Diversity of xerotolerant and xerophilic fungi in honey

E. Rodríguez-Andrade¹, A. M. Stchigel¹*, A. Terrab², J. Guarro¹ and J. F. Cano-Lira¹

Abstract

Fungi can colonize most of the substrata on Earth. Honey, a sugary food produced by bees (and other insects) has been studied little in terms of its fungal diversity. We have surveyed and evaluated the presence of xerotolerant and xerophilic fungi in a set of honey bee samples collected from across Spain. From 84 samples, a total of 104 fungal strains were isolated, and morphologically and phylogenetically characterized. We identified 32 species distributed across 16 genera, most of them belonging to the ascomycetous genera Aspergillus, Bettsia, Candida, Eremascus, Monascus, Oidiodendron, Penicillium, Skoua, Talaromyces and Zygossaccharomyces. As a result of this survey, eight new taxa are proposed: i.e. the new family Helicoarthrosporaceae, two new genera, Helicoarthrosporum and Strangyoarthrosporum in Onygenales; three new species of Eurotiales, Talaromyces affinitatimellis, T. basipetosporus, and T. bruneosporus; and two new species of Myxotrichaceae, Oidiodendron mellicola, and Skoua asexualis.

Keywords: Eurotiales, Fungi, Honey, New taxa, Onygenales, Osmophiles, Xerophiles

INTRODUCTION

Honey is a natural sweetener produced by honey bees (insects of the genus Apis of the order Hymenoptera) from nectar (blossom honey or nectar honey) or from carbohydrate-rich secretions of living green parts of plants or excretions of plant-sucking phytophagous aphids (insects of the family Aphididae, order Hemiptera) (honeydew honey) after combination with the bee’s specific substances, placement, dehydration, and storage in the honey comb to ripen and mature. Honey is mostly composed of monosaccharides (dextrose and fructose), at a concentration of not lower than 60% and a much lesser amount of oligosaccharides, organic acids, enzymes (amylases and α-glucosidase) and solid particles. Due to its particular physicochemical nature and biological origin, honey should be an ideal substratum for the development of xerotolerant and xerophilic fungi. However, little information has been gathered about these fungi and their relationships with honey and honey products. Nonetheless, most of the fungal species from honey had been reported as new for science.

Representative ascomycetous yeasts found in honey are Blastobotrys meliponae, Candida lundiana, C. magnoliae, C. sorbivorans, C. suthepensis, Schizosaccharomyces octosporus, Trichosporon mucoides, Zygossaccharomyces favi, Z. mellis, Z. richteri, Z. rouxii, and Z. siamensis (Lochhead & Farrell 1931; Ruiz-Argués & Rodriguez-Navarro 1975; Carvalho et al. 2010; Saksinchai et al. 2012a, b; Čadež et al. 2015; Crous et al. 2016). The obligate xerophiles Ascosphaera apis and Bettsia alvei have been reported in honey, as well as several xerotolerant species of Alternaria, Aspergillus, Cladosporium and Penicillium and a few mucoralean fungi (Snowdon & Cliver 1996; Kačáňiová et al. 2009; Pettersson & Leong 2011; Kačáňiová et al. 2012; Sinacori et al. 2014; Grabowski & Klein 2015). Recently, Monascus mellicola, Penicillium apimei, P. meliponae, P. mellis, and Talaromyces brasiliensis were reported from honey produced by stingless bees (Melipona scutellaris, family Apidae, order Hymenoptera) inhabiting Brazilian forests (Barbosa et al. 2017, 2018). Common environmental and plant pathogenic species of fungi have been reported in samples of honey collected in Spain (Pérez-Sánchez et al. 1997; Seijo et al. 2011; Magyar et al. 2016; Terrab et al. 2019) and Portugal (Martins et al. 2003). In another study, the yeast Metschnikowia reukaufii was.
surprisingly, the only fungus reported for floral honey from Portugal and Spain (Magyar et al. 2005). Although honey should be a substratum amenable for the development of xerotolerant and xerophilic fungi, few studies have intentionally targeted these fungi. Therefore, the main objective of this study was to assess the diversity of honey-associated fungi, by employing a selective culture medium to a set of samples collected predominantly in Spain, and to characterize the morphology, physiology and phylogeny of new isolates and those considered of taxonomic interest.

**MATERIALS AND METHODS**

**Fungal isolation**

A total of 83 samples of honeydew and blossom (nectar) honey from different locations in Spain (Fig. 1), and one from Argentina (San Martín, Buenos Aires province), have been processed. All samples were of the harvest in 2014, stored in settling tanks, and after a variable period of time clarified by filtration (with one exception, which was by centrifugation). Seventy-two of the Spanish samples corresponded to honeydew honeys, 45 from trading companies and 27 collected and processed by beekeepers. A few of the samples provided by commercial companies were categorized (according to the nature of the honeydew) as oak, holm oak and forest honey. The 11 samples of blossom honey were provided by beekeepers, and these were classified as multifloral. All samples provided by commercial companies were subjected to a thermal treatment, subjecting the honey at 45–55 °C for a few hours up to 2 days, or pasteurized (2 min at 80 °C). The samples provided by beekeepers have not undergone any heat treatment. For each sample, 10 g of honey was dissolved into 90 mL of sterile water in a sterile disposable plastic container, and 1 mL of such dilution (1:10) was aseptically plated onto two 90 mm diam. plastic Petri dishes and mixed with 15 mL of molten (at 50–55 °C) 18% glycerol agar (G18; DG18 [Hocking & Pitt 1980] without dichloran: 5 g peptone, 10 g dextrose, 1 g KH2PO4, 0.5 g MgSO4·7H2O, 15 g agar-agar, 1 L tap water, and supplemented with 250 mg/L of L-chloramphenicol). Once the medium had solidified, one of the Petri dishes was incubated in darkness at 15 °C and the other at 25 °C for up to 2 months. The colonies developed were examined under a stereomicroscope. Fungal structures from selected (representative of...
all morphological variety) colonies were transferred to 50 mm diam. Petri dishes containing G18 by using a sterile insulin-type needle and incubated in the same conditions to obtain pure cultures.

**Phenotypic study**

For cultural characterization, suspensions of spores from the isolates were prepared in a semi-solid medium (0.2% agar; 0.05% Tween 80), and 0.5 μL of such suspension was inoculated onto malt extract agar (MEA; Difco, Detroit, USA; Samson et al. 2010), oatmeal agar (OA; 30 g of filtered oat flakes, 15 g agar-agar, 1 L tap water; Samson et al. 2010), Czapek yeast extract agar (CYA; 30 g sucrose, 3 g NaNO₃, 5 g yeast extract, 1 g K₂HPO₄, 0.5 g KCl, 0.5 g MgSO₄·7H₂O, 0.01 g FeSO₄, 15 g agar-agar, 1 L tap water; Pitt 1979), yeast extract sucrose agar (YES; 20 g yeast extract, 150 g sucrose, 0.5 g MgSO₄·7H₂O, 20 g agar-agar, 1 L tap water; Frisvad 1981), creatine sucrose agar (CREA; 3 g creatine, 30 g sucrose, 1.6 g K₂PO₄·7H₂O, 0.5 g MgSO₄·7H₂O, 0.5 g KCl, 0.01 g FeSO₄·7H₂O, 0.05 g bromocresol purple, 20 g agar-agar, 1 L tap water; Frisvad 1981), G18, potato dextrose agar (PDA; Pronadisa, Madrid, Spain; Hawksworth et al. 1995), 25% glycerol nitrate agar (G25 N; 7.5 g Czapek concentrate, 0.75 g K₂HPO₄, 3.7 g yeast extract, 250 mL glycerol, 12 g agar-agar, 1 L tap water; Pitt 1979), bromocresol purple milk solids glucose agar (BCP-MS-G; 80 g skim milk powder, 40 g glucose, 10 mL of 1.6% of bromocresol purple in 95% ethanol, 30 g agar-agar, 1 L tap water; Kane & Smitka 1978), test opacity tween medium (TOTM; 10 g bacteriological peptone, 5 g NaCl, 1 g CaCl₂, 5 mL Tween 80, 15 g agar-agar, 1 L tap water; Slifkin 2000), phytoye extract agar (PYE; Becton, Dickinson & Co., Sparks, MD, USA; Carmichael & Kraus 1959), malt extract agar 70% fructose-glucose (MY70FG; 6 g malt extract, 6 g yeast extract, 10 g peptone, 350 g fructose, 350 g glucose, 12 g agar-agar, 1 L tap water; Beuchat & Hocking 1990), and blood agar (Becton, Dickinson & Co., Sparks, MD, USA). Colonies were characterized after three wk. at 25 °C in darkness. G18 medium was used to determine the minimum, optimal and maximum temperatures of growth. Christensen’s urea agar (EMD Millipore, Darmstadt, Germany; Christensen 1946) was inoculated and incubated during 4–7 days at 25 °C in darkness to detect the production of urease. Cycloheximide tolerance of the fungal strains was tested on Sabouraud dextrose agar (SDA; Pronadisa, Spain) supplemented with 0.2% of cycloheximide (Sigma, USA) after incubation at 30 °C for two wk. Fungal tolerance to NaCl was evaluated on SDA adding 3, 10 and 20% w/v NaCl, with the same incubation conditions as in the previous test. Colour notations were according to Kornerup & Wanscher (1978). The microscopic structures were characterized and measured from wet mountings of slide cultures, using water and 60% lactic acid. Photo micrographs were taken using a Zeiss Axio Imager M1 light microscope (Oberkochen, Germany) with a DeltaPix Infinity X digital camera, using Nomarski differential interference contrast. The samples for scanning electron microscopy (SEM) were processed according to Figueras & Guarro (1988), and SEM micrographs were taken at 15 keV with a JEOL JSM 840 microscope.

**DNA extraction, amplification and sequencing**

Total deoxyribonucleic acid (DNA) was extracted according to Marimon et al. (2006), and a fragment of the 28S nrRNA gene (LSU) was amplified and sequenced using the primer pair LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990). For some isolates the following markers were amplified and sequenced: ribosomal internal transcribed spacers (ITS) (ITS5/ITS4; White et al. 1990); and fragments of the beta-tubulin (BenA) (Bt2a/Bt2b; Glass & Donaldson 1995), calmodulin (CaM) (Cmd5/Cmd6; Hong et al. 2005) and RNA polymerase II subunit 2 (rpB2) (RPB2-5F/RPB2-7cR; Liu et al. 1999) genes. Amplicons were sequenced at Macrogen Europe (Macrogen, Amsterdam, The Netherlands). Consensus sequences were obtained using the SeqMan software v. 7 (DNAStar Lasergene, Madison, WI, USA). Sequences were generated were deposited in GenBank (Table 1).

**Phylogenetic analysis**

A preliminary molecular identification of the isolates was carried out with LSU sequences using Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and only the type sequences or reliable reference strains from GenBank were considered for identification, and a maximum level of identity (MLI) of ≥98% was used for identification at the rank of species and <98% at the rank of genus. BenA for to the genera Aspergillus, Penicillium, and Talaromyces, and ITS for the genera Monascus, Oidiodendron and Skoua were used for identification at the rank of species. An LSU tree was built to determine the phylogenetic relationships of all our isolates. Phylogenetic trees of ITS and a combination of ITS-2 were also built to distinguish the members of Myxotrichaceae and the genus Talaromyces, respectively. Cunninghamella bertholletiae (CBS 693.68), Mucor plumbeus (DAOM 220743), Mucor racemosus (ATCC 42647), and Rhizopus oryzae (CBS 112.07 and CBS 130416) were used as outgroup for the LSU tree; Aphanomyces keratinophilus (IMI 319010) for the Myxotrichaceae taxa tree; and Trichocoma paradoxa (CBS 247.57) for the Talaromyces tree. The sequence alignments and the maximum-
Table 1 Fungal taxa recovered with their nucleotide sequence accession number, and the geographic origin of the honey samples processed.

| Taxon                          | Culture collection accession number | EMBL/GenBank nucleotide sequence accession number | Geographic origin (province, community) |
|-------------------------------|-----------------------------------|-------------------------------------------------|----------------------------------------|
| *Alternaria multiformis*      | FMR 16018                         | LT963545 LT963546                               | Salamanca, Castilla y León             |
| *Ascosphaera atra*            | FMR 16318                         | LT964944 LT984552                               | Cáceres, Extremadura                  |
| *Aspergillus asperescens*     | FMR 16310                         | LT963510 LT986672                               | Zamora, Castilla y León               |
| *Aspergillus montevidensis*   | FMR 15994                         | LR027804 LT963466                               | Castellón, Valencia                   |
| *Aspergillus pseudoglaucus*   | FMR 9392                          | LT963510 LT963466                               | Castellón, Valencia                   |
| *Aspergillus pseudoglaucus*   | FMR 15992                         | LT963512 LT984695                               | Castellón, Valencia                   |
| *Aspergillus pseudoglaucus*   | FMR 16011                         | LT963515 LT984698                               | Ciudad Real, Castilla-La Mancha       |
| *Aspergillus pseudoglaucus*   | FMR 16011                         | LT963516 LT984699                               | Ciudad Real, Castilla-La Mancha       |
| *Aspergillus pseudoglaucus*   | FMR 16317                         | LT963517 LT984700                               | Zamora, Castilla y León               |
| *Bettsia alvei*               | FMR 15670                         | LT963566                                        | Castellón, Valencia                   |
| *Bettsia alvei*               | FMR 15672                         | LT963567                                        | Castellón, Valencia                   |
| *Bettsia alvei*               | FMR 15678                         | LT963568                                        | Castellón, Valencia                   |
| *Bettsia alvei*               | FMR 15681                         | LT963569                                        | Castellón, Valencia                   |
| *Bettsia alvei*               | FMR 15685                         | LT963570                                        | Castellón, Valencia                   |
| *Bettsia alvei*               | FMR 16111                         | LT963571                                        | Cáceres, Extremadura                  |
| *Bettsia alvei*               | FMR 16115                         | LT963572                                        | Toledo, Castilla-La Mancha            |
| *Bettsia alvei*               | FMR 16305                         | LT963574                                        | Ourense, Galicia                      |
| *Bettsia alvei*               | FMR 16313                         | LT963575                                        | Ourense, Galicia                      |
| *Bettsia alvei*               | FMR 16568                         | LT963573                                        | Cáceres, Extremadura                  |
| *Bettsia alvei*               | FMR 16570                         | LT963576                                        | Ourense, Galicia                      |
| *Candida magnoliae*           | FMR 16311                         | LT963487                                        | Ourense, Galicia                      |
| *Candida magnoliae*           | FMR 16314                         | LT963488                                        | Ourense, Galicia                      |
| *Candida magnoliae*           | FMR 16496                         | LT963486                                        | Ourense, Galicia                      |
| *Candida sorbosivorans*       | FMR 16278                         | LT963489                                        | Ourense, Galicia                      |
| *Cunninghamella bertholletiae*| FMR 16008                         | LT963490 LR215930                               | Salamanca, Castilla y León            |
| *Eremascus albus*             | FMR 16116                         | LT964975                                        | Cáceres, Extremadura                  |
| *Eremascus albus*             | FMR 16118                         | LT964976                                        | Cáceres, Extremadura                  |
| *Eremascus albus*             | FMR 16119                         | LT964977                                        | Toledo, Castilla-La Mancha            |
| *Eremascus albus*             | FMR 16493                         | LT964978                                        | Cáceres, Extremadura                  |
| *Helicoarthrosporum mellicola*| FMR 15673                         | LT978462                                        | Castellón, Valencia                   |
| *Helicoarthrosporum mellicola*| FMR 15679 = CBS 143838            | LT906535                                        | Castellón, Valencia                   |
| *Helicoarthrosporum mellicola*| FMR 16307                         | LT978463                                        | León, castilla y León                 |
| *Monascus pilosus*            | FMR 16306                         | LT963491 LT984551                               | Zamora, Castilla y León               |
| *Monascus purpureus*          | FMR 16283                         | LR215932                                        | Avila, Castilla y León                |
| *Monascus purpureus*          | FMR 16316                         | LT963493 LT984550                               | Cáceres, Extremadura                  |
| *Monascus purpureus*          | FMR 16321                         | LT963494 LR215933                               | Cáceres, Extremadura                  |
| Taxon                      | Culture collection accession number | EMBL/GenBank nucleotide sequence accession number | Geographic origin (province, community)                  |
|---------------------------|-----------------------------------|--------------------------------------------------|--------------------------------------------------------|
| Monascus ruber            | FMR 16284                         | LT963495 LT986673                                 | Zamora, Castilla y León                                |
| Mucor plumbeus           | FMR 16012                         | LT963539 LT984540                                 | Ciudad Real, Castilla-La Mancha                        |
| Mucor plumbeus           | FMR 16013                         | LT963540 LT984540                                 | Salamanca, Castilla y León                            |
| Mucor plumbeus           | FMR 16017                         | LT963541 LT984548                                 | Salamanca, Castilla y León                            |
| Oidiodendron mellicola   | FMR 15680                         | LT906540 LT978465                                 | Tarragona, Catalonia                                  |
| Oidiodendron mellicola   | FMR 15683 = CBS 143839            | LT906544 LT978464                                 | Castellón, Valencia                                   |
| Oidiodendron mellicola   | FMR 16016                         | LT978506 LT978470                                 | Salamanca, Castilla y León                            |
| Oidiodendron mellicola   | FMR 16028                         | LT978507 LT978471                                 | Toledo, Castilla-La Mancha                            |
| Oidiodendron mellicola   | FMR 16029                         | LT978509 LT978473                                 | Burgos, Castilla y León                               |
| Oidiodendron mellicola   | FMR 16274                         | LT978508 LT978472                                 | Toledo, Castilla-La Mancha                            |
| Oidiodendron mellicola   | FMR 16503                         | LT978504 LT978468                                 | Ciudad Real, Castilla-La Mancha                        |
| Oidiodendron mellicola   | FMR 16504                         | LT978505 LT978469                                 | Ourense, Galicia                                      |
| Penicillium camemberti    | FMR 16016                         | LT963578 LT984541                                 | Salamanca, Castilla y León                            |
| Penicillium citrinum      | FMR 16028                         | LT963451 LT984548                                 | Salamanca, Castilla y León                            |
| Penicillium corylophilum  | FMR 16010                         | LT963581 LT984538                                 | Asturias                                               |
| Penicillium corylophilum  | FMR 16027                         | LT963452 LT986674                                 | Asturias                                               |
| Penicillium corylophilum  | FMR 16030                         | LT963582 LT984547                                 | Cáceres, Extremadura                                   |
| Penicillium cravenianum   | FMR 16019                         | LT963580 LT984542                                 | Salamanca, Castilla y León                            |
| Penicillium cravenianum   | FMR 16020                         | LT963579 LT984549                                 | Cáceres, Extremadura                                   |
| Rhizopus oryzae           | FMR 16022                         | LT963543 LR215931                                 | Cáceres, Extremadura                                   |
| Schizosaccharomyces octosporus | FMR 16279                      | LT963544 LR215931                                 | Ourense, Galicia                                      |
| Skoua asexualis           | FMR 16497                         | LT964666 LR964665                                 | Cáceres, Extremadura                                   |
| Skoua asexualis           | FMR 16567                         | LT964666 LR964667                                 | Cáceres, Extremadura                                   |
| Skoua asexualis T         | FMR 16572 = CBS 144072            | LR585993 LR586005                                 | León, castilla y León                                  |
| Skoua fertilis            | FMR 10812                         | LR585990 LR586005                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 10813                         | LR585994 LR586006                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 10814                         | LR585995 LR586007                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 10815                         | LR585996 LR586008                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 15671                         | LR586000 LR586012                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 15672                         | LR586001 LR586012                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 15682                         | LR585998 LR586010                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 15686                         | LR585999 LR586011                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 15687                         | LR586000 LR586012                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 15689                         | LR586001 LR586012                                 | Castellón, Valencia                                   |
| Skoua fertilis            | FMR 16032                         | LR585989 LR586002                                 | Asturias                                               |
| Skoua fertilis            | FMR 16320                         | LR585990 LR586005                                 | Zamora, Castilla y León                                |
likelihood (ML) and Bayesian-inference (BI) phylogenetic analyses were performed as described previously (Valenzuela-Lopez et al. 2018). The final matrices used for the phylogenetic analysis were deposited in TreeBASE (www.treebase.org; accession number: S23122).

Growth at different water activities ($a_w$)
To test the capacity of growth in different water activities, media containing malt extract (1% w/w), yeast extract (0.25% w/w) and agar-agar (1% w/w) at pH 5.3 were adjusted at six different $a_w$ (0.97, 0.95, 0.93, 0.92, 0.88 and 0.82) by adding equal weights of fructose and glucose (corresponding to 22, 30, 40, 44, 48, and 55% w/w of sugars, respectively) (Pitt & Hocking 1977). Water activity was measured in duplicate by a water activity meter (Aqualab, Decagon Devices CX3 02734) with an accuracy of ±0.002 at 25 °C. Triplicate plates were inoculated at their centre with 5 μL of spore suspension of selected fungi, and incubated at 25 °C in darkness, with the exception of FMR 15880, FMR 15883 and FMR 16031, which were at 15 °C (because of their poor growth at 25 °C). The colony diam. was measured after 21 days.

RESULTS
Fungal diversity
All honey samples produced fungal colonies on G18 at 15 °C as well as at 25 °C. Table 1 summarizes the fungal strains identified phenotypically and molecularly. With

| Taxon                                      | Culture collection accession number | EMBL/GenBank nucleotide sequence accession number | Geographic origin (province, community) |
|--------------------------------------------|-------------------------------------|--------------------------------------------------|----------------------------------------|
| Skoua fertilis                            | FMR 16492                           | --                                               | Cáceres, Extremadura                   |
| Skoua fertilis                            | FMR 16571                           | LRS86002  LRS86013  LRS85992                      | Badajoz, Extremadura                  |
| Strongyloarthrosporum catenulatum $^T$     | FMR 16121 = CBS 143841              | --                                               | Toledo, Castilla-La Mancha            |
| Talaromyces affinitatimellis              | FMR 15674                           | LT965001  --                                      | Tarragona, Catalonia                  |
| Talaromyces affinitatimellis              | FMR 15675                           | LT965002  --                                      | Tarragona, Catalonia                  |
| Talaromyces affinitatimellis              | FMR 15677                           | LT965003  --                                      | Tarragona, Catalonia                  |
| Talaromyces affinitatimellis              | FMR 15684                           | LT965004  --                                      | Castellón, Valencia                   |
| Talaromyces affinitatimellis              | FMR 15688                           | LT906553  LT906550  LT906547  LT906538           | Castellón, Valencia                   |
| Talaromyces affinitatimellis $^T$          | FMR 15690 = CBS 143840              | LT906552  LT906549  LT906546  LT906543           | Castellón, Valencia                   |
| Talaromyces affinitatimellis              | FMR 16029                           | LT965005  --                                      | Cáceres, Extremadura                  |
| Talaromyces affinitatimellis              | FMR 16033                           | LT906554  LT906551  LT906548  LT906539           | Salamanca, Castilla y León            |
| Talaromyces affinitatimellis              | FMR 16114                           | LT965006  --                                      | Salamanca, Castilla y León            |
| Talaromyces affinitatimellis              | FMR 16125                           | LT965009  --                                      | Zamora, Castilla y León               |
| Talaromyces affinitatimellis              | FMR 16126                           | LT965012  --                                      | Zamora, Castilla y León               |
| Talaromyces affinitatimellis              | FMR 16276                           | LT965010  --                                      | Zamora, Castilla y León               |
| Talaromyces affinitatimellis              | FMR 16494                           | LT965007  --                                      | Cáceres, Extremadura                  |
| Talaromyces affinitatimellis              | FMR 16501                           | LT965008  --                                      | Cáceres, Extremadura                  |
| Talaromyces basipetosporus $^T$            | FMR 9720 = CBS 143836              | LT906563  --                                      | Buenos Aires, Argentina               |
| Talaromyces brunneosporus $^T$             | FMR 16566 = CBS 144320              | LT962483  LT962488  LT962485  LT962487           | Salamanca, Castilla y León            |
| Xerocystis xerophilum                     | FMR 15669                           | --                                               | Castellón, Valencia                   |
| Zygosaccharomyces gambellaensis           | FMR 16277                           | --                                               | Salamanca, Castilla y León            |
| Zygosaccharomyces gambellaensis           | FMR 16569                           | --                                               | Cáceres, Extremadura                  |
| Zygosaccharomyces mellis                  | FMR 16280                           | --                                               | Ourense, Galicia                      |
| Zygosaccharomyces siamensis               | FMR 16312                           | --                                               | Ourense, Galicia                      |
| Zygosaccharomyces siamensis               | FMR 16034                           | --                                               | Salamanca, Castilla y León            |

FMR = Faculty of Medicine of Reus culture collection; CBS = Westerdijk Fungal Biodiversity Institute (ex Centraalbureau voor Schimmelcultures). $^T$ = ex type

The results of the fungal diversity analysis for each honey sample are presented in Table 1. The table includes the fungal taxa recovered with their nucleotide sequence accession number, and the geographic origin of the honey samples processed.

Table 1 Fungal taxa recovered with their nucleotide sequence accession number, and the geographic origin of the honey samples processed (Continued)

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Fungal diversity
All honey samples produced fungal colonies on G18 at 15 °C as well as at 25 °C. Table 1 summarizes the fungal strains identified phenotypically and molecularly. With
the exception of a few ascomycetous yeasts and of Mucorales, most of the fungi were filamentous Ascomycota. From the latter, the highest number of strains corresponded to Skoua (syn. Eremascus) fertilis, Bettisia alvei, and Oidiodendron sp., followed by an unknown arthrosptic fungus, Eremascus albus and Skoua sp. Alternaria multiformis, Ascosphaeraatra, another unknown arthrosptic fungus and Xerochrysium xerophilum were isolated only once. Obligate xerophilic species of Aspergillus were not found, but the xerotolerant A. pseudoglaucus, A. asperescens and A. monteviendensis were isolated. Three species of Monascus were identified, i.e. M. pilosus, M. purpureus, and M. ruber. The isolates of Penicillium were classified as P. camemberti, P. citrinum, P. corylophilum, and P. cravenianum. Members of Talaromyces were classified at the rank of section, i.e. section Trachyspermi and section Purpurei. We only identified three species of Mucoromycota, viz. Cunninghamhella bertholletiae, Mucor plumbeus, and Rhizopus oryzae. Regardless of their geographical origin, type of honey (nectar or honeydew) and if honey was or not thermally treated, S. fertilis and B. alvei were present in all honey samples.

Molecular phylogeny

Our first phylogenetic study included 206 LSU sequences with a total of 606 characters, including gaps, 352 of them being parsimony informative. The ML analysis was congruent with that obtained in the BI analysis, both displaying trees with similar topologies. The isolates were distributed across two main clades (Fig. 2a-c), the first (100% BS / 1 PP) corresponding to the Ascomycota and including 99 isolates, and the second (100% BS / 1 PP) involving the rest of the isolates and pertaining to the Mucoromycota. The first main clade was divided into six subclades: A (82% BS / 1 PP), which represents Onygenales; B (75% BS / 0.96 PP), Eurotiales; C (100% BS / 1 PP); Pleosporales, D (unsupported) as incertae sedis; E (100% BS / 1 PP), Schizosaccharomyceutes, and F (94% BS / - PP), Saccharomyceutes. Subclade A contains seven well-supported groups, six of which represent the known families of Onygenales, i.e. Gymnosacccaeae (A1), Arthrodarmatacaceae (A3), Nannizziopiasiaceae (A4), Eremascaceae (A7), Ascosphaeracea (A8), and Spioromastigaceae (A9), and a seventh group (A5) composed of five of our strains probably representing a new family. The groups representing Ajellomyceceae (A6) and Onygenaceae (A2) were unsupported. Strains in subclade A were distributed as follows: the five mentioned above into A5, FMR 16121 into a separate branch of the Ajellomyceceae (A6), four strains conspecific with Eremascus albus (A7), and one (FMR 16318) identified as Ascosphaeraatra (A8). Thirty-nine strains were placed in Eurotiales (Subclade B). One (FMR 16566) was placed together with Talaromyces flavus and T. kabodanensis in an unsupported branch, and 16 strains near to T. miniolutes into a well-supported sister clade (B1). Into B2 (unsupported), which includes species of Aspergillus, eight of the strains were placed in a branch (99% BS / 1 PP) together with A. glaucus, A. monteviendensis and A. pseudoglaucus (sect. Aspergillus). For the final identification of these eight strains, we used BenA sequence comparison, which were found to be A. monteviendensis (one strain) and A. pseudoglaucus (seven strains). FMR 16310 was placed in a branch together with the ex-type sequence of A. asperescens (sect. Nidulantes). Seven strains grouped into the sister clade B3 (unsupported), representing five species of Penicillium. FMR 15669 was identified as Xerochrysium xerophilum (B4), and five strains were initially identified as Monascus spp. Based on the comparison of ITS sequences, these five strains were finally identified as M. pilosus (one strain), M. purpureus (three strains), and M. ruber (one strain). Strain FMR 16018 was located together with Alternaria multiformis (Subclade C, Pleosporales). Subclade D (unsupported) was divided into three groups: D1, representing the Myxotrichaceae; D2, the genus Skoua; and D3, the Pseudotrichaceae. This group had 38 strains, 10 among the genera Oidiodendron and Myxotrichum (D1), 17 together with Skoua fertilis (D2), and 11 within Bettisia alvei (D3). Subclade E (Schizosaccharomyceutes), grouped FMR 16279 together with the ex-type sequence of Schizosaccharomyceoctosporus. Subclade F (Saccharomyceutes), had nine strains belonging to Zygosaccharomyceetes spp. (five strains) and Candida spp. (four strains). Clade G had 5 strains, Mucorales, divided into three groups that comprised Mucor spp. (three strains), Cunninghamhella bertholletiae (FMR 16008) and Rhizopus oryzae (FMR 16022), respectively. Figures 3, 4 show the trees resulting from the phylogenetic analyses of Myxotrichaceae and Talaromyces, respectively. The phylogenetic tree based on the analysis of the ITS (Fig. 3), included 67 sequences belonging to Myxotrichaceae and Pseudotrichaceae, whose alignments encompassed a total of 547 characters, including gaps, 204 of which were parsimony informative. The ML and BI analyses showed a similar tree topology. It comprised a main clade of Myxotrichaceae, where 20 strains were located, 17 of Skoua (14 identified as S. fertilis), and the remaining three in a separate branch that might represent a new species of the genus. Finally, three strains phylogenetically distant from the others appeared in a separate branch close to Myxotrichum setosum and Oidiodendron truncatum. The tree based on four concatenated loci (BenA, CaM, rpb2 and ITS; Table 2; Fig. 4) was built to resolve the phylogenetic relationships of the Talaromyces strains. The dataset contained 123 sequences with a total of 2265 characters, including gaps, (520 of them for ITS, 377 for BenA, 516 for CaM and 852 for rpb2), of which
Members of Mucoromycota were chosen as out-group. Support in nodes is indicated above thick branches and is represented by posterior probabilities (BI analysis) of 0.95 and higher and/or bootstrap values (ML analysis) of 70% and higher. Fully supported branches (100% BS / 1 PP) are indicated in bold. T = ex type. Alignment length 606 bp. The sequences generated by us are in Table 1.
Fig. 3 ML phylogenetic tree based on the analysis of ITS nucleotide sequences of representative taxa of the families *Myxotrichaceae* (in grey background) and *Pseudeurotiaceae*. *Aphanoascus keratinophilus* IMI 319010 was chosen as out-group. Support in nodes is indicated above thick branches and is represented by posterior probabilities (BI analysis) of 0.95 and higher and/or bootstrap values (ML analysis) of 70% and higher. Fully supported branched (100% BS/1 PP) are indicated in bold. T = ex type. Alignment length 544 bp.
Fig. 4 ML phylogenetic tree built using the ITS, BenA, CaM and rpb2 concatenated dataset for species of the genus Talaromyces. Species of the section *Trachyspermi* are indicated in a blue background and those of the section *Purpurei* in yellow. *Trichocoma paradoxa* CBS 247.57 was chosen as out-group. Support in nodes is indicated above thick branches and is represented by posterior probabilities (BI analysis) of 0.95 and higher and/or bootstrap values (ML analysis) of 70% and higher. Fully supported branched (100% BS / 1 PP) are indicated in bold. T = ex-type strain.

Alignment length 2265 bp
| Species name            | Section          | Strain no.                  | GenBank accession #                  |
|------------------------|------------------|-----------------------------|-------------------------------------|
|                        |                  |                             | BenA | CalM | gap2 | ITS       |
| Talaromyces aculeatus  | Talaromyces      | CBS 289.48 = IMI 040588 = NRRL 2129 | KF741929 | KF741975 | KM023271 | KF741995 |
| Talaromyces adpressus  | Talaromyces      | CBS 140620 = CGMC3.18211 = DTO 317-G4 | KU866644 | KU866741 | KU867001 | KU866657 |
| Talaromyces alveolaris | Talaromyces      | UTHSC DI16–146             | LT559085 | LT795594 | LT795595 | LT558968 |
| Talaromyces amazonensis| Talaromyces      | CBS 140373 = IBT 23215 = DTO 093-F9 | KX011490 | KX011502 | –          | KX011509 |
| Talaromyces aberystalkiae| Talaromyces    | CBS 132696 = D TO 179-F5       | JX315623 | KF741937 | JX315698 | JX315660 |
| Talaromyces angelicae  | KACC 46611      |                             | KF183640 | KJ885259 | –          | KF183638 |
| Talaromyces apiculatus | CBS 312.59 = FRR 635 = IMI 068239 |                             | KF741916 | KF741950 | KM023287 | JN899375 |
| Talaromyces calidicanius| Talaromyces    | LT558968                   | –          | –          | –          | –          |
| Talaromyces caudicatus  | Talaromyces      | CBS 310.38 = IMI 197477 = NRRL 2098 | JX494305 | KF741959 | KM023282 | JN899327 |
| Talaromyces caudicatus  | Talaromyces      | CBS 311.34 = IBT 23221 = DTO 056-D9 | KX011489 | KX011501 | KX011510 | JN899342 |
| Talaromyces eucarpius   | Talaromyces      | CBS 140607 = CGMC3.18200 = DTO 317-D8 | KU866837 | KU866733 | KU866993 | JN899369 |
| Talaromyces flavovirens | Talaromyces      | CBS 102801 = IBT 27044       | JX091376 | KF741933 | –          | JN899319 |
| Talaromyces flavus      | Talaromyces      | CBS 112002                  | –          | –          | –          | –          |
| Talaromyces flavus      | Talaromyces      | CBS 310.38 = IMI 197477 = NRRL 2098 | JX494305 | KF741949 | JF417426 | JN899360 |
| Talaromyces galapagensis| Talaromyces      | CBS 751.74 = IFO 31796       | KJ865733 | KJ885271 | KM023303 | AB176617 |
| Talaromyces fusiformis  | Talaromyces      | CBS 140637 = CGMC3.18210 = DTO 317-F4 | KU866843 | KU866740 | KU867000 | KU866656 |
| Talaromyces galapagensis| Talaromyces      | CBS 113134 = IBT 23221 = DTO 056-D9 | KX011489 | KX011501 | –          | JN899342 |
| Talaromyces fusiformis  | Talaromyces      | CBS 140637 = CGMC3.18210 = DTO 317-F4 | KU866843 | KU866740 | KU867000 | KU866656 |
| Talaromyces fusiformis  | Talaromyces      | CBS 113134 = IBT 23221 = DTO 056-D9 | KX011489 | KX011501 | –          | JN899342 |
| Talaromyces intermedius | Talaromyces      | CBS 152.65 = BDUN 267 = IFO 31752 = IMI 100874 | JX091387 | KJ885290 | –          | JN899332 |
| Talaromyces kambodanensis| Talaromyces     | DI16–149                   | LT559088 | LT795598 | LT795599 | LT558971 |
| Talaromyces liani      | Talaromyces      | CBS 225.66 = IMI 098480 = NRRL 3380 = VAM F-301 | JX091380 | KJ885257 | –          | JN899395 |
| Talaromyces macrosporus| Talaromyces      | CBS 317.63 = FRR 404 = IMI 197478 | JX091382 | KF741952 | KM023292 | JN899333 |
| Talaromyces mangshanicus| Talaromyces      | CGMCC 3.18013             | KX447530 | KX447528 | KX447527 | KX447531 |
| Talaromyces marneffei   | Talaromyces      | CBS 388.87                 | JX091389 | KF741958 | KM023283 | JN899344 |
| Talaromyces muroii      | Talaromyces      | CBS 756.96 = PF 1153       | KJ865727 | KJ885274 | –          | JN899351 |
| Talaromyces neofusisporus| Talaromyces     | AS3.15415 = CBS 139516     | KP765381 | KP765383 | –          | KP765385 |
| Talaromyces nuijiae     | Talaromyces      | CBS 138208 = DTO 269-E8    | KJ775213 | KJ775425 | –          | KJ775720 |
| Talaromyces panamensis  | Talaromyces      | CBS 128.89 = IMI 297546    | HQ156948 | KF741936 | KM023284 | JN899362 |
| Talaromyces paucisporus | Talaromyces      | PF 1150 = IFM 53616       | –          | –          | –          | AB176603 |
| Talaromyces pinophilus  | Talaromyces      | CBS 631.66 = CECT 2809 = DSM 1944 = IAM 7013 = IMI 114933 | JX091381 | KF741964 | KM023291 | JN899382 |
Table 2  Talaromyces spp. nucleotide sequences employed to build a phylogram to locate phylogenetically our strains from honey (Continued)

| Species name | Section | Strain no. | GenBank accession # |
|--------------|---------|------------|---------------------|
| **BenA** | CaM | rpb2 | ITS |
| Talaromyces primulinus | **Talaromyces** | CBS 321.48 = CBS 439.88 = FRR 1074 = IMI 040031 = MUCL 31321 = NRRL 1074 | JX494305 | KF741954 | KM023294 | JN899317 |
| Talaromyces purgamentorum | **Talaromyces** | CBS 113145 = IBT 23220 = DTO 056-E1 | KX011147 | KX011500 – | KX011504 |
| Talaromyces purpureogenus | **Talaromyces** | CBS 286.36 = IMI 091926 | JX315639 | KF741947 | JX315709 | JN899372 |
| Talaromyces qii | **Talaromyces** | AS3.15414 = CBS 139515 = IMI 091926 | JX315629 | KF741938 | JX315700 | JX315662 |
| Talaromyces rapidus | **Talaromyces** | UTHSC DI16–148 = CBS 142382 T | LT559087 | LT795600 | LT795601 | LT558970 |
| Talaromyces ruber | **Talaromyces** | CBS 132704 = DTO 193-H6 = IBT 10703 = CBS 113137 | JX315629 | KF741938 | JX315700 | JX315662 |
| Talaromyces rubicundus | **Talaromyces** | CBS 342.59 = IMI 099723 = NRRL 3400 | JX494309 | KF741956 | KM023296 | JN899384 |
| Talaromyces sayulitensis | **Talaromyces** | CBS 138204 = DTO 245-H1 | KJ775206 | KJ775422 – | KJ775713 |
| Talaromyces siamensis | **Talaromyces** | CBS 475.88 = IMI 323204 | JX091379 | KF741960 | KM023279 | JN899385 |
| Talaromyces stipitatus | **Talaromyces** | CBS 375.48 = NRRL 1006 = IMI 39805 | KM111288 | KF741957 | KM023280 | JN899438 |
| Talaromyces stollii | **Talaromyces** | CBS 408.93 | – | JX315646 | JX315712 | JX315674 |
| Talaromyces thailandensis | **Talaromyces** | CBS 133147 = KUFC 3399 | JX494294 | KF741940 | KM023307 | JN899031 |
| Talaromyces verruculosus | **Talaromyces** | CBS 388.48 = DSM 2263 = IMI 040039 = NRRL 1050 | JX494192 | KF741944 | KM023306 | KF741994 |
| Talaromyces viridis | **Talaromyces** | CBS 114.72 = ATCC 22467 = NRRL 5575 | JX494310 | KF741935 | JN121430 | AF285782 |
| Talaromyces viridulus | **Talaromyces** | CBS 252.87 = FRR 1863 = IMI 288716 | JX091389 | KF741943 | JF417422 | JN899314 |
| Talaromyces aenigerinus | **Helici** | CBS 350.66 = BDUN 276 = IMI 105412 | KJ865736 | KJ885285 | JN121502 | AY753346 |
| Talaromyces bohemicus | **Helici** | CBS 545.86 = CCF 2330 = IAM 14789 | KJ865719 | KJ885280 | JN121532 | JN899400 |
| Talaromyces boninensis | **Helici** | CBS 650.95 = IBT 17516 | KJ865721 | KJ885263 | KM023276 | JN899356 |
| Talaromyces cinnabarinus | **Helici** | CBS 267.72 = NHL 2673 | AY753377 | KJ885256 | JN121477 | JN899376 |
| Talaromyces diversiformis | **Helici** | CBS 141931 = CGMCC3.18204 = DTO 317-E3 | KX961216 | KX961259 | KX961274 | KX961215 |
| Talaromyces georgiensis | **Helici** | UTHSC DI16–145 = CBS 142380 | LT559084 – | LT795600 | LT558967 |
| Talaromyces helicus | **Helici** | CBS 335.48 = DSM 3705 = IMI 040593 = NRRL 2106 | KJ865725 | KJ885289 | KM023273 | JN899359 |
| Talaromyces reverso-olivaceus | **Helici** | CBS 140672 = CGMCC3.18195 = DTO 317-C3 | KU866834 | KJ866730 | KU866990 | KU866646 |
| Talaromyces ryukyuensis | **Helici** | CBS 291.80 = NHL 2917 = DTO 176-16 | – | – | – | AB176628 |
| Talaromyces varians | **Helici** | CBS 386.48 = IMI 040586 = NRRL 2096 | KJ865731 | KJ885284 | KM023274 | JN899368 |
| Talaromyces cephalica | **Purpurei** | CBS 101419 = CAOM 233229 | JX755295 | KJ885287 | KM023309 | AY787844 |
| Talaromyces chlorolomus | **Purpurei** | DAOM 241016 = CV 2802 | GU385736 | KJ885265 | KM023304 | FJ160273 |
| Talaromyces coalescens | **Purpurei** | CBS 103.83 | JX091390 | KJ885267 | KM023277 | JN899366 |
| Talaromyces dendriticus | **Purpurei** | CBS 660.80 = IMI 216987 | JX091391 | KF741965 | KM023286 | JN899339 |
| Talaromyces pittii | **Purpurei** | CBS 139.84 = IMI 327871 | KJ865728 | KJ885275 | KM023297 | JN899325 |
| Talaromyces pseudostromaticus | **Purpurei** | CBS 470.70 = FRR 2039 | HQ156950 | KJ885277 | KM023298 | JN899371 |
| Talaromyces pychocondium | **Purpurei** | DAOM 241017 = CV 2808 = DTO 180-E7 | GU385733 | JX140701 | KM023278 | FJ160266 |
| Talaromyces purpureus | **Purpurei** | CBS 475.71 = FRR 1731 = IMI 181546 | GU385739 | KJ885292 | JN121522 | JN899328 |
| Species name                | Section       | Strain no. | GenBank accession # |
|-----------------------------|---------------|------------|---------------------|
| *Talaromyces rademirici*    | Purpurei      | CBS 140.84 | KJ865734            |
| *Talaromyces ramulosus*     | DAOM 241660   | CV 2837    | FJ753200            |
| *Talaromyces aerius*        | Trachyspermi  | CBS 133440 | KJ114778            |
| *Talaromyces assiutesis*    | Trachyspermi  | CBS 147.78 | KJ865720            |
| *Talaromyces atroroseus*    | Trachyspermi  | CBS 133442 | KJ114789            |
| *Talaromyces austrocalifornicus* | Trachyspermi | CBS 644.95 | KJ865732            |
| *Talaromyces convolutus*    | Trachyspermi  | CBS 100537 | KJ114773            |
| *Talaromyces diversus*      | Trachyspermi  | CBS 320.48 | KJ114783            |
| *Talaromyces erythromellis* | Trachyspermi  | CBS 644.80 | KJ114771            |
| *Talaromyces heineensis*    | CGMCC 3.18012 |          | X447525             |
| *Talaromyces minioluteus*   | Trachyspermi  | CBS 137.84 | KJ114798            |
| *Talaromyces minisotilis*   | Trachyspermi  | CBS 624.68 | KJ114799            |
| *Talaromyces mirabile*      | Trachyspermi  | CBS 264.72 | KJ114797            |
| *Talaromyces solicola*      | Trachyspermi  | CBS 373.4B | KJ114803            |
| *Talaromyces trachyspermus* | Trachyspermi  | CBS 162.67 | KJ114771            |
| *Talaromyces ucrainicus*    | Trachyspermi  | CBS 579.72 | KJ114706            |
| *Talaromyces yawatai*       | Bacillispori  | CBS 296.4B | AY753368            |
| *Talaromyces colombiensis*  | Bacillispori  | CBS 113151 | KX011488            |
| *Talaromyces emodensis*     | Bacillispori  | CBS 100536 | KJ865724            |
| *Talaromyces hachijoensis*  | Bacillispori  | PF 1174    | –                   |
| *Talaromyces mimosinus*     | Bacillispori  | CBS 659.80 | KJ865726            |
| *Talaromyces proteolyticus* | Bacillispori  | CBS303.67  | KJ865729            |
| *Talaromyces unicus*        | Bacillispori  | CBS 100535 | KJ865735            |
| *Talaromyces palmae*        | Subinflata    | CBS 442.8B | HQ156947            |
| *Talaromyces subinflatus*   | Subinflata    | CBS 652.95 | KJ865737            |
| *Talaromyces acaricola*     | Islandici     | CBS 137386 | JX091610            |
| *Talaromyces allahabadensis*| Islandici     | CBS 304.63 | KF984614            |
| *Talaromyces atricola*      | Islandici     | CBS 255.31 | KF984566            |
| *Talaromyces brunneus*      | Islandici     | CBS 227.60 | KF984719            |
| *Talaromyces cereus*        | Islandici     | CBS 140622 | KU866845            |
| *Talaromyces*               |              |            |                     |
1069 were parsimony informative (195 for ITS, 217 for BenA, 308 for CaM and 349 for rpb2). The sequence datasets did not show conflict in the tree topologies for the 70% reciprocal bootstrap trees, which allowed the multi-locus analysis. The ML analysis showed similar tree topology and was congruent with the Bayesian analysis. In this tree (Fig. 4), the five Talaromyces strains we obtained were located in two different clades: one corresponding to the section Trachyspermi (100% BS / - PP), with four strains phylogenetically distant from T. atroroseus, one of them (FMR 9720) in a separate branch; and the second corresponding to the section Purpurei (74% BS / - PP), where the fifth strain (FMR 16566) was located in a distant branch.

TAXONOMY

Subclade A: Onygenales

Based on the above phylogenetic analyses, we suggest the following novel taxonomic arrangements: Helicoarthrosporaceae fam. nov. (Fig. 2; sister clade A5), phylogenetically close to the family Gymnoascaceae, with Helicoarthrosporum gen. nov. as type genus and H. mellicola sp. nov. as the type species; based on the strain FMR 16121, we introduce Strongylothorosporum gen. nov. with S. catenulatum sp. nov. as its type species. These new taxa are described and illustrated below.

Helicoarthrosporaceae Stchigel, Rodr.-Andr. & Cano, fam. nov. MycoBank MB 832226.

Diagnosis: Differing from other families of Onygenales by the production of long, sinuous to helical chains of arthroconidia (which are shorter, right, curved or contorted in other taxa).

Type genus Helicoarthrosporum Stchigel et al. 2019.

Description: Hyphae hyaline, septate. Asexual morph reduced to sinuous, helical or zig-zag lateral branches, terminal part becoming fertile, disarticulating into conidia. Conidia hyaline, prismatic to cuboid, holom- and enterothecic conidia. Sexual morph not observed.

Helicoarthrosporum Stchigel, Cano & Rodr.-Andr., gen. nov. MycoBank MB 823584.

Etymology. From Greek ἔλικα-, helix, -άρθρωσι-, joint, and -σπόρα, spore, referring to the morphology of the conidiophores.

Table 2 Talaromyces spp. nucleotide sequences employed to build a phylogram to locate phylogenetically our strains from honey (Continued)

| Species name               | Section   | Strain no. | GenBank accession # |
|---------------------------|-----------|------------|---------------------|
|                           |           |            | BenA   | CaM   | rpb2 | ITS   |
| Chlamydocarpus             | Islandici | NRRL 58811 | KF196843 | KJ885288 | KM023270 | KJ865739 |
| Talaromyces columbinus     | Islandici | CBS 137381 = DTO 181-C5 = DAOM 241027 = IBT 32814 | JX091608 | JX140727 | KF84914 | JX091472 |
| Talaromyces crassus        | Islandici | CBS 137385 = DTO 182-12 = DAOM 241024 = IBT 32487 | JX091615 | JX140734 | KF849494 | JX091481 |
| Talaromyces islandicus     | Islandici | CBS 338.48 = IMI 040042 = MUCL 31324 = NRRL 1036 | KF984655 | KF984780 | KF985018 | KF984885 |
| Talaromyces loliensis      | Islandici | CBS 643.80 = FRR 1798 = IMI 216901 = MUCL 31325 | KF984658 | KF984783 | KF985021 | KF984888 |
| Talaromyces neorugulosus   | Islandici | CBS 140623 = CGMCC3.18215 = DTO 318-A8 | KJ866846 | KJ866743 | KJ867003 | KJ866659 |
| Talaromyces piceus         | Islandici | CBS 361.48 = IMI 040038 = NRRL 1051 | KF984668 | KF984680 | KF984899 | KF984792 |
| Talaromyces radicus        | Islandici | CBS 100489 = FRR 4718 | KF984599 | KF984773 | KF985013 | KF984878 |
| Talaromyces rotundus       | Islandici | CBS 369.48 = IMI 040589 = NRRL 2107 | KJ865730 | KJ885278 | KM023275 | JN899353 |
| Talaromyces rugulosus      | Islandici | CBS 371.48 = IMI 040041 = MUCL 31201 = NRRL 1045 | KF984575 | KF984702 | KF984925 | KF984834 |
| Talaromyces scorteus       | Islandici | CBS 340.34 = NRRL 1129 = FRR 1129 | KF984565 | KF984684 | KF984916 | KF984892 |
| Talaromyces subaurantius   | Islandici | CBS 137383 = DTO 181-12 = DAOM 241020 = IBT 32838 | JX091609 | JX140728 | KF849460 | LT558965 |
| Talaromyces tardifaciens   | Islandici | CBS 250.94 | KC20954 | KF984682 | KF984908 | JN899361 |
| Talaromyces tratensis      | Islandici | CBS 133146 = KUFC 3383 | KF984559 | KF984690 | KF984911 | KF984891 |
| Talaromyces wortmannii     | Islandici | CBS 391.48 = IMI 040047 = NRRL 1017 | KF984648 | KF984756 | KF984977 | KF984829 |
| Talaromyces yeleensis      | Islandici | DTO 268E5 | KJ775210 | – | – | KJ775717 |
| Trichocoma paradoxa        | –         | CBS 247.57 | JF417468 | JF417505 | JF417421 | JF417485 |
**Diagnosis:** Distinguished from other phylogenetically related genera by its long, sinuous to helical chains of prismatic to cuboid arthroconidia, and by its extreme xerotolerance.

**Type species:** *Helicoarthrosporum mellicola* Stchigel et al. 2019.

**Description:** Mycelium composed by hyaline, septate hyphae. Conidiophores consisting in fertile lateral branches and terminal part of the hyphae, sinuous, helical or zigzag, disarticulating in hyaline, mostly prismatic to cuboid, holo- and enteroarthric conidia. *Helicoarthrosporum mellicola* Stchigel, Cano & Rodr.-Andr., sp. nov. Fig. 5. MycoBank MB 823585.

**Etymology:** From Latin *mellis*-, honey, and *-cola*, to reside, referring to the habitat of the fungus.

**Diagnosis:** *Helicoarthrosporum mellicola* morphologically resembles *Scytalidium cuboideum* (syn. *Arthrographis cuboidea*), *S. ganodermophthorum*, and *S. sphaerosporum* in producing long chains of cuboid arthroconidia (Kang et al. 2010). *Helicoarthrosporum mellicola* grows slowly on PDA and shows a high xerotolerance, whereas *Scytalidium* spp. grow fast on PDA and do not show a xerotrophic habit; also, *S. ganodermophthorum* and *S. sphaerosporum* produce both asexual and sexual morphs, while *H. mellicola* only displays an asexual one.

**Type:** Spain: Valencia community: Castellón province, from decanted and filtered honey, 10 May 2014, A. Gómez Pajuelo (CBS H-23368 – holotype; CBS 143838 = FMR 15679 – ex-type cultures; LSU sequence GenBank LT906535).

**Description:** Colonies on G18 reaching 38–41 mm diam after 3 wk. at 25 °C, flattened, velvety, yellowish white (4A2) at the centre, margins regular, sporulation sparse; exudate absent; reverse pale yellow (4A3), diffusible pigment absent. Mycelium composed of hyaline to subhyaline, septate, smooth- and thin-walled hyphae, 1.5–4 μm wide; racquet hyphae present. Conidiophores reduced (mostly) to fertile side branches and to the terminal part

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![Image](image_url)
of a vegetative hyphae, sinuous to helical or in zig-zag, mostly simple, sometimes branched, 15–180 μm long, hyaline, disarticulating in conidia. *Conidia* mostly 1-celled, sometimes up to 4-celled, mostly holothallic, occasionally enterothallic, in chains of up to 30, mostly barrel-shaped, prismatic or cuboid, sometimes triangular and “Y”-shaped, smooth-walled, thicker than the hyphae, thicker at the ends, 2–8 × 2–5 μm, hyaline, disarticulating by schizolysis or rhexolysis from the conidiogenous hyphae. *Chlamydosporales* produced on OA, terminally on or intercalar in the fertile hyphae hyaline, one to multicellular, smooth- and thick-walled, globose, ovoid, pyriform, clavate or irregularly-shaped, truncate at the base or at both ends, to 10 μm long and 3–5 μm wide.

**Colonies** on G18 reaching 22–27 mm diam after 3 wk. at 15 °C, flat, velvet, yellowish-white (4A2), margins regular, sporulation sparse, exudate absent; reverse pale yellow (4A3), diffusible pigment absent; no growth on G18 over 35 °C; on PDA reaching 31–35 mm diam after 3 wk. at 25 °C, slightly elevated, velvet, slightly sulcate, yellowish (3A2) at the centre and white (3A1) at the edge, exudate absent; reverse reddish yellow (4A6) at the centre and pale orange (5A3) at the edge, diffusible pigment absent; on OA at 25 °C after 3 wk. very small, 7–8 mm diam, velvet, white (4A1), sporulation sparse, exudate absent; reverse pale orange (5A3), diffusible pigment absent.

Minimum, optimal and maximum temperature of growth on G18 are 15 °C, 25 °C, and 30 °C, respectively; no hemolysis observed on blood agar at 25 °C, and on BCP-MS-G casein hydrolyzed without pH changes. Lipase negative, urease positive. Inhibited by cycloheximide and 20% NaCl, but tolerant to 3% and to 10% NaCl on Sabouraud dextrose agar.

**Other specimens examined:** Spain: *Valencia community:* Castellón province, from decanted and filtered honey, 10 May 2014, A. Gómez Pajuelo (FMR 15673). *Castilla y León community:* León province, from decanted, filtered and thermally treated honey, 20 May 2014, A. Terrab (FMR 16307). *Castilla y León community:* Zamora province, from decanted and filtered honey, 5 Oct. 2014, A. Gómez Pajuelo (FMR 16308). *Extremadura community:* Cáceres province, from decanted, filtered and thermally treated honey, 16 May 2014, A. Terrab (FMR 16315).

**Etymology:** From Greek στρογγυλός, globose, ἀρθρώσις, joint, and -spora, spore, referring to the morphology of the conidia.

**Diagnosis:** Distinguished from other genera of Onygenales by the production of thick-walled globose arthroconidia, and because this fungus is an obligate xerophile.

**Type species:** *Strongyloarthrosporum catenulatum* Rodr.-Andr. et al. 2019.

**Description:** Mycelium of hyaline, septate hyphae. *Conidiophores* fertile lateral branches and part of the vegetative hyphae, disarticulating. *Conidia* enterothallic, hyaline, mostly globose.

*Strongyloarthrosporum catenulatum* Rodr.-Andr., Cano & Stchigel, sp. nov. Fig. 6. MycoBank MB 823588.

**Etymology:** From Latin catenulatus, in chains, referring to the disposition of the conidia.

**Diagnosis:** *Strongyloarthrosporum catenulatum* is phylogenetically close to the *Ajellomyctaceae*, a family of non-xerophilic fungi characterized by their thermally dimorphic nature and, consequently, pathogenic for animals. By contrast, *S. catenulatum* is an obligate xerophilic fungus with globose conidia sometimes disposed in chains.

**Type:** Spain: *Castilla-La Mancha community:* Toledo province, from decanted, filtered and thermally treated honey, 12 May 2014, A. Terrab (CBS H- 23371 – holotype; CBS 143841 = FMR 16121 – ex-type cultures; LSU sequence GenBank LT906534).

**Description:** Colonies on G18 reaching 20–21 mm diam after 3 wk. at 25 °C, elevated, velvet, sulcate, sporulation sparse, exudate absent, yellowish white (4A2) at the centre and white (3A1) at the edge; reverse orange-grey (5B2), diffusible pigment absent. Mycelium composed of hyaline, septate, smooth, thin- to thick-walled, anastomosing hyphae, 1.5–4 μm wide. *Conidiophores* reduced mostly to single fertile side branches and to the terminal part of the vegetative hyphae, 5–60 μm long, hyaline, disarticulating in conidia. *Conidia* hyaline, mostly one-celled, occasionally two-celled, holothallic, or produced in basipetal chains of up to ten conidia, smooth-walled, thicker than the hyphae, thicker at the ends, mostly globose, 3–6 μm diam, flattened or not at one or both ends, disarticulating by rhexolytic secession from the conidiogenous hyphae. *Chlamydosporales* and racquet hyphae absent.

**Colonies** on G25 N reaching 19–20 mm diam after 3 wk. at 25 °C, elevated, velvet, sulcate, exudate absent, sporulation sparse, light orange (5A4) at the centre and grey (5B1) at the edge; reverse greyish orange (5B5), diffusible
pigment absent; on MY70FG reaching 29–30 mm diam after 3 wk. at 25 °C, flat, floccose, margins entire, sporulation sparse, white; reverse light yellow (4A4), diffusible pigments absent. Minimum, optimal and maximum temperature of growth on G18 are 15 °C, 25 °C, and 35 °C, respectively, does not grow on blood agar, BCP-MS-G, Sabouraud dextrose agar with different NaCl concentrations, TOTM, OA, PYE nor on Christensen’s urea agar.

Subclade B: Eurotiales

Due to both LSU-based (Fig. 2; sister clade B1) and ITS-BenA-CaM-rpb2-based (Fig. 4) phylogenetic trees, four of our Talaromyces strains were placed in section Trachyspermi in a well-supported subclade divided in two branches, and one more strain was placed into the section Purpurei in a basal position (Fig. 4), phylogenetically distant and phenotypically different from other species of Talaromyces in this section, consequently, we propose the recognition of three new species of the genus.

Talaromyces basipetosporus Stchigel, Cano & Rodr.-Andr., sp. nov.

Etymology: After the morphological similarity to the asexual morph of Basipetospora (formerly applied to the asexual morph of Monascus).

Diagnosis: Differs from other species in sect. Trachyspermi in that the conidiogenesis is very similar to that of Monascus (syn. Basipetospora), characterized by retrogressively produced conidia, which have not been previously described in Talaromyces (see diagnosis of Talaromyces affinitatimellis).

Type: Argentina: Buenos Aires province: San Martín, from decanted, filtered and thermally treated honey, 1 Oct. 2007, M. A. Álvarez (CBS H-23365 – holotype; CBS 143836 = FMR 9720 – ex-type cultures; LSU sequence GenBank LT964940).

Description: Colonies on MEA reaching 10–11 mm diam after 3 wk. at 25 °C, slightly elevated, velvety to floccose,
margins entire, yellowish grey (4B2) at the centre and white (4A1) at the edge, exudate absent, sporulation sparse; reverse brownish red (8C8) at the centre and greyish orange (5B6) at the edge, diffusible pigments absent. Mycelium abundant, composed of subhyaline to pale brown, smooth to echinulate, thin-walled, septate, anastomosing hyphae, of 2–3 μm wide. Conidiophores mostly reduced to a single conidiogenous cell, sometimes slender and with an additional conidiogenous locus near the base, arising alternately or oppositely at both sides of the vegetative hyphae, mostly separate from the vegetative hyphae by a basal septum. Conidiogenous cells smooth-walled to echinulate, mostly cylindrical and occasionally slightly slender towards the apex, sometimes broadening below the apex, but also flask- or barrel-shaped, very variable in length, 3–20(−45) × 1–2.5 μm, conidiogenesis retrogressive. Conidia one-celled, hyaline and echinulate when young, becoming brown to dark brown and nearly smooth-walled with the age, formed basipetally, in false chains of up to ten conidia, mostly globose, 3.0–5.0 μm diam. Sexual morph not observed.

Colonies on DG18 reaching 13–14 mm diam after 3 wk. at 25 °C, colonies moderately elevated, texture floccose,
yellowish orange (4B7) with mycelium white (5A1) at edge, sporulation dense, exudate absent, diffusible pigments absent, reverse reddish golden (6C7) at centre and pale yellow (3A4) at edge; on G18 reaching 10–11 mm diam after 3 wk. at 25 °C, slightly elevated, velvety to floccose, margins regular, yellowish white (3A2), exudates uncolored, diffusible pigment absent, reverse pale orange (5A3) at the centre and white at the edge; on OA reaching 5–6 mm diam. After 3 wk. at 25 °C, flat, margins entire, mycelium grey, texture velvety to floccose, sporulation dense, diffusible pigments absent, exudate absent, colonies dark brown (5D4) at centre and grey with olive-brown (6B1–4E6) patches at edge; on PDA reaching 10–11 mm diam. After 3 wk. at 25 °C, elevated, velvety, brown (7E7) at the centre and brownish grey (4D2) at the edge, sporulation abundant, exudate absent, diffusible blackish olive (2G6) pigment present, reverse dark brown (7F4) at centre and brown (7E8) at the edge; on YES reaching 7–8 mm diam after 3 wk. at 25 °C, moderately elevated, sulcate, rough, sporulation strong, blackish brown (6G8), diffusible pigments absent, exudates absent, reverse yellowish brown (5E8).

Minimum, optimal and maximum temperature of growth on G18 are 15, 25, and 30 °C, respectively; does not grow on CYA, Czapek 20% sucrose, CREA, Starch agar, or MY70FG.

Talaromyces brunneosporus Rodr.-Andr., Cano & Stchigel, sp. nov.

Etymology: From Latin brunneus-, brown, and -sporarum, spore, in reference to the colour of the conidia.

Diagnosis: Distinguished from other species in sect. Purpurei, with the exception of T. purpurei (the type species of the section), by the production of solitary phialides and monoverticillate conidiophores (biverticillate conidiophores in the other species of the section). However, T. brunneosporus can be differentiated from T. purpureus because lack of a sexual morph (present in the latter species),

Fig. 8 Talaromyces brunneosporus CBS 144320. a Colonies from left to right (top row) CYA, MEA, DG18 and OA; (bottom row) CYA reverse, MEA reverse, YES and CREA. b, c Poorly-developed (single phialide) and well-developed (monoverticillate) conidiophores; the arrows indicate the conspicuous collarette at the top of the phialides. d A chain of globose, dark brown, verrucose conidia. Scale bar = 10 μm
and produces penicillate conidiophores (having an aspergil- late look in T. purpureus) and verrucose conidia (ornamen- ted with spiral ridges in T. purpureus).

**Type:** Spain: Castilla y León community: Salamanca province, from decanted, filtered and thermally treated honey, 1 Oct. 2014, A. Terrab (CBS H-23375 – holotype; CBS 144320 = FMR 16566 – ex-type cultures; LSU sequence GenBank LT964943).

**Description:** Colonies on MEA reaching 13–14 mm diam after 3 wk. at 25 °C, slightly elevated, velvety to floccose, margins irregular, yellowish white (4A3), exudate absent, sporulation sparse, reverse light brown (6D8) at the centre and yellowish brown (5D6) at the edge, diffusible yellowish brown (5E6) pigment present. *Mycelium* abundant, composed of subhyaline, smooth- and thin-walled, septate, anastomosing hyphae 2–3 μm wide. *Conidiophores* mostly stalked, monoverticillate, smooth- and thin-walled, bearing one to four conidiogenous cells at the top, frequently arising oppositely at both sides of the vegetative hyphae, sometimes reduced to a single conidiogenous cell, sessile or integrated to the vegetative hyphae (= adelophialides). *Conidiogenous cells* phialidic, smooth-walled, mostly slender towards the apex, flask-shaped, 8–12 × 2.5–3.5 μm, with a darkened apical area when the conidiogenous cells have produced several conidia, conidiogenesis enteroblastic. *Conidia* one-celled, globose, hyaline and smooth-walled when young, becoming brownish-green to dark brown and verrucose with the age, 3–4 μm diam, in long false chains of up to 25 conidia. Sexual morph not observed.

**Colonies** on CYA reaching 4–5 mm diam after 3 wk. at 25 °C, elevated, velvety, dark brown (8F4) at the centre and greyish-brown (7E3) at the edge, exude absent, sporulation abundant, reverse dark brown (8F6) at the centre and reddish brown (8E5) at the edge, diffusible brown (6E7) pigment present; on DG18 reaching 10–11 mm diam after 3 wk. at 25 °C, moderately elevated, floccose, margins irregular, yellowish white (4A2) at the centre and olive-brown (4D6) at the edge, exude absent, sporulation strong, reverse light brown (5D7), diffusible yellowish brown (5D5) soluble pigment present; on OA reaching 9–10 mm diam after 3 wk. at 25 °C, flat, floccose, margins entire, exude absent, sporulation strong, colonies blackish olive (2G6) at the centre and brown (6E6) at the edge, diffusible olive brown (4E8) pigment present; on YES reaching 8–9 mm diam after 3 wk. at 25 °C, flat, floccose, black at the centre and yellowish-brown (5E6) at the edge, exude absent, sporulation sparse, reverse dark violet (8E8), diffusible blackish brown (6G8) pigment present.

Minimum, optimal and maximum temperature of growth on G18 are 15, 25, and 30 °C, respectively; no growth on CYA at 37 °C nor on CREA at 25 °C.

**Notes:** *Talaromyces bruneosporus* and *T. purpureus* grow more slowly on CYA and MEA than other species of the section. However, *T. bruneosporus* produces dark brown colonies with a dark brown diffusible pigment on CYA, while the colonies of *T. purpureus* are pale beige and without diffusible pigments. Also, the colonies on OA and MEA are purplish in *T. purpureus* and pale coloured and dark brown in *T. bruneosporus*.

*Talaromyces affinitatimellis* Rodr.-Andr., Stschigel & Cano, sp. nov. Fig. 9. MycoBank MB 823591.

**Etymology:** From Latin *affinitatis*-, affinity, and -*mellis*, honey, after the substrate from which the fungus was isolated.

**Diagnosis:** Differing from all other species in sect. *Trachyspermi* (with the exception of *T. basipetosporus*) by the production of conidia by retrogressive conidiogenesis. *Talaromyces affinitatimellis* differs from *T. basipe- tosporus* by the production cylindrical, smooth-walled to echinulate conidiogenous cells ending in a greenish brown, broad collarette-like structure (conidiogenous cells irregularly-shaped, smooth-walled, and without such apical structure in *T. basipetosporus*).

**Type:** Spain: Valencia community: Castellón province, from decanted and filtered blossom honey, 10 May 2014, A. Gómez Pajuelo (CBS H-23370 – holotype; CBS 143840 = FMR 15690 – ex-type cultures; LSU sequence GenBank LT964939).

**Description:** Colonies on MEA reaching 29–30 mm diam. After 3 wk. at 25 °C, flat, floccose, not sulcate, margins entire, olive (3D3) at the centre and white (4A1) at edge, exude absent, sporulation sparse; reverse pale orange (5A3) at centre and pale yellow (4A3) at edge, diffusible pigment absent. *Mycelium* abundant, composed of subhyaline to pale brown, smooth- and thin-walled, se- rate, anastomosing hyphae, of 2–4 μm wide. *Conidiophores* hyaline to pale brown, reduced to a single conidiogenous cell, occasionally with an additional conidiogenous locus near the base or lateraly disposed, or short-stalked and bearing two conidiogenous cells, sometimes with an additional lateral conidiogenous cell arising alternately at both sides of the vegetative hyphae, separate from them by a basal septum. *Conidiogenous cells* hyaline to pale brown, smooth-walled, mostly cylin- drical and occasionally slightly slender towards the apex, sometimes ending in a greenish-brown, broad collarette-like structure, 3–20 × 1.5–3 μm, conidiogenesis retrogressive but
Conidial one-celled, hyaline and echinulate, becoming brown to dark brown and nearly smooth-walled with the age, produced basipetally in false chains of up to ten in number, mostly globose, 3.0–5.0 μm diam. Sexual morph not observed.

Colonies on DG18 reaching 13–14 mm diam after 3 wk. at 25 °C, moderately elevated, floccose, yellowish orange (4B7) with white (5A1) margins, exudates absent, sporulation strong; reverse reddish golden (6C7) at the centre and pale yellow (A4) at the edge, diffusible pigment absent; on G18 reaching 21–24 mm diam at 25 °C, slightly elevated, velvety to floccose, margins regular, yellowish white (4A4), exudates absent, sporulation abundant, reverse greyish orange (5B6), diffusible pigment absent; on OA reaching 12–13 mm diam after 3 wk. at 25 °C, flat, velvety to floccose, margins entire, black, exudates absent, sporulation abundant; colonies grey (7F1) at the centre and dark brown (6F4) to black at the edge, diffusible pigment absent; on PDA reaching 39–43 mm diam after 3 wk. at 25 °C, flat, velvety, margins slightly irregular, yellowish-brown (5F6) at the centre, grey (7F1) and yellowish brown (5E4) at the middle part, and light grey (5B1) at the edge, exudate absent, sporulation scarce, reverse dark brown (7F7) at the centre and brownish yellow (5C7) at the edge, diffusible pigment absent; on YES reaching 10–11 mm diam after 3 wk. at 25 °C, moderately elevated, floccose, white (4A1), exudate absent, sporulation sparse, reverse greyish orange (5B6), diffusible pigment absent.

Minimum, optimal and maximum temperature of growth on G18 are 15, 25, and 35 °C, respectively; no growth on CYA, Czapek 20% or CREA, or at 40 °C on all tested media.

Other specimens examined: Spain: Catalonia community: Tarragona province, from decanted and filtered blossom honey, 10 May 2014, A. Gómez Pajuelo (FMR 15674, FMR 15675, and FMR 15677); Valencia community: Castellón province, from decanted and filtered blossom honey, 10 May 2014, A. Gómez Pajuelo (FMR 15684 and FMR 15688); Extremadura community: Cáceres province, from decanted, filtered and thermally treated honeymdew honey, 16 May 2014, A. Terrab (FMR 16029, FMR 16499, and FMR 16501); Castilla y León community: Salamanca province, from decanted, filtered and thermally treated honeymdew honey.
honeydew honey, 01 Oct. 2014, A. Terrab (FMR 16033 and FMR 16114); Zamora province, from decanted, filtered and thermally treated honeydew honey, 05 Oct 2014, A. Terrab (FMR 16125, FMR 16126, FMR 16276, and FMR 16494).

**Subclade D: Incertae sedis**

Based on both LSU-based (Fig. 2; sister clade D1) and ITS-based (Fig. 3) phylogenetic trees, ten of our strains were located in a well-supported and separated branch related to species of the genera *Oidiodendron* and *Myxotrichum*, and phylogenetically distant from the most similar taxa included in the study, *M. setosum* and *O. truncatum* (Fig. 3). Recognition of all of these distinct strains was also supported by unique phenotypic characteristics; therefore, we propose the recognition of the new species *Oidiodendron mellicola*. Furthermore, because three of our strains were placed near *Skoua fertilis* in both LSU-based (Fig. 2; sister clade D2) and ITS-based (Fig. 3) phylogenies and because they showed different phenotypic features and enough phylogenetic distance relative to *S. fertilis*, we also propose the introduction of a further new species, *Skoua asexualis*.

_Oidiodendron mellicola_ Rodr.-Andr., Cano & Stchigel, sp. nov. Fig. 10. MycoBank MB 823586.

Etymology: From Latin _mellis_, honey, and _-cola_ dwelling on, referring to the habitat.

Diagnosis: Forming a terminal clade together with *O. truncatum* and *M. setosum* at a significant phylogenetic distance (5.3% from the other two species), and differing morphologically from other known species of _Oidiodendron_ and the asexual morphs of _Myxotrichum_ in the absence of well-differentiated conidiophores, and the slow growth.

Type: Spain: Valencia community: Castellón province, from decanted and filtered blossom honey, 10 May 2014, A. Gómez Pajuelo (CBS H-23369 – holotype; CBS 143839 = FMR 15683 – ex-type cultures; ITS sequence GenBank LT906544).

Description: Colonies on PDA at 15 °C reaching 15–16 mm diam after 3 wk., white (5A1), sporulation sparse

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**Fig. 10 Oidiodendron mellicola CBS 143839**. A Colonies on PDA at 15 °C and at 25 °C, and on G18 at 25 °C (left to right), surface and reverse (from top to bottom). B–D Conidiophores. E Disarticulating chains of conidia. Scale bar = 10 μm
(seen after 6 wk. of incubation), exudate absent, reverse orange-white (6A2) at the centre and orange-grey (6B2) at the edge, diffusible pigment absent. Mycelium composed of hyaline, septate, smooth- and thin-walled hyphae, 1–3 μm wide. Conidiophores reduced to fertile side branches and the terminal part of a vegetative hyphae, mostly simple or once branched near or at the base, 10–40 μm long, pale olive, disarticulating in conidia. Conidia one-celled, mostly holoarthric, sometimes enterocarathic, mostly in chains of up to ten, occasionally solitary and sessile, mostly barrel-shaped, sometimes cylindrical, conical or “Y”-shaped, 5–14 × 2.5–5 μm, pale olive, disarticulating by schizolytic or rhexolytic secession from the hyphae. Chlamydospores absent. Sexual morph absent.

Colonies on PDA reaching 10–11 mm diam. After 3 wk. at 25 °C, elevated, compact, velvety, margins irregular, olive brown (4E3), exudates absent, sporulation abundant; reverse olive brown (4E5) at the center, grey (5D1) at the edge, diffusible pigment absent. Colonies on G18 reaching 11–12 mm diam after 3 wk. at 25 °C, elevated, velvety to floccose, yellowish white (4A2) at the centre and white (4A1) at the edge, margins regular, sporulation absent, reverse pale yellow (4A3), diffusible pigment absent; on G18 at 15 °C reaching 12–15 mm diam after 3 wk., similar in aspect than at 25 °C; on MY70FG and MEA 2% at 25 °C after 3 wk. reaching 1–3 mm diam.

Minimum, optimal and maximum temperature of growth on G18 are 5, 15, and 25 °C, respectively; no growth on OA or PCA at 25 °C.

Other specimens examined: Spain: Catalonia community: Tarragona province, from decanted and filtered blossom honey, 10 May 2014, A. Gómez Pajuelo (FMR 15680); Castilla-La Mancha community, Ciudad Real province, from decanted, filtered and thermally treated honeydew honey, 10 May 2014, A. Terrab (FMR 16031, FMR 16117, and FMR 16503); Toledo province, from decanted, filtered and thermally treated honeydew honey, 15 May 2014, A. Terrab (FMR 16120 and FMR 16282); Galicia community: Ourense province, from decanted, filtered and thermally treated honeydew honey, 03 May 2014, A. Terrab (FMR 16504); Castilla y León community: Salamanca province, from decanted, filtered and thermally treated honeydew honey, 01 Oct. 2014, A. Terrab (FMR 16023); Burgos province, from decanted, filtered and thermally treated honeydew honey, 23 May 2014, A. Terrab (FMR 16274).

Skoua asexualis Rodr.-Andr., Cano & Stchigel, sp. nov. Fig. 11. MycoBank MB 824092.

Etymology: From Latin asexualis, without sex, because of lack of a known sexual morph.

Diagnosis: Differing from the other known species of the genus, S. fertilis, in asexual reproduction, as the latter only produces ascospores within globose asci arising from the mycelium.

Type: Spain: Castilla y León community: León province, from decanted, filtered and thermally treated honeydew honey, 1 Oct. 2014, A. Terrab (CBS H-23397 – holotype; CBS 144072 = FMR 16572 – ex-type cultures; ITS sequence GenBank LT964668).

Description: Colonies on PDA reaching 6–7 mm diam after 3 wk. at 25 °C, elevated, velvety, sporulation abundant, exudates absent, diffusible pigment absent, colonies brown (7E6) at the centre and whitish at the edge, reverse brownish orange (6C5) at the centre and greyish orange (5B3) at the edge. Mycelium composed of hyaline, repeatedly septate, smooth- and thin-walled hyphae, 2–6 μm wide. Conidiophores absent. Conidia mostly one-celled, occasionally two- to three-celled, hyaline, solitary or in short chains, smooth- and thick-walled, mostly globose, occasionally broadly ellipsoidal, pyriform, or irregular-shaped, truncate at one or both ends, 3–7 μm diam, conidiogenesis holoblastic when sessile or terminal, and holothallic when intercalary, disarticulating by rhexolytic secession; the holoblastic and holothallic conidia produce a succession of secondary holoblastic conidia, forming a big, radiating mass of cells of up to 50 μm diam, which eventually detach as complex asexual propagules from the fertile hyphae. Chlamydospores similar to the conidia but thicker, mostly non- or occasionally one-septate, intercalary or terminal. Sexual morph unknown.

Colonies on MEA reaching 3–4 mm diam after 3 wk. at 25 °C, colonies elevated, velvety to floccose, margins irregular, sporulation abundant, diffusible pigment absent, mycelium yellowish white (4A2), reverse pale yellow (4A3); on G18 reaching 4–5 mm diam after 3 wk. at 25 °C, elevated, floccose, margins irregular, sporulation sparse, diffusible pigment absent, exudates absent, colonies pale yellow (4A3) at the centre, reverse orange-grey (5B2).

Minimum, optimal and maximum temperature of growth on G18 are 15, 25, and 30 °C, respectively; no growth on CYA, CREA, OA, or YES at 25 °C.

Other specimens examined: Spain: Extremadura community: Cáceres province, from decanted, filtered and thermally treated honeydew honey, 16 May 2014, A. Terrab (FMR 16497 and FMR 16567).

DISCUSSION

This is the most comprehensive assessment of the diversity of the xerotolerant and xerophilic fungi of honey
intended for human consumption to date. We have isolated selectively and identified, by a polyphasic approach, six species of ascomycetous yeasts and 27 of filamentous ascomycetes, some representing new taxa, from honey samples. The yeasts, *Candida magnoliae*, *C. sorbosivorans*, *Schizosaccharomyces octosporus*, *Zygosaccharomyces barkeri*, *Z. mellis*, and *Z. gambellarensis*, had been reported from honey before, and *C. magnoliae* has also been associated with living honeybees (Gilliam et al. 1974b). All these yeasts have been described as osmophilic and able to grow at a w of 0.80 or lower (Tilbury 1967; van Eck et al. 1993; Ganthala et al. 1994; Erickson & McKenna 1999; Torriani et al. 2011). We found *C. magnoliae* and *C. sorbosivorans* were phylogenetically closely related (see Fig. 2), and it was reported that both differ only in a few physiological characteristics (James et al. 2001). To our knowledge, none of the species of *Aspergillus* that we isolated (*A. asperescens*, *A. montevidensis*, and *A. pseudoglaucus*) have previously been reported from honey. *Aspergillus asperescens* was originally isolated from soil and bat dung (Stolk 1954), but also from rotten wood and soybean seeds; however, most of the isolates were from cave soil (probably linked to bat dung). *Aspergillus montevidensis* and *A. pseudoglaucus* have been reported as the most important food-spoilage species of the genus (Pitt & Hocking 1977; Kozakiewicz 1989), but are known from extreme environments such as salterns (Butinar et al. 2005). *Aspergillus montevidensis* has been reported from various environmental samples (air, soil, etc.), and even on honeybees and bee larvae (http://gcm.wfcc.info/; Talice & Mackinnon 1931; Gilliam et al. 1974a); *A. pseudoglaucus* has been reported in air, paper and soil (http://gcm.wfcc.info/; Blochwitz 1929). *Aspergillus montevidensis* and *A. pseudoglaucus* are able to grow at a w values of 0.80 (Snow 1949; Armolik & Dickson 1956; Guynot et al. 2003). *Monascus* is a well-known genus with species (especially *M. purpureus* and *M. ruber*) of economic importance due to their use in production of foodstuffs, bioactive compounds, pigments and enzymes. Currently, *Monascus* is placed in *Aspergillaceae* (syn. *Trichocomaceae*) based on phylogenetic studies, and closely related to *Leiothecium ellipsoides* and *Xeromyces bisporus* (Houbraken & Samson 2011; Pettersson et al. 2011). Recently, three new species
were added, all of them associated with stingless bees: *M. flavipigmentosus*, *M. mellicola*, and *M. recifensis* (Barbosa et al. 2017). We found a small number of isolates, including *M. pilosus*, *M. purpureus*, and *M. ruber*. These species have been frequently reported in fermented and spoiled foods (van Tieghem 1884; Hesselhine 1965; Lin 1975; Hawksworth & Pitt 1983). *Monascus ruber* has also been found in soil and human clinical specimens (Hawksworth & Pitt 1983). Species of *Monascus* have been previously reported in honey by Snowdon & Cliver (1996) and by Barbosa et al. (2017). *Monascus pilosus*, *M. purpureus*, and *M. ruber* were reported previously (Hawksworth & Pitt 1983) as able to grow well on G25 N ($a_w = 0.93$). The species of *Penicillium* we found in honey included *P. camemberti*, *P. citrinum*, *P. corylophilum*, and *P. cravenianum*. The most common source of isolation of *P. camemberti* is blue cheeses, but it can also be found on a wide variety of substrata (Thom 1906; http://gcm.wfcc.info/). *Penicillium citrinum* was originally reported in milk and bread in the USA (Thom 1910), but it is found globally and easy to recover from spoiled foods and diverse environmental sources (www.cabri.org/collections.html) including honey, pollen and bee nests (Barbosa et al. 2018). *Penicillium corylophilum* (Dierckx 1901) mostly occurs in damp buildings in North America and Western Europe, but also in foods and mosquitoes (Da Costa & De Oliveira 1998; McMullin et al. 2014), and honey (Sinacori et al. 2014). The minimum $a_w$ reported for the growth of *P. camemberti*, *P. citrinum* and *P. corylophilum* was around 0.80 (Abellana et al. 2001; Fontana 2008; Kalai et al. 2017). *Penicillium cravenianum*, a species moderately xerotolerant (grows on G25 N), has only been reported in soil (Visagie et al. 2016). Notably, all the isolates of *Talaromyces* that we found in honey belonged to three unrecognized species. *Talaromyces basipetosporus* was recovered from a honey sample in Buenos Aires province, Argentina, and is characterized by simple conidiophores that mimic those of the asexual morph of *Monascus* (syn. *Basipetospora*), which develops conidia by a retrogressive mode of conidigenesis, a feature not previously reported in *Talaromyces*. *Talaromyces affinitatimellis* displays a similar conidigenesis to *T. basipetosporus* and both species are phylogenetically closely related but phenotypically differentiated as *T. affinitatimellis* grows faster and produces more complex conidiophores. *Talaromyces brunneosporus* differs from the other species of sect. *Purpurei*, apart from *T. purpureus*, in having monopha- lidic and monovercillate conidiophores (they are biver- cicillate in the other species). However, both species are distinguishable because *T. brunneosporus* produces peni- cillate conidiophores (not aspergillate as in *T. purpur- eus*), longer phialides, and verrucose conidia with a flattened base (*T. purpureus* conidia are ornamented by spiral ridges). *Talaromyces basipetosporus* has a high xerotolerance, with similar growth rates on MEA with sugars up to $a_w = 0.82$. Despite the decreasing growth rates of *T. brunneosporus* and *T. affinitatimellis* when sugar concentration increases, both fungi are able to grow at $a_w = 0.82$ (Fig. 12). *Xerochrysium xerophilum* (Pitt et al. 2013; syn. *Chrysosporium xerophilum*, Pitt 1966), is an extreme xerophile with a minimum $a_w$ for growth of 0.66 (Gock et al. 2003; Leong et al. 2011). This fungus, previously reported from chocolate, coconut, dried prunes, and stored corn (Pitt & Hocking 2009; Pitt et al. 2013), has not been found in honey until now. This species is phylogenetically close to *Monascus* (Pitt et al. 2013). Among the species of *Onygenales*, *Ascosphaera atra* and *Eremascus albus* were recovered once and four times, respectively. *Ascosphaera atra* (Skou & Hackett 1979) was originally reported from dead larvae of the alfalfa leafcutter bee covered in cysts of *Ascosphaera aggregata* (Skou 1975), and from pollen in the gut of healthy leafcutter larvae. This fungus was subsequently reported from grass silage (Skou 1986). *Ascosphaera atra* is homothallic and saprobic, probably being a common contaminant of pollen (Skou & Hackett 1979), which would explain its presence in honey samples. *Eremascus albus* is a well-known xerophilic fungus, with spores that can germinate at $a_w$ as low as 0.70 (Pitt 1965). This fun- gus has been reported to spoil malt extract (Eidam 1883), chocolate cake, dried fruits, and mustard powder (Harrold 1950), but never previously from honey. We identified several isolates belonging to the newly described family *Helicoarthroporaceae*, which only includes the new monotypic genus *Helicoarthroporus*, and a single strain belonging to the new monotypic genus *Strongyloarthroporus* (Ajellomyctaceae). The morphology of *Helicoarthroporus mellicola* resembles species of *Scytalidium* (*S. cuboides*, *S. ganoder- mophthorum*, and *S. sphaerosporum*) because of the production of cuboid arthroconidia in long chains. However, *Helicoarthroporus* is phylogenetically distant from *Scytalidium*, as the latter is related to *Mxylotricha- ceae*. *Strongyloarthroporus catenulatum* was found to be phylogenetically close to *Ajellomyctaceae*, whose members are thermally dimorphic and pathogenic to ani- mals (including the humans), and has never been re- ported as xerotolerant. However, having features not seen in that family, *S. catenulatum* is unequivocally a xerophilic fungus, only growing on G18, G25 N and MY70FG, and producing globose arthroconidia, either singly or in chains. The sole xerophilic fungus phylogen- etically close to *S. catenulatum* is *Eremascus albus* (*Ere- mascaceae*), but it only develops a sexual morph. Regarding the family *Mxylotrichaceae*, *Skoua fertilis*, which was detected in all honey samples, resembles *Ere- mascus albus* (Eidam 1883) in having naked asci arising.
directly out of the mycelium and formed by the fusion of two equal cells borne on short entwined hyphae. Both taxa can be only morphologically differentiated by the shape of the ascospores and by sexual reproductive details. While *S. fertilis* (syn. *E. fertilis*) belongs to Leotiomycetes, closely related to Myxotrichaceae (Wynns 2015), *E. albus* is located in Eurotiomycetes, closely related to Onygenales (Cai et al. 1996; Berbee 2001; Wynns 2015). *Skoua* was introduced for *E. fertilis* (i.e. *Skoua fertilis*) and has been reported on bee bread, honeycomb, dried prunes and spoiled moist prunes, green compost, and shortcake (www.cabri.org/collections.html; http://gcm.wfcc.info/; Harrold 1950), but not so far on honey. The minimum *a*<sub>w</sub> for growth and sporulation reported for *S. fertilis* was 0.77 (Pitt 1965; Wynns 2015), a similar value observed in all our strains (0.82). We isolated three strains of *Skoua* phylogenetically different from *S. fertilis*, and named them as *Skoua asexualis* because they form asexual spores instead of the sexual spores as observed in the type species of the genus. *Bettsia alvei* (Skou...
1972, 1975), the other fungus identified in all honey samples, belongs to *Pseudewortiaceae* and is characterized by dark, closed ascomata (usually called “spore cysts”) and hyaline globose ascospores, forming a sticky mass. *Bettsia alvei* has been isolated from hives in Europe as well as the USA (Burnside 1929), and from bakery products, spoiled chocolate, desiccated coconut, honeycomb, concentrated jelly, dried and spoiled prunes, pollen, table jelly, bee wax, and wine starters (www.cabri.org/collections.html; http://gcm.wfcc.info/). It was also isolated from chocolate in Austria (a$_w$ less than 0.3), but thus far had not been recorded from honey. The lowest a$_w$ tested for growth of this species was 0.88 (Beuchat & Pitt 1990) and 0.89 (Udagawa & Toyazaki 2000), similar values to those we found. All our isolates of *B. alvei* developed the chrysosporium-like asexual morph but failed in the production of the sexual morph. Among the most frequent species we isolated was an undescribed species of *Oidiodendron, O. mellicola*. Species of this genus are mostly recovered from soil and other substrata rich in cellulose, and are found worldwide (Domsch et al. 1980; Caldutch et al. 2004; Rice & Currah 2005). *Oidiodendron mellicola* is phylogenetically related to *O. truncatum* and *M. setosum*, the former characterized by well-differentiated dark conidiophores and barrel-shaped conidia with a dark scar at one or both ends (typical features of *Oidiodendron*), and the latter by hyaline conidiophores and conidia, and by dark brown to black, spinose, gymnothecial ascomata (typical of the genus *Myxotrichium*). Interestingly, *M. setosum* is reported as a common hive fungus in Europe (Burnside 1929). *Oidiodendron mellicola* is the only species of the genus reported from honey, and it can be distinguished morphologically from other species of the genus by its absence of stipitate conidiophores, and the production of long chains of conidia, which are pale, smooth, ellipsoidal to cylindrical, truncated (but not darkened, as in *O. truncatum*) at one or both ends, and by the slow growing colonies. Like most of the species of the genus, *O. mellicola* grows better at 15 °C than 25 °C. Other fungi rarely found in our study were *Alternaria multiformis*, *Mellicola grows better at 15 °C than 25 °C. Other fungi*... among other physicochemical and biological parameters. Honey is clearly one of the relatively unexplored habitats for the missing fungal diversity, especially as the new taxa we found came from samples from just two countries.

**CONCLUSION**

The application of G18 as a selective culture medium for isolation of xerotolerant/xerophilic fungi from honey samples enabled the recovery and identification of 13 genera and 29 species of *Ascomycota*, and three genera (one species for each) of *Mucoromycota*. Many of these fungi have never reported from honey before. Among them, we proposed a new family (*Helicoarthrosporaceae*), two new genera (*Strongyloarthrosporum* and *Helicoarthrosporum*) and seven new species (*Strongyloarthrosporum catenulatum*, *Helicoarthrosporum mellicola*, *Oidiodendron mellicola*, *Skoua asexualis Talaromyces basipetosporus, T. bruneosporus, and T. affinitatemellis*). All fungal taxa that we isolated from honey were able to grow at low water activity (up to 0.82), but only *Ascosphaera atra, Bettsia alvei* (two fungi strongly associated to honeybees and their life-style), *Eremascus albus, Strongyloarthrosporum catenulatum* (one of the new taxa we described) and *Xerochrysium xerophylum* can be considered obligate xerophiles. Also, because several of the honey samples were thermally treated, these fungi can be considered as hot-resistant. Honey is evidently a reservoir of xerotolerant and xerophilic fungi, which survives to the thermal treatment used to make honey non-crystallisable. Some of these fungi are related to the honeybee life-style; however, as in the case of the new taxa described here, the origin in nature remains unknown. In the latter case, flowers and aphids could play an important role as a source of such fungi. During the course of the study, the most important pathogenic fungi for honeybees, *Aspergillus flavus* and *Ascosphaera apis*, were not found. Several of the fungi found in honey samples (*Aspergillus* and *Pencillium* spp.) are potential producers of mycotoxins, but this does not mean that the honey may represent a risk to the health of the consumer, because (in general) the production of mycotoxins or the fungal growth are suppressed at water activities lower than 0.70 (Manna & Kim 2017), as is the case of honey (a$_w$ of 0.60 or less). Honey should be considered as a “living food” and, consequently, its “normal” mycobiota merits more extensive study. It is expected that such “normal” mycobiota may vary qualitatively and quantitatively, depending on the geographic origin, the botanical type and water activity of the honey, among other physicochemical and biological parameters. Honey is clearly one of the relatively unexplored habitats for the missing fungal diversity, especially as the new taxa we found came from samples from just two countries.

**Abbreviations**

a$_w$: water activity; BCP-MS-G: Bromcresol purple milk solids glucose agar; BEA: Bile esculin agar; BenA: fragment of the beta-tubulin gene; BI: Bayesian-inference; BLAST: Basic Local Alignment Search Tool; CaM: fragment of the calmodulin gene; CREA: Creatine sucrose agar; CYA: Cenatey extract agar; DG18: Dichloran 18% glycerol agar; DNA: Deoxyribonucleic acid; G18 DG18: without dichloran; G25 N 25%: glycerol nitrate agar; ITS: Ribosomal internal transcribed spacers; LSU: Large sub unit of the ribosomal genes; MEA: Malt extract agar; ML: Maximum-likelihood; MLI: Maximum level of...
identification; MY70FG: Malt extract yeast extract 70% fructose-glucose; nrrRNA: Nuclear ribosomal ribonucleic acid; OA: Oatmeal agar; PDA: Potato dextrose agar; PYE: Phytone yeast extract agar; rp2β: fragment of the RNA polymerase II subunit 2 gene; SEM: Scanning electron microscopy; TOTM Test opacity tween medium; TreeBASE: a repository of user-submitted phylogenetic trees and data used to build them; YES: Yeast extract sucrose agar

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Adherence to national and international regulations

The authors confirm that this manuscript respects the Nagoya Protocol to the Convention on Biological Diversity.

Authors’ contributions

ER-A performed all the experimental work, culturing the samples, isolating in pure culture the fungi and performing their phenotypic characterization, as the identification and taxonomy of the fungal strains, and reviewed the draft several times. JG contributed actively in the identification and taxonomy of the fungal strains, and reviewed the draft. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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