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ABSTRACT

A novel fungal species able to synthesize enzymes with potential synergistic actions in lignocellulose conversion was isolated from the biomass of *Arundo donax* during biodegradation under natural conditions in the Gussone Park of the Royal Palace of Portici (Naples, Italy). In this work, this species was subjected to morphological and phylogenetic analyses. Sequencing of its genome was performed, resulting in 28 scaffolds that were assembled into 27.05 Mb containing 9744 predicted genes, among which 396 belong to carbohydrate-active enzyme (CAZyme)-encoding genes. Here we describe and illustrate this previously unknown species, which was named *Talaromyces borbonicus*, by a polyphasic approach combining phenotypic, physiological, and sequence data.

INTRODUCTION

Arundo donax (common name "giant reed") is a nonedible, perennial herbaceous rhizomatous crop belonging to the family of Poaceae in the tribe Arundineae (Mariani et al. 2010). It is widespread in the Mediterranean area, and its adaptability to different soils and climatic conditions, high biomass productivity, and annual harvesting period make this crop an attractive lignocellulosic feedstock (Lewandowski et al. 2003; Corno et al. 2014). In the context of use of renewable biomass as an alternative to fossil sources, *A. donax* has been used in the production of biogas, bioethanol, and other biochemicals such as succinic acid by biological fermentation (Ask et al. 2012; Corno et al. 2015; Ventorino et al. 2017). Enzymatic treatment of lignocellulosic biomass for production of fermentable sugars represents the most preferred alternative to chemical hydrolysis, with the potential of higher selectivity, lower energy costs, and milder operating conditions than chemical processes (Yang et al. 2011). In nature, lignocellulosic biomass is degraded due to the synergistic action of cellulolytic, hemicellulolytic, and ligninolytic enzymes within complex enzymatic multistep processes (Voříšková and Baldrian 2013; Yang et al. 2014). These enzymes are produced by microbial communities that can be therefore used as a source of novel biocatalysts (Amore et al. 2013; Okeke et al. 2015; Daas et al. 2016) for improvement of lignocellulose conversion in green technologies.

During the screening of microbiota of *A. donax* biomass piles left to biodegrade under natural conditions in the Gussone Park of the Royal Palace of Portici (Naples, Italy), novel microorganisms were isolated and characterized for their activities in lignocellulose conversion (Ventorino et al. 2015, 2016). In this study, we describe a novel *Talaromyces* species selected from among these microorganisms for its ability to synthesize different enzymes with potentially synergistic actions in lignocellulose conversion, such as endo- and exo-cellulase, cellobiohydrolase, xylanase, pectinase, and laccase (Ventorino

⊕ Supplemental data for this article can be accessed on the publisher's Web site.

et al. 2015, 2016). This strain was also able to produce feruloyl esterase (FAE) activity measured using *p*-nitrophenyl ferulate as substrate. In addition to the description of the new species, we also report its draft genome sequence.

MATERIALS AND METHODS

Strain isolation and preservation.—A *Talaromyces* strain (A-T2C-71X), belonging to the microbial collection of Division of Microbiology, Department of Agricultural Sciences, University of Naples Federico II, was isolated from lignocellulosic biomass of *A. donax* during biodegradation under natural conditions (Ventorino et al. 2015) in the Gussone Park of the Royal Palace of Portici, Naples, Italy (40°48′50.1″N, 14°20′48.2″E).

Cultures and media.—*Talaromyces borbonicus* was cultivated in complete medium (de Vries et al. 2004). Mycelium was sampled after 48 h of growth, and genomic DNA was extracted with a cetyltrimethylammonium bromide (CTAB)-based extraction buffer (Hildén et al. 2005). RNA was extracted and purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany) from 6- and 9-d-old cultures grown on wheat bran, wheat bran with ferulic acid, destarched wheat bran, and glucose in minimal medium.

Morphological analysis.—Macroscopic analysis of the strain was performed on creatine agar (CREA), Czapek yeast extract agar (CYA), CYA supplemented with 5% NaCl (CYAS), dichloran 18% glycerol agar (DG18), malt extract agar (MEA; Oxoid, Hampshire, United Kingdom), oatmeal agar (OA), and yeast extract sucrose agar (YES). All Petri dishes were incubated at 25 C for 7 d and additional CYA plates were incubated at 30 and 37 C. Microscope preparations were made from 1–2-wk-old colonies grown on MEA. All media were investigated in order to determine whether the strain is able to produce ascomata. Details on the inoculation of the agar media, Ehrlich reaction, and the macro- and micromorphological analyses are given in Houbraken et al. (2014) and Yilmaz et al. (2014).

Sequencing and data analysis.—Concentration and quality of the DNA samples were determined with the Life Technology Qubit and a 0.6% agarose gel, whereas the quality of the RNA samples was checked with a Fragment Analyzer (Advanced Analytical Technologies,

Ankeny, Iowa). Genome and transcriptome sequencing were performed at GenomeScan. NEBNext Ultra DNA Library Prep Kit for Illumina (NEB E7370S/L; Ipswich, Massachusetts) and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB E7420S/L) were used according to the manual for library preparation. Quality and yield after sample preparation were measured with a Bioanalyzer (Agilent Technologies, Santa Clara, California).

Clustering and DNA sequencing with the Illumina cBot and HiSeq 2500 was performed according to the manufacturer's protocols with a concentration of 8.0 pM of DNA, standard Illumina primers, and HiSeq control software HCS 2.2.58. Image analysis, base calling, and quality check were performed with the Illumina data analysis pipeline RTA 1.18.64 and bcl2fastq 1.8.4. Reads were trimmed for adapter sequences and filtered for sequence quality with the in-house tool fastqFilter 2.05. The short-read genome assembler ABySS 1.3.7 (Simpson et al. 2009) was used for assembly. An optimization for k-mer length was performed using previous fungal genome assemblies. A length of 64 was found to give the best results, optimized for the lowest amount of scaffolds with a longer average length. Scaffolds shorter than 500 bp, unlikely to contain complete coding sequences, were removed.

The hidden Markov model (HMM)-based algorithm Glimmer 3.0.2 (Majoros et al. 2004) was trained for gene finding with the genome of *Talaromyces stipitatus* (Nierman et al. 2015). Furthermore, an evidence-based method of gene finding was performed with the CodingQuarry (Testa et al. 2015) software and the mapped mRNA-sequencing reads. GC content was assessed by QUAST (Gurevich et al. 2013).

Phylogenetic analysis.—The nuc rDNA ITS1-5.8S-ITS2 (ITS) barcode and sequences of genes for β-tubulin (*benA*), calmodulin (*caM*), and RNA polymerase II second-largest subunit (*rpb2*) of strain A-T2C-71X/CBS 141340 were extracted from the genome sequence and used in the phylogenetic analyses. The lengths of the data sets were 512 bp (*benA*), 578 bp (*caM*), 455 (ITS), and 851 bp (*rpb2*), and the best substitution models were HKY+G (*benA*), TN93+G+I (*caM, rpb2*), and T92+G+I (ITS). The phylogenetic relationship of this strain with other section *Helici* members was studied by maximum likelihood (ML) and Bayesian inference (BI) analyses. A sequence alignment was made with the extracted sequences and the reference sequences of section *Helici* (Yilmaz et al. 2014; Chen et al. 2016) using MAFFT (Katoh et al. 2005), and the optimal model was calculated in MEGA6 (Tamura et al. 2013). A

maximum likelihood (ML) tree was inferred with MEGA6, and the Bayesian inference (BI) analysis was performed in MrBayes 3.2.2 (Ronquist et al. 2012). Support of the nodes was calculated with 1000 rapid bootstrap replicates for the ML analysis, and in the Bayesian analysis, every 1000 generations were sampled and the first 25% of the samples were discarded. Bootstrap percentages and posterior probability values are presented at the nodes, and values less than 70% bootstrap support (BS) or 0.95 posterior probability (PP) are not shown. *Talaromyces adpressus* CBS 140620 was used as an outgroup. The alignments and phylograms from the ML analyses were deposited in TreeBASE (study no. 22208). GenBank accession numbers for sequences used in phylogeny are MG855687, MG855688, and MG855689.

CAZyme analysis.—The putative encoded protein sequences were first compared with the full-length sequences of the CAZy database (http://www.cazy.org/; Lombard et al. 2014) with BLAST (Altschul et al. 1990), and query sequences that produced an e-value >10^{-6} were discarded. Query sequences that had >50% identity with a sequence already classified in the CAZy database were automatically assigned to the same family as the subject sequence. The remaining query sequences were subjected to manual curation that involved BLAST searches against a library built with partial sequences corresponding to individual GH, PL, CE, and CBM modules and examination of the conservation of specific family patterns and features such as catalytic residues (where known). Subfamily assignments were performed with a set of hidden Markov models (Rabiner and Juang 1986) built after each subfamily.

RESULTS

Phylogenetic analysis.—The *Talaromyces* strain (A-T2C-71X) investigated in this study was named *T. borbonicus* because it was collected in Gussone Park, which is part of the Royal Palace of Portici and formerly belonging to the dynasty of the Bourbons of Naples. Its phylogenetic analysis showed that this fungus belongs to section *Helici* (FIG. 1).With the description of *T. borbonicus*, section *Helici* currently contains 10 species. Two main clades are present in section *Helici*: one clade includes *Talaromyces boninensis*, *Talaromyces borbonicus*, *Talaromyces helicus*, *Talaromyces reverso-olivaceus*, and *Talaromyces varians*, and the other clade contains *Talaromyces aerugineus*, *Talaromyces bohemicus*, *Talaromyces cinnabarinus*, *Talaromyces diversiformis*, and

Talaromyces ryukyuensis. *T. borbonicus* CBS 141340 clusters in all phylogenies with URM 7624, an unidentified strain from inside nests of *Melipona scutellaris* bees in Brazil.

Genome features.—Over 93 200 000 reads were obtained after quality filtering and trimming, with an average read length of 151 bp. The draft genome sequence of *T. borbonicus* has an estimated size of 27.05 Mb resulting from the assembly of 47 contigs (TABLE 1).The average genome coverage is 518×, and the GC content of the assembly is 46.1%.

The output from both Glimmer and CodingQuarry was combined into a single gene model of 9744 genes.

CAZyme analysis.—In order to identify putative genes and enzymes involved in the breakdown, biosynthesis, or modification of carbohydrates, the total predicted open reading frames (ORFs) in *T. borbonicus* genome were compared with the entries of the carbohydrate-active enzymes (CAZy) database (http://www.cazy.org/). A total of 396 putative CAZymes were identified in the *T. borbonicus* genome (TABLE 2).

The most abundant predicted CAZyme was from the glycoside hydrolase (GH) class (approx. 63% of all CAZymes), followed by glycosyltransferases (GTs) with approx. 23% of the total predicted CAZymes. The amounts of carbohydrate esterases (CEs; 5%), auxiliary activities (AAs; 5%), and polysaccharide lyases (PLs; 0.5%) were significantly smaller. ORFs coding for putative carbohydrate-binding modules (CBMs) having binding activity to carbohydrates corresponded to approx. 15% of the total CAZymes. In particular, 82% of the detected CBMs were in association with other CBMs or with GHs or CEs displaying a modular structure.

TAXONOMY

Talaromyces borbonicus Houbraken, sp. nov.   FIG. 2
MycoBank MB821643

In: *Talaromyces* section *Helici*.

Typification: ITALY. Naples, Gussone Park of the Royal Palace of Portici, *Arundo donax* biomass (after biodegradation under natural conditions), 2012, O. Pepe (**holotype** CBS H-22672). Ex-type culture: CBS 141340 = DTO 351-D3.

Etymology: Referring to dynasty of the Bourbons of Naples. The type location formerly belonged to the dynasty of the Bourbons of Naples.

Diagnosis: *Talaromyces borbonicus* produces brown-colored funicles with short, brown pigmented conidio-phores (10–40 × 2.5–3.5(–4.5) μm) and small-sized
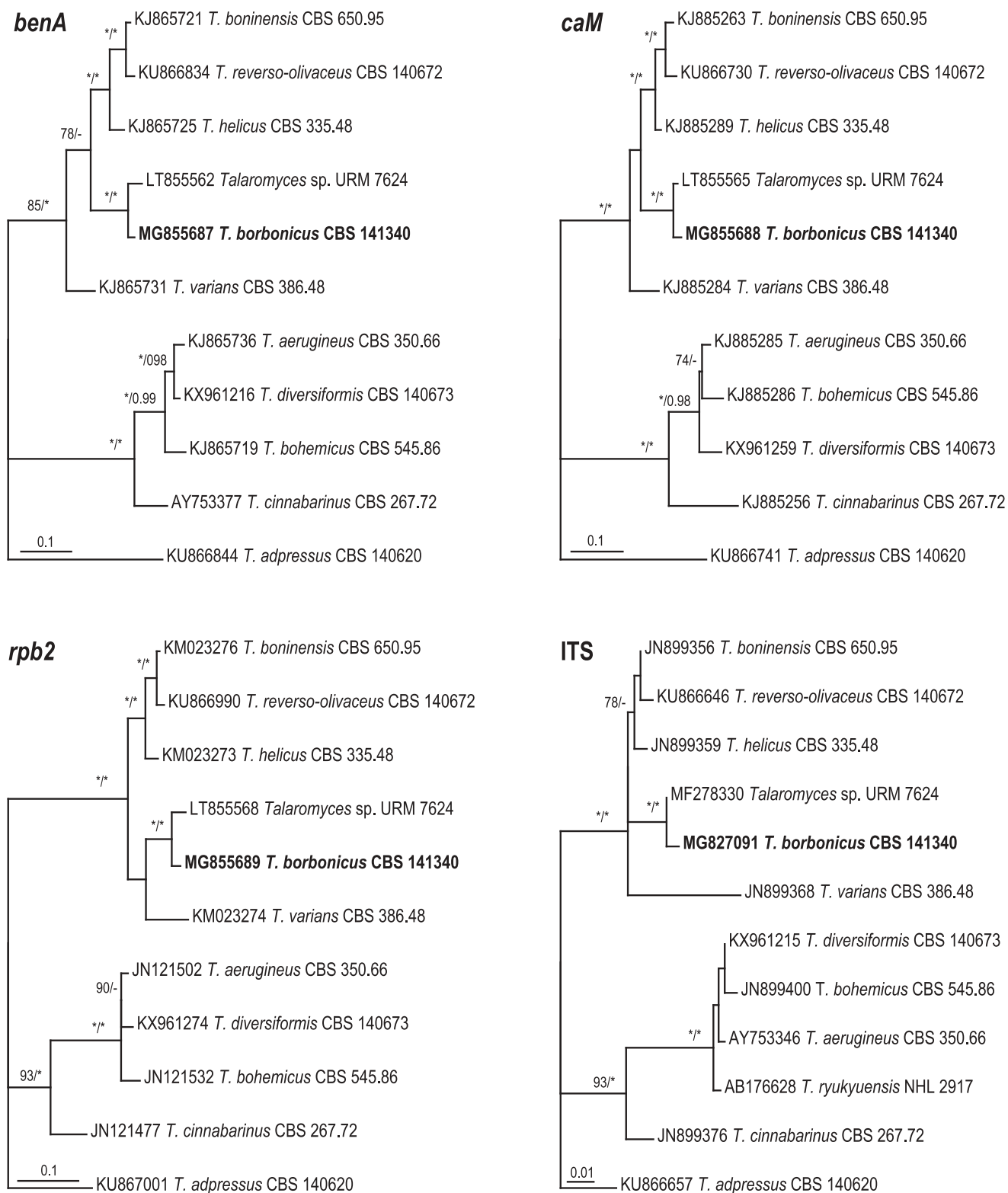
Figure 1. Phylogenetic trees showing the relationships among strains belonging to *Talaromyces* section *Helici*. Maximum likelihood bootstrap proportions (BS) and Bayesian posterior probabilities (PPs) are appended to nodes; only BS >70% and PPs >0.95 are shown; lower supports are indicated with a hyphen, whereas asterisks indicate full support (100% BS or 1.00 PP). The bars indicate the number of substitutions per site. The phylogram is rooted with *Talaromyces adpressus* CBS 140620.

Table 1. Genome features of *T. borbonicus*, sp. nov., A-T2C-71X (CBS 141340).

No. of reads	93 219 198
Paired-end read length (bp)	151
Genome assembly size (Mb)	27.05
No. of contigs	47
No. of scaffolds	28
Contig N50	1 406 961
Scaffold N50 (bp)	2 198 792
No. of exons per gene (average)	2.8
GC content (%)	46.1
No. of predicted genes	9744

Table 2. CAZyme contents of *T. borbonicus*.

	T. borbonicus	
CAZyme classification	ORFs	%
Glycosyltransferases (GTs)	91	23
Carbohydrate esterases (CEs)	20	5,1
Glycoside hydrolases (GHs)	250	63,1
Polysaccharide lyases (PLs)	2	0,5
Auxiliary activities (AAs)	19	4,8
Carbohydrate-binding modules (CBMs)	60	15,2
Expansins (EXPs)	6	1,5
Total CAZymes*	396	

*The total number of CAZymes is less than the sum (GTs + CEs + GHs + PLs +AAs + CBMs) due to the fact that some multimodular predicted proteins were detected.

conidia (1.5–2.0 µm). Furthermore, the species grows well on CYA incubated at 37 C (25–32 mm, 7 d).

Colony characters: Colony diam, 7 d, in mm at 25 C, unless stated otherwise: CYA 20–55; CYA at 30 C 28–32; CYA at 37 C 25–32; CYAS 5–12; DG18 13–16; MEA 22–28; YES 20–28; creatine agar 12–17, poor growth, good acid and no base compounds produced. Optimum growth temperature on CYA approx. 28–30 C.

CYA, 25 C, 7 d: Colonies centrally elevated, plane; sporulation poor, only in center; colony texture floc-cose; mycelium white, pale brown in center; exudate present as small droplets, clear to pale brown; soluble pigments absent; margin entire; conidia dull green; reverse concentric rings in different shades of brown. YES, 25 C, 7 d: Colonies low, randomly sulcate; sporulation moderate; mycelium white; exudate droplets absent; soluble pigments absent; margin entire; conidia dull green in center, brownish green at edges, reverse brown with dark brown center. MEA, 25 C, 7 d: Sporulation moderate to strong; colony texture floc-cose; mycelium white; exudate droplets absent; soluble pigments absent; conidia en masse dark green; reverse brownish yellow. DG18, 25 C, 7 d: Colonies slightly elevated; sporulation moderate; mycelium white; reverse pale brown, becoming brown in center. Ehrlich reaction negative.

Micromorphology: Ascomata and sclerotia absent. Conidiophores arising from dark brown pigmented funicles, predominantly biverticillate, occasionally with additional divergent branch; stipes smooth-walled, pale brown pigmented, short, 10–40 × 2.5–3.5(–4.5) µm; metulae pale brown pigmented, 3–8, 8.5–11 × 2.5–3.5 µm; phialides 2–7 per metula, pale brown pigmented, acerose, 9–11 × 2–3 µm; conidia broadly ellipsoidal or ellipsoidal, smooth-walled, 1.5–2 µm.

Notes: BLAST analyses on GenBank with ITS, *benA*, *caM*, and *rpb2* sequences of *T. borbonicus* revealed three similar sequences (FJ791130, KC506181, KT224828; 99% similarity, all ITS sequences). These sequences were generated from different specimens and different continents (FJ791130: old laid-paper document, Portugal; KC506181: mangrove sediment, Brazil; KT224828: *Dendrobium officinale*, China). ITS sequences can't be used for species recognition in *Talaromyces* (Yilmaz et al. 2014), and these sequences might therefore represent *T. borbonicus* or a closely related species.

DISCUSSION

The genus *Talaromyces* was initially described by Benjamin in 1955 as a sexual state of *Penicillium* producing yellow soft-walled ascomata covered by interwoven hyphae, with ovate to globose asci containing mostly spiny ascospores (Benjamin 1955). Due to the diverse characteristics of its members, the genus was divided into four sections based on the structure of the conidial state (Stolk and Samson 1972). However, Samson et al. (2011) and Yilmaz et al. (2014) redefined the taxonomy of *Talaromyces* by merging *Penicillium* subgenus *Biverticillium* into *Talaromyces* and proposing a new classification based on the ITS, *benA, rpb2*, and *caM* gene analyses together with phenotypic features including its morphology. The 88 accepted *Talaromyces* species were placed into seven sections, named *Bacillispori, Helici, Islandici, Purpurei, Subinflati, Talaromyces*, and *Trachyspermi*.

Strains belonging to the *Talaromyces* genus have an enormous biotechnological potential because of their ability to secrete a variety of small molecules and enzymes with different applications, such as xylanases for pulp bleaching (Maalej et al. 2009; Maalej-Achouri et al. 2009; Orencio-Trejo et al. 2016), cellulases for lignocellulosic biomass deconstruction (Inoue et al. 2014; Fujii et al. 2015; Schafhauser et al. 2015; Orencio-Trejo et al. 2016), feruloyl esterases for agro-food industries and tailored synthesis of pharmaceuticals (Crepin et al. 2003; Garcia-Conesa et al. 2004; Vafiadi et al. 2006; Mandalari et al. 2008; Watanabe et al. 2015) and pigments for the sustainable bioproduction of environmentally friendly dyes (Schafhauser et al. 2015). In
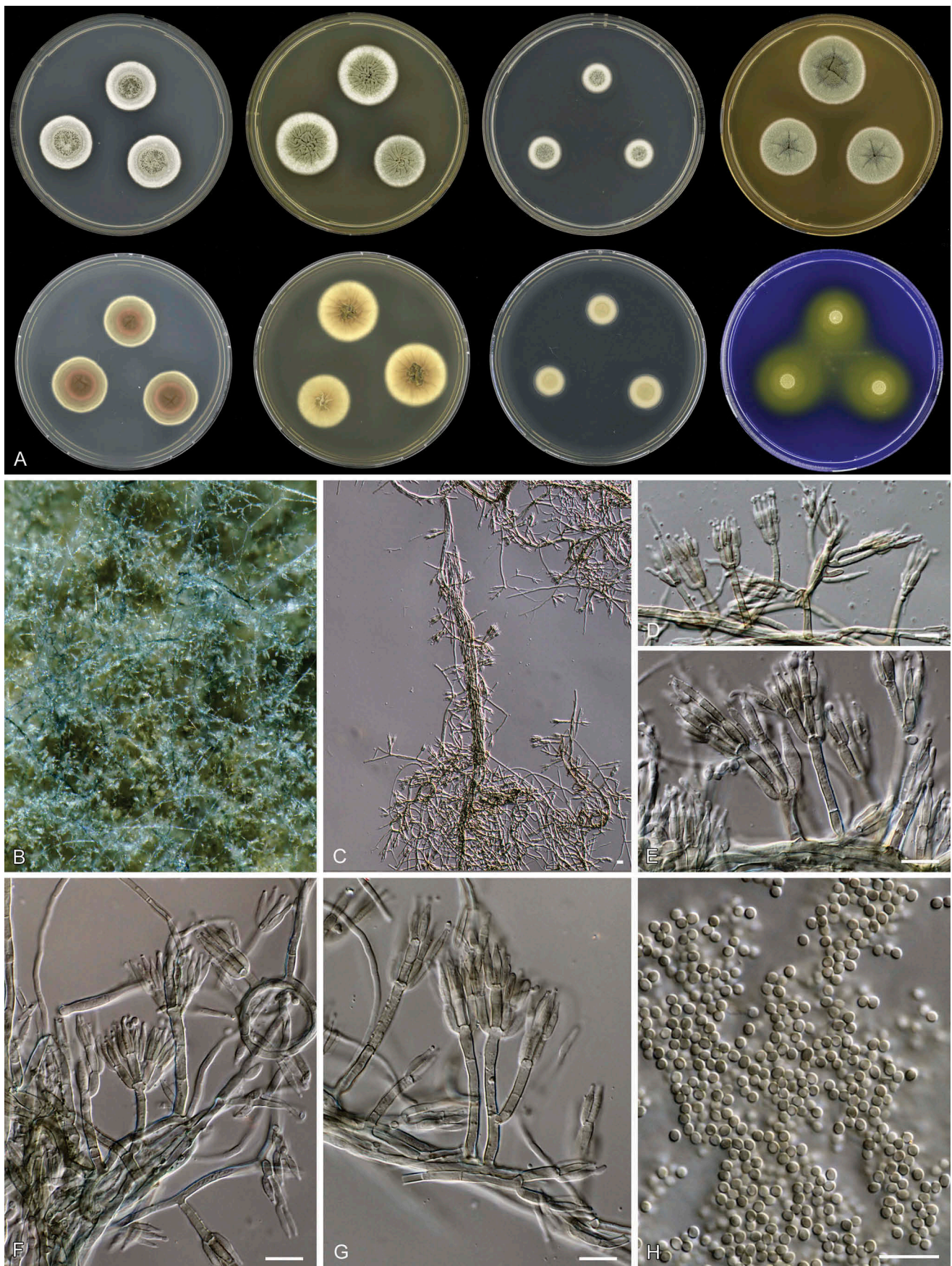
Figure 2. *Talaromyces borbonicus*. A. 7-d-old cultures, 25 C, left to right, first row: CYA, YES, DG18, MEA, all obverse; second row: CYA reverse, YES reverse, DG18 reverse, CREA obverse. B. Detail of colony on MEA. C–G. Conidiophores. H. Conidia. Bars = 10 μm.

Table 3. CAZyme contents of selected fungal genomes in number of genes per group.

Species	Strain	Total CAZymes	GHs		PLs		CEs		AAs		GTs	CBMs	EXPs
			Total	PBD	Total	PBD	Total	PBD	Total	PBD			
Talaromyces borbonicus	A-T2C-71X/CBS 141340	396	251	144	2	0	20	17	14	4	91	67	6
Penicillium rubens	Wisconsin 54-1255	426	222	120	9	9	20	20	22	9	101	51	1
*Penicillium chrysogenum**	unknown	481	234	125	9	9	20	20	50	10	110	56	2
*Penicillium subrubescens**	CBS132785	719	410	241	9	9	38	38	63	16	107	85	7
*Talaromyces stipitatus**	ATCC 10500	514	271	136	2	0	17	18	47	8	105	65	7
Aspergillus niger	NRRL3	542	252	137	9	9	22	22	65	19	119	72	3
Aspergillus oryzae	RIB40	600	304	174	23	20	27	26	69	16	119	54	4
Aspergillus nidulans	FGSC A4	572	275	161	23	21	28	28	57	16	97	90	2
*Trichoderma reesei**	QM6a	410	200	77	5	0	16	15	32	6	92	58	7
Neurospora crassa	OR74A	416	182	77	4	3	22	22	51	27	86	68	3

Note. PBD = plant biomass degradation–related genes; GHs = glycoside hydrolases; PLs = polysaccharide lyases; CEs = carbohydrate esterases; AAs = auxiliary activities; GTs = glycosyl transferases; CBMs = carbohydrate-binding modules; EXPs = proteins distantly related to expansins.
*Peng et al. 2017.

this work, a *Talaromyces* isolate from the lignocellulosic biomass *A. donax* during biodegradation under natural conditions (Ventorino et al. 2015) in the Gussone Park of the Royal Palace of Portici, Naples, Italy, was studied. Phylogenetic analysis has revealed that *T. borbonicus* belongs to section *Helici*, which currently comprises 10 species divided into two clades. *Talaromyces borbonicus* resides in a clade with *T.boninensis, T. helicus, T. reverso-olivaceus*, and *T. varians*.

Yilmaz et al. reported that section *Helici* members produce biverticillate conidiophores with stipes that are generally pigmented (Yilmaz et al. 2014). These features are shared by *T. borbonicus*. *T. borbonicus* is phylogenetically most closely related to *T. boninensis, T. helicus, T. reverso-olivaceus*, and *T. varians*. *Talaromyces boninensis* and *T. helicus* produce ascomata, and this feature is not observed in *T. borbonicus*. Furthermore, *T. borbonicus* and *T. boninensis* produce acid compounds on CREA, whereas no growth and/or acid is produced by *T. helicus, T. varians*, and *T. reverso-olivaceus*. The production of brown-colored funicles on MEA with short, brown pigmented conidiophores (10–40 × 2.5–3.5(–4.5) μm) is unique to *T. borbonicus*.

The strain *T. borbonicus* A-T2C-71X (CBS 141340) studied in this work was selected for its ability to synthesize different enzymes having potentially synergistic actions on lignocellulose conversion, such as endo- and exo-cellulase, cellobiohydrolase, xylanase, pectinase, laccase, and feruloyl esterase (Ventorino et al. 2015). Sequencing of *T. borbonicus* genome revealed an estimated size of 27.05 Mb resulting from the assembly of 47 contigs containing 9744 predicted genes. Genome analysis of *T. borbonicus* revealed the presence of 396 genes coding for enzymes devoted to degradation, modification, or creation of glycosidic bonds, corresponding to 4% of the total predicted genes. There are several studies on *Talaromyces* species producing enzymes involved in plant biomass

degradation (Inoue et al. 2014; Fujii et al. 2015; Schafhauser et al. 2015; Orencio-Trejo et al. 2016). An example is *Talaromyces cellulolyticus* (formerly *Acremonium cellulolyticus*), which is one of the best-characterized cellulase-producing fungi (Fujii et al. 2015).

T. borbonicus revealed a number of CAZyme genes analogous to other related species (TABLE 3; SUPPLEMENTARY TABLES 1, 2). In particular, genes belonging to families GH3, GH28, GH31, GH35, and GH43 are the most abundant, including genes that encode enzymes involved in plant cell wall degradation. Moreover, the number of predicted CAZyme genes in *T. borbonicus* genome is 40–50% higher than reported for *Thermomyces lanuginosus* (Winger et al. 2014), a unique cellulase-free fungus, producing high quantities of a GH11 xylanase. This presence of a broad set of genes falling into the class of plant biomass-degrading enzymes reveals the potential of the novel fungal strain *T. borbonicus* A-T2C-71X/CBS 141340 to produce enzymes with biotechnological use in the deconstruction of the lignocellulosic biomass.

Accession numbers.—This draft genome sequence of *T. borbonicus*, sp. nov., A-T2C-71X/CBS 141340 has been deposited at DNA Data Bank of Japan (DDBJ)/European Nucleotide Archive (ENA)/GenBank under accession number NBSA00000000 and consists of sequences NBSA01000001–NBSA01000028. The version described in this paper is version NBSA01000000. The BioProject in GenBank is PRJNA379116. The strain is available from the CBS culture collection (www.westerdijkinstitute.nl) housed at the Westerdijk Institute (Utrecht, The Netherlands). The genome is also available through the JGI fungal genome portal MycoCosm (Grigoriev et al. 2014).

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