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Unexpected *Nectriaceae* species diversity in cheese, description of *Bisifusarium allantoides* sp. nov., *Bisifusarium penicilloides* sp. nov., *Longinectria* gen. nov. *lagenoides* sp. nov. and *Longinectria verticilliforme* sp. nov.

Savary O[1], Coton M[1], Frisvad JC[2], Nodet P[1], Ropars J[3], Coton E[1] and Jany J-L[1#]

[1]Univ Brest, Laboratoire Universitaire de Biodiversité et Écologie Microbienne, F-29280 Plouzané, France
[2]Department of Biotechnology and Biomedecine, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark
[3]Ecologie Systématique et Evolution, Université Paris-Saclay, CNRS, AgroParisTech, 91400 Orsay, France

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Abstract

Investigation of various cheese rinds for *Bisifusarium domesticum* revealed a completely unexpected diversity of *Fusarium*-like fungi. Elongation factor 1-alpha gene sequence data, classically used for *Fusarium* spp. identification, suggested that some isolates corresponded to so far undescribed *Nectriaceae* species. In this context, a multi-gene phylogenetic analysis, based on ten loci, namely the large subunit of the ATP citrate lyase (*acl1*), alpha-actin (*act*), calmodulin (*cmdA*), histone H3 (*his3*), the internal transcribed spacer region and intervening 5.8S nrRNA (*ITS*), 28S large subunit (*LSU*), RNA polymerase II largest subunit (*rpb1*), RNA polymerase II second largest subunit (*rpb2*), translation elongation factor 1-alpha (*tef1*) and β-tubulin (*tub2*), was therefore performed to determine the phylogenetic position of ten cheese and one environmental isolates. Phylogenetic reconstructions were then done using a *Nectriaceae* sequence dataset. Additionally, morphological observations as well as metabolite profiling were performed. Results clearly revealed the existence of four novel cheese-associated species, namely *Bisifusarium allantoides*, *Bisifusarium penicilloides*, *Longinectria lagenoides*, *Longinectria verticilliforme*, the two latter belonging to a novel genus (*Longinectria* gen. nov.).

Keywords – Fungi – *Fusarium*-like – Phylogeny – Taxonomy

Introduction

Cheese making dates back at least 8000 years (Fox & Guinee 2020) and in some cheeses, fungi play a major role during cheese ripening by contributing to their organoleptic richness and diversity (Irlinger et al. 2015). The most well-known examples worldwide are probably moldy rind soft cheeses (e.g. Brie and Camembert) produced using *Penicillium camemberti* and *Geotrichum candidum,* and blue-veined cheeses (e.g. Roquefort, Gorgonzola, Stilton) produced with *Penicillium roqueforti* (Cheeseman et al. 2014, Gillot et al. 2017, Dumas et al. 2020). Beyond these emblematic examples, other moulds contribute to cheese making (e.g. *Mucor* spp., *Scopulariopsis* spp. or

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Corresponding Author: Jean-Luc Jany – e-mail – jean-luc.jany@univ-brest.fr

Bisifusarium domesticum) (Hermet et al. 2012, Morin-Sardin et al. 2016, Dupont et al. 2017, Lebreton et al. 2020). During the last decades, many studies have revealed that cheeses harbour substantial fungal diversity (Ropars et al. 2012, Wolfe et al. 2014, Hermet et al. 2014, Dugat-Bony et al. 2016). These fungi may be either indigenous, originating from the ripening rooms or cheese environment, or voluntarily inoculated as adjunct cultures. At least part of the main species associated with cheese making are thought to have undergone a domestication process (i.e. adaptive divergence under human pressure at a human time scale), as observed for the emblematic *P. camemberti* and *P. roqueforti* species (Ropars et al. 2015, 2016, 2017, 2020, Bodinaku et al. 2019, Dumas et al. 2020), or have been described as well adapted to the cheese substrate, such as *Mucor* spp. found in Tomme (Morin-Sardin et al. 2016). Besides the above-mentioned species, which have been extensively studied, common cheese taxa such as *Scopulariopsis* spp. or *B. domesticum* have been seldomly investigated (Ropars et al. 2012). The latter species is commonly used by cheesemakers as an adjunct culture for cheese productions (e.g. Saint-Nectaire, Reblochon), and in some cases, to prevent excessive surface stickiness (e.g. Raclette, Gruyère, Appenzeller), a property from which its trivial name "*Anticollanti*" is derived (Bachmann et al. 2003, 2005). To date, *B. domesticum* has been solely isolated from cheese and is the only species within the family *Nectriaceae* associated with this food matrix. It was originally identified as *Trichothecium domesticum,* then described as *Fusarium domesticum* by Bachman et al. (Bachmann et al. 2003, 2005). Recently, nomenclatural changes divided *Fusarium* into seven different genera (Lombard et al. 2015, Sandoval-Denis et al. 2018, 2019). *Fusarium domesticum* was therefore assigned to the new genus *Bisifusarium* (and named *B. domesticum*) along with six other taxa previously assigned to the *Fusarium dimerum* species complex (FDSC) (Schroers et al. 2009, Lombard et al. 2015), namely *B. biseptatum* (Schroers, Summerbell & O'Donnell) Lombard & Crous, 2015, *B. delphinoides* (Schroers, Summerbell, O'Donnell & Lampr.) Lombard & Crous, 2015, *B. dimerum* (Penz.) Lombard & Crous 2015, *B. domesticum* (Fr.) Lombard & Crous 2015, *B. lunatum* (Ellis & Everh.) Lombard & Crous 2015, *B. nectrioides* (Wollenw.) Lombard & Crous 2015 and *B. penzigii* (Schroers, Summerbell & O'Donnell) L. Lombard & Crous, 2015 (Lombard et al. 2015). An eighth species, *Bisifusarium tonghuanum* B.D. Sun, Y.G. Zhou & A.J. Chen, 2017, was also recently added to the genus (Sun et al. 2017). Species within the genus *Bisifusarium* were described as having short macroconidia with a maximum of three septa and short phialides arising as lateral pegs from the hyphae (Gerlach & Nirenberg 1982, Schroers et al. 2009, Lombard et al. 2015, Sun et al. 2017).

It is noteworthy that the circumscription of the *Fusarium* genus, and consecutively the definition of *Bisifusarium* has recently been highly debated (Crous et al. 2021 *vs* Geiser et al. 2021) and, so far, no consensus nomenclature has been provided.

Accessing a large number of isolates is crucial for studying a species of interest. We therefore investigated different cheeses to create a large working collection of *B. domesticum* (*F. domesticum sensu* Geiser et al. 2020) isolates which led to the isolation and identification of various *Fusarium*-like isolates. Sequencing of the translation elongation factor 1-alpha used for rapid isolate identifications highlighted an unexpected diversity within the *Nectriaceae* family for four putative novel taxa. In order to clarify the taxonomic position of these four taxa, a multi-gene phylogenetic analysis using the ten gene set described by Lombard et al. (2015) was performed. To complete this description, morphological characters and secondary metabolite profiles were determined.

Material & Methods

Fungal isolations from cheeses

In total, fifty-six cheeses were analysed. On the one hand, forty-six different cheeses (from France, Switzerland, Italy, Spain and Canada), including artisanal and industrial cheeses, were obtained for fungal isolations. For each cheese sample, 10 g of rind were removed using a sterile scalpel, transferred into 90 mL of Tween 80 (0.015% v/v) in a sterile stomacher bag equipped with a filter membrane and mixed for 180 seconds using a Stomacher homogenizer (AES, France). Serial dilutions of the homogenate were spread on dichloran glycerol chloramphenicol selective agar

(DG18, Millipore, Merck, Germany) and malt yeast extract agar (M2Lev) supplemented with antibiotics (20 g/L malt extract, 3 g/L yeast extract 15 g/L agar, 100 mg/L penicillin and 100 mg/L streptomycin). Agar plates were incubated at 25°C for 7 days. Fungal colonies harbouring typical macroscopic and/or microscopic characters of *B. domesticum* or *Fusarium*-like fungi were further isolated and purified on M2Lev medium. On the other hand, analysis of ten different cheeses by international colleagues allowed us to increase the number of *Fusarium*-like isolates. All isolates were conserved at -80°C in 10% glycerol (v/v).

DNA extraction, PCR amplification and sequencing

DNA extractions were performed on seven to ten-day monospore cultures grown on M2Lev medium using the FastDNA SPIN kit (MP Biomedicals, Fisher Scientific) according to manufacturer recommendations. Each DNA extract was diluted to 40 ng/µL and stored at -20°C until PCR amplification.

Firstly, sequencing of the translation elongation factor 1-alpha (*tef1*) was used for species identification. Generated sequence data was compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST). Then, a subset of thirteen representative isolates, including eleven unidentified ones, was selected and used for the phylogenetic study (Table 1).

Secondly, their phylogenetic position was assessed using the ten loci and same primers as those described by Lombard et al. (2015) which was the only set available at the beginning of this study. Pending adoption of a consensus taxonomy, we named taxa according to the nomenclature proposed by the same authors. The ten loci corresponded to the large subunit of the ATP citrate lyase (*acl1*), alpha-actin (*act*), calmodulin (*cmdA*), histone H3 (*his3*), the internal transcribed spacer region and intervening 5.8S nrRNA (*ITS*), 28S large subunit (*LSU*), RNA polymerase II largest subunit (*rpb1*), RNA polymerase II second largest subunit (*rpb2*), translation elongation factor 1-alpha (*tef1*) and β-tubulin (*tub2*) genes. PCR mixtures (Table 2) using the different primers are detailed in Table S1. Specific PCR amplifications were checked after migration of resulting amplicons on a 1% (w/v) agarose gel in 1X Tris-acetate-EDTA buffer (molecular biology grade, Promega). Sequencing was performed using the different forward and reverse primers by Eurofins Genomics platform (Ebersberg, Germany). Contig assembly was done using Geneious prime v2020.0.3. The sequences generated and used in the phylogenetic analysis were deposited in GenBank under accession numbers detailed in Table 1.

Dataset preparation

Since a preliminary phylogenetic reconstruction performed using *tef1* sequences nested all the isolates of interest within the IX to XII clades described in Lombard et al. (2015) (data not shown), for each of the ten analyzed loci, a first dataset, consisting of the sequences of the isolates of interest from our collection as well as 57 sequences corresponding to unique representative strains of each taxon from those four clades, was established (Table S2). An additional reduced dataset that included only *ITS*, *LSU*, *rpb1*, *rpb2*, *tef1* and *tub2* genes sequences from the first dataset identified as pertaining to *Bisifusarium*, based on the phylogenetic reconstruction obtained with the full dataset in the present study, was used to confirm that the four loci missing for *B. tonghuanum* had no impact on the positioning of several isolates (despite our best efforts, additional sequence data could not be obtained due to the unavailability of any *B. tonghuanum* strain).

Alignment and phylogenetic reconstruction

Sequences of the ten loci: *acl1, act, cmdA, his3, ITS, LSU, rpb1, rpb2, tef1* and *tub2* were aligned independently using MAAFT v7.450 (Kuraku et al. 2013, Katoh et al. 2019) with the Auto algorithm as implemented in Geneious prime v2020.0.3. Introns and exons were separated in each dataset. Alignment of the exonic region was ascertained by checking the deduced translated aminoacyl alignment and alignment of the intronic regions was edited using Gblocks b0.91b

(http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with all the options enabled to allow less stringent selection, discard highly ambiguous regions and ensure reproducibility. Since phylogenetic incongruence between the different genes was not detected (P = 0.001) by performing an Incongruence Length Difference (ILD) test on 1000 replicates as implemented in PAUP 4.0a166 (Swofford 2003), the whole dataset was used after concatenation of the ten individual alignments.

Table 1 Information for strains sequenced for the phylogenetic analyses

Species	Isolate	Substrate	Collector and/or Depositor	Locality	GenBank Accession No.									
					acl1	*act*	*cmdA*	*his3*	*ITS*	*LSU*	*rpb1*	*rpb2*	*tef1*	*tub2*
Bisifusarium allantoides	UBOCC-A-120035	Cheese (Manchego)	O. Savary	Spain	MW810987	MW811002	MW811017	MW811031	MW654536	MW654511	MW811046	MW811060	MW811075	MW811090
	UBOCC-A-120036^{T}	Soft cheese type	F. Deniel	France	MW810999	MW811014	MW811029	MW811044	MW654548	MW654523	MW811054	MW811072	MW811087	MW811102
	UBOCC-A-120037	Soft cheese type	F. Deniel	France	MW811000	MW811015	MW811030	MW811040	MW654549	MW654524	MW811055	MW811073	MW811088	MW811103
Bisifusarium biseptatum	CBS 110311^{T}	Soil	P. Nelson & W. Marasas	South-Africa	MW810998	MW811013	MW811028	MW811039	MW654547	MW654522	MW811053	MW811071	MW811086	MW811101
Bisifusarium dimerum	MNHN RF-05625^{T}	*Homo sapiens*	H. Ph. Endtz	Netherlands	MW810997	MW811012	MW811027	MW811038	MW654546	MW654521	MW811058	MW811070	MW811085	MW811100
Bisifusarium penicilloides	UBOCC-A-120021^{T}	Cheese (Mont d'Or)	O. Savary	France	MW810993	MW811008	MW811023	MW811035	MW654542	MW654517	MW811051	MW811066	MW811081	MW811096
	UBOCC-A-120034	Cheese (Mont d'Or)	O. Savary	France	MW810992	MW811007	MW811022	MW811034	MW654541	MW654516	MW811050	MW811065	MW811080	MW811095
	VTT-D-041022	Surface of sandstone building, Arbroat Abbey	Suihko et al.	Scotland, UK	MW810986	MW811001	MW811016	MW811041	MW654535	MW654510	MW811045	MW811059	MW811074	MW811089
Fusarium babinda	UBOCC-A-120044	Raw cow's milk cheese	O. Savary	France	MW810995	MW811010	MW811025	MW811037	MW654544	MW654519	-	MW811068	MW811083	MW811098
Fusarium solani s.l.	UBOCC-A-120045	Raw cow's milk cheese	O. Savary	France	MW810996	MW811011	MW811026	-	MW654545	MW654520	MW811052	MW811069	MW811084	MW811099
Longinectria lagenoides	ESE 00140	Cheese (Tilsit)	J. Ropars	France	MW810994	MW811009	MW811024	MW811036	MW654543	MW654518	MW811057	MW811067	MW811082	MW811097
	UBOCC-A-120038	Cheese (Raclette d'alpage)	O. Savary	France	MW810988	MW811003	MW811018	MW811042	MW654537	MW654512	MW811047	MW811061	MW811076	MW811091
	UBOCC-A-120039^{T}	Cheese (Vacherin Fribourgeois)	O. Savary	France	MW810990	MW811005	MW811020	MW811032	MW654539	MW654514	MW811048	MW811063	MW811078	MW811093

Table 1 Continued.

Species	Isolate	Substrate	Collector and/or Depositor	Locality	GenBank Accession No.									
					acl1	*act*	*cmdA*	*his3*	*ITS*	*LSU*	*rpb1*	*rpb2*	*tef1*	*tub2*
	UBOCC-A-120041	Raw cow's milk cheese	O. Savary	France	MW810989	MW811004	MW811019	MW811043	MW654538	MW654513	MW811056	MW811062	MW811077	MW811092
Longinectria verticilliforme	UBOCC-A-120043[T]	Cheese (Alpeggio)	O. Savary	France	MW810991	MW811006	MW811021	MW811033	MW654540	MW654515	MW811049	MW811064	MW811079	MW811094

Table 2 PCR amplification mix for the ten loci

	acl1, rpb1, rpb2, tub2	act	cmdA	his3	ITS, LSU	tef1
PCR Buffer (X)	1	1	1	1	1	1
MgCl2 (mM)	1.5*	2	2	2	2	1.5
Primers (µM)	0.2	0.5	0.2	0.08	0.2	0.15
dNTPs (mM)	0.2	0.2	0.08	0.2	0.2	0.3
GoTaq DNA polymerase (Promega) (U/µL)	0.025	0.05	0.02	0.025	0.025	0.025

*For some isolates, in order to obtain amplicons with rpb1 primers, the $MgCl_2$ concentration was adjusted to 3.5 mm

Phylogenetic analyses were based on Maximum Likelihood (ML) and Bayesian Inference (BI). For ML analysis, the best partition scheme and evolution models were assessed using PartitionFinder2 v2.1.1 using the "*greedy*" algorithm and selecting the best models for each partition using the corrected Akaike information criterion (AICc), restricting the search among the models available in RAxML2 v8.2.4.1 (Stamatakis 2014) and excluding the models with proportion of invariable (P-Invar) site estimates since it can cause analysis problems according to the RAxML manual (Yang 2006, Stamatakis 2016). The ML analysis was performed using RAxML2 v8.2.4.1 on the ABiMS platform (http://abims.sb-roscoff.fr/), using the default algorithm (-f d) and the GTRGAMMA model. The robustness of the analysis was indirectly evaluated by bootstrap support among 1000 replicates (-N 1000) and a majority-rule consensus tree was further built (-J MR). For BI analysis, the best models (among the namedExtended models of the bModelTest add on in Bayesian Evolutionary Analysis Utility -BEAUti2-) were estimated for each intronic and exonic regions of each gene (a total of 24 partitions) simultaneously with the BI analysis using the Bayesian Evolutionary Analysis Sampling Trees (BEAST)2 v2.6.2 package. The BI analysis was performed using the Monte Carlo Markov Chain method using the BEAST2 v2.6.2 package by performing eight independent repetitions of 100,000,000 generations each, with sampling a tree at every 1,000 generations and a burn-in was fixed at 10% (discard the first 10% trees). For both analyses, *Allantonectria miltina* (CBS 121121) was selected as an outgroup. Convergence of the independent BI analyses was checked using Tracer v1. 7.1. software (Rambaut et al. 2018) and a consensus tree was obtained using Logcombiner and Treeannotator. The two phylogenetic trees obtained with the ML and BI methods were compared using FigTree v1.4.4 (Rambaut 2006) (http://tree.bio.ed.ac.uk/). A tree based on the topology of ML tree with nodes with bootstrap support < 60% collapsed using Tree Collapser CL 4 (Hodcroft), and annotated with bootstrap and

posterior probability node supports was built. The number of informative sites was calculated using the web server DIVEIN (Deng et al. 2010). The exact same set of methods was applied to analyze the reduced dataset (six loci) focused on *Bisifusarium* isolates.

Morphological characters

Fungal isolates pertaining to putative new species were inoculated in a three-point position on Potato Dextrose Agar (PDA, Difco, Fisher Scientific) for morphological descriptions. Additional cultures were also performed on M2Lev and oatmeal agar (OA; 50 g/L oatmeal, 5 g/L sodium chloride and 12 g/L agar) media and incubated at 25°C, in the dark. Macroscopic morphological characters (e.g. pigment production, aerial hyphae) were examined after a minimum of 14 days. Moreover, as recommended by Leslie & Summerell (2006), synthetic nutrient-poor agar (*Spezieller Nährstoffarmer Agar*, SNA) prepared as described in the publication cited above, was tested for all strains while carrot agar medium (400 g/L carrot, 20 g/L agar) was used for the induction of sexual characters when isolates were mated using all possible combinations. Theses plates were incubated at 25°C, in the dark, for eight weeks.

In parallel, for isolates identified as new type strains, five µL of an inoculum concentrated at 10^6 conidia/mL were also inoculated on PDA medium (two biological and two technical replicates) and incubated at 15 and 25°C in darkness. Colony diameters were monitored for 40 and 21 days, respectively. Growth rates fitting each kinetic were estimated by the modified Gompertz equation (Zwietering et al. 1990) and using the MATLAB software (MATLAB. 9.5.0.944444. (R2018b), Natick, Massachusetts: The MathWorks Inc.). Two supplementary technical replicates were also deposited and incubated at 5, 10, 20, 30 and 37°C in darkness, then colony diameters were monitored during 14 days to determine growth limits as a function of temperature.

As for microscopic characteristics, descriptions were done based on PDA cultures as well as supplementary observations on M2Lev medium after 7 to 17 days growth. Fungal structures were mounted on glass slides using lactophenol cotton blue solution as a mounting fluid. Additional observations were performed after 40–46 days (fourteen days in the dark then exposure to natural light during a minimum of fourteen days) to potentially observe chlamydospores. An Olympus BX40 microscope equipped with an Olympus Digital Camera DP70 (Olympus, Tokyo, Japan) was used to observe microscopic characteristics (e.g. presence, shape and length of macroconidia and/or microconidia, phialidic pegs, chlamydospores) based on the recommendation of "The *Fusarium* laboratory manual" (Leslie & Summerell 2006). Several measurements were taken with a magnification of 400x and 1000x.

Then, conidia observations were performed on the isolates identified as new type strains using scanning electron microscopy. To do so, two mL of tween 80 (0.005 %, v/v) were added on each 10–12 days monospore culture on PDA medium to collect conidia suspensions. Concentrations were determined by counting spores in Malassez cells and were adjusted at 10^7 conidia per mL. One mL of each suspension was filtered on a 0.2 µm polycarbonate membrane filter (GTTP01300, Milipore, Merck, Germany) using a 13 mm filter holder (SX0001300, Milipore, Merck, Germany). The filter was transferred on a Whatman filter paper to absorb excess liquid, then a fragment was bonded on the observation plot. Finally, two µL of Hitachi ionic liquid HILEM IL 1000 (Hitachi High-Tech, Europe, GmbH) was deposited and the observations were directly performed by scanning electron microscopy (SEM; HITACHI S-3200N) at the PIMM microscopy platform (Université de Bretagne Occidentale, France), without any additional fixation or metallization steps.

Secondary metabolite production

The four holotype strains UBOCC-A-120021, UBOCC-A-120036, UBOCC-A-120039 and UBOCC-A-120043 were inoculated as three replicates using five µL of 10^6 conidia/mL suspensions, on PDA, malt extract agar (MEA: 20 g/L malt extract, 1 g/L peptone, 20 g/L glucose and 20 g/L agar), yeast extract sucrose agar (YES, pH 5.2) and skim milk agar (100 g/L Difco skim milk powder, 10 g/L yeast extract and 20 g/L agar) for 14 days at 25°C. Then, five 6 mm plugs were removed from the agar colonies for extractions with ethyl acetate / isopropanol (3:1) supplemented with 1%

(vol/vol) formic acid using ultra-sonication. After removal of the extracted plugs, the remaining liquid was evaporated and the extract re-dissolved in methanol. Three µL of the methanol extract were analyzed by high performance liquid chromatography (HPLC) using diode array (DAD) and fluorescence detection (emission 230 nm, excitation 333 nm & emission 230 nm and excitation 450 nm) (Thermo Scientific, Dionex, Sunnyvale, California, USA) as described by Frisvad & Thrane (1987). A bracketed alkylphenone retention index was calculated for each compound as described by Frisvad & Thrane (1987, 1993). The UV spectra of the compounds eluting were compared to authentic standards of known secondary metabolites as described by Nielsen et al. (2011), Klitgaard et al. (2014) and Kildgaard et al. (2014) by comparing retention time, retention index and UV spectra recorded from 200 – 600 nm (diode array detection). The water / acetonitrile gradient used in the HPLC analyses and the column and other conditions used are described by Nielsen et al. (2011).

Results

Cheese sampling and analysis

Among the 56 cheeses sampled to collect *B. domesticum* isolates, a large number of fungal isolates pertaining to different genera such as *Geotrichum*, *Mucor*, *Penicillium* or *Scopulariopsis* were observed (data not shown) along with the typical *B. domesticum* morphotypes (n = 9, one isolate per cheese). Interestingly, other isolates (n = 10, one macroscopically different isolate per cheese) exhibited *Fusarium*-like morphotypes. Identifications based on translation elongation factor 1-alpha sequencing confirmed that the isolates exhibiting the typical *B. domesticum* morphotypes indeed belonged to the latter species, while the sequences obtained for the *Fusarium*-like isolates could not be identified at the species level. Based on the observed blastn scores (closest identification to already described *Nectriaceae* species estimated to 83.5 to 85.9%, data not shown), the possibility of four putative new *Nectriaceae* species was highlighted. Among these isolates, two were macroscopically similar to an environmental strain (surface of sandstone building) described as *Trichothecium* sp. (94.9% similarity to *B. domesticum* based on ITS gene (Suihko et al. 2007)). ITS sequencing later confirmed the identity of this environmental strain with two of the obtained cheese isolates (data not shown), which was therefore included in this phylogenetic analysis (for a total of 11 unidentified *Fusarium*-like isolates). Noteworthy, during cheese investigation, other *Nectriaceae* species were identified, namely *F. babinda* (e.g. UBOCC-A-120044), *F. oxysporum* (e.g. ESE 00175), *F. culmorum* (e.g. ESE 00152) and *F. solani s.l.* (e.g. UBOCC-A-120045, ESE 00026, ESE 00073, ESE 01219), indicating that, beyond *B. domesticum*, members of this family can be associated with this food matrix although they may well be just environmental contaminants.

Phylogenetic analyses

A phylogenetic reconstruction (Fig. 1) was performed using a concatenated non-redundant dataset of ten loci: *acl1, act, cmdA, his3, ITS, LSU, rpb1, rpb2, tef1* and *tub2* sequences of 64 strains (Table 1, Supplementary Table 2) belonging to 19 genera (18 based on Lombard et al. nomenclature (2015) and a new genus proposed in this study), as well as *A. miltina* CBS 121121 as an outgroup. The multi-gene alignment length was 5134 nucleotides, including few gaps and missing data (*acl1* 487 bp, *act* 536 bp, *cmdA* 413 bp, *his3* 319 bp, *ITS* 533 bp, *LSU* 758 bp, *rpb1* 656 bp, *rpb2* 780 bp, *tef1* 351 bp and *tub2* 301bp). Moreover, this alignment contained 2226 informative sites (2122 without gaps). After alignment using the ten loci dataset, some of the unidentified isolates presented high identity levels between them, thus forming four distinct genetic groups, namely (i) UBOCC-A-120021, UBOCC-A-120034 and VTT-D-041022 (maximum divergence of 10/6441 nucleotides over the whole raw dataset), (ii) UBOCC-A-120035, UBOCC-A-120036 and UBOCC-A-120037 (maximum divergence of 2/6173 nucleotides), (iii) ESE-00140, UBOCC-A-120038, UBOCC-A-120039 and UBOCC-A-120041 (maximum divergence of 6/6187 nucleotides) and (iv) UBOCC-A-120043. Therefore, given the very low nucleotide divergence, only one representative sequence for each group, namely UBOCC-A-120021, UBOCC-A-120036, UBOCC-A-120039 and UBOCC-A-120043, was conserved for further phylogenetic analyses. The obtained phylogenetic tree (Fig. 1)

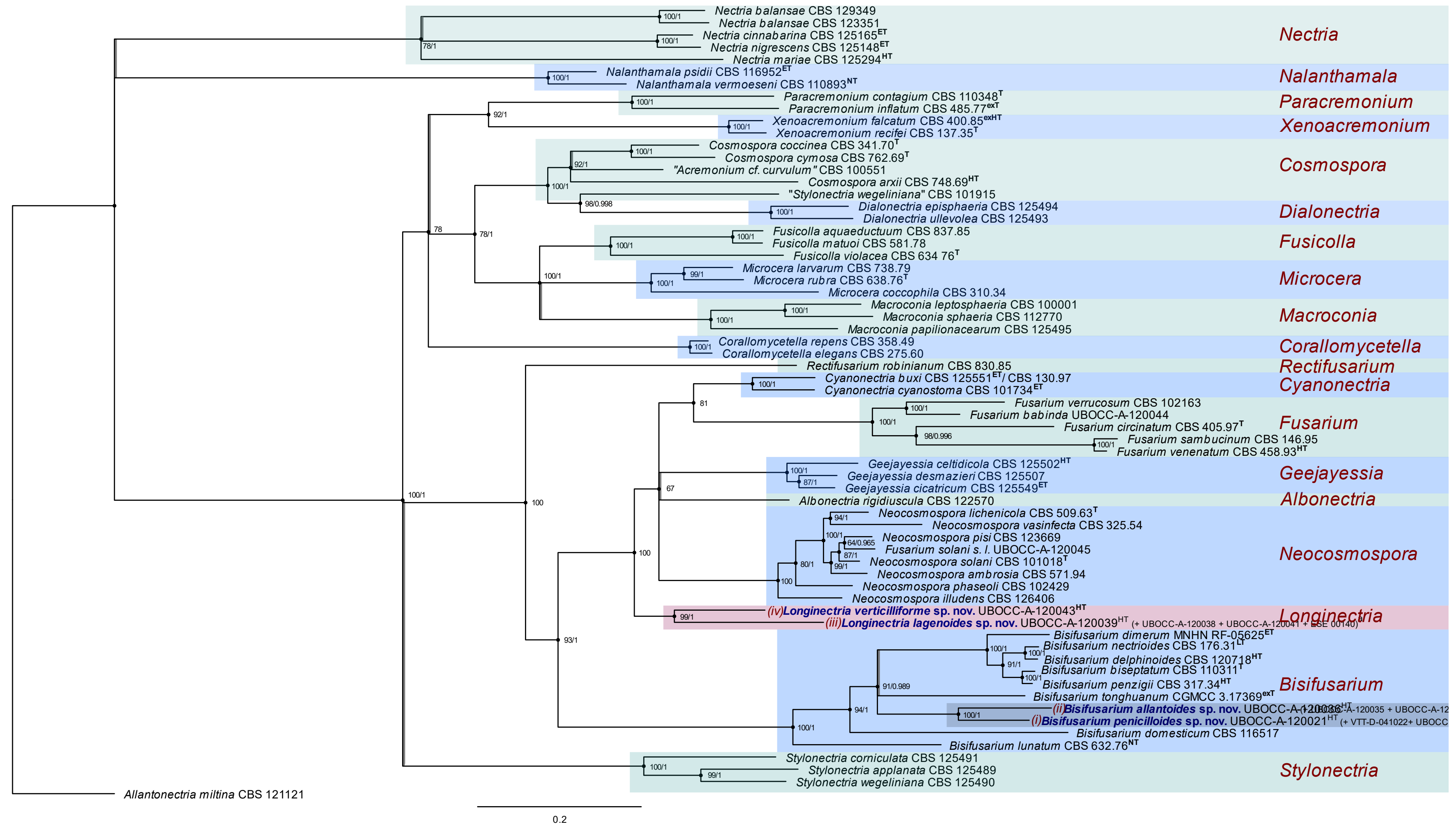

Figure 1 – Maximum-Likelihood phylogenetic consensus tree of Nectriaceae species inferred from the combined ten gene sequence subset (*acl1, act, cmdA, his3, ITS, LSU, rpb1, rpb2, tef1* and *tub2*). Maximum-Likelihood bootstrap supports (in percentage, 1000 replicates) and Bayesian posterior probabilities are represented at nodes (respectively right and left). The tree is rooted with *A. miltina* CBS 121121. "ET", "LT", "HT", "T", "exT", "exHT", "NT" respectively mean epitype, lectotype, holotype, type, ex-type, ex-holotype and neotype. The four new species are written in blue and bold. "a": Only the sequences of the type strain of each proposed species were used in the phylogenetic analysis given the very low nucleotide divergence between strains within the considered species.

indicated that the putative new species were not clustered with already described species within the *Nectriaceae* family. Interestingly, UBOCC-A-120021 and UBOCC-A-120036 were nested within the *Bisifusarium* genus (*Fusarium dimerum* species complex *sensu* Geiser et al. 2021) with nucleotide differences comprised between 566 to 676/5134 and 571 to 680/5134, respectively, with other already described *Bisifusarium* species (Supplementary Table 3) and therefore likely correspond to two new species within this genus. On the other hand, UBOCC-A-120039 and UBOCC-A-120043 constituted an independent sister clade with a group including different *Nectriaceae* genera (described by Lombard et al. (2015) as *Neocosmospora*, *Albonectria, Geejayessia, Cyanonectria* and *Fusarium*) with a minimum nucleotide divergence of 566/5134 bp with species from these clades (Supplementary Table 4) suggesting that they belong to a distinct genus from *Neocosmospora*, *Albonectria, Geejayessia, Cyanonectria* and *Fusarium,* and other more phylogenetically distant Nectriaceae. Indeed, this minimum nucleotide divergence are comparable to those observed between the genera within this group, or even higher as for *Neocosmospora* and *Albonectria* (539/5134 bp) or *Neocosmospora* and *Geeyassia* (560/5134 bp) or *Neocosmospora* and *Cyanonectria* (554/5134 bp) (Supplementary Table 4).

As the latest addition to the *Bisifusarium* genus (*B. tonghuanum*) was based only on six genes of the "Lombard et al." gene set, we wanted to confirm that the missing data had no impact on the positioning of putative *Bisifusarium* isolates. Therefore, a second phylogenetic reconstruction was performed using a concatenated non-redundant dataset of the six common loci (*ITS*, *LSU*, *rpb1*, *rpb2*, *tef1* and *tub2*) of the ten species belonging to the *Bisifusarium* genus (the eight already described and the two additional ones based on this study) and *F. venenatum* CBS 458.93^{T} as an outgroup. This alignment length was 2854 nucleotides long, including few gaps. It contained 400 informative sites (391 without gaps). The resulting ML and BI analyses were highly concordant (data not shown) and the position of UBOCC-A-120021 and UBOCC-A-120036 in the resulting phylogenetic tree (Fig. S1) was concordant with the one observed for the ten loci-based analysis. *Bisifusarium allantoides* sp. nov. and *B. penicilloides* sp. nov. were placed in a sister clade of *B. tonghuanum* with a nucleotide divergence of 225 and 193/2854 nucleotides, respectively with *B. tonghuanum* CGMCC 3.17369. Both multi-gene phylogenetic analyses were deposited in TreeBASE (Study ID 28488 and 28538).

Taxonomy

Bisifusarium allantoides O. Savary, M. Coton, E. Coton and J-L. Jany, sp. nov. Figs 2, 3

MycoBank number: MB840947

Etymology – From latin "*allantois*" referring to the allantoid-shape of the conidia.

Holotype – UBOCC-A-120036, CBS 147587

Macromorphology – Based on UBOCC-A-120036 (CBS 147587) colony growth for 16 days at 25°C, colony surface on PDA was pale orange (RGB [167, 137,106] to [175, 137, 90]) with many white powdery structures and cottony hyphae, more or less aerial, with an elevation at the colony center (Fig. 2A). Margins were smooth and less colored. After natural light exposure during several days, colony color became more pronounced. It can be noted that the mycelium was very well attached to the agar. The colony reverse on PDA was orange at the center (RGB [148, 100, 59]) and progressively less colored until the margins (RGB [144, 130, 103]) (Fig. 2B). No pigment diffusion in the agar was observed. Interestingly, on the M2Lev medium, the morphological aspect was highly similar to that on PDA with more cottony structures, a more pronounced colony color, and smooth and regular margins (Fig. 3).

Micromorphology – Ascomatal state unknown. Phialides were less abundant than in the other described *Bisifusarium* species. Monophialides formed terminally (Fig. 2C) or laterally on hyphae and were cylindrical, tapering toward the tip (81–127 µm). Formation of lateral phialidic pegs common on PDA (and on M2Lev) were observed (Fig. 2D). Polyphialides were not observed. Microconidia and macroconidia were difficult to distinguish (Fig. 2E–K) but observed macroconidia were typically allantoidal, straight or curved and cylindrical (size on average 12.41 x 3.2 µm, 9.5–23.6 x 2.72–4.1 µm). The apical cells were papillate while the basal cells were distinctly notched.

They were mostly 1-septate or more. Microconidia could be characterized by short conidia 0-septate or 1-septate with median septum or mostly an off-centered septum (size on average 7.6 x 2.5 µm, 4.7–9.7 x 1.5–3.2 µm). They were cylindrical, straight or curved, and slightly notched. After several weeks of growth, no chlamydospore was observed. SNA medium did not provide any supplementary information.

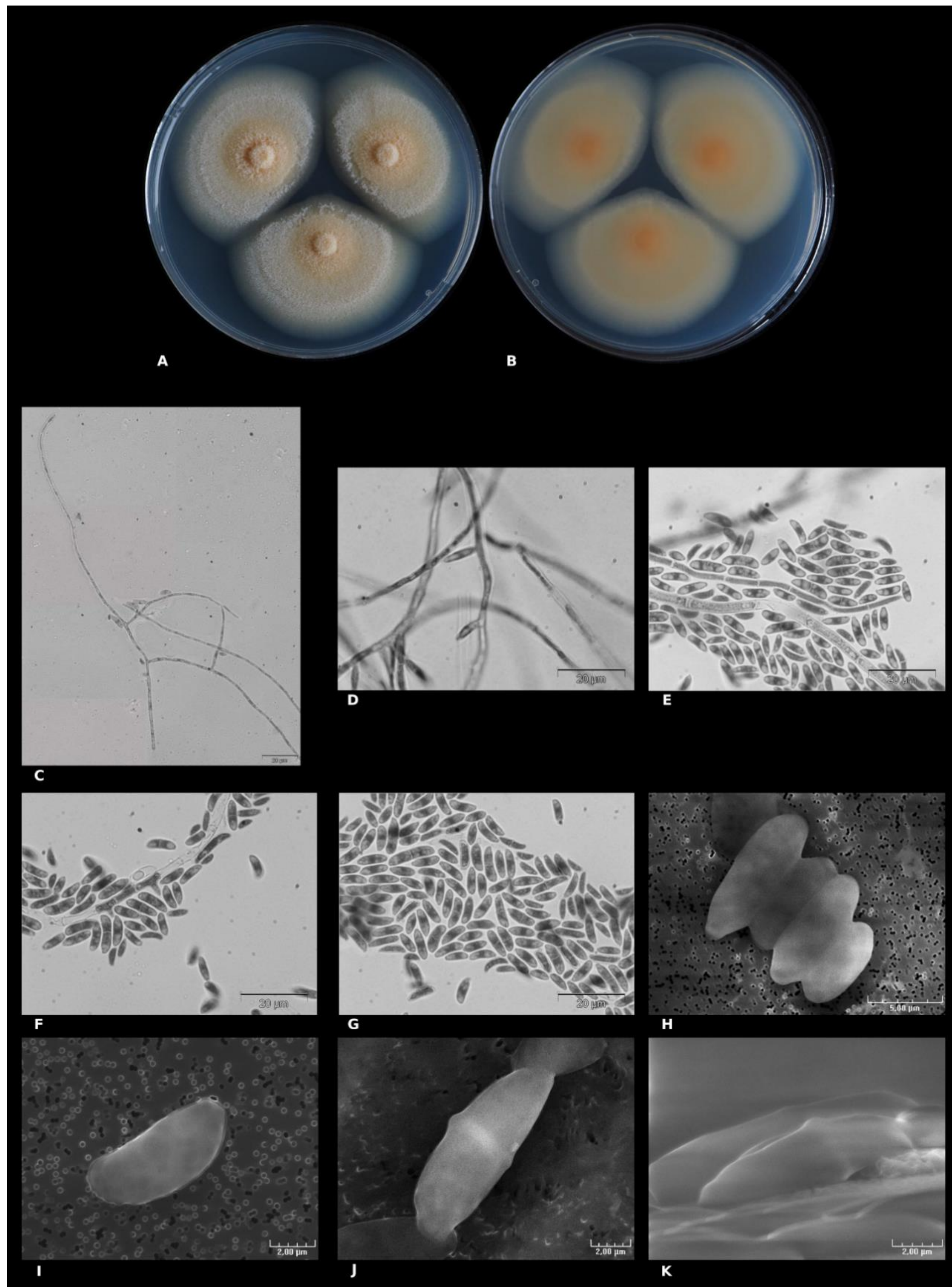

Figure 2 – Macroscopic morphological and microscopic characteristics of *Bisifusarium allantoides* (UBOCC-A-120036^{HT}). A colony on PDA (14 days at 25°C). B colony reverse on PDA (14 days at 25°C). C terminal monophialides. D phialidic pegs. E–K conidia. C–G microscopic acquisitions using classical microscopy. H–K microscopic acquisition using scanning electron microscopy.

Growth characteristics on PDA – Moderately fast growth was observed at 25°C, colony diameter measuring 70.5 ± 1.7 mm versus 43.5 ± 0.6 mm at 15°C and growth rates were estimated at 5.2 ± 0.3 mm/days versus 3.3 ± 0.1 mm/days, respectively. Moreover, *B. allantoides* growth was observed between 5°C and 30°C, colony diameters measuring at 14 days were 8.0 ± 0.0 mm at 5°C, 19.0 ± 0.0 mm at 10°C, 55.0 ± 4.2 mm at 20°C, 39.0 ± 2.8 mm at 30°C and no growth at 37°C.

Secondary metabolites – No known mycotoxins already described to be produced by *Fusarium*, *Penicillium*, *Aspergillus* or *Alternaria* spp. (e.g. fusaric acid, zearalenone, trichothecenes, butenolide or enniatins) were detected. For most detected compounds, except ergosterol, extrolites could not be identified but belonged to 47 chromophore families (Table 3). These secondary metabolites included the "emon" chromophore or corresponded to extra apolar free fatty acids (including linoleic acid and probably oleic acid), mid-cyclic lipopeptides with tyrosine in the cyclic peptide, indole alkaloids or 2-pyruvoylaminobenzamide-like molecules (Table 3).

Substrate and distribution – To date, *B. allantoides* has been solely isolated from European cheese.

Material examined – France, isolated from French soft cheese rind, F. Deniel, 11 Dec. 2013, holotype UBOCC-A-120036 = CBS 147587.

Additional material examined – France, isolated from French soft cheese rind, F. Deniel, UBOCC-A-120037, 11 Dec. 2013; Spain, isolated from Spanish cheese (Manchego), B. Mayo, before Nov. 2018, UBOCC-A-120035.

Note – On oatmeal agar media, sporodochia could be noted for the three *B. allantoides* strains (Fig. 3).

Bisifusarium penicilloides O. Savary, M. Coton, E. Coton and J-L. Jany, sp. nov. Figs 3, 4

MycoBank number: MB840948

Etymology – From latin "*penicillus*", referring to the penicillate organisation of polyphialides.

Holotype – UBOCC-A-120021, CBS 147586

Macromorphology – Based on UBOCC-A-120021 (CBS 147586) colony growth for 16 days at 25°C on PDA, short aerial mycelial strands with a fungal colony very well attached to the agar. Colony surface on PDA was slightly colored, beige (RGB [191,181,163]) with yellow shades (Fig. 4A) and color intensified during sporulation in natural light becoming orange (RGB [210,163,114]). No powdery structure was observed, but velvety and downy aspects were. A slight elevation could be noted in the center and lighter regular margins were observed. The colony reverse on PDA was pale (RGB [168,163,149]) (Fig. 4B), to orange pale (RGB [170,143,119) after natural light exposure for several days. No pigment production in agar was observed. On M2Lev medium (Fig. 3), a similar macroscopic profile was observed with slightly more aerial hyphae and more pronounced colony color than on PDA medium.

Micromorphology – Ascomatal state unknown. Several types of conidiogenous cells were observed. Indeed, while polyphialides with a penicillate configuration were frequently observed (Fig. 4C, D) (phialides length after bifurcation on average 15 µm, 9–25 µm) and appear as a species characteristic. several monophialides formed terminally (≥ 73–92 µm) or laterally on hyphae and lateral phialidic pegs (Fig. 4E) could be also noted. Phialides were cylindrical tapering towards the tip. Microconidia were not observed and macroconidia were mostly 0-septate or rarely 1-septate with a median septum (Fig. 4F–J) (size on average 6.5 x 2.7 µm, 5.6–7.2 x 2.1–3.2 µm). They were ellipsoidal, straight or curved presenting a lunate or reniform shape, and basal cells were distinctly notched. After several weeks of growth, globose chlamydospores were observed, typically intercalary, solitary or in chains that can be long. SNA medium did not provide any supplementary information.

Growth characteristics on PDA – Slow growth was observed at 25°C, colony diameter measuring 36.0 ± 1.8 mm versus 43.0 ± 0.8 mm at 15°C and the growth rates were estimated at 2.6 ± 0.2 mm/days versus 3.2 ± 0.1 mm/days, respectively. Moreover, *B. penicilloides* growth was observed between 5°C and 25°C, colony diameters measuring at 14 days 9.5 ± 0.7 mm at 5°C, 20.5 ± 0.7 mm at 10°C, 49.0 ± 1.4 mm at 20°C, and no growth at 30°C and 37°C.

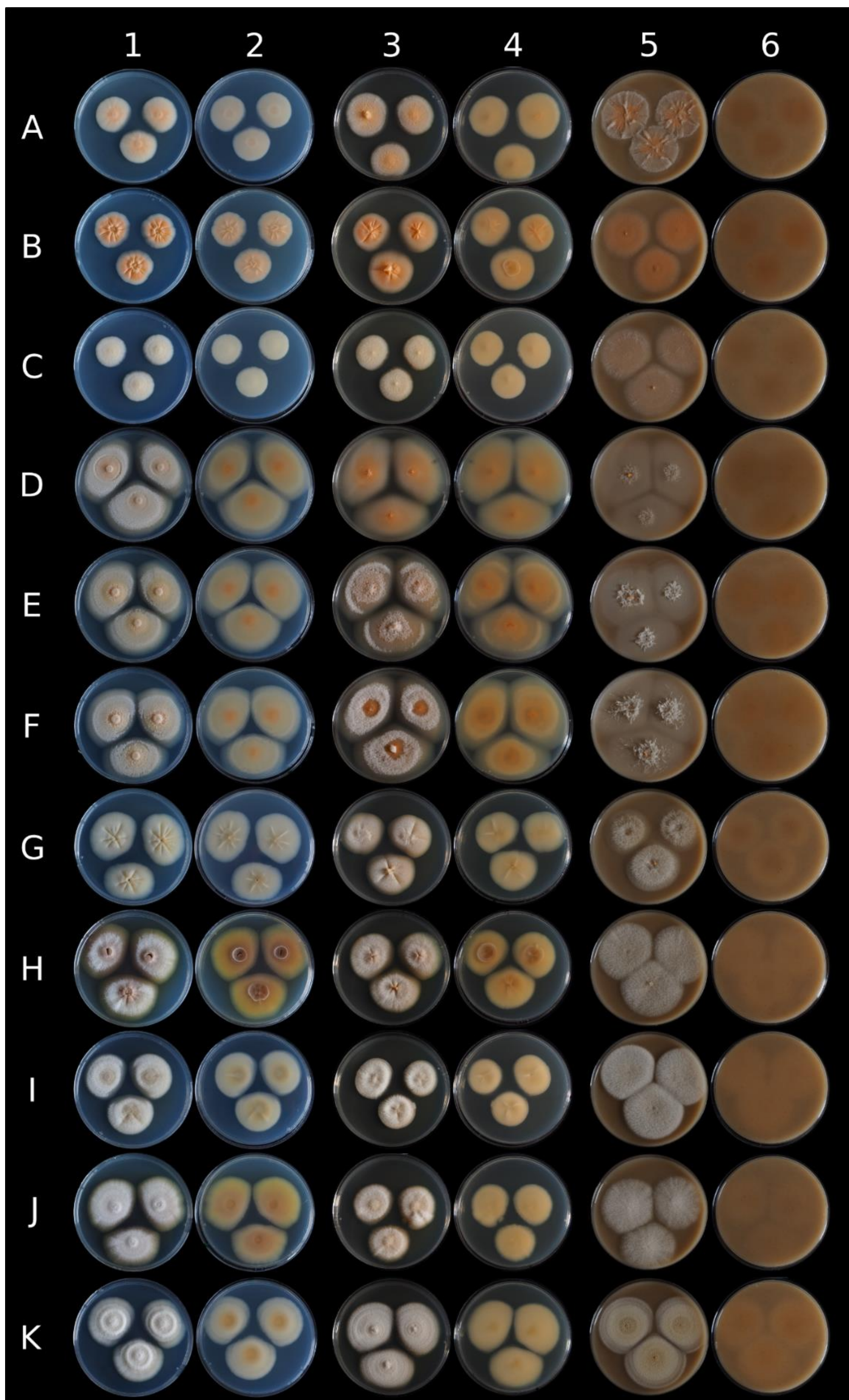

Figure 3 – Macroscopic comparison of eleven strains belonging to four new *Nectriaceae* species on different culture media. Lines A to K respectively correspond to *Bisifusarium penicilloides* VTT-D-041022, *B. penicilloides* UBOCC-A-120034, *B. penicilloides* UBOCC-A-120021, *Bisifusarium allantoides* UBOCC-A-120035, *B. allantoides* UBOCC-A-120036, *B. allantoides* UBOCC-A-120037, *Longinectria lagenoides* UBOCC-A-120038, *L. lagenoides* UBOCC-A-120039, *L. lagenoides* UBOCC-A-120041, *L. lagenoides* ESE-00140 and *Longinectria verticilliforme*

UBOCC-A-120043 grown during 14 days at 25°C on Potatoes Dextrose Agar (column 1 & 2: observe & reverse), M2Lev (column 3 & 4: observe & reverse) and oatmeal agar (column 5 & 6: observe & reverse).

Secondary metabolites – No known mycotoxins already described to be produced by *Fusarium*, *Penicillium*, *Aspergillus* or *Alternaria* spp. were detected. For most detected compounds, except ergosterol, extrolites could not be identified but belonged to 47 chromophore families (Table 3). These secondary metabolites included the "emon" chromophore, extra apolar free fatty acids (including linoleic acid and probably oleic acid), mid-cyclic lipopeptides with tyrosine in the cyclic peptide, indole alkaloids or 2-pyruvoylaminobenzamide-like molecules (Table 3).

Substrate and distribution – Two strains isolated from cheese rind, including the holotype. One strain (VTT-D-041022), previously identified as *Trichothecium* sp. (94.9% similarity to *B. domesticum*, based on ITS sequencing) was also obtained from the outer surface of a sandstone building, Arbroat Abbey (Suihko et al. 2007).

Material examined – France, isolated from French soft cheese (Mont d'Or), O. Savary, 22 Jan. 2019, holotype UBOCC-A-120021 = CBS 147586.

Additional material examined – France, isolated from French cheese (Mont d'Or), O. Savary, 21 Jan. 2019, UBOCC-A-120034; Scotland, isolated from outer surface of sandstone building, Arbroat Abbey, Suihko et al. (2007), before Nov. 2006, VTT-D-041022.

Note – A "yeasty" odour was clearly detected during fungal growth on PDA. A few macroscopic differences were observed among the three studied isolates (Fig. 3). Colony surface was more colorful during the first days of culture for the VTT-D-041022 and UBOCC-A-120034 isolates but after several days of exposure to natural light, all three fungal cultures presented the same intense color. While VTT-D-041022 and UBOCC-A-120021 had the same mycelium characteristics, UBOCC-A-120034 strain colonies were radially furrowed with a concave center and margins were lighter and irregular.

Longinectria O. Savary, M. Coton, E. Coton and J-L. Jany, gen. nov.

MycoBank number: MB840949

Etymology – From the latin *longus* = long, "Longi-" refers to the phialides length (on average 42 µm; 15–72 µm, some being very long – 153 to 237 µm) observed for the *Longinectria* species and "*-nectria*" refers to the *Nectriaceae* family.

Type species – *Longinectria lagenoides* O. Savary, M. Coton, E. Coton & J-L. Jany, sp. nov.

Ascomatal state unknown. Conidiophores are variable-length phialides, sometimes extremely long (e.g. 153–237 µm), lateral, sometimes verticillate, hyaline. Macroconidia straight to slightly curved, apical cell morphology blunt to papillate and a basal cell often notched, 0–3 septate, hyaline. Microconidia ovoid, ellipsoid to allantoid, 0–1 septate, hyaline. Chlamydospores absent to abundant, globose, single, in pairs or chains, intercalary or terminal.

Longinectria lagenoides O. Savary, M. Coton, E. Coton and J-L. Jany, sp. nov. Figs 3, 5

MycoBank number: MB840950

Etymology – From latin *lagoena* = bottle, refers to the observed phialide shape.

Holotype – UBOCC-A-120039, CBS 147588

Macromorphology – Based on UBOCC-A-120039 (CBS 147588), colony growth for 16 days at 25°C, colony surface on PDA was heterogeneously colored (Fig. 5A). The center was mainly brown (RGB [139,112,93]), the smooth margin was brown (RGB [104,95,78]) and intensified during growth. The intermediate zone appeared grey (RGB [164,167,173]) with brown shades because of the aerial white hyphae with powdery structures. Brown pigments were diffused in the agar around the colony. Colonies were radially furrowed with a convex center. Colony reverse was also heterogeneously colored (Fig. 5B), the center being brown (RGB [98,61,43]), then progressively clearer (RGB [128,90,51]) and highly visible on the reverse face, the fungal colony dug into the agar. Concerning fungal growth on M2Lev, colonies were beige with shades of brown and, in particular,

in the regular margins and convex center (Fig. 3). Very aerial hyphae were observed with powdery and/or cottony structures and the mycelium dug into agar.

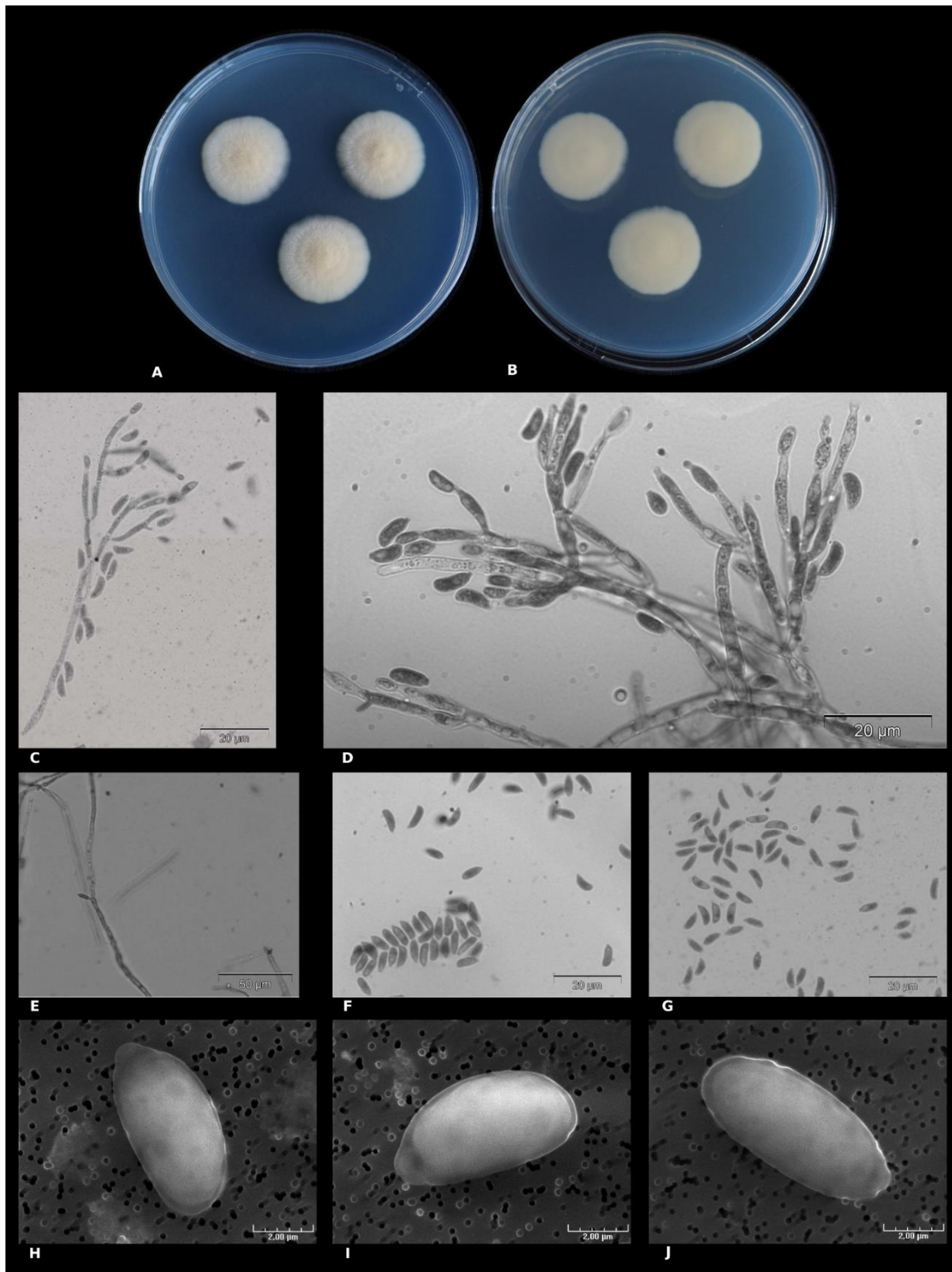

Figure 4 – Macroscopic morphological and microscopic characteristics of *Bisifusarium penicilloides* (UBOCC-A-120021^{HT}). A Colony on PDA (14 days at 25°C). B Colony reverse on PDA (14 days at 25°C). C–D polyphialides. E phialidic pegs. F–J conidia. C–G microscopic acquisitions using classical microscopy. H–J microscopic acquisitions using scanning electron microscopy.

Micromorphology – Ascomatal state unknown. Lateral phialides with different sizes were observed (on average 44 µm, 15–72 µm), some being very long (size 153–237 µm) (Fig. 5C–D)

while others are short and could even be assimilated to phialidic peg. Macroconidia 0–3 septate, very straight in shape with an apical form, blunt to papillate, and basal cell very pronounced and notched (Fig. 5E –5J). Their size is on average 17 x 4 µm (12–29 x 3–7 µm) length range. Microconidia 0–1 septate ovoid to allantoid shape (8–12 x 3–5), apical form blunt and basal cell slightly notched (Fig. 5E, G). After several weeks of growth, globoses chlamydospores were observed typically intercalary, or terminal with mainly ≥ 2 chlamydospores. SNA medium did not provide any supplementary information.

Growth characteristics on PDA – Slow growth was observed at 25°C, colony diameter measuring 53.0 ± 1.6 mm versus 32.3 ± 0.5 mm at 15°C and the growth rates were estimated at 3.7 ± 0.1 mm/days versus 2.3 ± 0.0 mm/days, respectively. Moreover, *L. lagenoides* growth was observed between 5°C and 25°C, colony diameters measuring at 14 days 4.5 ± 0.0 mm at 5°C, 13.0 ± 0.0 mm at 10°C, 44.5 ± 2.1 mm at 20°C, and no growth at 30°C and 37°C.

Secondary metabolites – No known mycotoxins already described to be produced by *Fusarium*, *Penicillium*, *Aspergillus* or *Alternaria* spp. were detected. For most detected compounds, except ergosterol, extrolites could not be identified but belonged to 38 chromophore families (Table 3). These secondary metabolites again included the "emon" chromophore or corresponded to extra apolar free fatty acids (including linoleic acid and probably oleic acid), mid-cyclic lipopeptides with tyrosine in the cyclic peptide, indole alkaloids, 2-pyruvoylaminobenzamide-like molecules but also an alkylphenone chromophore and atrovenetin chromophore (Table 3).

Substrate and distribution – To date, *L. lagenoides* has been solely isolated from European cheese.

Material examined – France, isolated from Swiss cheese, O. Savary, 22 Nov. 2018, holotype UBOCC-A-120039, CBS 147588.

Additional material examined – France, isolated from Swiss cheese, O. Savary, 20 Nov. 2018, UBOCC-A-120038; France, isolated from Swiss cheese, O. Savary, UBOCC-A-120041, 27 Nov. 2018; France, isolated from semi-hard Swiss cheese, J. Ropars, ESE 00140, 2018.

Note – Few macroscopically differences were observed among the four isolates observed on PDA (Fig. 3). Indeed, colony color was more or less pronounced (RGB [156,153,136] or [157,157,162]) depending on the considered strain), and in particular, one strain (UBOCC-A-120038) remained yellow/beige (RGB [151, 153, 148]) even with natural light exposure during several days. Radial furrows with a convex center were mainly pronounced for UBOCC-A-120038 and UBOCC-A-120039 but also slightly more observable for ESE-00140. The colony reverse could be beige (RGB [140,134,121]) to brown (RGB [143,109,80]) depending on the strains.

Longinectria verticilliforme O. Savary, M. Coton, E. Coton and J-L. Jany, sp. nov. Figs 3, 6

MycoBank number: MB840951

Etymology – Name refers to the phialides bifurcation as a verticille.

Holotype – UBOCC-A-120043, CBS 147589

Macromorphology – Based on UBOCC-A-120043 (CBS 147589) colony growth for 16 days at 25°C, colony surface on PDA was white (RGB [209,212,218]) to white grey (RGB [176,175,178]) (Fig. 6A) and did not change color after several days when exposed to natural light. Mycelium was very powdery and cottony, with more elevated circular zones of slightly aerial hyphae. Interestingly, few transparent exudate droplets have been observed at 25°C as well as at 15°C. Colony reverse on PDA was more colored in the center (RGB [153,124,85]) then lighter until the margin (RGB [147,141,129]) (Fig. 6B). No pigment production in agar was observed. Moreover, a powderier aspect was observed on M2Lev medium (Fig. 3) *versus* PDA and the margins, although smooth and regular, were beige instead of grey white.

Micromorphology – Ascomatal state unknown. Verticillate phialides (mainly three branches or more) which could be in terminal as well lateral position with successive bifurcations were observed (Fig. 6C–E). Lateral phialides were also noted, sometimes very long (on average, length after bifurcation: 39 µm, with variations from 27 to 58 µm), and frequently septate containing a probable formation of new phialide bifurcations (size 3–15 µm). Moreover, conidia clusters, similar to false

head structures, formed from the same isolated phialides were observed (Fig. 6F). Macroconidia 1–3 septate, mainly straight or slightly ellipsoidal with an apical cell morphology blunt to papillate and a basal cell distinctly notched, size on average 15.3 x 2.8 µm (9.4–26.9 x 2.3–3.2 µm) (Fig. 6G–J). Microconidia 0-1 septate, straight or curved, reniform, apical cells morphology is blunt while basal cell is notched, size on average 7.6–3.1 (5.5–9.5 x 2.0–4.1 µm) (Fig. 6I, K). No chlamydospores observed. SNA medium did not provide any supplementary information.

Growth characteristics on PDA – Slow growth was observed at 25°C, colony diameter measuring 49.5 ± 3.3 mm versus 28.6 ± 2.9 mm at 15°C and the growth rates were estimated at 3.4 ± 0.3 mm/days versus 2.1 ± 0.3 mm/days, respectively. Moreover, *L. verticilliforme* growth was observed between 5°C and 25°C, colony diameters measuring at 14 days 5.0 ± 0.0 mm at 5°C, 15.0 ± 0.0 mm at 10°C, 41.5 ± 0.7 mm at 20°C, and no growth at 30°C and 37°C.

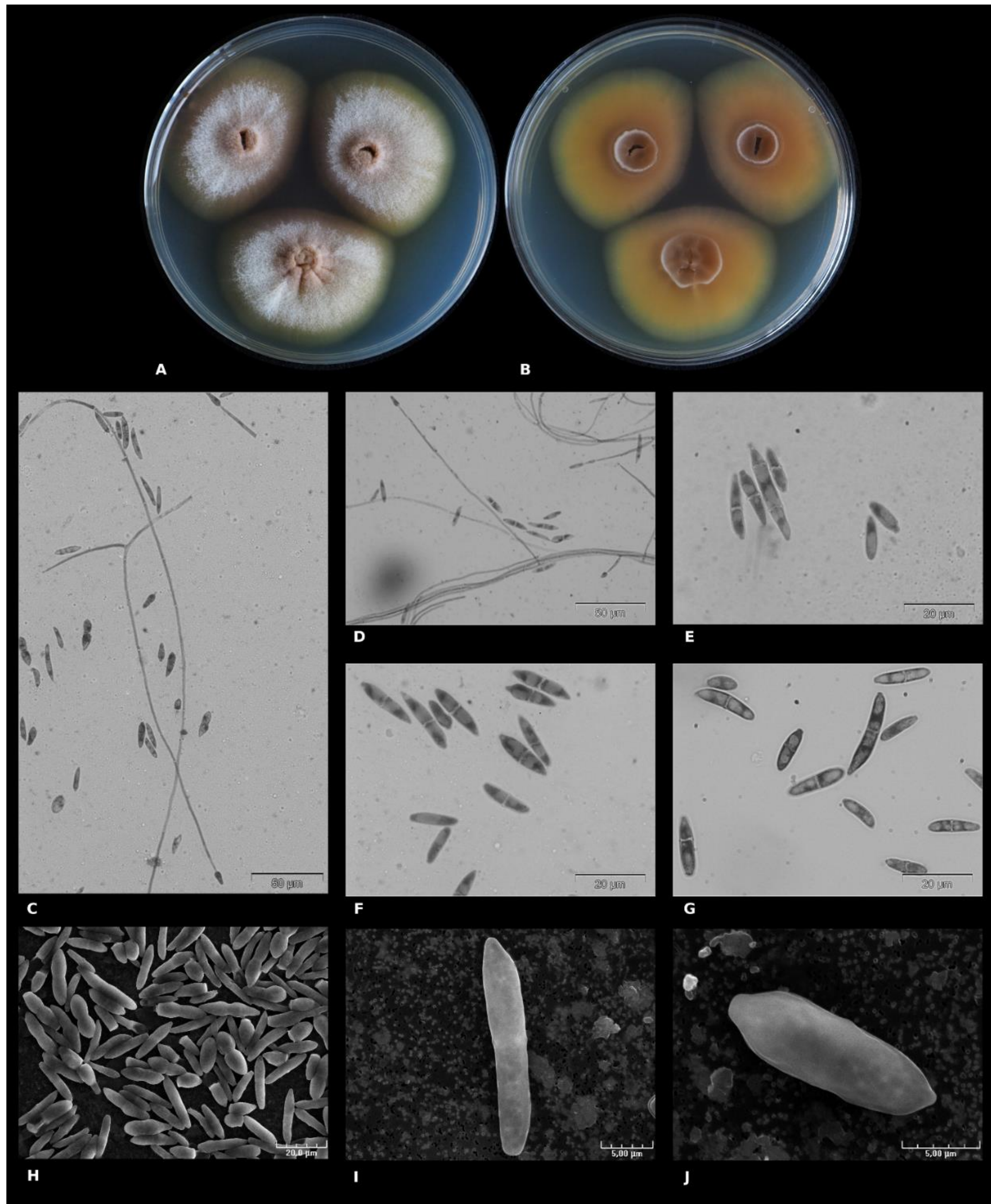

Figure 5 – Macroscopic morphological and microscopic characteristics of *Longinectria lagenoides* (UBOCC-A-120039[HT]). A Colony on PDA (14 days at 25°C). B Colony reverse on PDA (14 days at

25°C). C–D phialides. E–J micro- and macro- conidia. C–G microscopic acquisitions using classical microscopy, H–J: microscopic acquisition using scanning electron microscopy.

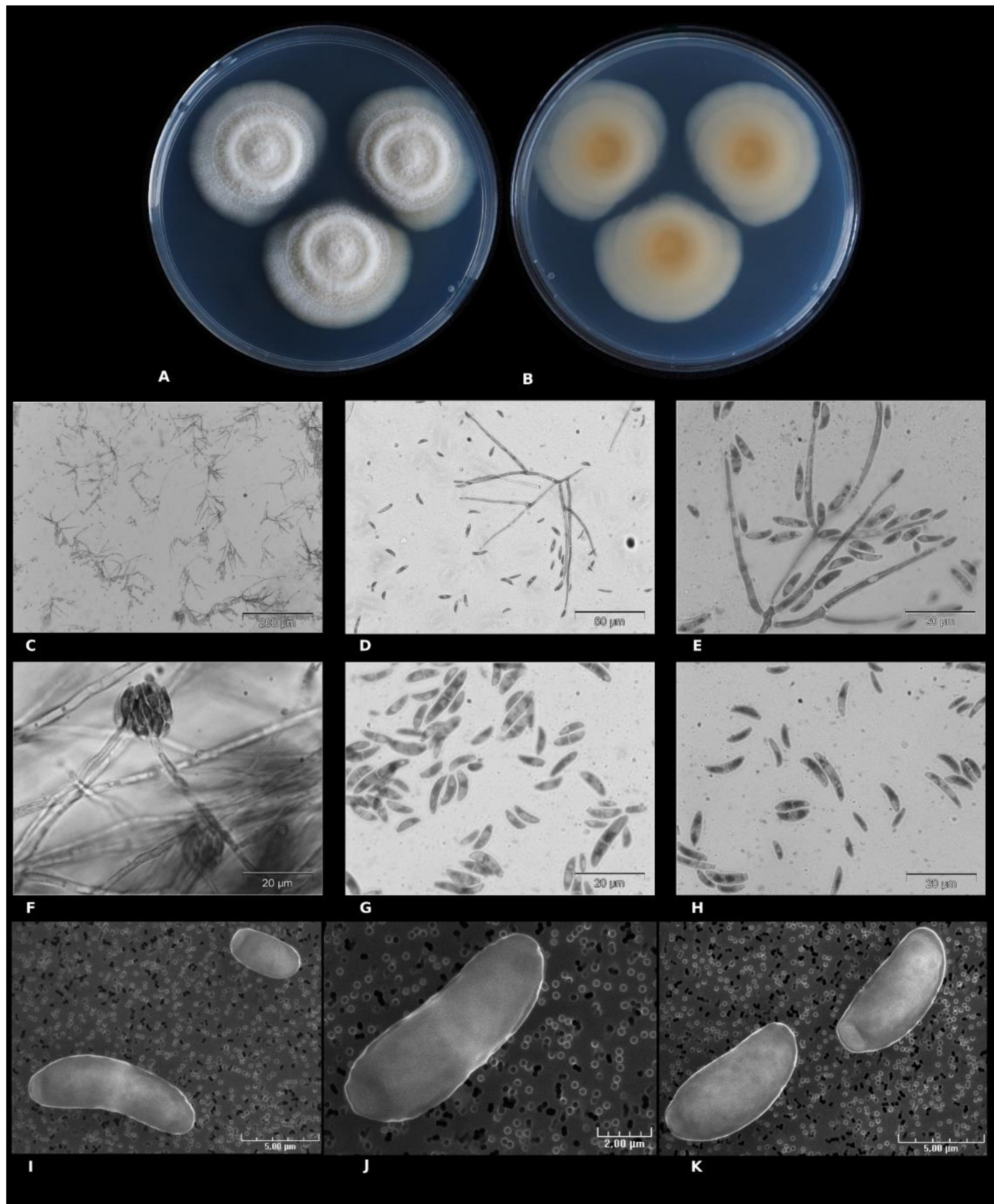

Figure 6 – Macroscopic morphological and microscopic characteristics of *Longinectria verticilliforme* (UBOCC-A-120043^{HT}). A Colony on PDA (14 days at 25°C). B Colony reverse on PDA (14 days at 25°C). C–E phialides. F–K micro- and macro- conidia. C–H microscopic acquisitions using classical microscopy, I–K: microscopic acquisitions using scanning electron microscopy.

Secondary metabolites – No known mycotoxins already described to be produced by *Fusarium*, *Penicillium*, *Aspergillus* or *Alternaria* spp. were detected. For most detected compounds, except ergosterol, extrolites could not be identified but belonged to 32 chromophore families (Table 3).

These secondary metabolites included the "emon" chromophore or corresponded to extra apolar free fatty acids (including linoleic acid and probably oleic acid), mid-cyclic lipopeptides with tyrosine in the cyclic peptide as well as RED chromophore (Table 3).

Substrate and distribution – To date, the only strain isolated for this species was from an Italian cheese.

Material examined – France, isolated from an Italian cheese (Alpeggio), O. Savary, 10 Dec. 2018, holotype UBOCC-A-120043, CBS 147589.

Table 3 Production of different chromophore groups of secondary metabolites by *Nectriaceae* spp.

Chromophore family	**Identity or chromophore**	***Bisifusarium allantoides*** **UBOCC-A-120036**[HT]	***Longinectria lagenoides*** **UBOCC-A-120039**[HT]	***Bisifusarium penicilloides*** **UBOCC-A-120021**[HT]	***Longinectria verticilliforme*** **UBOCC-A-120043**[HT]
Alk 1-3	indole alkaloid chromophore	+	+	+	-
Alk 4	indole alkaloid chromophore	-	+	-	-
Aspham	alkylphenone chromophore	-	+	-	-
Atrov	atrovenetin chromophore	-	+	-	-
Cyt	tyrosine chromophore	+	+	+	+
e1-12	no chromophore	+	+	+	+
e15-21	no chromophore	+	+	+	+
e38-48	fatty acid	+	-	+	-
Emon		+	+	+	+
Ergosterol	(common membrane constituent)	+	+	+	+
Ffa 1-4	fatty acid	+	+	+	+
Flit		-	+	-	-
Fluorescing compound at retention time 12.95		-	+	-	-
Linoleic acid	linoleic acid	+	+	+	+
Pal		-	-	+	-
Pyru	pyruvoylaminobenzamide chromophore	+	+	+	-
RED		-	-	-	+
Snor		+	-	+	-
Ulm		+	-	+	+
Vit		+	+	+	+
Vor		+	+	+	+
xx		+	-	-	+

The production and detection by HPLC-DAD of chromophore family is highlighted by the "+" symbol and its absence is represented by the "-" symbol

Discussion

The Ascomycete *Nectriaceae* (Hypocreales) family includes saprotrophic and/or plant and human pathogens. Interestingly, several species within the family have been used for industrial purposes (Lombard et al. 2015). One species, namely *Bisifusarium domesticum,* plays an essential role for many washed-rind cheeses to reduce surface stickiness (Bachmann et al. 2003, 2005). To date, the latter species has only been isolated from cheese and was the only *Nectriaceae* species clearly associated with this food matrix. However, this study reports the unexpected diversity of *Fusarium*-like species occurring in the cheese environment, and describes four new cheese-associated *Nectriaceae* species, including two that may pertain to a new genus.

While sampling washed-rind cheeses to obtain *B. domesticum* isolates on DG18 agar medium, we were firstly able to collect multiple *Fusarium* spp. isolates, rarely described in cheeses. In particular, isolates belonging to *Fusarium babinda*, *F. culmorum, F. oxysporum* and *Fusarium solani s.l.* were isolated from different cheeses although these species, considered as plant pathogens and/or soil saprophytes, are mainly recovered from soil and plant debris, but also the cause of some human infections (Leslie & Summerell 2006, Zhang et al. 2006, Geiser et al. 2013, Summerell 2019). Obviously, the question arises about their presence on cheeses, which is likely due to undesirable environmental contaminations. Secondly, in addition to these species, several isolates with a *Fusarium*-like morphology were also found and further grouped into four different taxa. Based on blastn analysis using *tef1* sequence, they could not be assigned to *Bisifusarium domesticum* or to any other known species within the *Nectriaceae* family.

Deciding whether or not these four taxa indeed corresponded to new species, i.e. “segments of evolutionary lineages” that evolve independently (de Queiroz 1998), is not a trivial task. Different criteria exist to detect lineages that have evolved independently. However, neither the Biological Species Concept (BSC, see Anderson & Ullrich 1979) nor the Genealogical Concordance Phylogenetic Species Recognition (Taylor et al. 2000) could be applied in this study. First, cross-mating between isolates collected in this study and other strains representative of phylogenetically close species was not possible since the teleomorphs for these species are not always known. Moreover, we did not collect a sufficient number of isolates of each putative new species to consider evaluating the concordance/incongruity of multiple unlinked genes to indicate a lack of genetic exchanges between lineages evolving independently as proposed by the GC-PSR criterium (Taylor et al. 2000) or even to observe the number of shared/unique single nucleotide polymorphisms (SNPs) among/within lineages. Therefore, this study focused on the use of multiple locus phylogenetic reconstructions that could identify independent clades and inform about the nucleotide divergences between taxa. By using the same set of loci as the one used by Lombard et al. (2015), that was publicly available at the beginning of this study, we were able to assess the phylogenetic placement of the four taxa representing putative species within the *Nectriaceae* family, and evaluate the nucleotide divergence between these taxa and the other phylogenetically close species and genera.

It is noteworthy that the circumscription of the *Fusarium* genus has recently been highly debated (Crous et al. 2021 vs. Geiser et al. 2021), mainly with two concepts: (i) a broad *Fusarium* concept (O’Donnell et al. 2020, Geiser et al. 2021) corresponding to a monophyletic group initiated at node F1 (node F1 in Geiser et al. 2013). This hypothesis was recently supported by a 19-gene phylogeny (Geiser et al. 2021) which showed a better phylogenetic support for node F1 than for other internal nodes. In addition, different practical arguments provided in O’Donnell et al. 2020 encourage the scientific community to use the broad *Fusarium* concept. (ii) A second concept with a *Fusarium* genus corresponding to a monophyletic group initiated at node F3 (node F3 in Geiser et al. 2013) that excludes the following genera: *Albonectria*, *Cyanonectria*, *Geejayessia*, *Neocosmospora*, *Rectifusarium* and *Bisifusarium*. The latter proposition brings forward the strongly supported monophyly of these different genera as well as its morphological, biochemical and ecological consistency that contrasts with the absence of synapomorphy when considering the broad *Fusarium* concept.

Pending adoption of a consensus taxonomy, we named taxa based on the nomenclature used by Lombard et al. (2015), although future studies may lead to genus name changes. This approach

also led to the proposal of a new genus (i.e. *Longinectria*) within the *Nectriaceae* family based on the definition of Crous et al. (2021), O'Donnell et al. (2020), Geiser et al. (2021).

In the context of this study, after a preliminary identification based on *tef1* sequencing, a phylogenetic analysis was performed comparing the ten-locus sequence set used by Lombard et al. (2015), namely *acl1, act, cmdA, his3, ITS, LSU, rpb1, rpb2, tef1* and *tub2,* from thirteen isolates, eleven corresponding to unidentified isolates with a *Fusarium*-like morphology and the two others where *F. babinda* and *F. solani s.l.* isolates. Finally, *B. biseptatum* CBS 110311^T, missing from Lombard et al. 2015 analysis, and *B. dimerum* MNHN-RF-05625^T, as an internal control, were also analysed. The obtained sequences were compared with the corresponding sequences from 57 strains issued from the dataset analyzed by the latter authors and supplemented with *B. tonghuanum* CGMCC 3.17369^T (Sun et al. 2017) sequences (6/10 loci available). A complementary analysis was then performed with the sequences of six loci, namely *ITS*, *LSU*, *rpb1*, *rpb2*, *tef1* and *tub2*, from the ten isolates identified as pertaining to the *Bisifusarium* genus. This was done to ensure that the missing sequence data for the recently described *B. tonghuanum* (Sun et al. 2017) had no impact on the positioning of some of our isolates as new *Bisifusarium* species.

Firstly, the ten loci analysis showed that six of the *Fusarium*-like cheese isolates clustered into two groups, the first one (i) containing UBOCC-A-120021, UBOCC-A-120034 and VTT-D-042022 strains, and the second one (ii) UBOCC-A-120035, UBOCC-A-120036 and UBOCC-A-120037 strains. These groups positioned within the supported *Bisifusarium* genus (Lombard et al. 2015) and were phylogenetically separated from other already described species within this genus as well as other *Fusarium* species. Moreover, the complementary analysis focused on the *Bisifusarium* genus (using six loci) confirmed the same topology. These two species formed a sister clade of *B. tonghuanum.* The nucleotide differences between these isolates and *B. tonghuanum* CGMCC 3.17369^T (299/5134 (90.2% identity) and 325/5134 (89.2% identity) nucleotides for *B. penicilloides* and *B. allantoides,* respectively), as well as with the other *Bisifusarium* species (e.g. 566/5134 (88.5% identity) and 571/5134 (88.4% identity) nucleotide differences with *B. biseptatum* CBS 110311^T), supports the existence of two new *Bisifusarium* species. Indeed, among *Bisifusarium*, several species have nucleotide differences that are 5-fold lower than between *B. allantoides* and *B. penicilloides* (e.g. 73/5134 nucleotides (98.5% identity) between *B. penzigii* CBS 317.34^T and *B. biseptatum* CBS 110311^T). Following morphological observations, the names *Bisifusarium allantoides* and *B. penicilloides* were proposed for these two new species. These two species were isolated from cheese rinds and their growth was confirmed at temperatures as low as 5°C thus compatible with cheese-making conditions. Interestingly, among *B. penicilloides* isolates, one strain was also isolated from the environment (sandstone building). This presumptive identification proximity based on macromorphological observation was confirmed using the ten loci sequencing phylogenetic analysis. This strain, isolated from the outer surface of a sandstone building, could provide key information about the environmental origin of this species. Indeed, this association to sandstone might suggest that this species is naturally found in some cheese, via its presence in ripening cellars (i.e. on walls or building surfaces). It is noteworthy that the genus *Bisifusarium* might actually include a larger number of species than the ten *Bisifusarium* spp. presented in this study. Indeed, among the five strains assigned to unnamed *Bisifusarium* species in Sun et al. (2017), a preliminary Maximum Likelihood analysis was carried out on a reduced dataset comprising the only sequences available for four loci for these strains (*ITS*, *LSU*, *tef1* and *tub2*). The results suggested that these strains might pertain to new *Bisifusarium* species (data not shown).

As for the other isolates, the position of the genetic cluster (iii) consisting of UBOCC-A-120038, UBOCC-A-120039, UBOCC-A-120041 and ESE-00140, on the one hand, and (iv) UBOCC-A-120043, on the other hand, that formed two branches within an independent sister clade of a clade grouping *Albonectria*, *Fusarium*, *Geejayessia*, *Cyanonectria* and *Neocosmospora* genera (as described by Lombard et al. 2015), is compatible with their affiliation to two new species within a new genus. The minimal nucleotide divergence (566/5134 nucleotides) existing between this clade and its sister clade, grouping *Albonectria*, *Fusarium*, *Geejayessia*, *Cyanonectria* and *Neocosmospora* genera, is similar or higher than those existing between genera of the latter clade (e.g. minimal

nucleotide divergence 554/5134 nucleotide between *Cyanonectria* and *Neocosmospora*). Furthermore, nucleotide differences (483/5134 nucleotides, 90.2% identity) occurring between UBOCC-A-120043 and UBOCC-A-120038 / UBOCC-A-120039 / UBOCC-A-120041 / ESE-00140 supported the fact that the two branches actually correspond to two different species within the genus. According to morphological characters of these five isolates, namely the observed length of the phialides, which appear to be longer than phialides commonly observed within the *Nectriaceae*, the name *Longinectria* (gen. nov.) is proposed in the present study for members of this genus. Then, following morphological observations, the names *Longinectria verticilliforme* and *L. lagenoides* were proposed for these two new species. Noteworthy, Crous et al. (2021) very recently described several new *Nectriaceae* genera, among which two (*Luteonectria* gen. nov. and *Nothofusarium* gen. nov.) are positioned close to the previously cited genus. However, the phylogenetic divergence between the *Longinectria* spp. (based on a Maximum Likelihood analysis using the accessible sequence, Supplementary Fig. 2) and these new genera, as well as morphological differences regarding phialides and conidia, agreed with the existence of another new genus (i.e. *Longinectria* gen. nov.) within the *Nectriaceae* family. Otherwise, temperature impact was investigated on these two species and, interestingly, growth was observed at 5°C while no growth occured at 30°C and 37°C on PDA. These observations are clearly in agreement with cheese production, as reported for *B. domesticum* for which a maximum growth temperature (T_{max}) was estimated to be 30°C (Schroers et al. 2009).

Overall, the present study clearly highlighted the unexpected diversity of *Nectriaceae* in cheeses which, up to now, were considered to be solely colonized by *B. domesticum* that is used as a technological auxiliary for its "*anticollanti*" ability (Bachmann et al. 2003, 2005). As shown in this study, one can note that even microbial ecosystems that have been associated with human activity for centuries, and quite extensively studied, are still sources to discover novel microbial diversity. The obtained data clearly suggests that cheeses, which is obviously the case for other fermented foods, might harbour much higher diversity than expected. By sampling a relatively small panel of washed-rind cheeses (n = 56), we identified four new species within the *Nectriaceae* family, a family that is best documented for pathogenic species (plant and/or human) (Lombard et al. 2015). This study is therefore a hint that, besides the most emblematic *Penicillium* species, there is still much to discover about cheese microbiota and more specifically the fungal communities of fermented foods. Regarding the four species described in the present study, it should be noted that the obtained results on extrolite production tend, so far, towards a safety status (in the sense of the European Qualified Presumption of Safety -QPS- or the US Generally Recognized As Safe -GRAS- statuses) of these isolates as no known mycotoxins, metabolites largely produced by *Fusarium* genus members (e.g. *F. graminearum* or *F. avenaceum*), were observed in the tested conditions. However, as some metabolites are unknown, this aspect should be further investigated. In the future, it could also be of interest to assess the respective functional diversity of these species to better understand their metabolic potential and to use comparative genomics to potentially identify cheese adaptation footprints and elucidate their role in cheese.

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Declarations

Conflicts of interest/Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material

The sequences generated and used in this phylogenetic analysis were deposited in GenBank under accession numbers detailed in this paper and the multi-gene phylogenetic analyses were deposited in TreeBASE. The holotype strains were made available at the UBOCC (Université de Bretagne Occidentale, France) and CBS-KNAW (Westerdijk Fungal Biodiversity Institute, The Netherland) culture collections.

Authors' contributions

EC, MC, and JLJ obtained the funding, supervised the study and designed the experiments. OS performed the experimental work and analysed all data. OS and JLJ performed phylogenetics analysis. OS and MC were involved in extrolite preparations while JCF performed all extrolite analyses on fungal cultures. JR performed some of the *Fusarium*-like fungi isolations and molecular identifications. PN supervised OS for microscopical observations and JLJ contributed to the new species morphological descriptions. OS drafted the manuscript and all co-authors edited and proofread the manuscript.

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