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Candida palmioleophila: Characterization of a Previously Overlooked Pathogen and Its Unique Susceptibility Profile in Comparison with Five Related Species[▽]

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***Candida palmioleophila* has previously been misidentified as *C. famata* or *C. guilliermondii*. We have investigated traditional and modern identification methods for the identification of this and related species. Forty-one clinical isolates previously identified as *C. famata* or *C. guilliermondii* and 8 reference strains were included. Color development on CHROMagar, growth temperature ranges, micromorphologies, carbon assimilation (ID32C), matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) profiles, and susceptibility profiles (mica- and anidulafungin and itra-, vori-, posa-, and fluconazole MICs were determined by EUCAST method EDef 7.1, and caspofungin MICs were determined by Etest) were determined, and results were compared to those of molecular identification (ITS1 and ITS2 sequencing). The following five different species were identified among the clinical isolates by sequencing, but no *C. famata* isolates were found: *C. guilliermondii* (22 isolates), *C. palmioleophila* (8 isolates), *C. fermentati* (6 isolates), *C. lusitaniae* (3 isolates), and *C. intermedia* (2 isolates). *C. palmioleophila* developed a distinct scintillating color of turquoise to rose, grew at 40°C, and failed to produce pseudohyphae within 14 days. The ID32C profile for 7/9 *C. palmioleophila* isolates was 5367352315, and all were unable to hydrolyze esculin (Esc). The six related species were well discriminated by MALDI-TOF MS. The susceptibility pattern for *C. palmioleophila* was unique, as the echinocandin MICs were low (range, 0.008 to 0.125 μg/ml) and fluconazole MICs were high (range, 8 to >16 μg/ml). Correct identification of *C. palmioleophila* is important due to its unique susceptibility profile. Identification is possible yet laborious with conventional techniques, whereas MALDI-TOF MS easily separated the related species.**

Modern molecular techniques and growing databases on fungal genome sequences have enabled reliable identification to the species level for species with indistinguishable phenotypic characteristics (11, 23, 24, 26, 34). One example is the discovery of *Candida dubliniensis* among *C. albicans* isolates in 1995 (41). Likewise, molecular taxonomic studies have led to the identification of *C. nivariensis* (1) and *C. bracarensis* (12), within the *C. glabrata* complex, and of *C. metapsilosis*, *C. orthopsilosis*, and *Lodderomyces elongisporus* (20, 43), within the *C. parapsilosis* complex. Due to unique susceptibility profiles of some species, accurate identification is important because treatment strategies are often guided by the species identification (5, 8, 16, 30–32). Thus, misidentification may lead to inappropriate treatment, particularly if accurate susceptibility testing is not performed (7, 8, 28, 35). We show here that this may be the case for *C. palmioleophila*, which appears to be an emerging species in Denmark (2). *C. palmioleophila* was first described by Nakase et al. in 1988 (25) and subsequently reported in 1999 as an opportunistic pathogen causing intravenous catheter-associated fungemia (39). This species has notoriously been misidentified as *C. famata* (*Debaryomyces hansenii*) (14, 29), which again is phenotypically indistinguishable from *C. guilliermondii* (*Pichia guilliermondii*) (27, 44, 49). On this background, we investigated the prevalence of *C. palmioleophila* in a Danish collection of clinical isolates previously identified as *C. famata* or *C. guilliermondii*, using internal transcribed spacer (ITS) sequencing, and subsequently assessed susceptibility profiles and identification results by using routine identification methods and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (33).

(Part of this study was presented at the Interscience Conference on Antimicrobial Agents and Chemotherapy [ICAAC], Boston, MA, September 2010.)

MATERIALS AND METHODS

Strains. Forty-one clinical isolates (including 31 blood culture isolates) from 37 patients, identified as either *C. famata* or *C. guilliermondii* at the mycology reference laboratory at Statens Serum Institut during the period 1949–2009, were included (Table 1). Five strains obtained from the Centraalbureau voor Schimmelcultures (CBS; Utrecht, Netherlands) (*C. palmioleophila* CBS 7418, *C. guilliermondii* CBS 6021, *C. fermentati* [*Pichia caribbica*] CBS 9966, *C. intermedia* CBS 572, and *C. famata* CBS 796) and three isolates received as external quality assessment strains from the United Kingdom National External Quality Assessment Service (UKNEQAS) (*P. guilliermondii* UKNEQAS 4447, *P. guilliermondii* UKNEQAS 8112, and *C. lusitaniae* [*Clavispora lusitaniae*] UKNEQAS 4620) were included as reference strains. All isolates had been stored in 10% glycerol broth at −80°C.

Phenotypic characterization. All isolates were inoculated on corn meal agar (CMA; SSI Diagnostika, Hillerød, Denmark) and incubated at 25°C, and micromorphology was evaluated on days 2, 7, and 14. The ability to grow at 37°C, 40°C, and 42°C was observed on day 2 after inoculation on preheated Sabouraud agar (pH 4; SSI Diagnostika, Hillerød, Denmark), and growth was categorized as either present (+), weak (w), or absent (−). Colony color on CHROMagar (SSI Diagnostika, Hillerød, Denmark) was examined on days 1, 2, and 3 after incubation at 37°C. Carbon assimilation patterns were obtained using commercially

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TABLE 1. Phenotypic characteristics of 8 reference strains and 41 clinical isolates originally identified as either *C. guilliermondii* or *C. famata*[a]

Strain (accession no.)	Original species identification	Growth at 37/40/42°C[b]	Formation of pseudohyphae on CMA[c]	Color on CHROMagar	ATB strip (ID32C) profile	Species identified by ITS sequencing
CBS 7418	*C. palmioleophila*	+/+/−	B	Turquoise	5367352315E−	*C. palmioleophila*
W38018-09	*C. famata*	+/+/−	B	Turquoise/rose	5367352315E−	*C. palmioleophila*
W27955-07	*C. guilliermondii*	+/+/−	B	Turquoise/rose	5367352315E−	*C. palmioleophila*
T47982-09	*C. famata*	+/+/−	B	Turquoise/rose	5367352315E−	*C. palmioleophila*
M45383-09	*C. famata*	+/+/−	B	Turquoise/rose	5367352315E−	*C. palmioleophila*
W29590-09	*C. famata*	+/+/−	B	Turquoise/rose	5367352315E−	*C. palmioleophila*
M62963-09	*C. guilliermondii*	+/+/−	B	Turquoise/rose	5367352315E−	*C. palmioleophila*
M67303-09	*C. famata*	+/+/−	B	Turquoise/rose	7367352315E−[d]	*C. palmioleophila*
F27811-07	*C. guilliermondii*	+/+/−	−	Turquoise/rose	5367352215E−[d]	*C. palmioleophila*
UKNEQAS 4447	*C. guilliermondii*	+/+/−	−	Dark purple	7577755715E+	*C. guilliermondii*
UKNEQAS 8112	*C. guilliermondii*	+/+/−	−	Light purple	7577752517E+	*C. guilliermondii*
CBS 6021	*C. guilliermondii*	+/+/−	+	Purple	7577377315E+	*C. guilliermondii*
A3970-87	*C. guilliermondii*	+/+/−	−	Purple	5577752717E+	*C. guilliermondii*
F15415-06	*C. guilliermondii*	+/+/−	+	Dark purple	7177352117E−	*C. guilliermondii*
W63031-08	*C. guilliermondii*	+/+/−	−	Purple	7577352117E+	*C. guilliermondii*
T51653-07	*C. famata*	+/+/−	−	Light purple	7177352117E−	*C. guilliermondii*
F38277-07	*C. guilliermondii*	+/+/−	−	Light purple	7577352117E+	*C. guilliermondii*
A173-94	*C. guilliermondii*	+/+/w	+	Light purple	7577352117E+	*C. guilliermondii*
A336-99	*C. guilliermondii*	+/+/−	−	Light purple	7577357317E+	*C. guilliermondii*
A331-75	*C. guilliermondii*	+/+/w	+	Light purple	7577752113E+	*C. guilliermondii*
A194-53	*C. guilliermondii*	+/+/w	−	Purple	7577752117E+	*C. guilliermondii*
A2189-86	*C. guilliermondii*	+/+/−	−	Purple	7577350515E+	*C. guilliermondii*
A2551-86	*C. guilliermondii*	+/+/−	−	Purple	5577350117E+	*C. guilliermondii*
W56260-07	*C. guilliermondii*	+/+/−	−	Light purple	7177352117E−	*C. guilliermondii*
A221-73	*C. guilliermondii*	+/+/−	−	Purple	7577352117E+	*C. guilliermondii*
PF6934-86	*C. guilliermondii*	+/+/−	−	Purple	7577352117E+	*C. guilliermondii*
A149-49	*C. guilliermondii*	+/+/−	−	Purple	7577750115E+	*C. guilliermondii*
A2745-86	*C. guilliermondii*	+/+/−	−	Purple	7577752115E+	*C. guilliermondii*
F44185-06	*C. guilliermondii*	+/+/−	−	Purple	7577752117E+	*C. guilliermondii*
A1499-76	*C. guilliermondii*	+/+/−	+	Purple	7577752317E+	*C. guilliermondii*
A1469-70	*C. guilliermondii*	+/+/−	+	Purple	7577750315E+	*C. guilliermondii*
M8464-08	*C. guilliermondii*	+/+/−	−	Light purple	7577750315E+	*C. guilliermondii*
H31147-09	*C. guilliermondii*	+/+/−	−	Purple	7577352117E+	*C. guilliermondii*
T38215-06	*C. guilliermondii*	+/+/−	−	Purple	7577350117E−	*C. guilliermondii*
CBS 9966	*C. fermentati*	+/+/−	−	Purple	5577350115E+	*C. fermentati*
F49572-08	*C. guilliermondii*	+/+/−	−	Purple	7577350117E+	*C. fermentati*
M10611-04	*C. guilliermondii*	+/+/−	−	Purple	7577350117E+	*C. fermentati*
W63245-01	*C. guilliermondii*	+/+/−	+	Purple	7577750717E+	*C. fermentati*
T38768-04	*C. guilliermondii*	+/+/w	−	Purple	7577350117E+	*C. fermentati*
T32779-07	*C. guilliermondii*	+/+/w	−	Purple	7577750117E+	*C. fermentati*
M39632-09	*C. guilliermondii*	+/+/w	−	Dark purple	7577752317E−	*C. fermentati*
UKNEQAS 4620	*C. lusitaniae*	+/+/+	−	Purple	5157370317E+	*C. lusitaniae*
H45593-09	*C. famata*	+/+/+	+	Purple	5377370317E+	*C. lusitaniae*
H27507-04	*C. famata*	+/+/+	−	Light red	5777770317E+	*C. lusitaniae*
F47819-04	*C. famata*	+/+/+	−	Light red	5757770317E+	*C. lusitaniae*
CBS 572	*C. intermedia*	−/−/−	+	Purple	5377340733E+	*C. intermedia*
A933-78	*C. famata*	−/−/−	+	Light purple	5177360137E+	*C. intermedia*
F61108-07	*C. famata*	−/−/−	+	Dark purple	5177360137E+	*C. intermedia*
CBS 796	*C. famata*	−/−/−	−	Light red	5577755117E+	*C. famata*

[a] Growth at 37°C, 40°C, and 42°C, the presence of pseudohyphae on corn meal agar, and the colony color on CHROMagar were analyzed. All isolates were analyzed biochemically by 2nd day ID32C reads, and the ITS1/ITS2 region was sequenced.

[b] w, weak growth.

[c] B, budding present, but no pseudohyphae present.

[d] Unacceptable ATB strip profile. Digits in gray differ from the consensus *C. palmioleophila* ID32C profile.

available ATB strips (ID32C; bioMérieux, Marcy l'Etoile, France), which were read on day 2.

Susceptibility testing. Susceptibilities to anidulafungin (MIC range, 0.008 to 4 μg/ml), caspofungin (two batches [TEK0010 and VEK0090]; MIC range, 0.008 to 4 μg/ml), micafungin (MIC range, 0.008 to 4 μg/ml), fluconazole (MIC range, 0.125 to 16 μg/ml), itraconazole (MIC range, 0.03 to 4 μg/ml), posaconazole (MIC range, 0.03 to 4 μg/ml), and voriconazole (MIC range, 0.03 to 4 μg/ml) were determined using the EUCAST microdilution method (13). MICs for caspofungin were also determined using Etest with RPMI–2% glucose medium (AB bioMérieux, Solna, Sweden) following the manufacturer's instructions.

MALDI-TOF MS. All clinical isolates were subjected to MALDI-TOF MS by following the instructions of the manufacturer and following previously described guidelines for yeast identification (22, 45). Prior to sample preparation, all yeasts were grown for 2 days at 37°C (or 25°C if growth was absent at 37°C) on CHROMagar, subjected to 70% ethanol fixation, and either submitted to MALDI-TOF preparation or stored at −18°C until used. Measurements were performed with a Microflex mass spectrometer (Bruker Daltonics, Germany) using Flexcontrol, version 3.0, and spectra were imported and analyzed using Maldi Biotyper (version 2.0; Bruker Daltonics, Germany). Spectra were calibrated using *Escherichia coli* ribosomal proteins and evaluated against the Bio-

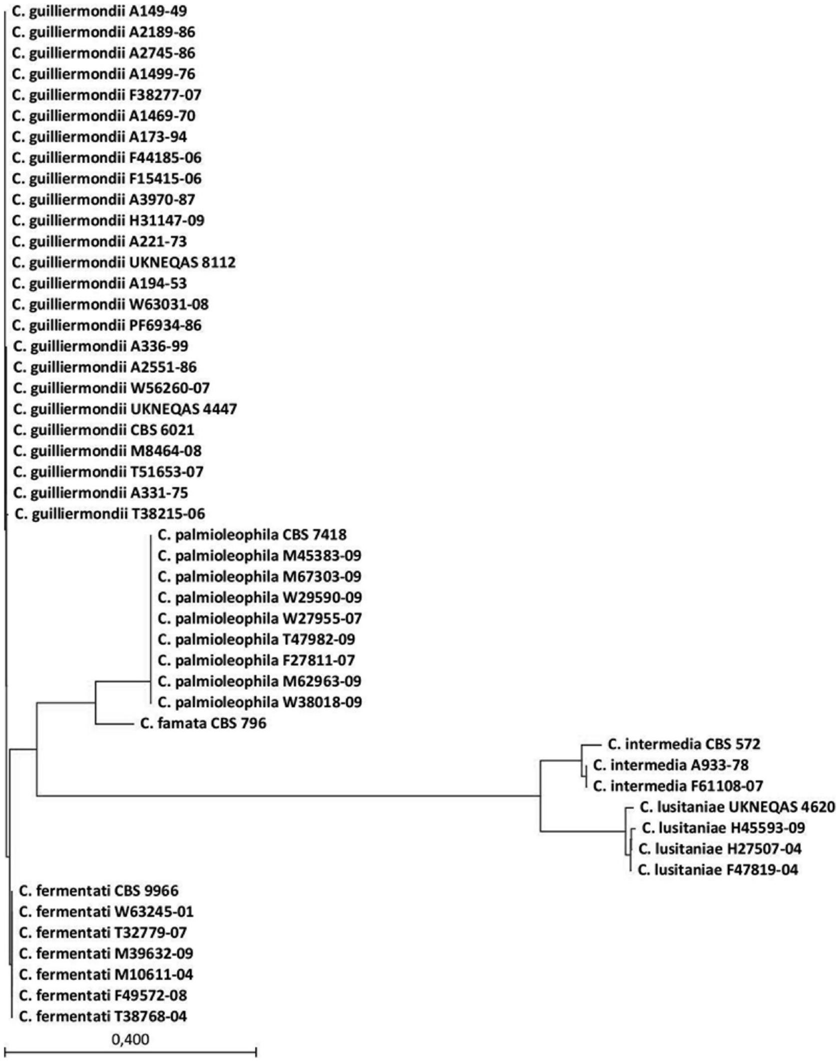

FIG. 1. Unrooted phylogenetic tree based on ITS sequence alignment, using the neighbor-joining algorithm (default) of Saitou and Nei (36), with 100 replicate bootstraps (CLC DNA Workbench). The scale bar represents the bootstrap distance. All isolates were named based on the individual BLAST result, followed by accession number and the year of isolation.

typer spectrum database by the default pattern-matching algorithm. Results are expressed as log values ranging from 0 to 3, where values of >1.7 are generally used for reliable genus identification and score values of >2.0 are used for reliable species identification (38). The original database did not contain reference spectra for *C. palmioleophila* or *C. fermentati*, so the MALDI-TOF profiles of our reference isolates were added manually to the library for a reevaluation of all obtained spectra (38). A MALDI-TOF score-oriented dendrogram was created using default settings in Biotyper (with distances measured by correlation with average linkages).

Molecular identification. Single yeast colonies were transferred to sterile 1.5-ml Eppendorf tubes. DNA was extracted by a 2-step buffer extraction approach as previously described (9, 10), using colony material as a replacement for clinical specimens. The universal fungal primers ITS1 (CGTAGGTGAACCTG CGG) and ITS4 (TCCTCCGCTTATTGATATGC) (48) were employed to amplify the ribosomal ITS1, 5.8S, and ITS2 regions by conventional PCR, using 5 μl extracted DNA in 25-μl reaction mixtures, applying REDExtract-N-Amp PCR Readymix (R4775; Sigma-Aldrich, Denmark) containing deoxynucleoside triphosphates (dNTPs), $MgCl_2$, and a hot start polymerase. The PCRs were performed in a Primus HT thermal cycler (MWG Biotech) with the following program: 5 min at 95°C, 10 touchdown cycles (94°C for 30 s, 58°C for 15 s [with a decrease of 1°C/cycle], and 75°C for 90 s), 30 cycles with a constant annealing temperature of 48°C, and a final elongation step at 75°C for 5 min. PCR products were purified on Qiagen spin columns (QIAquick PCR purification kit; Qiagen, Denmark) and sequenced by Macrogen, South Korea, applying the ITS1 and ITS4 primers for sequencing of both strands. Sequence analysis, alignments, and phylogenetics were performed with the bioinformatic software CLC DNA Workbench (CLC Bio, Denmark). Sequences were used for BLAST searches of sequence databases available through NCBI for species identifications, aligned to typed reference strains, and compared by phylogenetic analysis using the neighbor-joining algorithm (default) of Saitou and Nei (36), with 100 replicate bootstraps, based on alignment of the obtained ITS sequences.

Nucleotide sequence accession numbers. The sequences of the clinical isolates have been deposited in GenBank under the following accession numbers: for strain F27811-07, HQ693769; M45383-09, HQ693770; M62963-09, HQ693771; M67303-09, HQ693772; T47982-09, HQ693773; W27955-07, HQ693774; W29590-09, HQ693775; W38018-09, HQ693776; F49572-08, HQ693777; M10611-04, HQ693778; M39632-09, HQ693779; T32779-07, HQ693780; T38768-04, HQ693781; W63245-01, HQ693782; A933-78, HQ693783; F61108-07, HQ693784; F47819-04, HQ693785; H27507-04, HQ693786; H45593-09, HQ693787; A1469-70, HQ693788; A149-49, HQ693789; A1499-76, HQ693790; A173-94, HQ693791; A194-53, HQ693792; A2189-86, HQ693793; A221-73, HQ693794; A2551-86, HQ693795; A2745-86, HQ693796; A331-75, HQ693797; A336-99, HQ693798; A3970-87, HQ693799; F15415-06, HQ693800; F38277-07, HQ693801; F44185-06, HQ693802; H31147-09, HQ693803; M8464-08, HQ693804; PF6934-86, HQ693805;

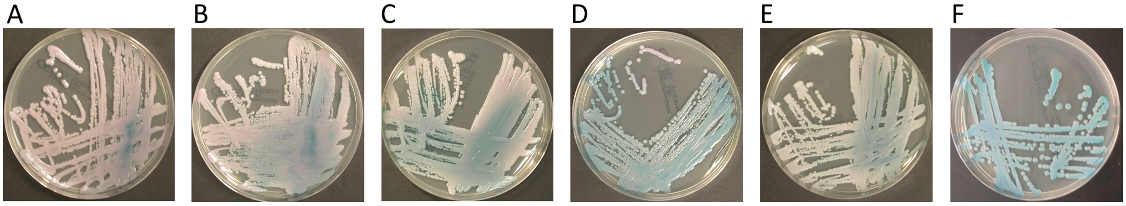

FIG. 2. Mosaic of six clinical isolates and the CBS reference strain of *C. palmioleophila* photographed on day 3 after incubation at 37°C on CHROMagar. (A) M45383-09; (B) W27955-07; (C) W38018-09; (D) F27811-07; (E) T47982-09; (F) CBS 7418. The clinical isolates scintillate from turquoise to rose, whereas the reference isolate develops a uniform turquoise color.

T38215-06, HQ693806; T51653-07, HQ693807; W56260-07, HQ693808; and W63031-08, HQ693809.

RESULTS

***Candida palmioleophila* discovered by molecular identification.** Sequencing of the rRNA ITS regions revealed that a total of 20 of 41 clinical isolates (49%) (Table 1) previously identified as *C. guilliermondii* or *C. famata* were misidentified by conventional methods. Among these, eight *C. palmioleophila* isolates (19.5%) were found (all from blood; two from 2007 and six from 2009). None of 11 isolates previously identified as *C. famata* were confirmed as this species, since 5 isolates were reidentified as *C. palmioleophila*, 3 as *C. lusitaniae*, 2 as *C. intermedia*, and 1 as *C. guilliermondii*. For comparison, among the 30 isolates initially identified as *C. guilliermondii*, 21 were confirmed by sequencing, whereas 6 were reidentified as *C. fermentati* (*Pichia caribbica*) and 3 were reidentified as *C. palmioleophila*. Phylogenetic analysis (Fig. 1) illustrated a genetic relatedness between *C. guilliermondii* and *C. fermentati*, while *C. palmioleophila* and the remaining three species clustered in separate groups.

Phenotypic characteristics of *C. palmioleophila* are distinct. The nine *C. palmioleophila* isolates (reference strain included) showed uniform growth patterns (Table 1), a maximum growth temperature of 40°C, intense budding but no pseudohyphae after 14 days, and color development on CHROMagar from turquoise (reference isolate only) to a distinct scintillating turquoise to rose (all clinical isolates) (Fig. 2). For comparison, only *C. lusitaniae* isolates were consistently able to grow well at 42°C, *C. famata* and *C. intermedia* were unable to grow at 37°C, and *C. intermedia* was the only species producing pseudohyphae on day 2. Moreover, all species other than *C. palmioleophila* developed various but uniform shades of purple or red.

Apart from a single isolate (M67303-09) tolerating cycloheximide (ACT) and one isolate (F27811-07) unable to assimilate D-melezitose (MLZ), the nine *C. palmioleophila* isolates had identical ID32C profiles and were all unable to hydrolyze esculin (Table 1). Consequently, ATB strip reads for *C. palmioleophila* gave either a 91.7% match for *C. famata* or an unacceptable profile. ID32C profiles for the other species were generally less uniform, but all were able to assimilate D-cellobiose, in contrast to *C. palmioleophila*.

MALDI-TOF MS accurately discriminates the six related species. The obtained MALDI-TOF mass spectra were evaluated against the original spectrum database and against the updated version, which included the additional reference spectra. Evaluation against the original database gave 27 isolates with a correct best match, including 21 with a score of >2 and 6 with a score of 1.7 to <2.0. Unreliable species identifications were clearly noticed by low score values (<1.5), indicating the absence of appropriate reference spectra (8/8 *C. palmioleophila* isolates and 6/6 *C. fermentati* isolates). Evaluation against the updated database yielded 41 of 41 isolates with a correct best match and 37 (90%) isolates with a spectral score value of >2. Two isolates of *C. palmioleophila* scored 1.99, and

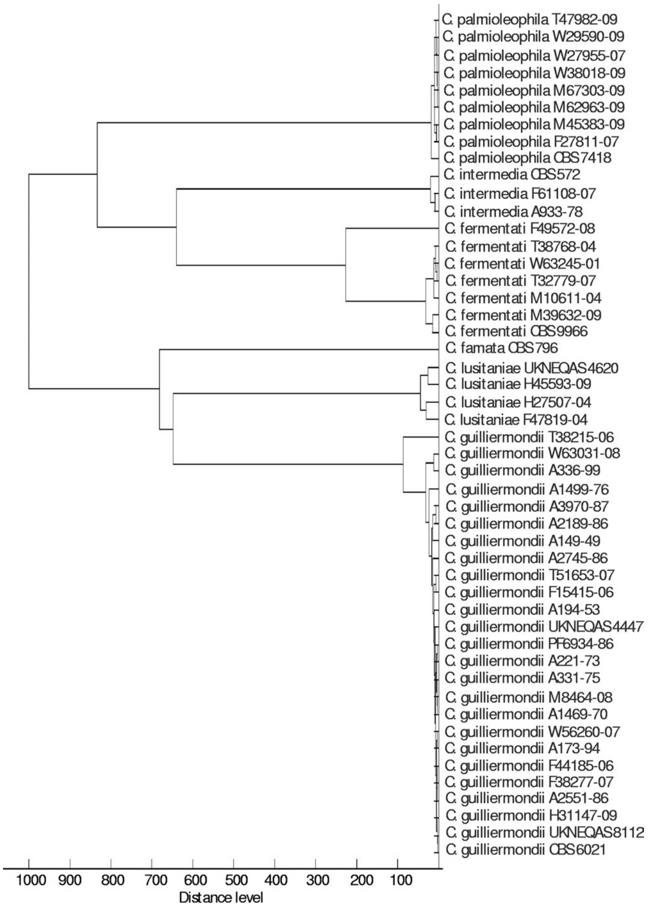

FIG. 3. Score-oriented dendrogram using Euclidian squared distances and average linkages to cluster the MALDI-TOF mass spectra obtained for all included isolates. All samples were named based on molecular identification, illustrating the complete agreement between ITS sequencing and the MALDI-TOF spectra.

TABLE 2. Selected susceptibility results by species for the 41 clinical isolates and 8 reference strains[a]

Strain (accession no.)	Species	MIC (μg/ml)				
		Fluconazole	Voriconazole	Anidulafungin	Micafungin	Caspofungin
CBS 7418	*C. palmioleophila*	8	0.06	0.008	0.008	0.032
W38018-09	*C. palmioleophila*	16	0.125	0.016	0.016	0.125
W27955-07	*C. palmioleophila*	16	0.25	0.03	0.03	0.125
T47982-09	*C. palmioleophila*	16	0.125	0.03	0.016	0.125
M45383-09	*C. palmioleophila*	>16	1	0.016	0.016	0.125
W29590-09	*C. palmioleophila*	>16	0.5	0.03	0.016	0.125
M62963-09	*C. palmioleophila*	>16	0.125	0.03	0.016	0.064
M67303-09	*C. palmioleophila*	>16	0.25	0.03	0.016	0.064
F27811-07	*C. palmioleophila*	>16	1	0.06	0.016	0.125
UKNEQAS 4447	*C. guilliermondii*	2	≤0.03	0.5	0.25	0.25
UKNEQAS 8112	*C. guilliermondii*	4	0.06	1	0.25	0.5
CBS 6021	*C. guilliermondii*	4	≤0.03	0.5	0.25	0.5
A3970-87	*C. guilliermondii*	2	0.06	0.125	0.06	1
F15415-06	*C. guilliermondii*	2	≤0.03	2	0.25	0.25
W63031-08	*C. guilliermondii*	2	0.06	0.5	0.125	0.5
T51653-07	*C. guilliermondii*	4	0.125	2	0.5	0.5
F38277-07	*C. guilliermondii*	4	0.06	2	0.5	0.5
A173-94	*C. guilliermondii*	4	≤0.03	2	0.25	0.5
A336-99	*C. guilliermondii*	4	0.06	2	0.25	0.25
A331-75	*C. guilliermondii*	4	0.125	1	0.25	0.5
A194-53	*C. guilliermondii*	4	0.06	2	0.25	2
A2189-86	*C. guilliermondii*	8	0.06	0.5	0.125	0.5
A2551-86	*C. guilliermondii*	8	0.06	0.5	0.25	1
W56260-07	*C. guilliermondii*	8	0.125	2	0.5	0.5
A221-73	*C. guilliermondii*	8	0.125	2	0.25	0.5
PF6934-86	*C. guilliermondii*	8	0.125	1	0.25	0.5
A149-49	*C. guilliermondii*	8	≤0.03	2	0.25	0.5
A2745-86	*C. guilliermondii*	8	0.06	2	0.25	0.5
F44185-06	*C. guilliermondii*	8	0.125	2	0.5	0.5
A1499-76	*C. guilliermondii*	8	0.06	4	0.25	0.5
A1469-70	*C. guilliermondii*	>16	0.5	1	1	1
M8464-08	*C. guilliermondii*	>16	0.5	1	0.25	0.25
H31147-09	*C. guilliermondii*	>16	0.5	1	0.25	1
T38215-06	*C. guilliermondii*	>16	1	2	1	0.5
CBS 9966	*C. fermentati*	2	≤0.03	2	0.25	0.5
F49572-08	*C. fermentati*	>16	2	2	0.5	0.5
M10611-04	*C. fermentati*	1	≤0.03	1	0.125	0.5
W63245-01	*C. fermentati*	1	0.06	0.5	0.125	0.25
T38768-04	*C. fermentati*	1	≤0.03	0.5	0.125	0.25
T32779-07	*C. fermentati*	1	0.06	1	0.25	0.25
M39632-09	*C. fermentati*	4	0.125	2	0.25	0.25
UKNEQAS 4620	*C. lusitaniae*	0.25	≤0.03	0.03	0.016	0.5
H45593-09	*C. lusitaniae*	≤0.125	≤0.03	0.06	0.06	0.25
H27507-04	*C. lusitaniae*	0.25	≤0.03	0.03	0.03	0.5
F47819-04	*C. lusitaniae*	0.5	≤0.03	0.06	0.06	0.25
CBS 572	*C. intermedia*	≤0.125	≤0.03	0.06	0.008	0.25
A933-78	*C. intermedia*	1	≤0.03	0.03	0.008	0.125
F61108-07	*C. intermedia*	2	≤0.03	0.03	0.008	0.125
CBS 796	*C. famata*	≤0.125	≤0.03	0.03	0.008	0.032

[a] MICs for fluconazole, voriconazole, anidulafungin, and micafungin were determined by EUCAST methodology, and those for caspofungin were determined using Etest.

a third isolate scored 1.90, whereas a single *C. guilliermondii* isolate scored 1.93.

A score-oriented dendrogram was created based on the spectra (Fig. 3), and it shows all isolates clustering with the corresponding reference strains, in agreement with ITS sequence identification.

C. palmioleophila **displays a unique susceptibility profile.** The clinical isolates of *C. palmioleophila* were highly susceptible to echinocandins (anidulafungin and micafungin MICs of ≤0.03 μg/ml and caspofungin Etest MICs of ≤0.125 μg/ml), less susceptible to itraconazole (MIC range, 0.125 to 1 μg/ml), posaconazole (MIC range, 0.06 to 0.25 μg/ml), and voriconazole (MIC range, 0.125 to 1 μg/ml), and resistant to fluconazole (MICs of ≥16 μg/ml). For comparison, echinocandin MICs were considerably higher for *C. guilliermondii* and *C. fermentati* isolates (e.g., anidulafungin MIC_{50}s [ranges] of 2 μg/ml [0.125 to 4 μg/ml] and 1 μg/ml [0.5 to 2 μg/ml], respectively), and azole MICs were notably lower for *C. lusitaniae*, *C. intermedia*, and *C. famata* (Table 2). The MIC values for caspofungin determined by the EUCAST method were not in agreement with the Etest MICs. Thus, given by species and as MIC_{50}s (MIC ranges), the values were as follows for both

TABLE 3. Summary of key phenotypic and biochemical characteristics of included *Candida* isolates

Species	Growth at 37/40/42°C	Formation of pseudohyphae[a]	Color on CHROMagar	Esculin hydrolysis[a]	D-Cellobiose assimilation	MIC range (μg/ml)		
						Anidulafungin	Fluconazole	Voriconazole
C. palmioleophila	+/+/−	−	Turquoise/rose	−	−	0.008–0.06	8–>16	0.06–1
C. guilliermondii	+/+/−	V	Purple	V[b]	+	0.125–4	2–>16	≤0.03–1
C. fermentati	+/+/−	V	Purple	V[c]	+	0.5–2	1–>16	≤0.03–2
C. lusitaniae	+/+/+	V	Purple/red	+	+	0.03–0.06	≤0.125–0.5	≤0.03
C. intermedia	−/−/−	+	Purple	+	+	0.03–0.06	≤0.125–2	≤0.03
C. famata	−/−/−	−	Light red	+	+	0.03	≤0.125	≤0.03

[a] V, variable.

[b] A total of 20/25 isolates were able to hydrolyze esculin.

[c] A total of 6/7 isolates were able to hydrolyze esculin.

caspofungin lots: for *C. palmioleophila*, 0.5 (0.5 to 1); for *C. guilliermondii*, 1 (0.5 to 2); for *C. fermentati*, 1 (0.5 to 1); for *C. lusitaniae*, 1 (1); for *C. intermedia*, 0.5 (0.5 to 1); and for *C. famata*, 0.25 (0.25).

DISCUSSION

In this work, we report on *Candida palmioleophila* as a previously overlooked fungal pathogen, characterize its unique susceptibility profile, and evaluate classical and new identification tools for correct identification of this species.

Within the collection of clinical isolates previously identified as *C. famata* or *C. guilliermondii*, four species were discovered: *C. palmioleophila* was discovered among both sets, *C. fermentati* among "*C. guilliermondii*" isolates only, and *C. lusitaniae* and *C. intermedia* solely among "*C. famata*" isolates. Overall, almost half of the isolates were originally misidentified by conventional diagnostics, even though the tests were performed at a specialized laboratory, and notably, no clinical isolates were confirmed to be *C. famata*. This suggests that *C. famata* may be an even more uncommon human pathogen than initially thought, if not totally absent. Our findings are in agreement with the work of Desnos-Ollivier et al. (14), who were able to confirm only 3 of 26 clinical isolates as *C. famata* by molecular methods, with only 1 confirmed as an invasive isolate. Interestingly, they also found a panel of misidentified species, including three clinical *C. palmioleophila* isolates misidentified as *C. famata.* However, in contrast to our findings, no *C. intermedia* isolates were found, but *Pichia jadinii* and *C. haemulonii* type II isolates were identified, again emphasizing the challenges for discriminating related species within the *C. guilliermondii*/*C. famata* group and the geographical variation in species distribution.

As shown in this study, correct identification could be obtained for *C. palmioleophila*, *C. lusitaniae*, *C. intermedia*, and *C. famata* by carefully combining traditional routine identification methods, whereas *C. guilliermondii* and *C. fermentati* remain notoriously inseparable (Table 3) (6, 17, 21, 37). Discriminating *C. palmioleophila* from *C. guilliermondii* is highly clinically relevant because the latter has reduced susceptibility to echinocandins due to a naturally occurring polymorphism at a locus that reduces susceptibility (Fks1p M642) (David Perlin, personal communication), whereas *C. palmioleophila* was shown to be highly sensitive. Thus, echinocandins would be an excellent choice for *C. palmioleophila* but less so for *C. guilliermondii* (21). *C. lusitaniae* is important to identify correctly because it is often misidentified (14, 42), but it should be regarded as a poor target for amphotericin B despite being classified as susceptible based on MIC determinations (5). Overall, compared to species complexes such as the *C. parapsilosis* complex or the *C. glabrata* complex, accurate identification within the *C. guilliermondii*/*C. famata* group has more clinical importance due to the remarkably diverse susceptibility profiles of the group members (18–20, 40). The challenges in discriminating *C. fermentati* and *C. guilliermondii* have been assessed previously, and the need for molecular methods for accurate identification was demonstrated (6, 17, 37, 46). Nevertheless, although interesting from an epidemiological perspective, the discrimination of these two species is clinically less crucial due to their equivalent susceptibility patterns.

Evaluation of caspofungin MICs with the EUCAST method gave a uniform and narrow MIC range of 0.25 to 2 μg/ml across the included species and both caspofungin lots (TEK0010 and VEK0090). This finding contrasts with the diverse susceptibility patterns suggested by the anidulafungin and micafungin EUCAST MIC results and the caspofungin Etest end points. We previously reported on variability in caspofungin microdilution MIC values across microdilution methods, time, and country (3, 4). So far, no data suggest a variable susceptibility to the three echinocandins, and we believe that the elevated caspofungin EUCAST MIC ranges for the most anidulafungin- and micafungin-susceptible species in this study reflect an *in vitro* phenomenon rather than true differences in susceptibility.

Conventional identification methods often require several phenotypic and biochemical assays, which are time-consuming and still insufficient for precise discrimination of some species (28, 29, 42, 47). On the other hand, molecular methods are rapid and highly discriminative identification tools, yet they are often expensive and may require skilled technicians (15, 22, 44). MALDI-TOF has recently proved a useful and powerful identification tool for several yeast and mold species and has been introduced over recent years to many routine clinical microbiology laboratories (22, 33, 38, 45). We therefore examined the performance of this method with this group of closely related *Candida* species. As shown in this study, the accuracy of the MALDI-TOF analysis and spectrum evaluation against the updated database was significant, yielding only four spectra which should be considered in the borderline range for reliable species identification (spectral scores of 1.9 to <2.0). However, a correct best match was obtained for all clinical isolates.

Moreover, phylogenetic analysis based on the obtained MALDI-TOF spectra depicted an excellent discriminatory power of this analysis, since all isolates were clustered with the corresponding reference strain and distinct from the other species. To our knowledge, this is the first report to describe the excellent performance of MALDI-TOF for the identification of *C. palmioleophila* and related cryptic species.

In conclusion, *Candida palmioleophila* is an emerging pathogen in Denmark and is often misidentified as *C. guilliermondii* or *C. famata*. Due to its unique susceptibility profile, correct identification has a high clinical importance. By conventional mycological methods, identification requires several tests and technician experience, yet MALDI-TOF MS and ITS sequencing may provide rapid and powerful alternatives to conventional identification techniques.

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