INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Ouhibi *et al.*, *Int J Syst Evol Microbiol* 2018;68:3217–3225
DOI 10.1099/ijsem.0.002962

MICROBIOLOGY SOCIETY

# *Penicillium tunisiense* sp. nov., a novel species of *Penicillium* section *Ramosa* discovered from Tunisian orchard apples

Salma Ouhibi,[1,2] Carla Santos,[2] Ridha Ghali,[1] Célia Soares,[2] Abderrazek Hedhili,[1] Russell Paterson[2] and Nelson Lima[2,*]

**Abstract**

Two similar *Penicillium* isolates could not be identified as previously described species in a survey of orchard apples from Tunisia for patulin-producing fungi. These isolates are described as novel species using multilocus DNA sequence analysis of partial β-tubulin, calmodulin and nuclear ribosomal internal transcribed spacer regions; and morphological, physiological and biochemical characteristics. The isolates were considered negative for patulin production since the *IDH* gene fragment was not detected and the compound detected at the same retention time of patulin (14.9 min) showed a different UV spectrum using U-HPLC/UV-DAD. In terms of phylogeny, the two isolates clustered with *Penicillium* section *Ramosa* and are closely related to *Penicillium chroogomphum*, *Penicillium lenticrescens* and *Penicillium soppii*. Furthermore, their macro- and micromorphological traits differed from these species. Hence, the isolates represent a novel species in *Penicillium* section *Ramosa* and the name *Penicillium tunisiense* sp. nov. is proposed, with the type strain MUM 17.62$^T$ (=ITEM 17445$^T$).

## INTRODUCTION

Several fungal species cause various apple rots [1]. Some penicillia are responsible for apple spoilage particularly within *Penicillium* subgenus *Penicillium*. *Penicillium expansum* causes blue rot of apples and produces the mycotoxin patulin that contaminates apples and apple products [2–5], which may be harmful to human health [6, 7]. Other contaminants of apples include *Penicillium solitum* and *Penicillium crustosum* [8, 9]. However, other fungi-spoil food and accurate identification of species are necessary to effectively control the quality of apple and apple-based products.

Traditionally, fungal identification relied on culture-based and morphological analyses. This often resulted in misidentifications since such techniques were mostly dependent on subjective characteristics. Although there are some morphological and phenotypical traits that differentiate closely related species such as the ones from section *Brevicompacta* and *Ramosa*, for example, how the rami are arranged (more or less appressed) or the species extrolite profile [9], these traits may not be sufficient. New solutions to *Penicillium* taxonomy are crucial to mycotoxin research and health [10], and a practical approach for *Penicillium* identification based on mycotoxin traits was proposed by Paterson *et al.* [11]. Currently, highly specific molecular biology techniques allow this differentiation in a more robust way. Phylogenetic analysis of *Penicillium* species is normally based on the *ITS* or *benA* genes and might include additional markers such as *CAL* and *RPB2* genes [12]. The thorough phylogeny of *Penicillium* described by Visagie *et al.* [13] provides solid information for the identification of new isolates based on molecular techniques.

As part of a study that investigated the presence of patulin-producing fungi in apples purchased in Tunisian city markets, two *Penicillium* isolates could not be assigned to previously described species. Here we report the multilocus phylogenetical, biochemical, physiological and morphological characterisation that was applied to describe *Penicillium tunisiense*, a novel species within *Penicillium* section *Ramosa*.

## METHOD

### Fungal isolates

A total of 56 *Penicillium* fungi were isolated from 270 orchard apples randomly collected from public markets in three different Tunisian towns: Gafsa (South, *n*=90);

**Author affiliations:** [1]Mahmoud Yaacoub Center of Urgent Medical Assistance of Tunis, Laboratory of Toxicology and Environment (LR12SP07) and El Manar University, Tunis, Tunisia; [2]CEB-Centre of Biological Engineering, Micoteca da Universidade do Minho (MUM), University of Minho, Braga, Portugal.
***Correspondence:** Nelson Lima, nelson@ie.uminho.pt
**Keywords:** phylogeny; taxonomy; β-tubulin; calmodulin; *ITS* region; MLSA.
**Abbreviations:** CSN, creatine sucrose neutral; CYA, Czapek yeast autolysate; G25N, glycerol nitrate; MEA, malt extract agar; MUM, Micoteca da Universidade do Minho; OA, oatmeal agar; YES, yeast extract sucrose.
The GenBank accession numbers of strain MUM 17.62$^T$ are *ITS* region sequence, MG586956; *benA* gene sequence, MG586970; *CAL* gene sequence, MG586974; and *TEF-1α* gene sequence, MG586972.
One supplementary figure is available with the online version of this article.

**Table 1.** Details of the primers used and target zone

| Target zone | Primer | Sequences | Reference |
|---|---|---|---|
| Beta-tubulin gene (*benA*) | Bt2a | 5′-GGTAACCAAATCGGTGCTGCTTTC-3′ | [33] |
| | Bt2b | 5′-ACCCTCAGTGTAGTGACCCTTGGC-3′ | |
| | Bt1b | 5′-GACGAGATCGTTCATGTTGAACTC-3′ | |
| Calmodulin (*CAL*) | Cl1 | 5′-GARTWCAAGGAGGCCTTCTC-3′ | [34] |
| | Cl2a | 5′-TTTTTGCATCATGAGTTGGAC-3′ | |
| Translation elongation factor 1-alpha (*TEF-1α*) | EF1c | 5′-TCGTCGTTATCGGCCACGTC-3′ | [35] |
| | EF6 | 5′-CTTSTYCCARCCCTTGTACCA-3′ | |
| Nuclear ribosomal internal transcribed spacer regions (*ITS*) | ITS1 | 5′-TCCGTAGGTGAACCTGCGG-3′ | [36] |
| | ITS4 | 5′-TCCTCCGCTTATTGATATGC-3′ | |
| Isoepoxydon dehydrogenase gene (*IDH*) | IDH1 | 5′-CAATGTGTCGTACT GTGCCC-3′ | [30] |
| | IDH2 | 5′-ACCTTCAG TCGCTGTTCCTC-3′ | |
| M13 fingerprinting | M13 | 5′-GAGGGTGGCGGTTCT-3′ | [37] |

Kasserine (Centre, *n*=90); and Tunis (North, *n*=90). The two strains of the proposed new species (MUM 17.62$^T$ and MUM 17.80) were isolated from the surface of Aziza apples from Tunis. Both isolates are deposited in the Micoteca da Universidade do Minho (MUM) culture collection, Braga, Portugal. The ex-type strain (MUM 17.62$^T$) is also deposited at ITEM Microbial Culture Collection of ISPA, Italy, with the accession number ITEM 17445$^T$.

## Molecular characterisation

The isolates were subjected to DNA extraction using a method adapted from Rodrigues *et al.* [14]. DNA was dissolved in 50–100 µl of ultrapure water, depending on the yield, and stored at −20 °C.

As an initial step to identify the isolates, amplification of the partial *benA* gene was performed using 0.2 µM Bt2a and Bt2b primers (Table 1), 1×VWR Taq DNA Polymerase Master Mix with 1 mM $MgCl_2$ (VWR) and approximately 50 ng template DNA in a 50 µl reaction volume. The PCR cycling protocol is described in Table 2. Obtained amplicons were purified using the NZYGelpure kit (NZYtech) and sent for Sanger sequencing to Stab Vida Lda (Madan Parque, Caparica, Portugal). The obtained *benA* gene sequences were compared with those in the NCBI database using BLAST to determine fungal species that have similar DNA sequences.

To further characterize the samples, amplification reactions of *ITS*, *CAL* and *TEF-1α* were prepared as described above using the corresponding primer pairs detailed in Table 1 and PCR cycling protocols in Table 2. PCR amplicons were sequenced as described above.

Sequences obtained for *benA*, *ITS* and *CAL* were aligned with type strain sequences from different *Penicillium* sections (Table 3) retrieved from the NCBI database (www.ncbi.nlm.nih.gov/). Alignment was performed using CLUSTAL_W [15] followed by visual inspection and, when necessary, manual correction using MEGA7.0 [16]. Data sets were concatenated in Seaview version 4.6.3 [17] in order to perform a multigene phylogeny. The most suitable model was determined in MEGA 7 based on the lowest Bayesian information criterion. Maximum-likelihood analyses were performed through an automatic calculation of the initial tree by applying the neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum-composite-likelihood approach, and then selecting the topology with superior log likelihood value. This was followed by a heuristic search with the nearest-neighbour-interchange method. Bootstrap analysis [18] was performed on 1000 replicate samples in order to support the nodes. All positions containing gaps and missing data were eliminated.

DNA fingerprinting of the two isolates by the M13 probe was performed using 0.8 µM primer (Table 1), 1×VWR Taq DNA Polymerase Master Mix with 1 mM $MgCl_2$ and 50 ng of template DNA Master in a 25 µl reaction volume. The PCR cycling protocol is described in Table 2. Products were separated by electrophoresis on 1.5 % agarose gel in TAE buffer at a constant voltage of 60 V for 90 min. Fingerprinting band patterns were compared using BioNumerics

**Table 2.** PCR cycling programs used for amplification

| Gene | Initial denaturing | Cycles | Denaturing | Annealing | Elongation | Final elongation |
|---|---|---|---|---|---|---|
| *benA*, *ITS*, *IDH* | 95 °C, 5 min | 35 | 95 °C, 1 min | 56 °C, 45 s | 72 °C, 90 s | 72 °C, 10 min |
| *CAL* | 95 °C, 10 min | 35 | 95 °C, 50 s | 55 °C, 50 s | 72 °C, 1 min | 72 °C, 7 min |
| *TEF-1α* | 94 °C, 5 min | 42 | 94 °C, 1 min | 62 °C, 30 s | 72 °C, 90 s | 72 °C, 10 min |
| M13 | 94 °C, 2 min | 40 | 94 °C, 2 s | 50 °C, 1 min | 72 °C, 2 s | 72 °C, 6 min |

**Table 3.** Strains used for phylogenetic analysis of the new *Penicillium* species isolated from Tunisian orchard apples

With the exception of *Trichocoma paradoxa*, all correspond to type strains of the indicated species. GenBank accession numbers of the *benA*, *ITS* and *CAL* sequences used to reconstruct the phylogenetic tree presented in Fig. 1 are listed here.

| Section | Species | Culture collection number | GenBank accession number | | |
|---|---|---|---|---|---|
| | | | *benA* | *ITS* | *CAL* |
| *Brevicompacta* | *Penicillium astrolabium* | MUM 06.161=CBS 122427=NRRL 35611 | DQ645793 | DQ645804 | DQ645808 |
| | *Penicillium bialowiezense* | CBS 227.28=IBT 23044=IMI 092237=LSHBP 71=NRRL 865 | AY674439 | EU587315 | AY484828 |
| | *Penicillium brevicompactum* | CBS 257.29=ATCC 10418=ATCC 9056=DSM3825=FRR 862=IBT 23045=IMI 040225=LSHBP 75=MUCL 28647=MUCL 28813=MUCL 28935=MUCL 30240=MUCL 30241=MUCL 30256=MUCL 30257=NRRL 2011=NRRL 862=NRRL 864=QM 7496 | AY674437 | AY484912 | AY484813 |
| | *Penicillium buchwaldii* | CBS 117181=IBT 6005=IMI 304286 | JX313182 | JX313164 | JX313148 |
| | *Penicillium fennelliae* | CBS 711.68=ATCC 22050=ATCC 52492=FRR 521=IHEM 4389=IMI 151747=MUCL 31322 | JX313185 | JX313169 | JX313151 |
| | *Penicillium kongii* | AS 3.15329 | KC427171 | KC427191 | KC427151 |
| | *Penicillium neocrassum* | MUM 06.160=CBS 122428=NRRL 35639 | DQ645794 | DQ645805 | DQ645809 |
| | *Penicillium olsonii* | CBS 232.60=IBT 23473=IMI 192502=NRRL 13058=NRRL 13716 | AY674445 | EU587341 | DQ658165 |
| | *Penicillium spathulatum* | CBS 117192=IBT 22220 | JX313183 | JX313165 | JX313149 |
| | *Penicillium tularense* | CBS 430.69=ATCC 22056=FRR 899=IFO 31740=IMI 148394=NRRL 5273=AS 3.14006 | KC427175 | AF033487 | JX313135 |
| *Ramosa* | *Penicillium chroogomphum* | CBS 136204=KCTC 46041=JZB 2120005 | KP684056 | KC594043 | KP684057 |
| | *Penicillium jamesonlandense* | CBS 102888=DAOM 234087=IBT 21984=IBT 24411 | DQ309448 | DQ267912 | KJ866985 |
| | *Penicillium kojigenum* | CBS 345.61=ATCC 18227=CCRC 31515=FRR 3442=IFO 9581=IMI 086562=LSHBBB394=MUCL 2457=NRRL 3442=QM 7957 | KJ834463 | AF033489 | KJ867011 |
| | *Penicillium lanosum* | CBS 106.11=ATCC 10458=FRR 2009=IFO 5851=IFO 6099=IMI 040224=LSHBP 86=MUCL 29232=NRRL 2009=QM 7591 | DQ285627 | DQ304540 | FJ530974 |
| | *Penicillium lenticrescens* | CBS 138215=DTO 129A8 | KJ775168 | KJ775675 | KJ775404 |
| | *Penicillium raistrickii* | CBS 261.33=ATCC 10490=FRR 1044=IFO 6104=IMI 040221=LSHBB100=NRRL 1044=NRRL 2039=QM 1936=VKMF-337 | KJ834485 | AY373927 | KJ867006 |
| | *Penicillium ribium* | CBS 127809=DAOM 234091=IBT 16537=IBT 24431 | DQ285625 | DQ267916 | KJ866995 |
| | *Penicillium scabrosum* | CBS 683.89=FRR 2950=IBT 3736=IMI 285533=DAOM 214786 | DQ285610 | DQ267906 | FJ530987 |
| | *Penicillium simile* | CBS 129191=ATCC MYA-4591 | FJ376595 | FJ376592 | GQ979710 |
| | *Penicillium soppii* | CBS 226.28=ATCC 10496=FRR 2023=IFO 7766=IMI 040217=MUCL 29233=NRRL 2023=QM 1964=IBT 18220 | DQ285616 | AF033488 | KJ867002 |
| | *Penicillium swiecickii* | CBS 119391=FRR 918=IBT 27865=IMI 191500=NRRL 918 | KJ834494 | AF033490 | KJ866993 |
| | *Penicillium tunisiense* | MUM 17.62$^{T}$=ITEM 17445$^{T}$ | MG586970 | MG586956 | MG586974 |
| | *Penicillium tunisiense* | MUM 17.80 | MG586971 | MG586957 | MG586975 |
| | *Penicillium virgatum* | CBS 114838=BBA 65745 | KJ834500 | AJ748692 | KJ866992 |
| *Chrysogena* | *Penicillium chrysogenum* | CBS 306.48=ATCC 10106=ATHUM2889=CCRC 30564=FRR 807=IBT 5233=IMI 024314=IMI 092208=LSHBAd 3=LSHBP 19=MUCL 29079=MUCL 29145=NCTC 589=NRRL 807=NRRL 810=QM 7500 | AY495981 | AF033465 | JX996273 |
| | *Penicillium rubens* | CBS 129667=NRRL 792=IBT 30129=ATCC 9783 | JF909949 | JX997057 | JX996263 |
| *Fasciculata* | *Penicillium commune* | CBS 311.48=ATCC 10428=ATCC 1111=CCRC 31554=DSM2211=IBT 6200=IFO 5763=IMI 039812ii=IMI 039812iii=NRRL 890=QM 1269=VKMF-3233 | AY674366 | AY213672 | KU896829 |
| | *Penicillium crustosum* | CBS 115503=ATCC 52044=FRR 1669=IBT 5528=IBT 6175=IMI 091917=NCTC 4002 | AY674353 | AF033472 | DQ911132 |
| | *Penicillium polonicum* | CBS 222.28=IBT 12821=IMI 291194=MUCL 29204=NRRL 995 | AY674305 | AF033475 | KU896848 |
| | *Penicillium solitum* | CBS 424.89=ATCC 9923=CBS 288.36=FRR 937=IBT 3948=IFO 7765=IMI 039810=IMI 092225=LSHBP 52=MUCL 28668=MUCL 29173=NRRL 937 | AY674354 | AY373932 | KU896851 |

**Table 3.** cont.

| Section | Species | Culture collection number | GenBank accession number | | |
|---|---|---|---|---|---|
| | | | *benA* | *ITS* | *CAL* |
| | *Penicillium verrucosum* | CBS 603.74=ATCC 48957=ATHUM2897=CECT 2906=FRR 965=IBT 12809=IBT 4733=IMI 200310=IMI 200310ii=MUCL 28674=MUCL 29089=MUCL 29186=NRRL 965 | AY674323 | AY373938 | DQ911138 |
| *Penicillium* | *Penicillium expansum* | CBS 325.48=ATCC 7861=ATHUM2891=CCRC 30566=FRR 976=IBT 3486=IBT 5101=IMI 039761=IMI 039761ii=MUCL 29192=NRRL 976=VKMF-275 | AY674400 | AY373912 | DQ911134 |
| | *Penicillium italicum* | CBS 339.48=ATCC 10454=DSM2754=FRR 983=IBT 23029=IMI 039760=MUCL 15608=NRRL 983=QM 7572 | AY674398 | KJ834509 | DQ911135 |
| Outgroup | *Trichocoma paradoxa* | CBS 788.83 | KF984556 | JN899398 | KF984670 |
| | *Talaromyces rugulosus* | CBS 371.48=ATCC 10128=CCRC 31518=IMI 040041=LSHB Ad27=MUCL 31201=NCTC 592=NRRL 1045=QM 7661 | KF984575 | JN899374 | KF196868 |

version 7.6.2 (Applied Maths NV; www.applied-maths.com).

Analysis of the *IDH* gene was performed through amplification of the IDH1–IDH2 primer pair (Table 1) in conjunction with a longer fragment of the *benA* gene as an internal amplification control (primers Bt2a and Bt1b, Table 1). The amplification reaction and PCR cycling protocol were the same as described above for the *benA* gene. PCR products were separated by electrophoresis on 1 % agarose gel in TAE buffer at a constant voltage of 80 V for 45 min.

### Extraction and analysis of patulin

Patulin extraction was performed for each fungal isolate as described by Morales *et al.* [19]. Samples were analysed using a U-HPLC equipped with a Shimadzu UV-DAD detector (276 nm). Patulin content was determined by chromatographic separation performed on a reverse phase C18 column (Brisa LC2 Teknocroma; 250×4.6, 5 µm) with a 5 % acetonitrile/water mobile phase, pumped at 1 ml $min^{-1}$. The retention time of patulin was 14.9 min.

### Morphological characterisation

Morphological characterisation of MUM 17.62$^T$ and MUM 17.80 was performed based on Visagie *et al.* [12]. The following culture media and incubation temperature were used: Czapek yeast autolysate (CYA) agar at 15, 25, 30 and 37 °C, malt extract agar (MEA), oatmeal agar (OA), yeast extract sucrose (YES) agar, glycerol nitrate (G25N) agar and creatine sucrose neutral (CSN) agar at 25 °C. After 7 days, digital images of colonies were obtained as described in Simões *et al.* [20] and macro- and micromorphological characters were examined under light optical microscopy and scanning electron microscopy. Colours and codes in descriptions are those from the *Methuen Handbook of Colour* [21].

### Light microscopy

The slides for light optical microscopic observations were prepared using tissue removed from 7 days old colonies grown on MEA and mounted in lactic acid and lactophenol cotton blue. Excess conidia were washed using drops of 96 % ethanol. Morphologies were observed using an optical microscope (Leica DM5000B). Length and width of stipe, metulae, phialides and two perpendicular diameters of the conidia were measured and rounded off to 0.5 µm.

### Scanning electron microscopy

Scanning electron microscopy was performed on samples fixed for 18 h at 4 °C using 3 % glutaraldehyde in PBS buffer followed by overnight incubation at 4 °C with 1 % aqueous osmium tetroxide, dehydrated in increasing ethanol series (50, 70, 80, 90 and 100 %) for 10 min and dried in a desiccator. The samples were added to aluminium pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs) and sputter-coated with gold. Observation was performed using an ultrahigh resolution field-emission scanning electron microscope NanoSEM-FEI Nova 200 (FEI).

## RESULTS AND DISCUSSION

### Molecular characterisation

Fifty-six *Penicillium* isolates were obtained during the current survey of orchard apples from Tunisia. Although *ITS* is the recommended DNA barcode for fungi [22], this marker is not variable enough for distinguishing all closely related species in *Penicillium* [22–24]. For that reason and considering that $\beta$-tubulin is a better discriminative marker that allows identification of *Penicillium* to species level [12, 23], partial *benA* gene sequencing of the 56 isolates was performed. BLAST results showed that the majority were *P. expansum* (sequence identity ≥99 %), with some isolates being classified as *P. solitum*, *P. crustosum*, *P. polonicum* and *P. bialowiezense*. However, two isolates were significantly different from other species included in the NCBI database (sequence identity <90 %) with the closest alignments indicating a possible connection with *Penicillium* section *Ramosa*. Those two isolates were designated *Penicillium* species with culture collection numbers MUM 17.62$^T$ and MUM 17.80.

To confirm their phylogenetic placement within *Penicillium*, combined sequences (*ITS*, *benA* and *CAL*) were aligned against those of type strains belonging to section *Ramosa*

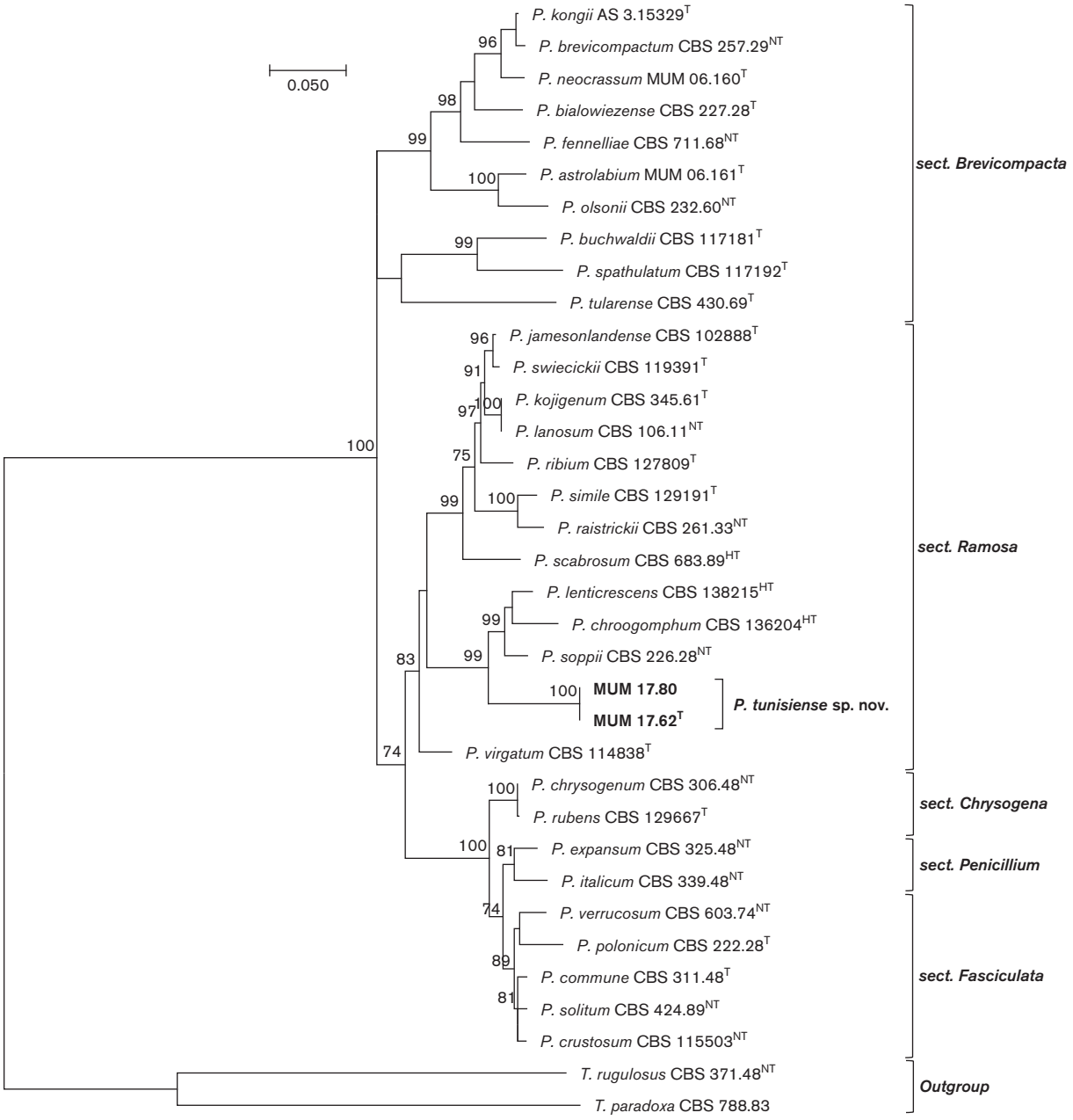


**Fig. 1.** Combined phylogeny for *ITS*, *benA* and *CAL* sequence data of the two *P. tunisiense* isolates (MUM 17.62$^T$ and MUM 17.80) with other *Penicillium* species detailed in Table 3. *Trichocoma paradoxa* (CBS 788.83) and *Talaromyces rugulosus* (CBS 371.48) were used as outgroups. Model selected: TN93+G+I. The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. T, ex-type strain; HT, holotype strain; NT, neotype strain.

and the closely related section *Brevicompacta*. Furthermore, members of sections *Chrysogena*, *Fasciculata* and *Penicillium*, the majority of which were found in the current survey or have been previously described as primary causing agents of blue mould in apples and pears [1], were also included. The analysis comprised 35 nucleotide sequences and a total of 955 positions in the final data set. In the obtained phylogenetic tree (Fig. 1), the two unidentified isolates are placed in a monophyletic clade that is composed exclusively by section *Ramosa* strains and is supported by 83 % of the bootstrap samples. Therefore, the two isolates are classified as *Penicillium* section *Ramosa* clustering with *P. chroogomphum*, *P. lenticrescens* and *P. soppii* (supported by 99 % of the bootstrap samples). The close relationship between these

**Table 4.** Comparison of *Penicillium tunisiense* MUM 17.62$^T$ with the related taxa

| | *P. tunisiense* | *P. chroogomphum** | *P. lenticrescens*† | *P. soppii*‡ |
|---|---|---|---|---|
| CYA at 25 °C: | | | | |
| Diameter (mm, in 7 days) | 11–13 | 7–8 | 12–24 | 25–35 |
| Colonial colour | Greenish grey | Light green | Greyish green | Dull green |
| CYA at 30 °C | No growth | Weak growth | No growth | NA |
| YES at 25 °C: | | | | |
| Diameter (mm, in 7 days) | 16–19 | 7–10 | 17–18 | NA |
| Conidiophores on MEA | Borne from aerial hyphae | Borne from aerial hyphae | NA | Borne from surface or aerial hyphae |
| Stipe (µm) | 46–245×1.5–2. | 50−150×2.5–3.5 | 150−415×3–4 | 300−450×2.5–4 |
| Phialides (µm) | 5.5–10×1–2 | 8−12×3–4 | 7.5–10.5×2.5–3.5 | 8−12×2.5–3 |
| Conidia (µm) | 1.5–3.5×1–3.5 µm | 4−5×3–4 | 2.5–3.5×2.5–3 | 2.5–3 |
| | Finely rough | Smooth | Smooth | Smooth and roughened or very delicately roughened |
| | Ellipsoidal, with a minor proportion subglobose | Ellipsoidal | Subglobose, with a minor proportion ellipsoidal | At first elliptical and becoming globose to subglobose at maturity |

NA, Not available in the original description.

*Data derived from Rong *et al.* [25].

†Data derived from Visagie *et al.* [13].

‡Data derived from Raper and Thom [38] and Pitt [28].

three previously described species was also reported by Rong *et al.* [25]. Furthermore, the molecular phylogenetic data presented herein confirms the separation of MUM 17.62$^T$ and MUM 17.80 from *P. chroogomphum*, *P. lenticrescens* and *P. soppii*, as they are placed in an individual branch supported by 100 % of the bootstrap samples. Previous studies have shown that, despite the close relationship, strains from sections *Brevicompacta* and *Ramosa* can be separated from each other [13, 25, 26]. In the analyses presented in Fig. 1, the majority of section *Brevicompacta* strains are clustered in an independent monophyletic branch (supported by 99 % of the bootstrap samples) with the exceptions being *P. spathulatum*, *P. buchwaldii* and *P. tularense*. This is an expected situation since they share morphological and phenotypic characteristics with section *Ramosa* species [27], which can explain the greater distance between these three species and the others in section *Brevicompacta*.

The *TEF-1α* gene was not included in the phylogenetic analysis as the complete dataset was not congruent due to unavailability of sequences in GenBank for all the strains considered in this study. Nevertheless, the *TEF-1α* sequence was informative as it had a nucleotide difference between the two novel strains (Fig. S1a, available in the online version of this article). This is a synonymous change, that is, it does not affect the amino acid sequence and so it is unlikely to have biological significance. It gives, however, a clear indication that the two isolates correspond to individual strains and not clones, a result confirmed by the DNA fingerprinting profile obtained using the M13 primer (Fig. S1b).

## Morphology and physiology analyses

Molecular results showed that two isolates considered in this study belong to *Penicillium* section *Ramosa*. Nevertheless, is also important to consider morphological and physiological criteria. Detailed morphological descriptions are given in the species description.

MUM 17.62$^T$ and MUM 17.80 conidia have slight colour differences depending of the medium used (greenish grey in CYA, MEA and G25N agar, greyish green in YES agar and CSN agar, dull green in OA). Furthermore, the two isolates sporulate abundantly, whereas *P. lenticrescens* and *P. soppii* sporulate sparsely and *P. chroogomphum* moderately after 7 days of growth [13, 25]. The reverse plates of our isolates show dark pigmentation, but no production of soluble pigments, including on CSN agar where both strains appear to be neutral. The lack of soluble pigmentation also occurs in *P. chroogomphum* and *P. lenticrescens* as well as no production of acid. The two isolates do not produce sclerotia while *P. soppi* does. Microscopically, members of section *Ramosa* have divergent rami [27], as observed in *P. soppii* and in MUM 17.62$^T$ and MUM 17.80. However, there are evident morphological differences (Table 4) such as the more conspicuous ellipsoidal conidia than observed in *P. chroogomphum*. The conidia are also characterised as being small and finely rough, which is more similar to *P. soppii* than to the other species. In addition, the conidiophores of the two isolates bear four to five metulae with four to six phialides, whereas *P. chroogomphum* has two to three metulae with three to eight phialides [25] and *P. soppii* has three to five metulae with five to eight phialides [28].

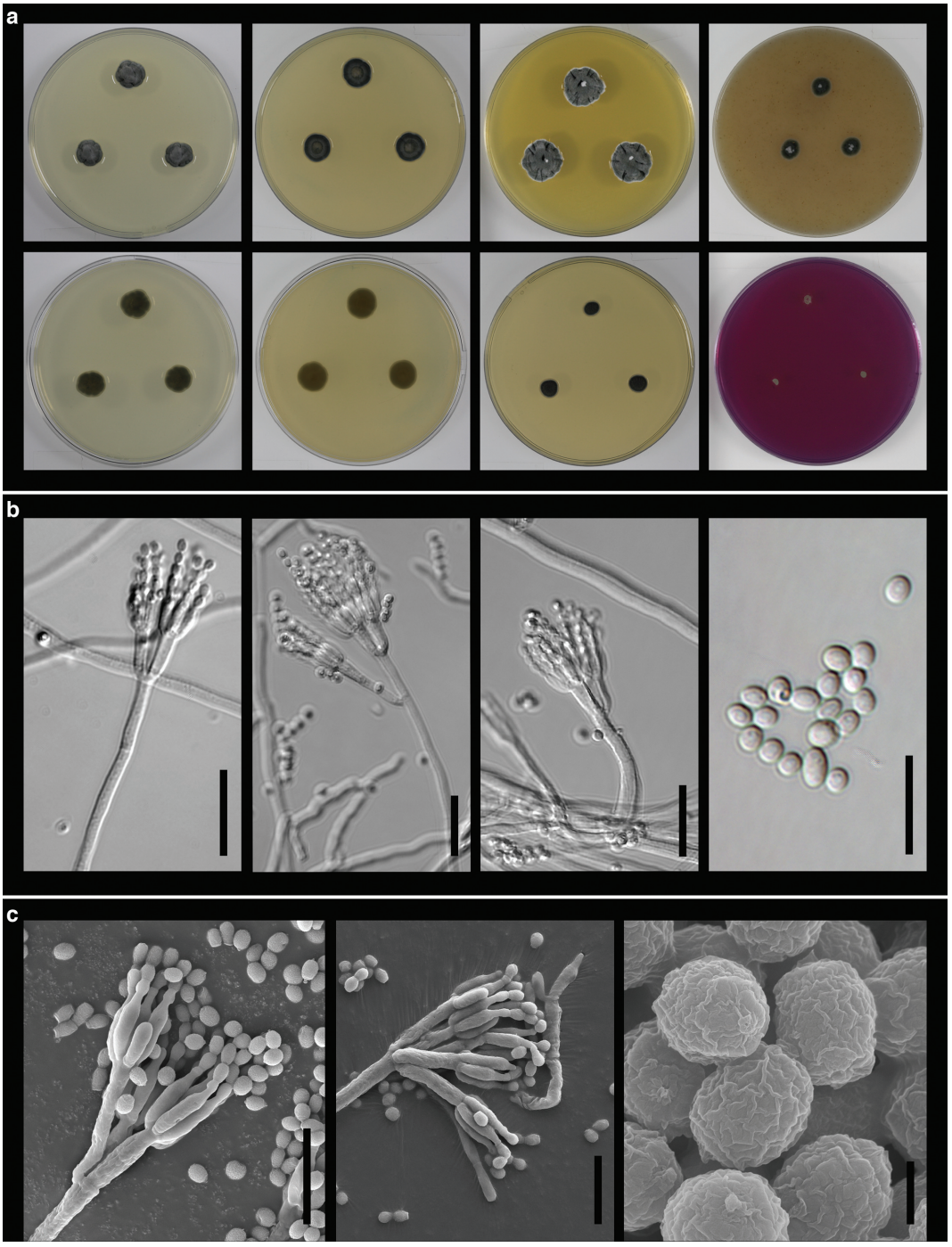


**Fig. 2.** *Penicillium tunisiense* sp. nov. MUM 17.62$^{T}$. (a) Colonies at 25 °C, 7 days: top row, left to right, obverse CYA, MEA, YES agar and OA; bottom row, left to right, reverse CYA, reverse MEA, obverse G25N agar and CSN agar. (b) Conidiophores (bar, 20 µm) and conidia (bar, 10 µm) under light microscopy. (c) Conidiophores (bar, 10 µm) and conidia (bar, 1 µm) as observed using scanning electron microscopy.

The two strains were isolated from the North of Tunisia, a Mediterranean country, with an average temperature of around 12 °C in the coldest month and 27 °C in the hottest. It is common for food-borne penicillia to grow in colder environments even if they are from warm, temperate and subtropical regions. However, the number of *Penicillium* species that grow better at 15 °C than at 25 °C is limited [27]. This new species corresponded well with this group

since its colony diameter average (12.8 mm) is wider at 15 °C on CYA with good sporulation leading to the assumption that its optimal temperature is lower than 25 °C with a colony diameter average of 11.6 mm. Species that grow and sporulate well at 25 °C, but have lower optimum growth temperature may be called psychrotolerant [29]. Although the closely related species *P. soppii* is considered to be psychrotolerant [28], it does not sporulate well at 25 °C. Furthermore, both isolates do not grow at 30 °C, a shared characteristic with *P. lenticrescens*. However, there is no indication if *P. lenticrescens* can grow at lower temperatures in the literature. The more recently described *P. chroogomphum*, forms larger colonies at 16 °C but differs from the novel isolates and *P. lenticrescens*, due to its ability to grow, even if slowly, at 30 °C (Table 4). When compared with the most common isolated species of this survey (*P. expansum*) this novel species has no ability to rot (results not shown) and produce patulin in apples.

### Patulin production

Both strains were *IDH* negative (results not shown), i.e. they lack the ability to convert isoepoxydon to phyllostine during the patulin biosynthetic pathway [30]. In addition, Paterson *et al.* [31] found that these strains produce a compound that has the same retention time as patulin but a different UV spectrum using U-HPLC/UV-DAD. These results lead to discussion that different secondary fungal metabolites may have identical analytical data (e.g. the same retention time and the same molecular mass). Frisvad [32] indicates that 95 secondary metabolites, including members of the patulin biosynthetic pathway, have the same molecular mass of patulin (M≈154). *Penicillium* patulin producers have been found in section *Ramosa* [32] but combining the molecular and UV spectrum results both strains are considered patulin-negative.

## DESCRIPTION OF *PENICILLIUM TUNISIENSE* SP. NOV.

*Penicillium tunisiense* (tu.ni.si.en′se. N.L. neut. adj. *tunisiense* pertaining to Tunisia) Fig. 2.

*Typus.* Tunisia, Tunis, fruits of *Malus pumila* Mill. (orchard apples, also known as table apples) isolated by Salma Ouhibi as N132 S1 on April 2014 (holotype MUM-H 17.62, culture ex-type MUM 17.62).

*Diagnosis.* Slow growth on general media, no growth at 30 °C, conidiophores biverticillate and terverticillate in older colonies, producing finely rough walled stipes and finely rough ellipsoidal conidia.

*Description.* Colony diameter, 7 days (mm): CYA 25 °C, 11–13; CYA 15 °C, 12–14; CYA 30 °C, no growth; CYA 37 °C, no growth; MEA 25 °C, 11–12; YES agar 25 °C, 16–18; G25N agar 25 °C, 6–8; OA 25 °C, 9–10; CSN agar 25 °C, 4–6.

*Colony characteristics*. CYA 25 °C, 7 days: colonies raised, margins low, narrow, entire; mycelia white; texture velutinous; sporulation dense, conidia *en masse* greenish grey (28C2); soluble pigments absent; scarce clear exudates; reverse dull green (30E4). MEA 25 °C, 7 days: colonies raised in the centre; radially sulcate, margins low, narrow, entire; mycelia white; texture velutinous; sporulation dense, conidia *en masse* greenish grey (26C2); soluble pigments absent; exudates absent; reverse olive (2D4). YES agar 25 °C, 7 days: colonies raised, sunken at centre, sulcate; margins low, narrow, irregular; mycelia white; texture velutinous; sporulation dense, conidia *en masse* greyish green (27C3); soluble pigments absent; exudates absent; reverse khaki to olive brown (4D5-E5). G25N agar 25 °C, 7 days: colonies raised; margins low, narrow, entire; mycelia white; texture velutinous; sporulation dense, conidia *en masse* greenish grey (29C2); soluble pigments absent; exudates absent; reverse greyish to olive to khaki (3E5-4D5). OA 25 °C, 7 days: colonies low, plane, white mycelia appeared in the centre; margins low, moderately wide, entire; mycelia white; texture velutinous; sporulation moderately dense, conidia *en masse* dull green (28D4); soluble pigments absent; exudates absent. CSN agar 25 °C, 7 days: Colonies low, plane; margins low, moderately wide, entire; mycelia white; texture velutinous; sporulation moderately dense, conidia *en masse* greyish green (27C3); neutral response with no production of acid or alkaline compounds.

*Micromorphology.* Conidiophores biverticillate, sub-terminal branching occasionally observed and terverticillate structures in older cultures; stipes finely rough walled, 46–245×1.5–2.5 µm; metulae divergent, moderately swollen at apex up to 4 µm, 9–12.5×2.5–3 µm (with a mean and standard deviation of 11±1×3.5±0.5 µm); vesicles 4–5 µm; phialides ampulliform, sometimes more slender and elongated, 5.5–10×1–2 µm (with a mean and standard deviation of 7 ±1.5×1.5±0.5 µm); average length metula/phialide 1.5; conidia finely rough, ellipsoidal, with a minor proportion subglobose, 1.5–3.5×1–3.5 µm (with a mean and standard deviation of 2.5±0.5×1.5±0.5 µm), average width/length=0.5, *n*=100.

*ITS barcode.* MG586956 (alternative markers: *benA*=MG586970; *CAL*=MG586974; *TEF-1α*=MG586972).

*Other isolate examined.* MUM 17.80 isolated by Salma Ouhibi as N293 S21 on May 2014 from the surface of orchard apples (*M. pumila*) from Tunis, Tunisia. *ITS* barcode. MG586957 (alternative markers: *benA*=MG586971; *CAL*=MG586975; *TEF-1α*=MG586973).

MycoBank number: MB823626.

Funding information
This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684) and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte.

Acknowledgements
We acknowledge the support of the Tunisian Ministry of Education and Scientific Research and the Environment – Toxicology Research Laboratory LR12SP07.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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