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Article

Candida Massiliensis sp. nov. Isolated from A Clinical Sample

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Abstract: The majority of *Candida* species are known as non-pathogenic yeasts and rarely involved in human diseases. However, recently case reports of human infections caused by non-*albicans Candida* species have increased, mostly in immunocompromised hosts. Our study aimed to describe and caracterise as thoroughly as possible, a new species of the *Candida* genus, named here *Candida massiliensis* (PMML0037), isolated from a clinical sample of human sputum. We compared genetic data based on the sequences of four genetic regions: "Internal Transcribed Spacers" of rRNA, D1/D2 domains (28S large subunit rRNA) and part of the genes encoding Translation Elongation Factor 1-α and β-tubulin2, to morphological characters, from scanning electron microscopy (TM 4000 Plus, SU5000), physiological, including the results of oxidation and assimilation tests of different carbon sources by the Biolog system, and chemical mapping by Energy-Dispersive X-ray Spectroscopy. Lastly, the *in vitro* antifungal susceptibility profile was performed using the E-test™ exponential gradient method. The multilocus analysis supported the genetic position of *Candida massiliensis* (PMML0037) as a new species of the genus *Candida*, and the phenotypic analysis highlighted its unique morphological and chemical profile when compared to other *Candida* species included in the study.

Keywords: biolog phenotypic technology; *Candida;* energy-dispersive X-ray spectroscopy; genotype; multilocus DNA sequencing; one new taxon; yeast

1. Introduction

The genus *Candida* belongs to the class *Saccharomycetes* and the family *Saccharomycetaceae*. *Candida* spp. are widespread in different environments and have always been attributed biological and biotechnological importance, as well as potential applications in agriculture, fermentation and chemical industries [1]. In recent decades, the health-care associated infections have increased, in particular invasive candidiasis, which has been reported in the literature as an important cause of nosocomial bloodstream infections associated with a high morbidity and fatality [2-4]. Among more than 200 identified *Candida* species, only a few have been involved in human disease [5]. Even though *Candida albicans* is the mostly incriminated yeast in *Candida* infections, according to recent studies, the incidence of other non-*albicans* species is rapidly rising. Among them, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei* (now *Pichia kudriavzevii*), and *C. dubliniensis* are considered as frequent opportunistic human pathogens [2, 6-11]. The prolonged hospitalisation of immunocompromised patients undergoing advanced medical treatment has led to an increase in the incidence of fungal infections, a large proportion of which is due to non-*albicans Candida* species, particularly in patients in haematology, organ-transplant and intensive care units [3, 4, 12-15].

In this present study we isolated a new species of *Candida* closely related to *Candida intermedia* and *Candida pseudointermedia* species, that are rarely reported as a human pathogens. *C. intermedia* is recognised as a member of the fungal community of the cheese surface and the human oropharyngeal cavity. This species has been recorded in a number

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of fungemia cases, but no extensive clinical and microbiological information has been supplied [16-19]. Descriptions of *C. intermedia* are mostly provided in basic clinical publications [18, 20]. *C. pseudointermedia* was first isolated from fish-paste, and described by Nakase *et al.* (1976). The yeast has also been isolated from different other substrates including, lake, coffee, human body, coconut water, rice phylloplane, tap water and spoiled ready-to-eat meals. To the best of our knowledge, it has not been involved in human diseases. The previous studies have focused on ecological, taxonomic and phylogenetic features, and there is no detailed phenotypic characterisation of this species [21]. The purpose of the present study is to suitably describe and to characterise, both genetically and phenotypically, a new species of *Candida* isolated from a clinical sample of human sputum and the two type strains of *Candida intermedia* ATCC 14,439 and *Candida pseudointermedia* ATCC 60126.

2. Materials and methods

2.1. Fungal strains

The novel *Candida* strain was isolated from a clinical sample of human sputum from a 7 years-old female patient followed for cystic fibrosis at the laboratory of Mycology in the Institut Hospitalo-Universitaire Méditerranée Infection in Marseille, France. The phenotypic features of *Candida massiliensis* PMML0037 were compared to those of two type strains; *C. intermedia* ATCC 14,439 and *C. pseudointemedia* ATCC 60126. The genotypic features of these three species were also compared with those of several other reference strains belonging to the *Candida* genus, collected from GenBank database (accession numbers are listed in Table 1).

2.2. Macroscopic characterisation

In order to study the temperature growth profiles and macroscopic features such as colonies time of growth, morphology and aspect as well as the surface/reverse colour, the three strains have been cultivated on Sabouraud Dextrose Agar (SDA) supplemented with Gentamicin and Chloramphenicol (GC) plates for five days, after being subcultured on other SDA GC plates that were incubated at distinct temperatures: 4, 25, 30, 37, 40, and 45°C.

2.3. Microscopic characterisation

In order to compare the microscopic characteristics of the different fungal structures, fresh cultures of the three strains cultured on SDA GC were firstly observed by Optical Microscopy (OM). The microscopic slides were prepared using the cellophane adhesive tape method with Lactophenol Cotton Blue (LCB) and pictures have been taken with the DM 2500 camera (LEICA CAMERA SARL, Paris, France). The second microscopic observation was achieved using the Scanning Electron Microscopy (SEM). The microscopic slides were prepared by fixing fresh fungal colonies in 400 μl of Glutaraldehyde 2.5% in 0.1M Sodium Cacodylate buffer and stored at 30°C for 30 minutes. Photographs were taken with the TM4000 Plus microscope settled at 15 kV lens mode 4 with a Back-Scattered Electron detector (BSE) and SU5000 microscope settled at 10 kV (Hitachi High-Technologies, Tokyo, Japan).

2.4. MALDI-TOF MS identification

The three *Candida* strains were inoculated on SDA GC at 30 °C for five days. After growth, strains were identified with Matrix-Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS) using the protein extraction procedure described in Cassagne *et al*. (2016) [22].

2.5. Antifungal Susceptibility Testing (AFST)

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We evaluated the *in vitro* activity of nine antifungal drugs: Amphotericin B (AMB), Anidulafungin (AND), Caspofungin (CAS), 5-fluorocytosine (5-FC), Fluconazole (FL), Itraconazole (ITC), Micafungin (MIC), Posaconazole (POS) and Voriconazole (VOR) against the three *Candida* species applying the Sensititre YeastOne™ (ThermoFisher Scientific, Dardilly, France) microdilution system. After the isolates were grown on SDA GC, the broth microdilution method was performed as detailed in the CLSI M27-A2 document. Summarily, the inoculum was suspended in 2 ml of saline and turbidity was settled to 0.5 McFarland to acquire an inoculum of ~1.5×10^8 CFU/ml. Then, 20 µl of this solution was supplemented to 10 ml of YeastBroth™ (ThermoFisher Scientific, France). Finally, 100 µl of this final solution were deposited into each Sensititre™ YeastOne™ YO09 (ThermoFisher Scientific, France) plate well. The MICs were read after 48h of incubation at 35°C.

2.6. Physiological analysis

2.6.1. EDX (Energy-Dispersive X-ray Spectroscopy)

The fixation of the three strains cultures in Glutaraldehyde solution of 2.5% and the cytospin protocol were processed as detailed in Kabtani *et al.* (2022) [23]. EDX was assessed with an INCA X-Stream-2 detector (Oxford Instruments) connected to the TM4000 Plus microscope and AztecOne software (Oxford instruments, Pasadena, CA, USA). The chemical mapping was carried out blind and took into account all the chemical elements. Weight and atomic percentages of chemical elements were subjected to a Principal Component Analysis (PCA) with the XLSTAT software (Addinsoft, Paris, France).

2.6.2. Biolog™ phenotypic analysis

The phenotypic analysis was assessed with the Biolog™ advanced phenotypic technology. The oxidation and carbon sources assimilation tests were performed using the YT MicroPlates™ (Gen III) (Biolog Catalog #1005) and assay was performed in triplicate. The three strains have been cultivated on Biolog Universal Yeast™ (BUY) Agar medium (Biolog Catalog #70005) and after three to five days of incubation all colonies were well developed. The preparation of the fungal suspension and the transmittance adjustment were done as described before in [23]. Plates have been incubated at 26 °C during seven days and have been read using the Biolog MicroStation™ Reader. The results were displayed as a heat map, realized with the XLSTAT™ software (Addinsoft).

2.7. DNA extraction and sequencing

The DNA extraction was achieved with the Qiagen® Tissue kit after five days of incubation at 30°C on SDA GC medium. Few colonies were added in bead tubes with 600 μl of lysis buffer G2 (provided with the Qiagen® Tissue kit). The FastPrep™-24 Instrument was used for a mechanical lysis; 40 seconds at 6 m/sec, then a centrifugation for one minute at 10,000 rpm. The extraction was carried out using the EZ1 Advanced XL instrument (Qiagen, les Ulis, France) depending on the instructions of the manufacturer. Four genes were amplified, the Internal Transcribed Spacer 1 and 2 (ITS1/ITS2) in the RNA ribosomal small-subunit (SSU), a fragment of β-tubulin gene (TUB2), a fragment of the Translation Elongation Factor 1-alpha gene (TEF-1-α) and the D1/D2 domains of the ribosomal DNA large-subunit (LSU). The DNA amplification and sequencing were performed as previously processed in [23]. The PCR Mix was prepared with 12.5 μl Ampli Taq Gold® (360 Master Mix, Applied Biosystems®), 6 μl sterile water DNase/RNase free and 0.75 μl (20 μM) Forward/Reverse primer in addition to 5 μl of DNA template to obtain a total volume of 25 μl per well. The PCR program for all genes amplification was as followed: a denaturation step at 95°C for 15 min, followed by 39 cycles of 95°C for 1 min, 56°C for 30 sec, 72°C for 1 min, and 72°C for 5 min. The resulting sequences were assembled and treated with ChromasPro 2.0. (Technelysium Pty Ltd) and were then compared with the

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reference sequences from the GenBank database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool nucleotide (BLASTn).

2.8. Phylogenetic analysis

We built five phylogenetic trees. The first tree was a multilocus based on the concatenation of the nucleotide sequences of four genetic regions (ITS, D1/D2, TEF-1α and B-tub2). The four other trees were single locus, each tree constructed with the nucleotide sequences of only one locus. All trees included the new species, two type strains, and several other *Candida* species collected from the GenBank database (accession numbers are detailed in Table 1). *Malassezia restricta* CBS 7877 was used as the outgroup. All phylogenetic trees were computed using the Maximum of Parsimony statistical model (MP) with the default settings. Estimation of branch robustness was evaluated with 1000 Bootstrap replications with Molecular Evolutionary Genetics Analysis (MEGA) software version 11 [24].

Table 1. GenBank accession numbers of the reference strains used for the phylogenetic analysis.

Species	Collection ID	GenBank accession numbers			
		ITS	D1/D2	TEF1	TUB2
Candida massiliensis	PMML0037	OM952170	OM952176	OP688587	OP688584
C. intermedia	ATCC 14439	OM952171	OM952177	OP688588	OP688585
C. pseudo-intermedia	ATCC 60126	OM952172	OM952178	OP688589	OP688586
C. albicans	ATCC 18804	NR_125332	NG_054826	AY497649	NA*
C. albicans	SC5314	NA	NA	NA	XM_019475574
C. tropicalis	CBS 94	NR_111250	OV701636	MN231084	NA
C. parapsilosis	ATCC 22019	NR_130673	NG_054833	KT694039	NA
C. parapsilosis	ATCC 90018	NA	NA	NA	MH352134
C. krusei	ATCC 6258	NR_131315	NA	NA	NA
C. krusei	ATCC 14243	NA	HQ199090	NA	NA
C. krusei	PW4166	NA	NA	LC425577	NA
C. africana	CBS 8781	NR_138276	NA	NA	NA
C. africana	VPCI 301/P/13	NA	KF741790	NA	NA
C. glabrata	CBS 12,440	KJ009319	NA	NA	NA
C. glabrata	CG1	NA	EF056321	NA	NA
C. glabrata	CBS 138	NA	NA	AY497636	NA
C. guilliermondii	ATCC 6260	AY939792	KU729151	NA	NA
C. guilliermondii	mmRL 1759	NA	NA	AY497663	NA
C. dubliniensis	CBS 7987	NR_119386	NA	AY497659	NA
C. dubliniensis	NRRL Y-17841	NA	NG_054869	NA	NA
C. dubliniensis	CD36	NA	NA	NA	XM_002416698
C. sake	CBS 159	NR_151807	NA	NA	NA
C. sake	NRRL Y-1622	NA	NG_055231	NA	NA
C. rugosa	ATCC 10,571	FJ768914	NA	NA	NA
C. rugosa	UTHSC 05-205	NA	HE716784	NA	NA
C. inconspicua	CBS 180	NR_111116	NA	KM252819	NA
C. inconspicua	NRRL Y-2029	NA	NG_055114	NA	NA
C. saraburiensis	CBS 11,696	KY102390	NG_054769	NA	NA
C. tetrigidarum	CBS 10,457	KY102438	NA	NA	NA
C. tetrigidarum	ATCC MYA-4369	NA	NG_042507	NA	NA
C. metapsilosis	CBS 10,907	KY102203	NA	NA	NA
C. metapsilosis	ATCC 96,144	NA	NG_054815	NA	NA
C. frijolesens	NRRL Y-48060	EF658666	NA	NA	NA
C. labiduridarum	NRRL Y-27940	EF658664	NA	NA	NA
C. labiduridarum	ATCC MYA-4368	NA	NG_042506	NA	NA
C. neerlandica	NRRL Y-27057	EF658662	NG_054776	NA	NA
C. cetoniae	IMB1R2	KC118129	NA	NA	NA
C. bohioensis	CBS 9897	KY101972		NA	NA

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C. bohioensis	ATCC MYA-4363	NA	NG_042503	NA	NA
C. blackwelliae	CBS 10,843	KY101962	NG_081287	NA	NA
C. wuzhishanensis	CBS 10,850	EU570111	NA	NA	NA
C. pseudocylindracea	CBS 10854	EU570109	NG_079493	NA	NA
C. orthopsilosis	ATCC 96,139	NR_130661	NG_054816	NA	NA
C. orthopsilosis	378	NA	NA	NA	MH352145
C. orthopsilosis	Co 90-125 Cam1	NA	NA	XM_003868467	NA
C. chauliodes	ATCC MYA-4356	FJ623621	NG_042500	NA	NA
C. famata var. flareri	CBS 1796	NA	NA	NA	AM990641
C. silvicultrix	CBS 6269	KY102394	KY106756	NA	JQ734924
C. silvicultrix	NRRL Y-7789	NA	NA	EF552562	NA
C. oxycetoniae	KGF56	NA	NA	XM_049324123	XM_049326316
C. oxycetoniae	CBS 10844	NR_111472.1	NG_081288	NA	NA
C. subhashii	J8A68	NA	NA	XM_049404981	XM_049405952
C. subhashii	UAMH 10744	NR_073356	EU836708	NA	NA
C. viswanathii	CBS 4024	NA	NA	AY497645	NA
C. blankii	CBS 1898	NA	NA	GU597341	NA
C. ponderosae	NRRL YB-2307	NA	NA	EF552569	NA
C. solani	NRRL Y-2224	NA	NA	EF552560	NA
C. ulmi	NRRL YB-2694	NA	NA	EF552519	NA
C. quercuum	NRRL Y-12942	NA	NA	EF552516	NA
C. montana	NRRL Y-17326	NA	NA	EF552499	NA
C. rugopelliculosa	NRRL Y-17079	NA	NA	EF552462	NA
C. ethanolica	NRRL Y-12615	NA	NA	EF552449	NA
C. blattae	NRRL Y-27698	NA	NA	MG050961	NA
C. boidinii	NRRL Y-2332	NA	NA	JQ699032	NA
C. heveicola	NRRL Y-48716	NA	NA	MG050956	NA
C. succiphila	NRRL Y-11998	NA	NA	EU014706	NA
C. arabinofermentans	NRRL YB-2248	NA	NA	EU014745	NA
C. suzukii	NRRL Y-27593	NA	NA	EU014719	NA
C. boidinii	NRRL Y-2332	NA	NA	EU014707	NA
C. chilensis	NRRL Y-7790	NA	NA	EU014765	NA
C. fluviatilis	ATCC 38621	NA	NA	KC507441	NA
Malassezia restricta	CBS 7877	NR_103585	NA	KF706471	KC573807
Malassezia restricta	GDMUZJ2015043	NA	KX808619	NA	NA

NA*, not available.

3. Results

3.1. Macroscopic characterisation

All three *Candida* species showed a rapid growth time on the SDA GC, between 48h and 72h (Figure 1). The yeasts were fast growing at all temperatures except at 4 °C, 40 °C and 45 °C, and the optimum temperature was 30 °C. All colonies were smooth, flat and dry, with a white to beige colour on both sides. However, colonies of *C. intermedia* ATCC 14,439 appeared much larger than those of *C. pseudointermedia* ATCC 60,126 and *C. massiliensis*PMML0037.

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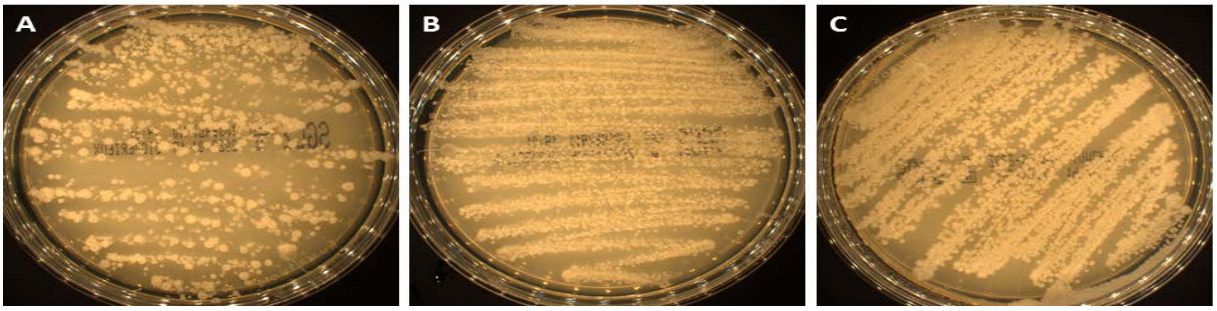

Figure 1. Culture growth on SDA GC after four days of incubation at 30 °C. The colonies colour of both recto and verso was white to beige. (**A**) *Candida intermedia* ATCC 14439. (**B**) *Candida pseudointermedia* ATCC 60126. (**C**) *Candida massiliensis* PMML0037.

3.2. Microscopic characterisation

The microscopic visualization revealed the presence of ovoid and ellipsoidal cells for all isolates, appearing single, budded or short-chained. *C. massiliensis* PMML0037 presented mostly ovoid cells (2.5 - 5 µm) and rarely ellipsoidal (2 – 7 µm), with a smooth surface and an absence of pseudohyphae, while *C. intermedia* ATCC 14,439 and *C. pseudointermedia* ATCC 60,126 both showed abundant pseudohyphae. Cells were ellipsoidal in form (2 - 8 µm) and rarely ovoid (2 - 4 µm) with a rough surface appearance.

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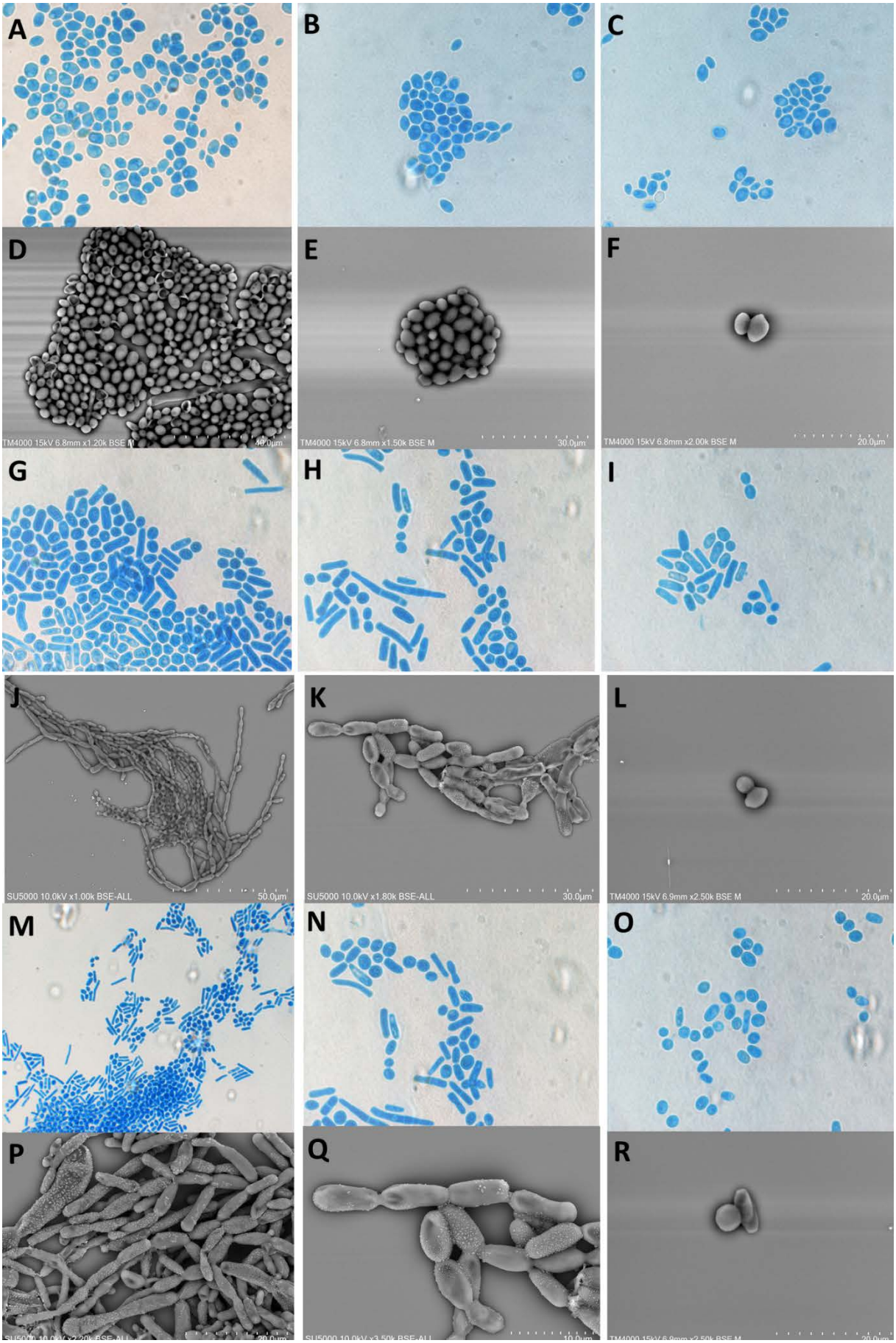

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Figure 2. Morphology of *Candida* spp. (**A-F**) Single ovoid, budded and short-chained cells of *C. massiliensis* (PMML0037). (**G–L**) Single ovoid, budded and short-chained cells of *C. pseudointermedia* (ATCC 60126). (**M–R**) Single ovoid, budded and short-chained cells of *C. intermedia* (ATCC 14439). Optical microscopy (magnification ×1000). Scale bars: 50 µm. Scanning electron microscopy; TM4000 Plus (15 KeV lens mode 4), SU5000 (10 kV). Scale bars: **Q** = 10 µm; **F, L, P, R** = 20 µm; **E, K** = 30 µm; **D** = 40 µm; **J** = 50 µm.

3.3. MALDI-TOF MS identification

The MALDI-TOF MS identification score of the new isolate *Candida massiliensis* (PMML0037) was irrelevant with a log score under the limit necessary to claim a good identification (log score <1.90). The new species was only identified at the genus level as belonging to *Candida sp*. Furthermore, all the mass spectra of the three strains were collected and deposited in the MALDI-TOF MS database.

3.4. Antifungal susceptibility testing (AFST)

The minimum inhibitory concentrations (MICs) values for all antifungal drugs tested are displayed in (Table 2). The MICs endpoints for the three species were defined as the lowest concentrations inhibiting the growth of 90% of the yeasts and were interpreted following the Clinical and Laboratory Standards Institute (CLSI) M27-S4 guidelines [25]. The MICs of AMB, AND, CAS, 5-FC, and MIF were low for all three strains. The new species *C. massiliensis* and the type strain of *C. pseudointermedia* showed high MICs for FL, ITC, and VOR. However, *C. massiliensis* demonstrated a low MIC for POS and *C. pseudointermedia* a high MIC.

Table 2. Results of *in vitro* antifungal susceptibility testing.

	Minimum inhibitory concentrations (mg/l) read at 48h								
	AMB	AND	CAS	MIF	5-FC	FL	ITC	POS	VOR*
Candida massiliensis PMML0037	4	0,25	0,5	0,06	0,06	>32	>32	2	>32
Candida intermedia ATCC 14439	1	0,5	1	0,06	0,12	1	0,25	0,06	0,015
Candida pseudointermedia ATCC 60126	2	0,12	0,5	0,06	0,5	>32	>32	>32	>32

* AMB, amphotericin B; AND, anidulafungin; CAS, caspofungin; 5-FC, 5-fluorocytosine; FL, fluconazole; ITC, itraconazole; MIF, micafungin; POS, posaconazole; VOR, voriconazole.

3.5. Physiological analysis

3.5.1. EDX (Energy-Dispersive X-ray Spectroscopy)

The principal component analysis (PCA) was constructed with the atomic weights and percentages of the chemical elements resulting from the chemical mapping conducted on the three *Candida* strains. The PCA (Figure 3) revealed highly heterogeneous chemical profiles; each *Candida* species appeared to be quite distant from the others.

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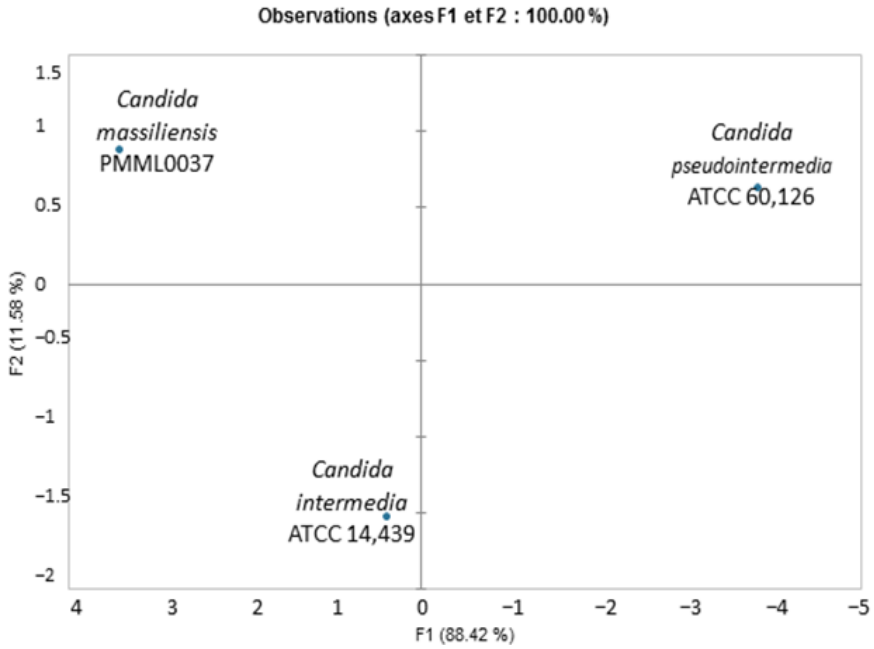

Figure 3. Principal component analysis (PCA) generated with the XLSTAT software of the energy-dispersive X-ray spectroscopy chemical mapping profile, realized for the novel species *Candida massiliensis* (PMML0037) and two *Candida* type strains. The principal components F1 and F2 explained 100% of the chemical mapping profile variance.

3.5.2. Biolog™ phenotypic analysis

The results of the oxidation and assimilation tests were depicted using heat maps (Figures 4 and 5). The two heat maps exhibited different records but were both consistent with the conclusion that *C. massiliensis* PMML0037 appeared more closely associated with *C. intermedia* ATCC 14,439 than *C. pseudointermedia* ATCC 60126.

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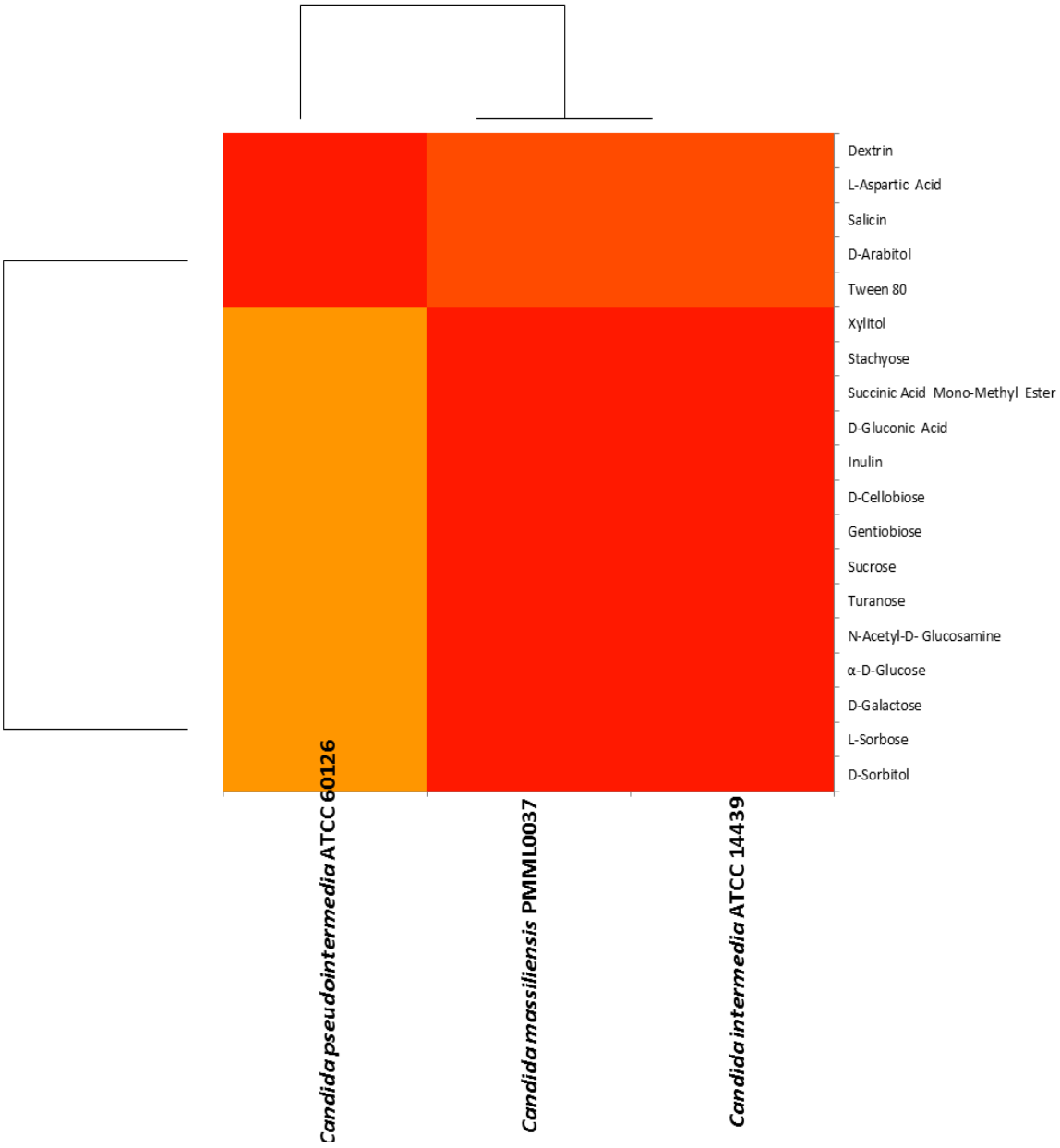

Figure 4. Heat map generated with XLSTAT software for the oxidation of carbon sources by the BiologTM system for the new species *Candida massiliensis* (PMML0037) and two type strains of the genus. Interpretation of the colour gradient: the most oxidised substrates are represented in light orange and the least oxidised substrates are represented in red.

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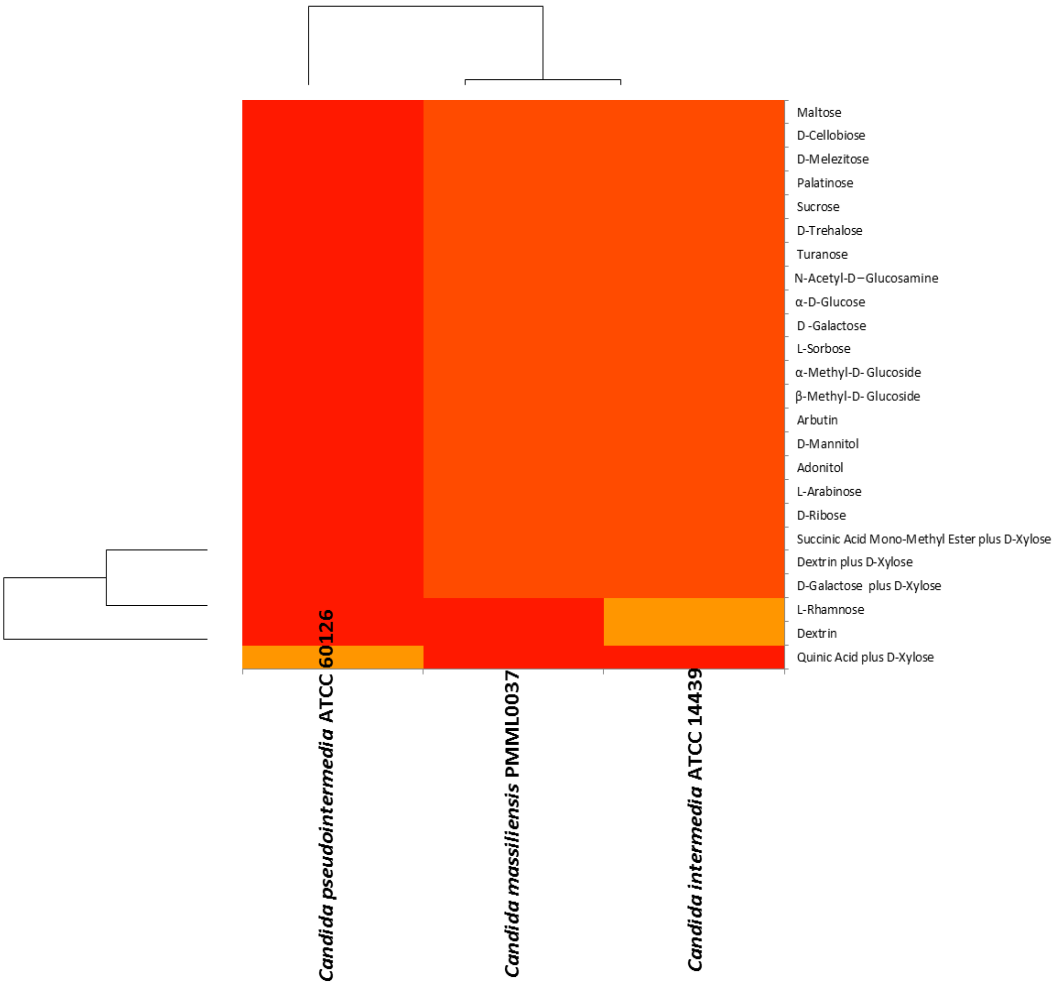

Figure 5. Heat map generated with XLSTAT software for the assimilation of carbon sources by the BiologTM system for the new species *Candida massiliensis* (PMML0037) and two type strains of the genus. Interpretation of the colour gradient: the most assimilated substrates are represented in light orange and the least assimilated substrates are represented in red.

3.6. DNA sequencing

The sequences were treated using the BLASTn tool with the reference data available from the GenBank database of NCBI. The BLASTn of the new isolate showed a percent identity ≤98 % for the four gene markers. Notably, the best-match pairwise identity was 96.15% for *C. intermedia* CBS 572 with ITS (NR111248), 95.94% for *C. blattae* with TEF-1α (MG050961), 98.79% for *C. intermedia* NNRL Y-981 with D1/D2 (NG055404), and 74.60% for *Ambrosiella xylebori* CBS 110,61 with B-tub2 (EU977469).

3.7. Phylogenetic analysis

The multilocus analysis of the first phylogenetic tree (Figure 6) constructed with the assembled sequences of the ITS, D1/D2, TEF-1α and B-tub2 genetic regions revealed that the newly isolated species *Candida massiliensis* (PMML0037) clustered within the *Candida intermedia* clade and seemed quite distinct from all other *Candida* species. The four other single-locus trees (Figure 7) included additional *Candida* species. Within each tree, *Candida massiliensis* (PMML0037) clustered in the *Candida intermedia* clade with high branch support. The five trees were in line and could clearly position the new species (PMML0037) in the *Candida* genus as a new distinct species closely related to *Candida intermedia* and *Candida pseudointermedia*.

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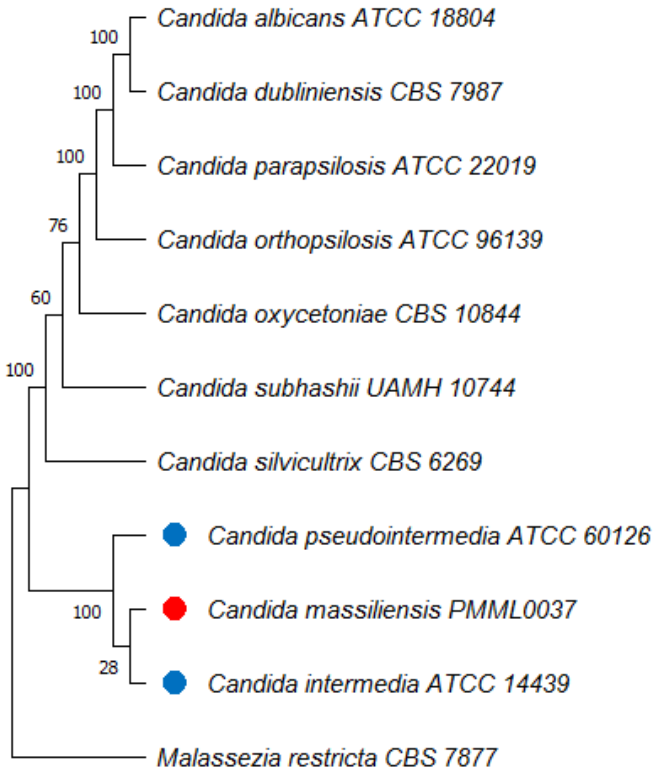

Figure 6. Multilocus phylogenetic tree of the new species *Candida massiliensis* PMML0037 (indicated with red dots) and 10 reference strains (type strains are indicated with blue dots), based on the concatenated ITS, D1/D2, TEF-1α and B-tub2 sequences. *Malassezia restricta* CBS 7877 was used as the outgroup. The maximum-parsimony tree was generated using the MEGA 11 software, with 1000 replication bootstrap values.

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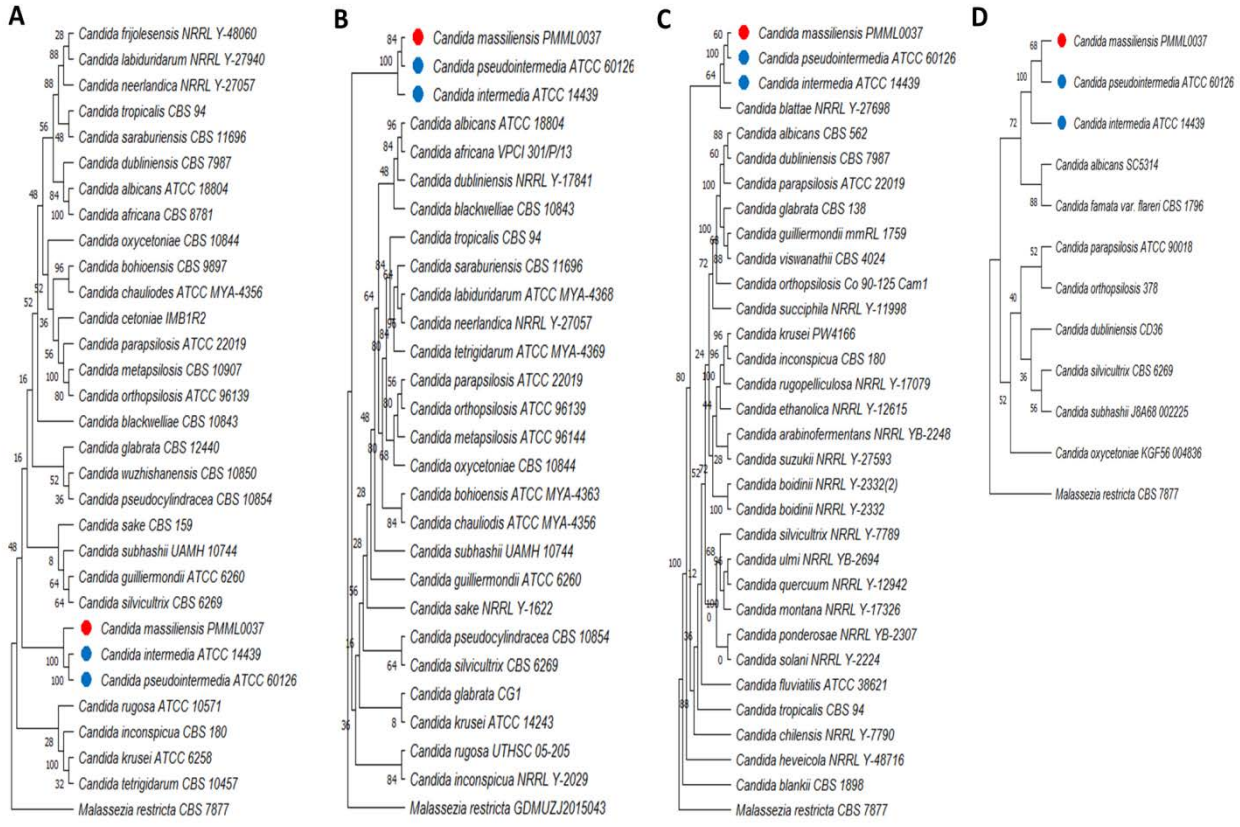

Figure 7. Four single-locus phylogenetic trees using the ITS (**A**), D1/D2 (**B**), TEF-1α (**C**), and B-tub2 (**D**) genetic regions. The species included in each tree differ because the sequences for each locus were not available for all strains. The red dots indicate the new species *Candida massiliensis* PMML0037 and blue dots indicate type strains. *Malassezia restricta* CBS 7877 was used as the outgroup. The maximum-parsimony tree was generated using the MEGA 11 software, with 1000 replication bootstrap values.

3.8. Taxonomy

Candida massiliensis Kabtani J. & Ranque S. sp. nov.

MycoBank: 845931

(Figure 2A–F).

Etymology: Named in honour of Marseille (France), the city where it was isolated.

Diagnosis: Closely similar to the other two *Candida* species examined, showing approximately the same macroscopic aspect. However, based on macroscopic features, it differs from *C. intermedia* and *C. pseudointermedia* by the predominantly presence of ovoid shaped cells and rarely ellipsoidal, the smooth surface aspect and the absence of pseudohyphae.

Type: France: Marseille. Human body (sputum), 14 May 2020. (Holotype IHEM 28560/PMML0037, stored in a metabolically inactive state.) GenBank: OM952170 (ITS), OP688584 (B-tub2), OP688587 (TEF-1a), OM952176 (D1/D2).

Description: the macroscopic analysis were characterised by a rapid time of growth on SDA GC medium at an optimal temperature of 30 °C. However, *Candida massiliensis* was not able to grow at 4 °C and 45 °C. Colonies were smooth, flat and dry with a white to beige colour on both sides. The microscopic features were characterised by the presence of abundant ovoid cells (2.5 - 5 µm) and rarely ellipsoidal shaped (2 – 7 µm), that appeared

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single, budded or short-chained, with a smooth surface and an absence of pseudohyphae. The Biolog™ carbon-source assimilation profile revealed that *C. massiliensis* PMML0037 can assimilate different carbon substrates, such as Palatinose, Sucrose, Turanose, L-Sorbose, Adonitol, Xylose, D-ribose, D-Manitol, Maltose, D-Cellobiose and many other substrates. Based on this phenotypic advanced analysis, *C. massiliensis* PMMFL0037 seems relatively close to *C. intermedia* ATCC 14439.

Host: Human

Additional specimen examined: *C. intermedia*: Type: United States; Puerto Rico. Human: Feces, before 1928. (ATCC 14439/CBS 572.) GenBank: OM952171 (ITS), OP688585 (B-tub2), OP688588 (TEF-1a), OM952177 (D1/D2).

Additional specimen examined: *C. pseudointermedia*: Type: Japan. Food: Kamaboko, before 1976. (ATCC 60126/CBS 6918) GenBank: OM952172 (ITS), OP688586 (B-tub2), OP688589 (TEF-1a), OM952178 (D1/D2).

4. Discussion

Recently, infections with non-*albicans* species have been reported with high frequency. *Candida intermedia* and *Candida pseudointermedia* have always been recognised as non-pathogenic yeasts and therefore rarely implicated in human disease. However, lately several cases of fungemia attributed to *C. intermedia* have increased. Since very little descriptive information is available for these two yeasts, an appropriate characterisation is recommended.

The MALDI-TOF MS was only able to achieve species identification for *C. intermedia* ATCC 14,439 and *C. pseudointermedia* ATCC 60,126 with a relevant log scores >2.0. The new isolate *C. massiliensis* PMML0037 was only identified to the genus level as *Candida* sp. This misidentification, due to the absence of relevant reference spectrum in the databse, led to conduct a DNA-based identification of this isolate by targeting four discriminant genes: the internal transcribed spacer (ITS1/ITS2) and the D1/D2 domains of the large subunit (LSU) of the ribosomal RNA genes, a fragment of the translation elongation factor 1-alpha gene (TEF-1-α), and a fragment of the β-tubulin gene (B-tub2).

The multilocus phylogenetic tree (Figure 6) constructed with the assembled sequences of ITS, D1D2, TEF-1-α and B-tub2, as well as the single-locus phylogenetic trees (Figure 7) constructed with each genetic region separately, all indicated that the newly isolated yeast *Candida massiliensis* (PMML0037) clustered within the same group as the type strains of *C. intermedia* and *C. pseudointermedia*, suggesting a small genetic distance. Based on this phylogeny, this new isolate appears closely related to the *C. intermedia* clade and distant from the other known *Candida* taxa present in the trees.

The susceptibility profiles of all three strains showed relatively low MICs for AMB, AND, CAS, 5-FC, and MIF. Besides, *C. massiliensis* and *C. pseudointermedia* displayed high MICs for FL, ITC, and VOR, but contrasting MICs for POS. While *C. intemedia* presented low MICs for all nine tested antifungal drugs in accordance with the results previously reported by Ruan *et al.* (2010) [26] and Taj-Aldeen *et al.* (2014) [27].

The macroscopic observation revealed a fast-growing phenotype for all *Candida* species on SDA (GC). We additionally observed smooth, flat and dry colonies with a white to beige colour on both sides. Whereas, *C. intermedia* ATCC 14,439 presented colonies of much larger diameter than *C. pseudointermedia* ATCC 60,126 and *C. massiliensis* PMML0037.

Furthermore, we microscopically observed the presence of single ovoid, budded and short-chained cells with a smooth surface and absence of pseudohyphae in the new isolate (PMML0037). In contrast, type strains of *C. intermedia* and *C. pseudointermedia* presented abundant pseudohyphae, ellipsoidal cells but rarely ovoid with a rough surface appearance. The impurities on the cell surface have already been described and explained by Paš *et al.* (2004) [28] as the secretion of macromolecules like extracellular polysaccharides.

The microscopic characterisation was very valuable for species differentiation and supported that the new species was morphologically quite distinct from the two type strains.

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Moreover, the physiological analysis using the EDX (energy-dispersive X-ray spectroscopy) revealed different chemical mapping profiles and was therefore more convincing in distinguishing the three species than the Biolog™ phenotypic advanced technology which demonstrated similar carbon-source oxidation/assimilation profiles between the new isolate and *C. intermedia*.

In conclusion, the clinically isolated yeast PMML0037 is described here as *Candida massiliensis*, a new species easily distinguishable from the other *Candida* species due to distinct genetic sequences and chemical and physiological profiles.

Author Contributions: Conceptualisation, K.J. and R.S.; methodology, R.S., M.M., C.C. and K.J.; formal analysis, K.J. M.M. and B.F.; writing—original draft preparation, K.J.; writing—review and editing, C.C. and R.S. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The *Candida massiliensis* holotype is available in the IHU MI (no. PMML0037) and IHEM (no. 28560) strain collections. The nucleotide sequences are available in GenBank (accession nos. OM952170 and OM952176). The datasets analysed in the current study are available from the corresponding author upon reasonable request.

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Conflicts of interest: The authors have no conflicts of interest to declare regarding this study.

Abbreviations

5-FC	5-fluorocytosine
AMB	Amphotericin B
AND	Anidulafungin
BLASTn	Basic Local Alignment Search Tool nucleotide
CAS	Caspofungin
DNA	Deoxyribonucleic acid
EDX	Energy-dispersive X-ray spectroscopy
FL	Fluconazole
GC	Gentamycin and chloramphenicol
IHU Méditerranée Infection	Institut Hospitalo-Universitaire Méditerranée Infection
ITC	Itraconazole
ITS	Internal transcribed spacers of the rRNA
SDA	Sabouraud Dextrose Agar
LSU	Large subunit of rRNA
MALDI-TOF MS	Matrix-assisted laser desorption/ionization–time of flight mass spectrometry
MEGA	Molecular Evolutionary Genetics Analysis
MICs	Minimum inhibitory concentrations
MIF	Micafungin
NA	Not available
NCBI	National Center for Biotechnology Information
POS	Posaconazole

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rRNA	Ribosomal ribonucleic acid
SSU	Small subunit of rRNA
TEF-1α	Partial translation elongation factor 1-alpha gene
B-tub2	Partial β-tubulin 2 gene
VOR	Voriconazole

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