroceratosphaeria* and *Pisorisporium* represents a distinct taxonomic group at the ordinal level within the *Sordariomycetes* based on the evidence of molecular sequence data. However, its relationship with other orders could not be elucidated with good statistical support. *Pisorisporiales* is nested in an unsupported clade as sister to the *Lulworthiales* and *Koralionastetales* situated basal to the *Hypocreomycetidae*.

The placement of the *Lulworthiales*, including *Spathulosporales*, and *Koralionastetales* within the *Sordariomycetes* based on DNA data had been ambiguous (Eriksson & Winka 1997, Spatafora et al. 1998, Kohlmeyer et al. 2000, Jones et al. 2009). Their sister relationship with the *Hypocreomycetidae* is supported in the 3-, 4- and 6-gene phylogenies by BI and ML methods (Schoch et al. 2007, Spatafora et al. 2007, Zhang et al. 2007), whereas the maximum parsimony, weighted parsimony and ML methods of the 4-gene analysis support their placement as a basal group in the *Sordariomycetes* (Zhang et al. 2007). The current position in combination with the *Pisorisporiales* may suggest a new subclass lineage in the *Sordariomycetes*.

Without molecular data, it is, in fact, challenging to place *Pisorisporium* in any of the accepted families and genera of the *Sordariomycetes*. Members of *Pisorisporium* grow on decaying deciduous wood submersed in fresh water. They are characterised by minute, immersed ascomata arranged in small groups or in rows oriented with the grain of wood, gradually erumpent by water erosion of the substrate and becoming superficial. Ascomata are upright but often grow obliquely or almost horizontally, which may be caused by the water flow. Paraphyses are fragile, arranged parallel among the asci, continuously tapering, becoming sparsely branched and intertwined above the ascal apices (Fig. 4). In ascospore and to some extent ascus morphology, *Pisorisporium* resembles members of *Ceratosphaeria*, *Ceratosphaerella* and *Pseudohalonectria* of the *Magnaporthaceae*. They are similar in overall morphology of fusiform, cylindrical to cymbiform, multiseptate, hyaline ascospores, but the three latter genera differ from *Pisorisporium* in a non-amyloid reaction of the apical annulus, long, sometimes flexuous protruding necks of ascomata and asexual morphs, i.e. harpophora-like and phialophora-like asexual morphs experimentally linked to *Ceratosphaeria* and the presumed *Didymobotryum*-like asexual morph of *Ceratosphaerella* (Shearer 1989, Réblová 2006, Huhndorf et al. 2008). Moreover, species of *Pseudohalonectria* and *Ceratosphaeria phialidica* differ from *Pisorisporium* by cylindrical to cymbiform asci with ascospores arranged in a fascicle or rarely 4-seriately, while in *Pisorisporium*, *Ceratosphaeria* and *Ceratosphaerella* the ascospores are predominantly 2-seriate within the ascus.

The amyloid reaction of the apical annulus is not quite consistent among orders of ascomycetes; in the *Sordariomycetes* it occurs predominantly in members of the *Xylariales*, i.e. *Amphisphaeriaceae*, *Diatrypaceae* and *Xylariaceae*. The positive blue to dark reaction of iodine solutions, i.e. Lugol and Melzer's reagents, due to the presence of starch-like polysaccharides in fungal microscopic structures is generally termed amyloid or euamyloid. The apical annulus of both species of *Pisorisporium* can be termed amyloid; it turns blue in Melzer's reagent and in Lugol's solution irrespective of whether a pre-treatment with KOH was applied. Regarding the amyloidity of the ascal apical structures and chemical reactions with other dyes like Congo red, toluidine blue or blue ink, we noticed a difference between *Pisorisporium* on one hand and members of the *Xylariaceae* and other taxa on the other. Only in *Pisorisporium* the apical annulus is readily stained by these chemicals (Fig. 4). However, such coloration, commonly encountered in many sordariaceous genera with chitinoid (non-amyloid) apical annulus, does not occur in genera with a known amyloid apical annulus. Our observation may imply that the apical annulus of *Pisorisporium* is composed of other components than commonly encountered in members of the *Xylariales*. Clarification of the chemical compounds responsible for this discrepancy is beyond the scope of the present paper. However, the fact itself is interesting and worth being reported.

Two genera of the *Amphisphaeriaceae*, *Crassoascus* and *Iodosphaeria*, can be compared with *Pisorisporium* based on morphology of ascospores, asci and the amyloidity of the apical annulus (Samuels et al. 1987, Barrasa et al. 1993). Members of *Crassoascus* differ from *Pisorisporium* by the flat apical annulus and fusiform, multiseptate, versicolorous ascospores with brown middle cells and hyaline end-cells, sometimes with hyaline cap-like appendages (Barr 1993, Barrasa et al. 1993, Catania & Romero 2012). *Iodosphaeria* can be distinguished from *Pisorisporium* by non-papillate ascomata associated with a repent, spreading network of brown hyphae, with a flat top from which radiate numerous, flexuous, unbranched hairs, asci with a flat apical annulus and suballantoid, rarely ellipsoid, non-septate ascospores and asexual morphs belonging to *Ceratosporium* and *Selenosporella* (Samuels et al. 1987, Barr 1993, Hsieh et al. 1997, Catania & Romero 2012).

Iodosphaeria aquatica is the only species that does not conform to the description of that genus; it resembles *Pisorisporium* with regard to the aquatic habitat, glabrous ascomata and septate, fusiform ascospores arranged 2–3-seriately within the ascus (Hyde 1995). *Iodosphaeria aquatica* differs from *Pisorisporium* by ascomata that are immersed beneath a blackened clypeus, the ascomatal wall, which is composed of thin-walled, brown angular cells and by 1-septate ascospores that have mucilaginous appendages at each pole. Molecular analysis of partial nuc28S rDNA sequences of *I. aquatica* revealed that the fungus is unrelated to the *Sordariomycetes* and it is preliminarily placed in the *Dothideomycetes* among genera with ascolocular development of the ascomata (strain HKUCC 166, nuc28S GenBank accession: AF452044, Jeewon et al. 2003). However, no information is available about whether this is a sequence obtained from the ex-type strain or the DNA was isolated from different material of *I. aquatica*.

Based on morphological characters and habitat, it is difficult to find similarities among members of the *Koralionastetales*, *Lulworthiales* and *Pisorisporiales*. *Lulworthiales* was established by Kohlmeyer et al. (2000), when it was discovered that the *Halosphaeriales* are polyphyletic comprising two distinct evolutionary lineages of marine fungi with terrestrial ancestors (Spatafora et al. 1998). Members of the *Lulworthiales* are predominantly marine ascomycetes, but some also inhabit niches in estuarine environments. They include saprobes on driftwood and intertidal wood, sea grasses, saltmarsh plants, coral rocks or parasites of uncalcified *Rhodophyta* or *Phaeophyta*. They are characterised by filiform, one- to multiseptate ascospores with apical mucus-containing chambers or gelatinous sheath (except species of *Lindra*), early deliquescing asci and the absence of a hamathecium in mature ascomata, while young ascomata contain pseudoparenchyma (Kohlmeyer 1997, Nakagiri & Tadayoshi 1997, Campbell et al. 2005, Koch et al. 2007).

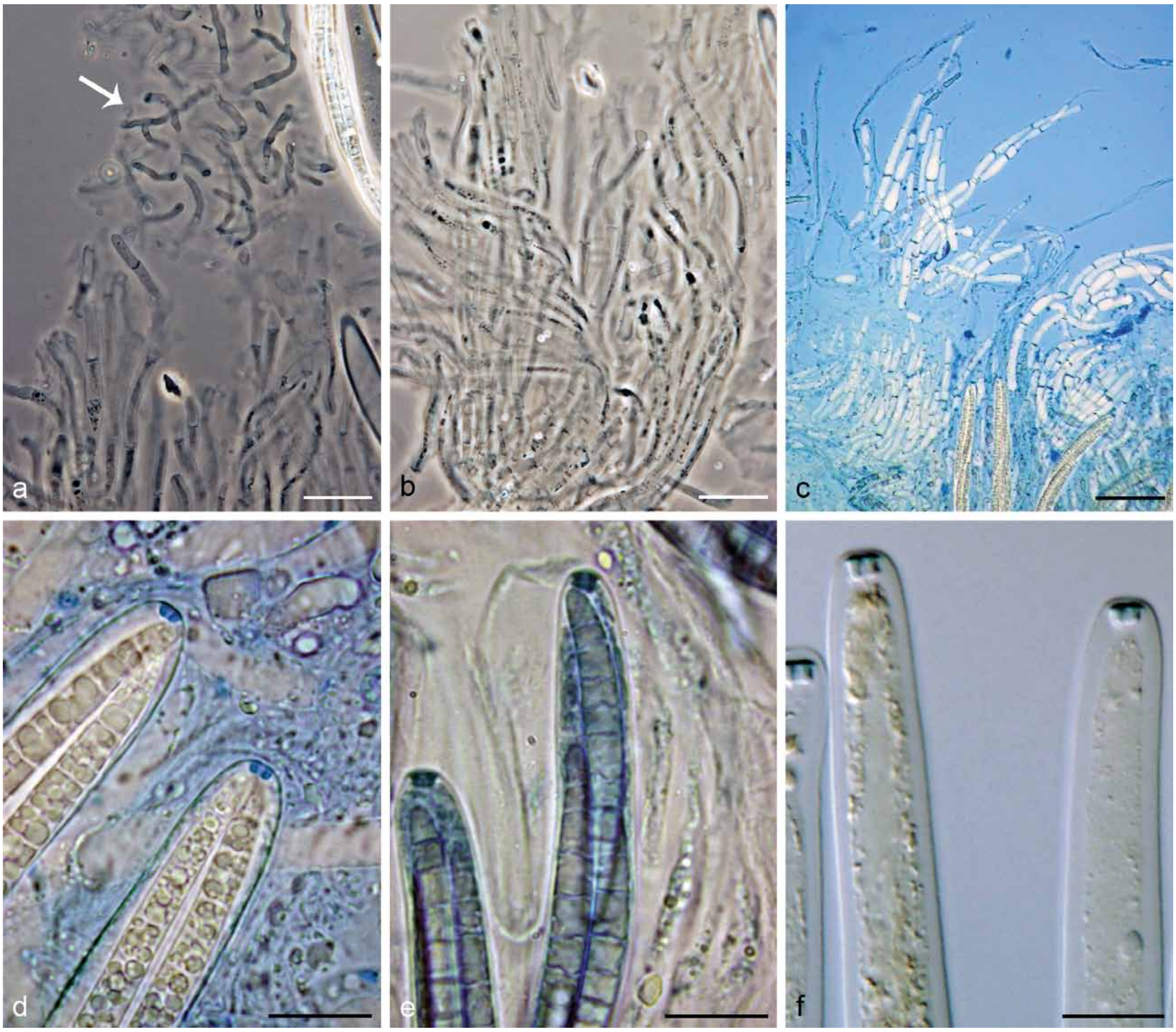

Fig. 4 *Pisorisporium* spp. a–d. *Pisorisporium glaucum*. a, b. paraphyses in Melzer's reagent, arrow indicates filaments that are sparsely branched and intertwined above ascal apices; c. paraphyses and three asci in blue waterman ink; d. apical annulus in blue waterman ink. — e, f. *Pisorisporium cymbiforme*. e. apical ring in black waterman ink; f. apical annulus in Melzer's reagent (a–d from PRM 924380; e, f from PRM 924378); a, b: PC; c–f: DIC. — Scale bars: a–c = 20 μm; d–f = 10 μm.

Asexual morphs of *Lulworthiales* belong to nine hyphomycetous dematiaceous genera with usually coiled conidia; they were assigned to the order based on molecular DNA data or the link between sexual and asexual morph was proven experimentally (Nakagiri & Tubaki 1983, Nakagiri 1984, Campbell et al. 2005, Jones et al. 2008, 2009, Abdel-Wahab et al. 2010).

The *Koralionastetales* were separated from the *Lulworthiales* by Campbell et al. (2008) to include fungi occurring obligatorily in marine habitats. They are characterised by a centrum containing paraphyses and periphyses and ellipsoid, fusiform to filiform ascospores without any apical structures and with typical formation of antheridia on germ tubes. Members of *Koralionastes* live on coral rocks, while *Pontogeneia* is a parasite of marine *Phaeophyta*. Their asexual morphs are unknown.

The type species of *Achroceratosphaeria* (*A. potamia*) and *Pisorisporium* (*P. cymbiforme*), originate in the same territory in the Ariège department in Midi-Pyrénées less than 1 km apart. The La Maille locality, where *P. cymbiforme* was repeatedly collected in 2013 and 2014, is a deep valley bordering steep slopes at the foot of the Arize massif, with a thick deciduous forest with high humidity. Trees and shrubs grow densely also along the shadowy La Maille brook. Decaying branches, twigs and larger logs fall regularly in the water flow, which provide a rich substrate in this locality. The La Maille brook may dry up at the end of season, leaving the otherwise submerged wood and driftwood exposed to air for several weeks or even months. Not far from here, in the Le Baup stream, of which the La Maille brook is a tributary, was collected *A. potamia*, another member of the *Pisorisporiales*.

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