

***Tritirachium candoliense* sp. nov., a novel basidiomycetous fungus isolated from the anoxic zone of the Arabian Sea**

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Research highlights

- New *Tritirachium* species
- First description of facultative anaerobe *Tritirachium*
- First description from anoxic ocean
- Morphological and ultrastructural dimorphism under anoxic stress

Abstract

A fungal culture (FCAS11) was isolated from coastal sediments of the Arabian Sea during the anoxic season. Multigene phylogenetic analyses confidentially place the organism as a novel species within the recently defined class *Tritirachiomycetes*, subphylum *Pucciniomycotina*, phylum *Basidiomycota*. We named the new species *Tritirachium candoliense* and provide the first description of a member of this class from a marine environment. DNA sequences and morphological characters distinguish *T. candoliense* from previously described *Tritirachium* species. Its growth characteristics, morphology and ultrastructural features showed that under anoxic conditions the species grows slowly and produces mainly hyphae with only few blastoconidia. Electron microscopy revealed differences when the culture was exposed to anoxic stress. Notable ultrastructural changes occur for example in mitochondrial cristae, irregularly shaped fat globules and the presence of intracellular membrane invaginations. We assume that the growth characteristics and substrate utilization patterns are an adaptation to its source location, the seasonally anoxic environment of the Arabian Sea.

Keywords

Marine, anoxic, Arabian Sea, facultative anaerobe fungus, Basidiomycota, morphological dimorphism, *Tritirachium candoliense* sp. nov.

1. Introduction

Fungi in marine ecosystems can be grouped into obligate marine fungi, which grow and sporulate in the marine environment and facultative or marine-derived fungi that originate from terrestrial or freshwater habitats, but thrive in marine habitats (Jones 2011a). The majority of obligate fungi from marine habitats described were found in association with marine plants, such as salt marsh plants, mangroves, sea grass, and animals such as sponges, or other substrata such as driftwood (Kohlmeyer and Kohlmeyer 2003; Abdel-Wahab and Nagahama 2012). Not many isolates of marine fungi have been identified from marine sediments or the water column. This could have been largely because the original definition for marine fungi required culturing of fungi directly from the marine substrates (Kohlmeyer and Kohlmeyer 1979). However, recent interests among ecologists to study the role of fungi in the marine environment and the availability of molecular tools have helped us to understand that there is a vast diversity of marine-derived fungi and many novel fungal groups have been identified only as environmental phylotypes (Jebarajet al. 2010; Jebarajet al. 2012; Richards et al. 2012).

Cultivation-independent studies of fungi from marine habitats has aided in the identification of a large number of these environmental clusters from various marine habitats. Some of the major clusters include the deep-sea fungal group (DSF Group-I) identified within the Ascomycota, Saccharomycotina, based on the environmental phylotypes retrieved from deep-sea sediments (Nagano et al. 2010; Bass et al. 2007) and oxygen-depleted marine environments (Takishita et al. 2007b). A soil clone group (SCGI) is also identified that clusters between Taphrinomycotina and Saccharomycotina as a monophyletic clade within Ascomycota (Nagahama et al. 2011). A novel environmental clade, the hydrothermal and/or anaerobic fungal group, was delineated within the Basidiomycota, Ustilagomycotina (López-García et al. 2007). Clusters of environmental sequences within Chytridiomycota have been identified from a number of marine environments such as *Rozella* and LKM 11 cluster (Bass et al. 2007; Takishita et al. 2007a; Stock et al. 2009; Nagahama et al. 2011). This group, which clusters close to the chytrid *Rozella* sp. has been identified as a new phylum (Cryptomycota), which has a wide range of distribution (Jones et al. 2011). These findings eventually lead to a reassessment of the number of marine fungi which has increased to approximately 10,000 taxa or phylotypes (Jones 2011b), compared to earlier reports with estimates of only about 1500 species (Jones and Mitchell 1996). To reveal the morphological identity, the physiology, biotechnological potential and ecological role of this largely unexplored reservoir of biological diversity, current efforts are ongoing to isolate, cultivate and investigate fungi from a variety of marine habitats.

Successful examples of these efforts include (but are not restricted to) divergent *Aspergillus* species reported from marine sediments of the Central Indian basin (Singh et al. 2012) and that are

also found in association with marine sponges (Ding et al. 2011). Repeated occurrences of fungal cultures, which are divergent from described taxa such as *Scopulariopsis* sp., are also reported (Gao et al. 2008; Ding et al. 2011; Wiese et al. 2011). New yeast species (Agaricomycotina and Pucciniomycotina) are reported from deep-sea sediments (Nagahama et al. 2001; Nagahama et al. 2003a) and in association with deep-sea tubeworms and clams (Nagahama et al. 2003b). Yeasts are one of the dominant groups in the marine habitat and a vast diversity of these unicellular fungi have been recovered from across the globe (Jones and Fell 2012, Fell 2012). Studies on the physiological adaptations of some of these isolates clearly showed that these marine-derived fungi are distinct from terrestrial forms (Burgaud et al. 2009; Singh et al. 2012). Recovery of these marine-derived fungi from diverse marine habitats suggests that further studies on their morphological, molecular and physiological characters can shed light on their functional importance in the marine ecosystem (Jones 2011b).

To add to these efforts, we set out to isolate and cultivate fungi from coastal regions of the Arabian Sea. In total, we obtained 54 different marine-derived fungi (Jebaraj and Raghukumar 2009) and phylogenetic analyses of their SSU rDNA revealed that many of these cultures were similar to known fungal taxa. However, the taxonomic marker genes of some cultures were highly divergent from deposited fungal sequences in public databases (Jebaraj et al. 2010). In this paper we describe and characterize one of these divergent fungal isolates (FCAS11, Jebaraj et al. 2010) as a new species, *Tritirachium candoliense* sp. nov., belonging to the recently identified class *Tritirachiomycetes* Aime and Schell (Schell et al. 2011) within the subphylum *Pucciniomycotina*, phylum *Basidiomycota*, subkingdom *Dikarya*, kingdom *Fungi*.

2. Material and methods

2.1 Isolation methods

The fungal culture (FCAS11), described in this study, was isolated from anoxic coastal sediments off Goa, India, using the particle plating technique (Jebaraj et al. 2009). The pure culture of the fungus was maintained on Malt extract agar (MEA, HiMedia, Bangalore, India) slants at 5°C, and sub-cultured and regularly checked for purity in the laboratory.

2.2 Light Microscopy

Colony and microscopic morphology of FCAS11 was studied using streak / line inocula on regular media, viz. Malt extract agar (MEA), Oat meal agar (OA), Corn Meal agar (CMA), Yeast extract peptone glucose agar (YPGA) (CBS 2001). To study the growth of the culture under anoxic conditions, streak-inoculated plates were placed in an anoxic jar (Anoxomat) that contained a mixture of 85% N₂, 15% CO₂ and H₂ gas in anoxic plastic bags (Merck, Anaerocult®). These bags with Anaeroclip® clips and Anaerotest® indicator were incubated at 25°C, according to the

manufacturer's instructions, either directly or after oxidic pre-incubation for 24 or 48 h. Growth was inspected at regular intervals up to one year.

2.3 Electron Microscopy

For scanning electron microscopy (SEM) selected samples from 5-day-old oxic and 3-month-old anoxic colonies of isolate FCAS11 were fixed in 3 % (v/v) glutaraldehyde / 0.15M phosphate buffered saline, pH 7.2, for two days at 4°C; post-fixed in 1% aqueous osmium tetroxide at 4°C for 16h; and dehydrated in a series of ethanol. Thereafter the ethanol was replaced in three steps with anhydrous acetone (Conway and Kiernan, 1998). The samples were dried beyond critical point in a CPD-030 (Leica Microsystems, Vienna, Austria). The dried specimens were mounted on stubs and subsequently coated with 6 nm Pt/Pd in a sputter coater 208HR (Cressington Scientific Instruments Ltd., Chalk Hill Watford, England, UK). Thereafter the samples were viewed in an XL30 scanning electron microscope equipped with a field emission gun (FEI Europe, Eindhoven, The Netherlands) at an acceleration voltage of 5 kV.

For transmission electron microscopy (TEM) selected samples from 5-day-old oxic colonies and 3-month-old anoxic colonies of isolate FCAS11 were high-pressure frozen (Leica EM HPF, Leica Microsystems, Vienna, Austria), and subsequently subjected to freeze-substitution in a mixture of 1 % osmium tetroxide, 3 % glutaraldehyde, 0.3 % uranyl acetate in anhydrous methanol (CS auto-substitution apparatus, Leica Microsystems, Vienna, Austria) at -85 °C for 7 days, after which the temperature was raised to 0 °C at a speed of 5 °C h⁻¹ (Sitte et al. 1985). After rinsing with methanol, and subsequently with anhydrous acetone, the samples were finally embedded in Spurr's resin (Electron Microscopy Sciences, Hatfield, USA; Spurr 1969). Sections of 90 nm were contrasted with 4 % (w/v) aqueous uranyl acetate for 20 min and 0.4 % (w/v) aqueous lead citrate for 2 min (Venable and Coggs 1965), and viewed with a transmission electron microscope Tecnai 12 (FEI Europe, Eindhoven, The Netherlands) at an acceleration voltage of 120 kV. All the organic solvents used were of HPLC/analytical grade.

2.4 Nutritional requirements and growth

Nutritional requirements were investigated by the ability to utilize various carbon and nitrogen compounds according to Kurtzman et al. (2011), as was growth at various temperatures, growth with 3.5, 10, 16, 18 and 36% NaCl, growth with 30, 40, 50, 60 and 70% glucose, and growth with 0.01 and 0.1 % cycloheximide. Auxanograms with nitrogen compounds were also placed in anoxic jars and inspected for growth up to five months. All preparations for light microscopy were made in water and viewed with a Zeiss Axioskop microscope using phase contrast optics. Staining of the nuclei with DAPI was done according to Boverset et al. (2006).

2.5 DNA isolation and gene amplification

Isolate FCAS11 was grown in 50 ml of malt extract broth in a 250-ml Erlenmeyer flask on a stationary platform for 5 – 7 days at room temperature. The mycelial mats were then harvested and about 200 mg of the freeze dried mycelia was used for total genomic DNA extraction using a high salt concentration extraction buffer (100mM TrisHCl, pH 8, 100mM sodium phosphate buffer pH 8, 1.5 M NaCl, 100mM EDTA pH 8, 1% CTAB) followed by precipitation with isopropanol (Jebaraj et al. 2010). The purity and identity of the culture was determined by comparing the SSU sequence with that reported in our earlier work (Jebaraj et al. 2010) that is deposited in GenBank. The internal transcribed spacer region (ITS) and large subunit (LSU) of the nuclear rDNA, translation elongation factor 1 α (EF1 α) and DNA directed RNA polymerase II subunit 2 (RPB2) were amplified. The basic PCR protocol employed an initial 3 min at 97 °C followed by 30 s at 97 °C, 30s at the specific annealing temperature for each primer set (Table 1), 1 min at 72 °C, followed by 7 min extension at 72 °C. The PCR amplicons were purified using GenEluteTMGel Extraction kit (Sigma Aldrich, USA) and cloned individually for each gene using the TA cloning kit (Invitrogen, USA) according to the manufacturer's instructions. A representative clone with the insert was sequenced bidirectional in a 3730 DNA Stretch Sequencer, with the XL Upgrade using ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). The sequences generated from this study are deposited in the NCBI's GenBank nucleotide database (<http://ncbi.nlm.nih.gov>) under accession numbers KC879157-KC879160.

2.6 Phylogenetic analyses

The SSU (obtained from the GenBank database, Jebaraj et al. 2010), ITS, LSU, RPB2 and EF1 α sequences of FCAS11 were individually subjected to BLASTn analyses (Altschul et al. 1990) in the GenBank database. For a multigene phylogeny, sequences of related taxa were retrieved and supplemented to the datasets published in Schell et al. (2011) (Table 2). The ITS dataset included 14 representatives within *Tritirachiomycetes* along with the FCAS11 sequence; *Sporobolomyces coprosmae* and *Sakaguchia dacryoidea* were used as an outgroup. The sequences were aligned using the MUSCLE algorithm in SeaView version 4 (Gouyet al. 2010) and refined with the G-Blocks program (<http://www.phylogeny.fr>). The resulting alignments were manually checked and subjected to Maximum Likelihood (ML) analyses using the RaxML cluster module available in the Computational Center at the University of Kaiserslautern (Germany). The ML algorithm employed the GTRGAMMAI model and bootstrapping was performed with 1000 replicates. Additionally, Neighbour Joining (NJ) trees were constructed in SeaView version 4. Robustness of NJ trees in the analyses was assessed with 1000 NJ bootstrap replicates. The alignments (including individual gene alignments) are deposited in TreeBase under submission ID 14277.

3. Results

3.1 Phylogenetic analyses

Without exception and with significant support, phylogenetic analyses of the concatenated multigene and the ITS (and also individual gene phylogenies of SSU, LSU, RPB2 and EF1 α , results not shown but available from the authors) place isolate FCAS11 within the recently described monophyletic class *Tritirachiomycetes* within subphylum *Pucciniomycotina* (Fig. 1 and Supplemental Fig. 1). The isolate FCAS11 forms an individual branch within the genus *Tritirachium* clearly distinct from *T. dependens* (= *T. egeum*), *T. oryzae*, *T. cinnamomeum*, *T. roseum* or any of the cryptic species which are yet to be described. Moreover, FCAS11 occurs separately from the lineage containing *Paratritirachium cylindroconium*. Pairwise alignment of the FCAS11 SSU, ITS and LSU sequences with its respective BLASTn hits within the *Tritirachium* genus shows only 97.61 % similarity with *T. oryzae* (CBS164.67), 83.88 % with *T. oryzae* (NRRL 2018) and 90.43 % with *T. oryzae* (CBS164.67). From these results we can confidently conclude that FCAS11 represents a new species.

3.2 Taxonomy

Tritirachium candoliense Manohar, Boekhout, Müller and Stoeck sp. nov.

MycoBank: MB 804034

Etym.: “*candoliense*” was derived from the name of the coastal village “Candoli”, which is the Konkani (regional language of Goa) name for “Candolim”.

Diagnosis: Colonies of *T. Candoliense* are farinose, pinkish and vinaceous in comparison to *T. oryzae*, *T. roseum* and *T. sp.* (CBS 473.93), which were reported to have pale vinaceous-lilac to light vinaceous-lilac colors (Schell et al. 2011). The reverse coloration is dark purplish, almost similar to that of *T. dependens* (IHEM 3497). Hyphae are uninucleate, contrasting to other members of *Tritirachiomycetes*, which are reported to have multinucleate hyphae. Conidiophores are erect, ascendant, smooth-walled, tapered, brownish, but paler to base, with sworls of two or three conidiogenous cells at regular distances. The arrangement of conidiogenous cells in *T. Candoliense* is similar to that of *T. dependens*, *T. cinnamomeum* and *Tritirachium sp.* (CBS 265.96). The conidiogenous cells are 1-2 celled, slightly ventricose near the base and tapered towards the apex like other members of *Tritirachium spp.*, but different from those in *T. cinnamomeum* that do not have tapered conidiogenous cells. The conidia measure 2.5-3.5 x 1.6-2.5 μm and the mean cell size is different from all other members of *Tritirachium spp.* The conidiogenous cells are flask-shaped similar to *T. dependens*, *T. Oryzae* and *Tritirachium sp.* CBS 473.93. These morphological similarities and differences of *T. candoliense* with other members of *Tritirachium spp.* clearly show that *T. candoliense* is morphologically distinct different from any of the reported *Tritirachium sp.*

(Table 3). In addition and as indicated above significant differences were observed in SSU, LSU, RPB2 and EF1 α DNA sequences.

Holotype: Dried culture preserved in H-CBS as CBS H-21328; ex-type cultures preserved in the Microbial Type Culture Collection and Gene Bank, Chandigarh, India as MTCC9381 and CBS Fungal Biodiversity Centre, Utrecht, The Netherlands as CBS123151.

Growth under oxic and anoxic conditions: The colony morphology (Fig. 2), conidiophore formation (Fig. 3) and the ultrastructural details (Fig. 4) of *T. Candoliense* was studied under oxic (Figs 2A, 3A, 4A-C) and anoxic conditions (Figs 2B, 3B, 4D-F).

Under oxic growth, streak colonies on CMA, after five days at room temperature 2-3 mm width, somewhat elevated and irregular through transversal ridges, pinkish vinaceous, farinose, with an eroded margin, reverse dark purplish brown. The culture exudes a dark purplish pigment into the agar on CMA and PDA, but not on MEA and YMA. After three months under oxic conditions the colony was dimorphic, up to 8 mm width, with center 3-4 mm, flat to somewhat elevated, velvety to farinose, under lens covered with fine hairs, pinkish red–vinaceous, dull, near margin paler, with strong hydrophobic surface (Fig. 2A), near margin with hyphal mat on agar surface, sectors may be present, reverse dark reddish brown; exudes dark pink to purplish pigment into agar; on MEA reddish pink exudation droplets are formed on colony surface.

Hyphae in older cultures were straight to somewhat undulating, septate, with cells measuring 15-40 x 1-2 μ m, without clamp connections; conidiophores erect, ascendent, smooth-walled, tapered, brownish, but paler to base, with sworls of two or three conidiogenous cells at regular distances. Conidiogenous cells 1-2-celled, slightly ventricose near base and tapered towards apex, 15-50 x 0.8-2.0 μ m, with a distinct sympodial rachis of up to 70 μ m long on which blastoconidia are formed, much like a bunch of grapes. Conidia 2.5-3.5 x 1.6-2.5 μ m, one-celled, ellipsoidal to clavate-ellipsoidal, with a rather broad base, pale brownish; conidia germinate with a short germ tube of 4-9 μ m long or with small secondary blastoconidia; submerged hyphae irregularly branched, with reddish brown cell walls. Staining of nuclei with DAPI revealed that the conidia and hyphal cell were uninucleate (data not shown). SEM analysis revealed hyphae with erect conidiophores with sworls of two or three conidiogenous cell with a terminal sympodial rachis on which the one-celled conidia are formed (Fig.3A). TEM analysis of hyphal cells grown in oxic conditions showed the presence of tapering hyphal septa with a central narrow pore with a putative pore-occluding cystosome (Fig. 4A). The cells contained significant numbers of fat globules (Fig. 4B), and mitochondria with well-developed cristae (Fig. 4C).

Under anoxic growth after approximately three months in an anoxic jar at 25°C, slow and weak growth with a whitish colony was observed on plates (Fig. 2B). Microscopically, hyphae are loose to dense, more or less dichotomously branched, with cells 9-35 x 1-2 μ m and the hyphal tips

may be somewhat swollen. Conidiogenous cells and conidia were almost absent, but few blastoconidia were present (Fig. 3B). Blastoconidia are obovoid to ellipsoidal, 2-3.5 x 2.3 – 2.8 μm , pale brownish, and with a rather broad base. SEM analysis yielded mainly sterile hyphae on which blastoconidia are formed occasionally, while the conidiophores and conidiogenous cells were observed rarely (Fig. 3B). TEM analysis showed that hyphal cells formed membrane loops from the plasmamembrane (plasmalemmasomes) into the cytoplasm (Fig. 4D, arrow), whereas cystosomes were present as well. The anoxic cells contained irregularly shaped to jagged fat globules (Fig. 4E, arrow) and mitochondria had both intact and degraded cristae (Fig. 4F, arrow).

Growth requirements: Most of the 47 tested carbon compounds, that included pentoses, hexoses, di- and trisaccharides, alcohols and polyols, amines and organic acids, were utilized by FCAS11 under oxic conditions indicating a versatile nature of the fungus. Growth on quinic acid and glucono-D-lactone was delayed and weak, whereas galacturonate and citrate could not be utilized (Suppl. Table 1). Nitrate (with an inhibition zone directly near the compound on the agar surface, and only growth starting at 5cm distance from the compound), ethylamine, L-lysine and glucosamine were utilized, but growth was absent with nitrite, cadaverine, creatin, creatinin, and imidazole. In anoxic jars, growth was not present with nitrate and nitrite. Under oxic conditions growth was present at 10-35°C, but not at 42°C. After one week good growth occurred on plates with 30, 40 and 50% glucose, whereas slow growth occurred [i.e. like a thin film] occurred after 20 days on 70% glucose; fermentation of glucose was absent. The isolate grew well on plates with 3.5, 10, 12.5, 16, 18 and 36% NaCl. Thus, isolate FCAS11 is osmophilic and halophilic, which may further explain its presence in the marine environment. The growth was variable with 0.01% cycloheximide and absent with 0.1% cycloheximide.

Distribution: Arabian Sea, coastal waters of India during the anoxic season. No further records about the distribution of this species are available.

Specimens examined: India, Candolim area, ca. 20 km offshore (15° 30' 522" N, 733° 39' 00" E), anoxic marine sediment, 24 m water depth. Cathrine Sumathi Manohar, ex-type cultures in Microbial Type Culture Collection and Gene Bank, Chandigarh, India, MTCC 9381, and CBS Fungal Biodiversity Centre (CBS-KNAW), Utrecht, The Netherlands, CBS123151.

4. Discussion

We here describe a novel fungus (isolate FCAS11) isolated from sediments in the seasonally oxygen minimum zone of the Arabian Sea, off Goa, India. Phylogenetic analyses identified FCAS11 as a novel species in the genus *Tritirachium*. Until recently, this genus was included within *Ascomycota*, but detailed phylogenetic analyses with an increased taxon sampling showed that the members of this genus form a significantly supported clade within the *Basidiomycota*. This clade was

assigned a separate class *Tritirachiomycetes*, order *Tritirachiales* and family *Tritirachiaceae* based on these analyses (Schell et al. 2011). So far the class *Tritirachiomycetes* includes four well-defined species (*T. dependens* (= *T. egeum*), *T. oryzae*, *T. cinnamomeum*, *T. roseum*), two cryptic species within the genera *Tritirachium*, and *Paratritirachium cylindroconium* (Schell et al. 2011; Beguin et al. 2012). *T. dependens* was first isolated from the dead roots of *Yucca treculeana* (Agavaceae) in Cuba (Limber 1940) and *T. cinnamomeum* was isolated from an unidentified fly in the Netherlands (Beyma 1942). *T. egeum* was found on a Rodac contact plate filled with malt extract agar in an indoor study associated with a *Penicillium* species (Beguin 2010). Phylogenetic analysis, however, has shown that *T. egeum* is probably a synonym of *T. dependens* (Schell et al. 2011). *T. roseum* and *T. oryzae* are saprotrophs and they are also associated with human infections (Rodrigues and Laibson 1975; Moraes et al. 2010). *T. oryzae* is the most commonly reported air-borne fungus, which was also detected in packing materials (de Hoog 1972) and was identified as the causative agent for toe-nail, corneal and scalp infection (Moraes et al. 2010; Rodrigues and Laibson 1975). Isolates CBS 265.96 and CBS 473.93 are classified as cryptic species within *Tritirachiomycetes* and are not yet described due to the lack of adequate information to characterize these isolates (Schell et al. 2011).

Paratritirachium cylindroconium is the only representative species for which the rDNA loci are available and that belongs to another genus recently identified within the class *Tritirachiomycetes*. This species was originally isolated as *Nodulisporium cylindroconium* and assigned to Ascomycota (Beyma 1942). This species was considered morphologically similar to *T. cinnamomeum* (de Hoog 1972), but phylogenetic analyses based on the SSU rDNA sequence suggest a separate generic status for this species within the class *Tritirachiomycetes* (Beguin et al. 2012). An increased taxon sampling for phylogenetic analyses will show the validity of the genus *Paratritirachium*.

The new isolate branches within the monophyletic genus *Tritirachium* (in all analyses with significant support, Fig. 1 and Suppl. Fig. 1, as well as individual gene trees, data not shown) but as an isolated branch distant to described and sequenced species. Therefore, we feel confident to describe isolate FCAS11 as a new species in the genus *Tritirachium*, considering the concept of monophyly for a fungal species in phylogenetic analyses. We name the new species *T. candoliense* after the small coastal village Candolim. Off the coast of this village a time series station has been established, which is part of the sampling program to study the coastal anoxic condition developing seasonally between April and October each year in this oceanic region (Naqvi et al. 2006). This new fungal culture was isolated from sediment collected in one of the time series stations during the anoxic season. *T. candoliense* is the only *Tritirachium* sp. described from a marine habitat has been reported before by us based on SSU sequences (Jebaraj et al. 2010), and that was subsequently mentioned by Richards et al. (2012). Interestingly, in spite of numerous molecular environmental

eukaryote diversity surveys in a variety of aquatic (mostly marine) habitats, no sequence was retrieved so far that falls within this genus. Not even a molecular diversity survey of fungi using both fungal-specific PCR primers and domain-specific eukaryote primers conducted in the same sampling area has uncovered a *Tritirachium*-related sequence (Jebaraj and Raghukumar 2009; Jebaraj et al. 2010). This indicates that environmental sequencing is no panacea when it comes to recording undescribed and unseen microbial diversity in our oceans and other environments. Combining methods from the modern (meta)genome era with traditional cultivation approaches is mandatory if we are to uncover and characterize the diversity hidden in the microbial world.

The sampling location of *T. candoliense* is the oxygen minimum zone (OMZ) of the Arabian Sea with seasonal anoxia (Jebaraj and Raghukumar, 2009) at times of anoxic conditions in the sediment and bottom water. *T. candoliense* is capable of slow growth under the absence of oxygen (Fig. 2 A, B) under laboratory conditions. Interestingly, the fungus produces very few spores under these circumstances, which could be a successful general survival strategy for fungi under anaerobic conditions. Fungal dimorphism induced by anaerobic stress has been noted in some fungi. In the ascomycetous yeast *Yarrowia lipolytica* anaerobic stress resulted in the formation of mycelial cells (Ruiz-Herrera and Sentandreu 2002) and in the zygomycetous fungus *Mucor* spp. anaerobiosis resulted in the formation of yeast-like cells (Bartnicki-Garcia and Nickerson 1962). The latter observations date back to the 1870s when a number of scientists, such as Pasteur, Reess, Fitz and Brefeld, observed that dimorphism of *Mucor* was either regulated by low oxygen or high carbon dioxide levels [cited in Bartnicki-Garcia and Nickerson, 1962]. Next to anaerobiosis, other factors such as elevated levels of carbon dioxide, pH, composition of the growth medium and temperatures, are known to impact fungal dimorphism (Bartnicki-Garcia and Nickerson 1962; Rogers et al. 1974; Ruiz-Herrera and Sentandreu 2002; Klein and Tebbets 2007). The inability of *T. candoliense* to sporulate under anoxic conditions as abundantly as under oxic conditions shows that the organism is not ideally adapted to anoxia and it remains to be seen how the almost exclusive hyphal growth at anoxic conditions is regulated. A deficiency in adaptation to anoxia becomes evident in the ultrastructure showing vestigial possibly malfunctioning mitochondria (Fig. 4), which would also explain the slow growth in the absence of molecular oxygen. Changes from oxic to anoxic conditions also resulted in poor mitochondrial ultrastructure in the yeast *Saccharomyces cerevisiae* (Postmus et al. 2011). Examples of terrestrial fungi growing in the presence and also in absence of oxygen include species of the ascomycote genus *Fusarium*. *Fusarium oxysporum*, a plant pathogen grows well without molecular oxygen by utilizing H₂ and possessing hydrogenase (Gunner and Alexander 1964). Further efforts will be in order to study the metabolic processes that enable *T. candoliense* for anaerobic proliferation. It is reasonable to assume that the hitherto unknown anaerobic physiology of this new isolate provides insufficient energy for strong growth under exclusion of oxygen. *F. oxysporum* possesses two nitrate reductases for assimilatory

and dissimilatory processes, which are active in aerobic and anerobic conditions, respectively (Kurakovet al. 2000). This remains to be studied for *T. candoliense*. The fungus was also able to grow at elevated concentrations of sugar and salt levels that may allow it to grow/survive in the marine environment. Thus its ability to grow in media with low water activities and under anoxia contributes to its presence in anoxic marine habitats. The detailed interplay between these two physiological factors and the growth or survival of the fungus, however, needs to be determined.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at xxxxxx

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Figure legends

Fig 1. Most likely phylogenetic tree of FCAS11 (*Tritirachium candoliense*) based on maximum likelihood analysis of the concatenated SSU, ITS, LSU, EF1 α and RPB2 sequences of *Tritirachium* species (phylum Basidiomycota). Values at nodes indicate bootstrap support from 1000 replicates from ML/NJ analyses.

Fig 2. Growth of FCAS11 (*Tritirachium candoliense*) in: A. oxic growth condition after three days, malt extract agar at 25 °C, also note the hydrophobic nature of the colony surface and B. in anoxic growth condition after three months, Malt extract agar at 25°C. Bar represents 5 mm.

Fig 3. SEM images of FCAS11 (*Tritirachium candoliense*) A. grown in oxic condition showing many blastoconidia formed on a sympodial rachis; B. grown in anoxic condition with very few blastoconidia (arrow). Bar represents 5 μ m.

Fig 4. TEM analyses of FCAS11(*Tritirachium candoliense*) hyphae grown under oxic (A-C) and anoxic (D-F) conditions showing: A. septum with cystosome (arrow; B. fat droplets (arrows); C. mitochondria (arrows) with normal cristae; and TEM analyses of hyphae grown under anoxic condition showing D. membrane invaginations ; E. irregular shaped fat droplet; F. degraded/ malformed mitochondrion (arrow). Bar represents 0.5 μ m.

Suppl Fig 1. Phylogenetic tree of FCAS11 (*Tritirachium candoliense*) based on the maximum likelihood analysis of the ITS sequences of the class Tritirachiomycetes. Values at nodes indicate bootstrap support from 1000 replicates from ML/NJ analysis above 50.

Table 1. List of PCR primers used in this study for the amplification of taxonomic marker genes of *T.candoliense* sp. nov..

Primer set	Primer	Primer Sequence (5' -3')	Annealing Temp (°C)	Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGG	58	White et al. 1990
	ITS4	TCCTCCGCTTATTGATATGC		
LSU	LR0R	GTACCCGCTGAACTTAAGC	50	Vilgalys et al. 1990
	LR7	TACTACCACCAAGATCT		
RPB2	5F	GAYGAYMGWGATCAYTTYGG	55	Liu et al. 1999
	11bR	CAATCWCGYTCCATYTCWCC		
EF1 α	983F	GCYCCYGGHCAYCGTGAYTTYAT	56	Rehner and Buckley 2005
	2218R	ATGACACCRACRGCACRGTYTG		

Table 2. List of sequences used for phylogeny studies.

S.No.	Taxon	SSU	ITS	LSU	RPB2	EF-1 α
1	<i>Agaricostilbum hyphaenes</i> (Har. & Pat.) Oberw. & Bandoni	AY665775	AF444553	AY634278	AY780933	AY879114
2	<i>Auricularia</i> sp.	DQ234542	DQ200918	AY634277	DQ366278	DQ408144
3	<i>Bannoa</i> sp.	DQ631899	DQ631900	DQ631898	DQ631901	DQ631902
4	<i>Bensingtonia yuccicola</i> (Nakase & M. Suzuki) Nakase & Boekhout	DQ234543	NR_073280	AY745727	n/a	DQ028582
5	<i>Calocera cornea</i> (Batsch) Fr.	AY771610	AB712437	AY701526	AY536286	AY881019
6	<i>Chionosphaera apobasidialis</i> D.E. Cox	U77662	NR_073325	AF177407	n/a	n/a
7	<i>Cintractia limitata</i> G.P. Clinton	DQ645507	DQ645508	DQ645506	DQ645509	DQ645511
8	<i>Classicula fluitans</i> R. Bauer, Begerow, Oberw. & Marvanová	AY124478	n/a	AY512838	n/a	n/a
9	<i>Coprinus comatus</i> (O.F. Müll.) Pers.	AY665772	AY854066	AY635772	AY780934	AY881026
10	<i>Cryptomycocolax abnormis</i> Oberw. & R. Bauer	n/a	n/a	AY512841	n/a	n/a
11	<i>Endocronartium harknessii</i> (J.P. Moore) Hirats.	AY665785	DQ206982	AY700193	DQ234551	DQ234567
12	<i>Helicobasidium mompa</i> Tanaka	U77064	AY292429	AY254179	n/a	EF100614
13	<i>Helicogloea lagerheimii</i> Pat.	AY124476	n/a	AY512849	n/a	n/a
14	<i>Helicogloea variabilis</i> K. Wells	U78043	n/a	L20282	n/a	n/a
15	<i>Jaculispora submersa</i> H.J. Huds. & Ingold	AY124477	n/a	AY512853	n/a	n/a
16	<i>Leotia lubrica</i> (Scop.) Pers.	AY544687	DQ491484	AY544644	DQ470876	DQ471041
17	<i>Leucosporidium scottii</i> Fell, Stätzell, I.L. Hunter & Phaff	AY707092	AF444496	X53499	n/a	DQ059761
18	<i>Microbotryum violaceum</i> (Pers.) G. Deml & Oberw.	U77062	AY588099	AY512864	n/a	n/a
19	<i>Mixia osmundae</i> (Nishida) C.L. Kramer	D14163	DQ831010	AB052840	n/a	n/a
20	<i>Paratritirachium cylindroconium</i> (de Hoog) Beguin, Pyck & Detandt	JF797220	n/a	n/a	n/a	n/a
21	<i>Platygløea vestita</i> Bourdot & Galzin	AY124480	n/a	AY512872	n/a	n/a
22	<i>Sakaguchia dacryoidea</i> Y. Yamada, K. Maeda & Mikata	DQ832206	AF444597	AF189972	DQ832208	DQ832209
23	<i>Septobasidium canescens</i> Burt	AY373389	DQ241446	DQ241479	n/a	DQ648060
24	<i>Sphacelotheca polygoni-persicariae</i> G. Deml & Oberw.	DQ832220	AF444593	AF189974 ^b	n/a	DQ832224
25	<i>Sporisorium reilianum</i> (J.G. Kühn) Langdon & Full.	DQ832229	n/a	DQ832228	DQ832231	DQ832233
26	<i>Sporobolomyces coprosmae</i> Hamam. & Nakase	D66880	NR_073317	AF189980	n/a	AB127087

27	<i>Tritirachium candoliense</i> sp. nov. (CBS 123151)	GQ120154	KC879157	KC879158	KC879159	KC879160
28	<i>T. cinnamomeum</i> (CBS 182.42)	JF7972225 ^c	JF779667	n/a	n/a	n/a
29	<i>T. dependens</i> (<i>T. egenus</i>) (IHEM 3497)	JF263567	JF779666	n/a	n/a	n/a
30	<i>T. oryzae</i> (CBS 116300) ^a	n/a	JF779657	n/a	n/a	n/a
31	<i>T. oryzae</i> (CBS 164.67)	JF779647	GQ329853	GQ329853	JF779648	JF779645
32	<i>T. oryzae</i> (CBS 388.39) ^a	n/a	JF779661	n/a	n/a	n/a
33	<i>T. oryzae</i> (CBS 442.70) ^a	n/a	JF779659	n/a	n/a	n/a
34	<i>T. oryzae</i> (CBS 837.71) ^a	n/a	JF779658	n/a	n/a	n/a
35	<i>T. oryzae</i> (CBS 896.70) ^a	n/a	JF779660	n/a	n/a	n/a
36	<i>T. oryzae</i> (NRRL 1210) ^a	n/a	JF779662	n/a	n/a	n/a
37	<i>T. oryzae</i> (NRRL 2018) ^a	n/a	JF779663	n/a	n/a	n/a
38	<i>T. roseum</i> (CBS 183.42)	JF779655	JF779669	n/a	JF779656	n/a
39	<i>T. species</i> (CBS 265.96)	JF779653	JF779668	JF779652	JF779654	n/a
40	<i>T. species</i> (CBS 473.93)	n/a	JF779664	JF779649	JF779651	JF779646
41	<i>T. depends</i> (BPI 414589) ^a	n/a	JF779665	n/a	n/a	n/a
42	<i>Taphrina wiesneri</i> (Ráthay) Mix	AY548293	AB505445	AY548292	n/a	DQ479936
43	<i>Tilletia goloskokovii</i> Schwarzman	DQ832247	DQ832248	AY818999	DQ832249	Q83225Z
44	<i>Ustanciosporium standleyanum</i> (Zundel) M. Piepenbr.	DQ846889	DQ846890	DQ846888	DQ846891	DQ846893

n/a not available

^a taxon was not included for multi-gene analysis

^b *Sphacelotheca* cf. *koordersiana*

^c IHEM 3714

Table 3. Comparison of morphological characters of *T. candoliense* sp. nov. with other described *Tritirachium* species (after Schell et al. 2011)

Species	type	Colony color	Conidial size, mean (mm)	Conidiogenous cell strongly tapered	Conidiogenous cell flask-shaped	Conidiophore branches often terminate in whorls of conidiogenous cells
<i>T. dependens</i>	BPI 414589	Tan	3.2 X 2.5	Yes	Yes	Yes
<i>T. dependens</i>	IHEM 3497	Tan	3.3 X 2.7	Yes	Yes	Yes
<i>T. cinnamomeum</i>	CBS 182.42	Tan	4.2 X 2.9	No	No	Yes
<i>T. sp.</i>	CBS 265.96	Tan	3.6 X 2.8	No	No	Yes
<i>T. roseum</i>	CBS 183.42	Vinaceous	2.8 X 2.0	Yes	No	No
<i>T. oryzae</i>	CBS 896.70	Vinaceous	2.5 X 2.1	Yes	Yes	No
<i>T. sp.</i>	CBS 473.93	Vinaceous	2.5 X 2.1	Yes	Yes	No
<i>T. candoliense</i> sp. nov.	CBS 123151	Vinaceous	3.0 X 2.05	Yes	Yes	Yes

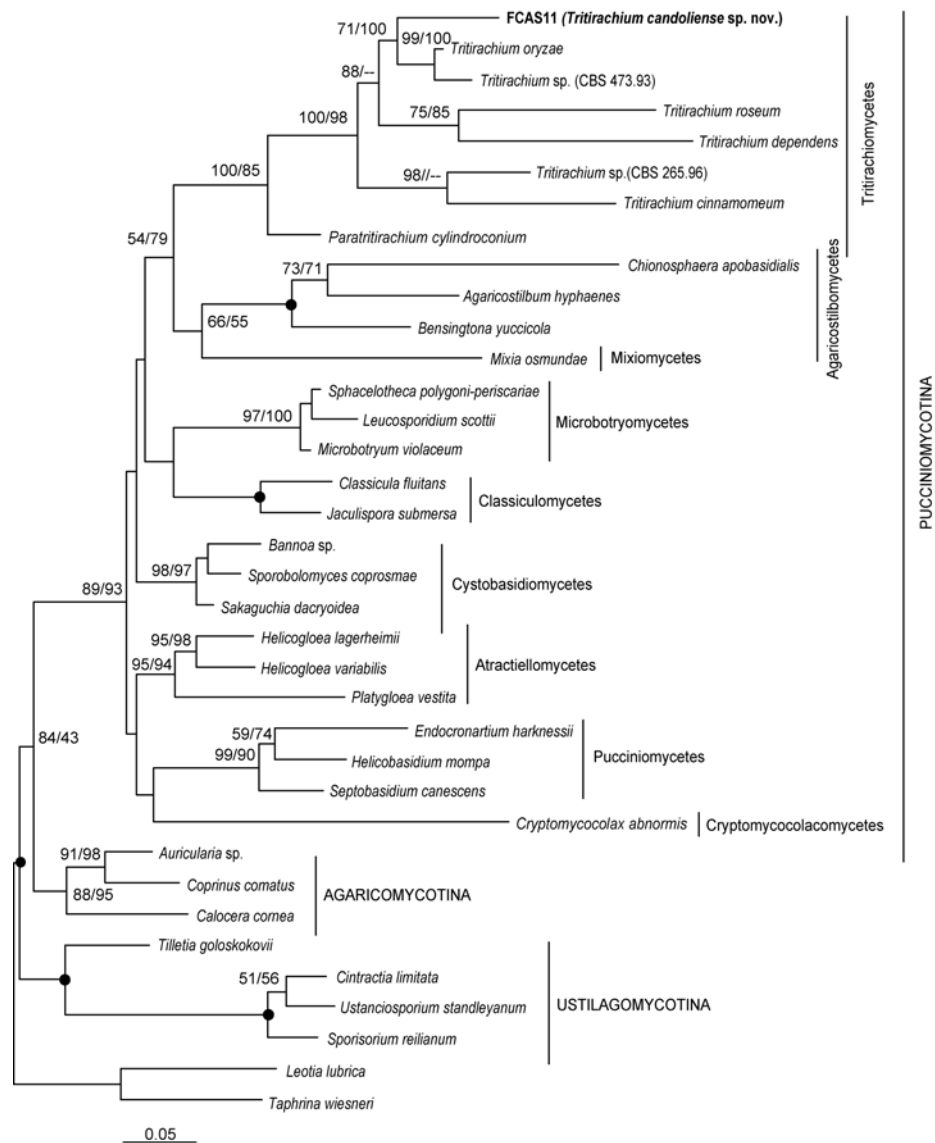


Fig 1. Most likely phylogenetic tree of FCAS11 (*Tritirachium candoliense*) based on maximum likelihood analysis of the concatenated SSU, ITS, LSU, EF1 α and RPB2 sequences of *Tritirachium* species (phylum Basidiomycota). Values at nodes indicate bootstrap support from 1000 replicates from ML/NJ analyses.

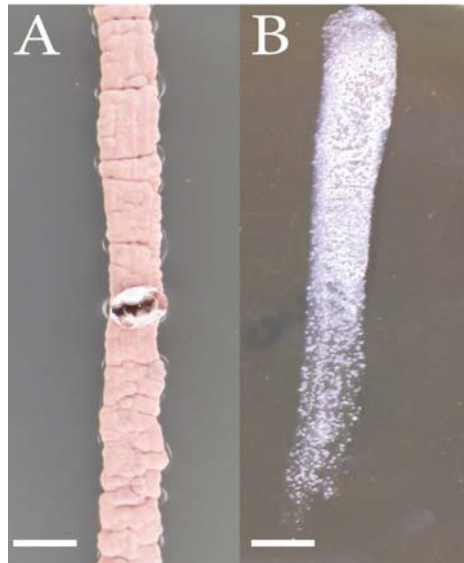


Fig 2. Growth of FCAS11 (*Tritirachium candoliense*) in: A. oxic growth condition after three days, malt extract agar at 25 °C, also note the hydrophobic nature of the colony surface and B. in anoxic growth condition after three months, Malt extract agar at 25°C. Bar represents 5 mm.

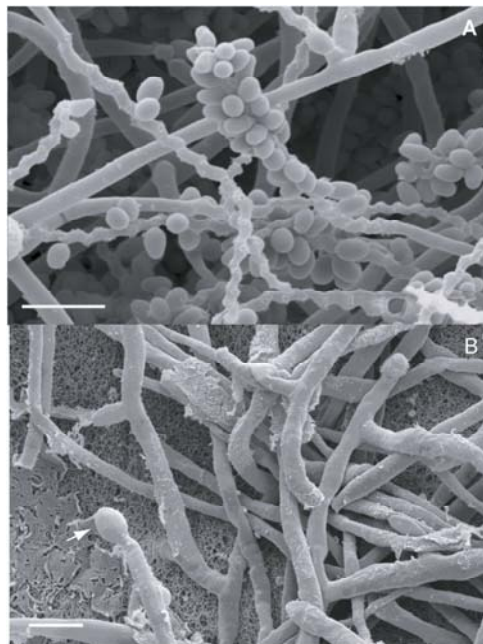


Fig 3. SEM images of FCAS11 (*Tritirachium candoliense*) A. grown in oxic condition showing many blastoconidia formed on a sympodial rachis; B. grown in anoxic condition with very few blastoconidia (arrow). Bar represents 5 μ m.

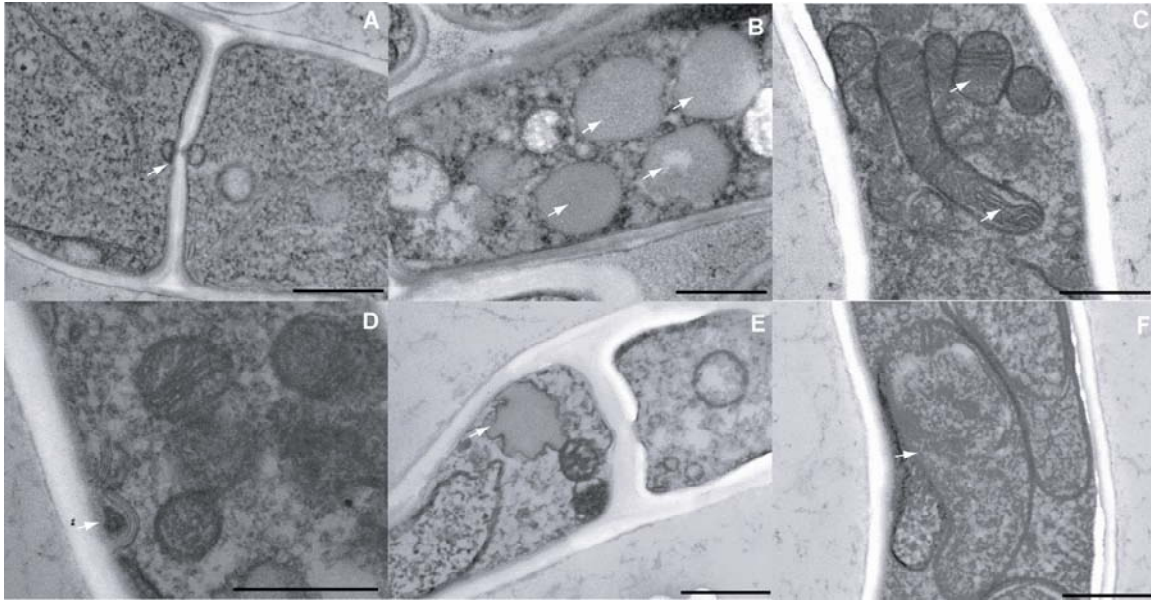


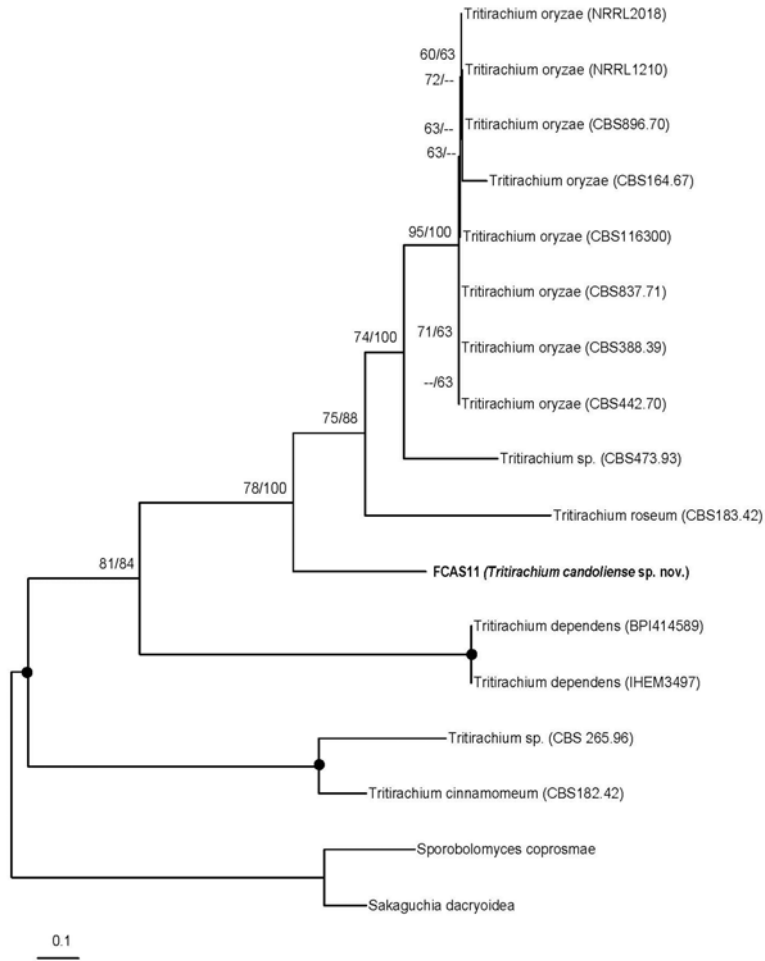
Fig 4. TEM analyses of FCAS11(*Tritirachium candoliense*) hyphae grown under oxic (A-C) and anoxic (D-F) conditions showing: A. septum with cystosome (arrow; B. fat droplets (arrows); C. mitochondria (arrows) with normal cristae; and TEM analyses of hyphae grown under anoxic condition showing D. membrane invaginations ; E. irregular shaped fat droplet; F. degraded/ malformed mitochondrion (arrow). Bar represents 0.5 μm .

Suppl Table 1. Nutritional growth characteristics of FCAS11
(*Tritirachium candoliense* sp. nov.)

Fermentation	
Glucose	-
Carbon sources	
D-glucose	+
D-galactose	+
L-sorbose	+
D-glucosamine	+
D-ribose	+
D-xylose	+
L-arabinose	+
D-arabinose	+
L-rhamnose	+
Sucrose	+
Maltose	+
α,α Trehalose	+
Methyl- α -D-glucoside	+
Cellobiose	+
Salicin	+
Arbutin	+
Melibiose	+
Lactose	+
Raffinose	+
Melezitose	+
Inulin	+
Soluble starch	+
Glycerol	+
mesoErythritol	+
Ribitol	+
Xylitol	+
L-Arabinitol	+
D-Glucitol	+
D-Mannitol	+
Galactitol	+
myo-Inositol	+
Glucono- δ -lactone	w/d
D-Gluconate	+
D-glucuronate	+
D-galacturonate	-
DL-Lactate	+
Succinate	+
Citrate	-
Methanol	d/+
Ethanol	d/+
Propane 1,2diol	d/+
Butane 2,3diol	d/+

Quinic acid	w/d
Saccharate	+
Galactonic acid	+
Nitrogen compounds	
Nitrate	+
Nitrite	-
Ethylamine	+
L-Lysine	+
Cadaverine	-
Creatine	-
Creatinine	-
Glucosamine	+
Imidazole	-
Salt	
3.5%	+
10%	+
16%	+
18%	+
36%	+
Glucose	
30%	+
40%	+
50%	+
70%	V
Cycloheximide	
0.01%	V
0.10%	-
Temperatures	
10°C	+
25°C	+
30°C	+
35°C	+
42°C	-

The symbols denotes the culture showed ability to utilize (+), inability to utilize (-), was viable (V), weak and delayed growth (w/d), delayed growth (d/+) with different nutritional supplements and conditions.



Suppl Fig 1. Phylogenetic tree of FCAS11 (*Tritirachium candoliense*) based on the maximum likelihood analysis of the ITS sequences of the class Tritirachiomycetes. Values at nodes indicate bootstrap support from 1000 replicates from ML/NJ analysis above 50.