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Candida inconspicua and *Candida norvegensis*: New Insights into Identification in Relation to Sexual Reproduction and Genome Organization

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***Candida inconspicua* and *Candida* (*Pichia*) *norvegensis* are two emerging pathogenic species that exhibit reduced susceptibility to azole derivatives. Conventional (biochemical) approaches do not readily differentiate between the two species. The first aim of this work was to analyze the performance of biochemical, proteomic (matrix-assisted laser desorption ionization–time of flight [MALDI-TOF]), and molecular approaches in the precise identification of these species. These results then led us to sequence 3 genomic loci, i.e., the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA), the D1/D2 domain of the 28S rDNA, and the elongation factor 1α (EF-1α) gene, either directly or following cloning, of 13 clinical isolates and 9 reference strains belonging to the 5 species included in the *Pichia cactophila* clade, namely, *Pichia cactophila*, *Pichia insulana*, *C. inconspicua*, *C. norvegensis*, and *P. pseudocactophila*. Finally, isolates of *C. inconspicua* were challenged for sexual reproduction on the appropriate medium. Our results show that EF-1α sequencing and proteic profiling by MALDI-TOF are the two most efficient approaches to distinguish between *C. norvegensis* and *C. inconspicua*. As a characteristic of the *P. cactophila* clade, we found multiple alleles of the rDNA regions in certain strains belonging to the tested species, making ITS or D1/D2 sequencing not appropriate for identification. Whatever the method of identification, including MALDI-TOF and EF-1α sequencing, none could differentiate *C. inconspicua* from *P. cactophila*. The results of phylogenetic analysis and the generation of asci from pure cultures of all *C. inconspicua* strains both support the identification of *P. cactophila* as the teleomorph of *C. inconspicua*.**

Candida spp. remain the predominant cause of invasive fungal infections (1). The incidence of candidemia, the main clinical form of invasive candidiasis, significantly increased in the 1980s (2). Concomitantly, non-*C. albicans* species have emerged among the causative agents of candidiasis (3). The reasons for this emergence remain unclear, but selective pressure due to a larger use of azole derivatives has been suggested (4). Indeed, in addition to *C. glabrata* and *C. krusei*, the two main fluconazole-resistant species isolated from patients with candidemia, a number of fluconazole-resistant species are now regularly isolated from deep-seated infections. Together, these "rare" species may account for as much as 10% of the etiologic agents in some medical centers (5).

Candida inconspicua and *Candida norvegensis* (teleomorph [tel.]: *Pichia norvegensis*) are among those fluconazole-resistant emerging species that are more frequently isolated from invasive infections (6). In addition to this increasing concern, these two species are known to be difficult to differentiate from each other using routine techniques such as biochemical panels. Indeed, auxanograms such as ID32C differentiate the two species only according to the hydrolysis of esculin, a phenotypic trait whose variability is well known (7). These quite similar biochemical profiles may reflect the close phylogenetic relationship between these 2 species. Indeed, both species belong to the *Pichia cactophila* clade, which, in addition to *C. inconspicua* and *C. norvegensis*, encompasses three teleomorphic stages, namely, *P. cactophila*, *Pichia insulana*, and *Pichia pseudocactophila* (8, 9).

For such "difficult-to-identify" yeast species, the molecular approach, mainly using sequence analysis of the internal transcribed spacer (ITS) or D1/D2 domain of the large subunit of the ribosomal DNA (rDNA), is considered the reference method for a definitive identification. However, direct sequencing of rDNA fragments amplified from *C. norvegensis* and *C. inconspicua* is frequently unsuccessful (personal data).

Here, we report on the performance of biochemical, proteomic (matrix-assisted laser desorption ionization–time of flight [MALDI-TOF] mass spectrometry), and molecular approaches, including sequencing of a fragment of the transcription elongation factor 1α (EF-1α) gene, for the specific identification of clinical isolates of *C. inconspicua* and *C. norvegensis*. Sequence-based identification allowed us to better characterize the rDNA content

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TABLE 1 Biological characteristics of 13 clinical isolates and 8 CBS reference strains belonging to species of the *P. cactophila* clade[a]

Strain	Identification result(s)		Molecular analysis					
	ID32C (esculin hydrolysis) identification	MALDI-TOF (no. of replicates with LS > 2)	ITS	Best hit	D1/D2	Best hit(s)	EF-1	Best hit(s)
SA952	*C. norvegensis* (Pos)	*P. cactophila* (4)	PSC	NI	USC	NI	HPM	*C. inconspicua*
SA1635	*C. inconspicua* (Neg)	*P. cactophila* (4)	PSC	NI	USC	NI	HPM	*C. inconspicua*
SA1729	*C. inconspicua* (Neg)	*C. inconspicua* (2), *P. cactophila* (2)	PSC	NI	PSC	NI	HPM	*C. inconspicua*
SA1730	*C. norvegensis* (Pos)	*C. inconspicua* (2), *P. cactophila* (2)	PSC	NI	HPM	*C. inconspicua*, *P. cactophila*	HPM	*C. inconspicua*
SA1457	*C. inconspicua* (Neg)	*C. inconspicua* (3), *P. cactophila* (1)	PSC	NI	USC	NI	HPM	*C. inconspicua*
SA1458	*C. inconspicua* (Neg)	*P. cactophila* (4)	PSC	NI	USC	NI	HPM	*C. inconspicua*
SA1460	*C. norvegensis* (Pos)	*P. norvegensis* (3)	PSC	*P. norvegensis*	USC	NI	CC	*C. norvegensis*
SA1459	*C. inconspicua* (Neg)	*P. cactophila* (4)	PSC	NI	USC	NI	HPM	*C. inconspicua*
SA1461	*C. inconspicua* (Neg)	*P. cactophila* (3)	CC	*C. inconspicua*	USC	NI	HPM	*C. inconspicua*
SA1462	*C. inconspicua* (Neg)	*C. inconspicua* (2), *P. cactophila* (2)	PSC	NI	USC	NI	HPM	*C. inconspicua*
SA1463	*C. inconspicua* (Neg)	*C. inconspicua* (2), *P. cactophila* (2)	CC	*C. inconspicua*	USC	NI	HPM	*C. inconspicua*
SA2546	*C. norvegensis* (Pos)	*P. norvegensis* (4)	USC	NI	HPM	*P. norvegensis*	CC	*C. norvegensis*
SA2185	*C. inconspicua* (Neg)	*P. cactophila* (4)	PSC	NI	PSC	NI	HPM	*C. inconspicua*, *P. cactophila*
CBS6926	*C. inconspicua* (Neg)	*P. cactophila* (4)	USC	NI	HPM	*C. inconspicua*, *P. cactophila*	HPM	*C. inconspicua*
CBS7059	*C. inconspicua* (Neg)	*P. cactophila* (4)	PSC	NI	PSC	NI	HPM	*C. inconspicua*
CBS7103	*C. inconspicua* (Neg)	*P. cactophila* (4)	PSC	NI	PSC	NI	HPM	*C. inconspicua*
CBS2155	NI (Neg)	*P. cactophila* (4)	CC	*C. inconspicua*	PSC	NI	HPM	*C. inconspicua*
CBS6928	*C. inconspicua* (Neg)	*P. cactophila* (4)	CC	*C. inconspicua*	HPM	*C. inconspicua*, *P. cactophila*	HPM	*C. inconspicua*
CBS180	*C. inconspicua* (Neg)	*P. cactophila* (4)	CC	*C. inconspicua*	HPM	*C. inconspicua*, *P. cactophila*	HPM	*C. inconspicua*
CBS6564	*C. norvegensis* (Pos)	*P. norvegensis* (4)	CC	*P. norvegensis*	HPM	*P. norvegensis*	CC	*P. norvegensis*
CBS6929	NI (Neg)	NI	PSC	NI	HPM	*P. pseudocactophila*	CC	*P. pseudocactophila*
CBS11169	*C. inconspicua* (Neg)	NI	PSC	*C. inconspicua*	HPM	*P. insulana*	CC	*P. cactophila*, *C. inconspicua*, *P. pseudocactophila*

[a] LS, log score; Pos, positive; Neg, negative; NI, nonidentified; PSC, partially superimposed chromatograms; USC, uninterpretable superimposed electropherograms; HPM, heterozygous point mutations; CC, clear chromatograms.

of these species. This, along with a study of the sexual reproduction of these yeasts, led us to suggest that *P. cactophila* is the sexual morph of *Candida inconspicua*.

MATERIALS AND METHODS

Strains. Thirteen clinical isolates identified using a biochemical panel (ID32C; bioMérieux, Marcy l'Etoile, France) as either *C. inconspicua* ($n = 9$) or *C. norvegensis* ($n = 4$) were used in this study (Table 1). In addition, 9 reference strains of *P. cactophila* ($n = 5$), *C. inconspicua* ($n = 1$), *P. norvegensis* ($n = 1$), *P. insulana* ($n = 1$), and *P. pseudocactophila* ($n = 1$) were included. Detailed references of the strains used in this study are presented in Table S1 in the supplemental material.

Biochemical-phenotypic identification. All strains were identified with the use of an ID32C auxanogram panel (bioMérieux), according to the manufacturer's recommendations. Particular attention was paid to the esculin hydrolysis, the unique trait thought to be able to distinguish between *C. inconspicua* (lack of hydrolysis) and *C. norvegensis* (presence of hydrolysis). The percentage of identification based on the relative similarity between the biochemical profile of the tested strain and the analyzed profiles was recorded. According to the manufacturer, the identification is considered very good above 99%, good between 90% and 99%, and acceptable between 80% and 90%. It is worth noting that *P. cactophila*, *P. insulana*, and *P. pseudocactophila* are not included in the ID32C database.

MALDI-TOF mass spectrometry. Strains were analyzed by MALDI-TOF mass spectrometry using a Bruker Microflex mass spectrometer (Bruker, Wissembourg, France). Briefly, strains were cultured on CHROMagar *Candida* plates (BD, Pont-de-Claix, France) during 48 h at 37°C. Proteins from two yeast colonies were extracted using an ethanol-acetonitrile-formic acid treatment following the manufacturer's recommendations. Extracts were then spotted in quadruplicate onto a MALDI-TOF anchor chip and submitted for analysis. Bruker Biotyper version 2.0 was used for identification. A log score of >2 is considered to represent confidence for species identification according to the manufacturer's recommendations. Based on previous experiments (personal data), we also considered a species identification to be correct when at least 3 of the 4 spectra reached a log score of >1.7 for the same species. Bruker database version 2.0 contains representatives of *C. inconspicua* (one strain), *P. norvegensis* (five strains), and *P. cactophila* (three strains). Neither *P. pseudocactophila* nor *P. insulana* is included in the database.

Molecular analysis. All strains were subjected to direct sequencing of the D1/D2 domain and the ITS region of the ribosomal DNA and a fragment of the EF-1α gene. Briefly, DNA from isolated colonies obtained after 48 h was extracted using a heat shock-Chelex resin protocol (10) on CHROMagar *Candida* medium incubated at 37°C. Amplification was performed in a 50-μl reaction mixture containing a 0.5 μM concentration of each primer (Eurogentec, Angers, France), a 0.25 mM concentration of

TABLE 2 Alignment of 6 different alleles of the ITS locus found in *P. cactophila* reference strains after cloning and sequencing

	ITS sequence[a]	No. of clones					
		CBS2155 ($n = 10$)	CBS6926 ($n = 10$)	CBS6928 ($n = 20$)	CBS7059 ($n = 7$)	CBS7103 ($n = 15$)	CBS2155 ($n = 10$)
ITSa	CCGTCTTTCGGTGGCTCCCCCGAAATGGAACGATTGCGGGCTTAG		3		3	8	
ITSb	CC---TTTGGGTGGCTCCCCCGAAATGGAACGATTGCGGGCT-AG					2	
ITSc	CC---TTTGGGTGGCTCCCCCGAAATGGAACGATTGCGGGCTTAG	10	6			5	10
ITSd	CCAC--TTCGGTGGCTCCCCCGAAATGGAACGATTGCGGGCT-AG		1				
ITSe	CCGCC--TCGGTGGCTCCCCCGAAATGGAACGATTGCGGGCT-AG			20	1		
ITSf	CCGCC--TCGGTGGCTCCCCCGAAATGGAACGATTGCGGGCTTAG				3		
	..***.***.....................................*..						

[a] Data represent nucleotide sequences starting at position 308 of the ITS sequence of the CBS180 strain (GenBank accession number AB179767). Asterisks represent nucleotide divergences between alleles.

each deoxynucleoside triphosphate (equimolar concentrations of dATP, dCTP, dGTP, and dTTP) (New England BioLabs, Evry, France), 0.5 U of DreamTaq polymerase (Fermentas, Saint Rémy-lès-Chevreuse, France), and 5 μl of DNA. The ITS1-ITS4 and NL1-NL4 primer pairs used for the amplification of the ITS region and the D1/D2 domain, respectively, have been published previously (11, 12). A specific primer pair (EF1_F [GTG TCGGTGAATTCGAAGCTGGTA] and EF1_R [GGTGGGTATTCAGT GAAAGC]) was designed to amplify a ca. 780-bp fragment of the EF-1α gene of the considered species.

Direct sequencing was performed using a BigDye Terminator V3.1 kit (Life Technologies, Saint Aubin, France) and run in a 3500xL Dx genetic analyzer (Life Technologies). Chromatograms were then edited and manually corrected using BioEdit software version 7.0.9.0.

Cloning of the rDNA intergenic spacer region. Because the chromatograms of the ITS region obtained through direct sequencing frequently resulted in superimposed traces, we decided to clone those amplicons before sequencing. The PCR products were purified using a MinElute PCR purification kit (Qiagen, Courtaboeuf, France) and then cloned into the plasmid vector PCR II Topo (Invitrogen). The plasmid was used to transform chemocompetent NeB 5-alpha *Escherichia coli* cells (New England BioLabs), according to the manufacturer's instructions. For each amplicon (i.e., strain), 7 to 20 bacterial clones were further sequenced with the primers used for amplification. After plasmid DNA extraction using a PureLink Quick Plasmid Miniprep kit (Life Technologies), recombinant plasmids were sequenced in both directions using a BigDye Terminator v3.1 kit (Sanger ABI 3730xl; GATC Biotech, Mulhouse, France).

Phylogenetic analysis. Sequences were aligned using Clustal X software (version 2.0.10) (13) and were used to draw trees using the maximum parsimony and neighbor-joining methods implemented in the MEGA5.2.2 software (14).

Sexual reproduction. In order to investigate the potential sexual reproduction of *C. inconspicua*, strains were grown on Sabouraud chloramphenicol gentamicin agar (Bio-Rad, Marnes la Coquette, France) and were then spotted onto potassium acetate agar plates (yeast extract [0.25%], glucose [0.1%], potassium acetate [10%], agar [20%]) and incubated at 30°C for 1 week. Strains were spotted individually on the potassium acetate plates but also in combination with every other strain used in this study. As it readily produced asci, *P. cactophila* CBS6926 was used alone as positive control. Each day, a thin smear of the culture was stained using the Wirtz stain that distinguishes the blastoconidia (stained in pink) from the ascospores (in green) (15).

Nucleotide sequence accession numbers. Accession numbers of the new sequences described in this study are registered in GenBank under accession numbers KM252801 to KM252842.

RESULTS

Molecular analysis. Analysis of the sequences of the ITS region is shown in Table 1 and in Table S3 in the supplemental material. Traces for one clinical isolate (SA2546) and one *P. cactophila* reference strain (CBS6926) were repeatedly unreadable throughout their length. In contrast, for six strains, a complete chromatogram was obtained. BLASTN comparison of the sequences obtained from *C. inconspicua* (CBS180) and *P. cactophila* (CBS2155 and CBS6928) reference strains and two clinical isolates retrieved *C. inconspicua* as the best hit with 99% identity (or 100%, omitting heterozygous point mutations). The sequence obtained from the *P. norvegensis* reference strain (CBS6564) matched with a *P. norvegensis* sequence in GenBank (99% identity). Five reference strains and 11 clinical isolates presented superimposed chromatograms that were partially interpretable. Sharp analysis of these chromatograms suggested the superimposition of multiple staggered traces (see Fig. S1 in the supplemental material). Thus, ITS amplicons from 5 *P. cactophila* reference strains were cloned into the PCR II Topo plasmid vector and 7 to 20 clones were sequenced for each amplicon. Unambiguous chromatograms were obtained in all cases. Six different alleles, ITSa to ITSf, were identified (Table 2). Alignment of these sequences showed insertions or deletions at positions 310 and/or 350. One to three different alleles were identified per strain, in accordance with the superimposed traces obtained from direct sequencing (Fig. 1).

The analysis of the D1/D2 domain revealed somewhat similar results (Table 1; see also Table S4 in the supplemental material). Eight strains among 22 had chromatograms that could be read on both strands, on a length of 571 to 604 bp. BLASTN comparison of the sequences obtained from 2 *P. cactophila* (CBS6926 and CBS6928) and the *C. inconspicua* (CBS180) reference strains, as well as a clinical isolate (SA1730), returned *C. inconspicua* and *P. cactophila* as best hits with similar E values. One clinical isolate (SA2546) showed 99% nucleotide identity with *P. norvegensis*. BLASTN comparison of the sequences from the *P. norvegensis*, *P. insulana*, and *P. pseudocactophila* reference strains returned adequate identification results in all cases. In contrast, 9 clinical isolates presented direct traces that were fully uninterpretable. In addition, 3 reference strains and 2 clinical isolates had a clear chromatogram over only a limited sequence portion (51 to 108 bp). Sharp analysis of these chromatograms suggested, again, a combination of multiple staggered traces.

In contrast, clear chromatograms of EF-1α sequence were obtained for all the strains tested (Table 1; see also Table S2 in the supplemental material). For the 6 *C. inconspicua* and *P. cactophila* reference strains, and for 11 clinical isolates, BLASTN comparisons to GenBank data returned 2 best hits of either *P. cactophila* or

C. inconspicua with similar E values. In these cases, 3 to 13 coincident peaks were found in the chromatograms of the forward and reverse sequences, suggestive of heterozygous point mutations in diploid cells. *P. norvegensis* was the best hit for the *P. norvegensis* reference strain and for 2 clinical isolates.

Phylogenetic analysis of the *P. cactophila* clade. For each locus, we used our own sequences and sequences retrieved from GenBank to build phylogenetic trees using the neighbor-joining method (Fig. 1). In each case, *Candida krusei* (tel. *Issatchenkia orientalis*) ATCC 24210 served as the outgroup.

Overall, the topologies of the trees were similar whatever the locus analyzed. The *P. norvegensis* strains formed a cluster strongly supported by a bootstrap value of between 89% and 100%. Similarly, the *P. insulana*, *C. inconspicua*, and *P. cactophila* strains grouped into a cluster with a bootstrap value at 99.7% to 99.9%. The differentiation of a cluster gathering *P. cactophila* and *C. inconspicua* was variably supported according to the locus: 84.7% for the D1/D2 domain and 68% for the ITS region. Finally, *P. cactophila* strains can always be differentiated from *C. inconspicua* strains.

Interspecies and intraspecies variability. The same sequences were used to estimate interspecies and intraspecies sequence divergence according to the locus (see Table S5 in the supplemental material). The interspecies variability between *P. pseudocactophila*, *P. norvegensis*, and *P. insulana* and an "artificial" group comprising the *P. cactophila* and *C. inconspicua* strains ranged between 0.015 and 0.093 according to the locus. In contrast, the interspecies variability between *C. inconspicua* and *P. cactophila* ranged between 0.001 and 0.011, values equivalent to those found for intraspecies variability, estimated for the other species tested in this study (see Table S5).

Sexual reproduction of *C. inconspicua* and *P. cactophila*. Since both molecular and spectrometric approaches failed to clearly delineate *C. inconspicua* and *P. cactophila*, we hypothesized that these 2 species may represent the anamorph and the teleomorph of the same species, respectively. Subcultured on potassium acetate agar, in a pure culture or a culture mixed with any other strain, all the clinical isolates identified as either *C. inconspicua* or *P. cactophila* and the *C. inconspicua* CBS180 reference strain were able to form asci containing 1 to 4 ascospores within 1 week (Fig. 2). Similarly, we found sexual reproduction for *P. pseudocactophila* and *C.* (*Pichia*) *norvegensis* strains.

Performance of routine identification methods. The performances were evaluated by comparison to the identification obtained through the EF-1α locus sequencing, considered the reference in this study (Table 1).

ID32C returned an identification of *C. inconspicua* for 14 strains molecularly identified as *C. inconspicua* or *P. cactophila*. Three *C. norvegensis* strains (one reference strain and two clinical isolates) were correctly identified. One *P. cactophila* reference strain (CBS2155) was not identified, and two clinical isolates of *C. inconspicua*, molecularly confirmed, were identified as *C. norvegensis*.

Reference strains of *C. inconspicua* ($n = 1$) and *P. cactophila* ($n = 5$) were identified as *P. cactophila* using MALDI-TOF analysis. For the 11 clinical strains molecularly identified as either *C. inconspicua* or *P. cactophila*, MALDI-TOF analysis returned an identification as either *P. cactophila* ($n = 6$; 55%) or *P. cactophila*-*C. inconspicua* ($n = 5$; 45%). All the *C.* (*Pichia*) *norvegensis* strains were correctly identified using MALDI-TOF analysis. Similar results were obtained using Sabouraud chloramphenicol gentamicin as the primary medium for isolation (data not shown).

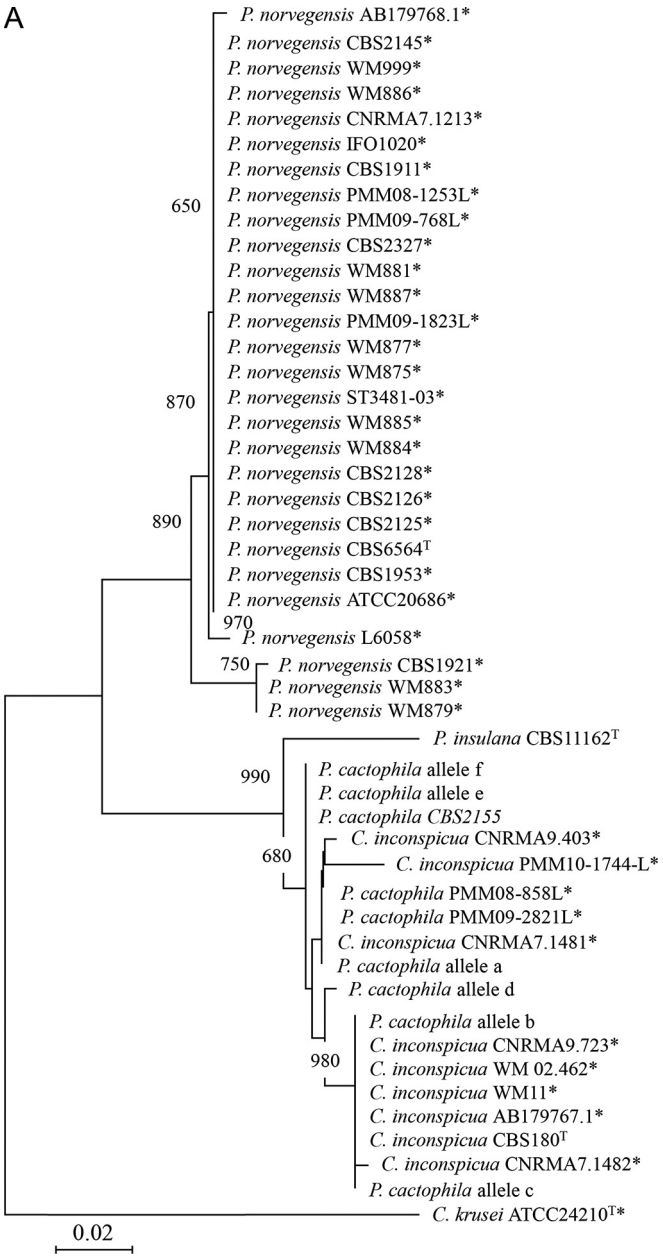

FIG 1 Phylogenetic trees built with the neighbor-joining method using sequences of clinical and reference strains belonging to the *P. cactophila* clade. *Candida krusei* was used as an outgroup. Bootstrap values are from 1,000 replicates and are given at branch nodes. Only bootstrap values over 750 are indicated. The tree is drawn to scale with branch lengths in the units of the number of nucleotide changes over the whole sequence. For the ITS locus, *P. cactophila* alleles a to f correspond to the different alleles characterized after cloning and sequencing the ITS region from different *P. cactophila* strains (see Table 2).

DISCUSSION

Candida inconspicua and *C. norvegensis* are two emerging species whose specific identification is frequently incorrect (7). The first aim of this study was to compare the performances of biochemi-

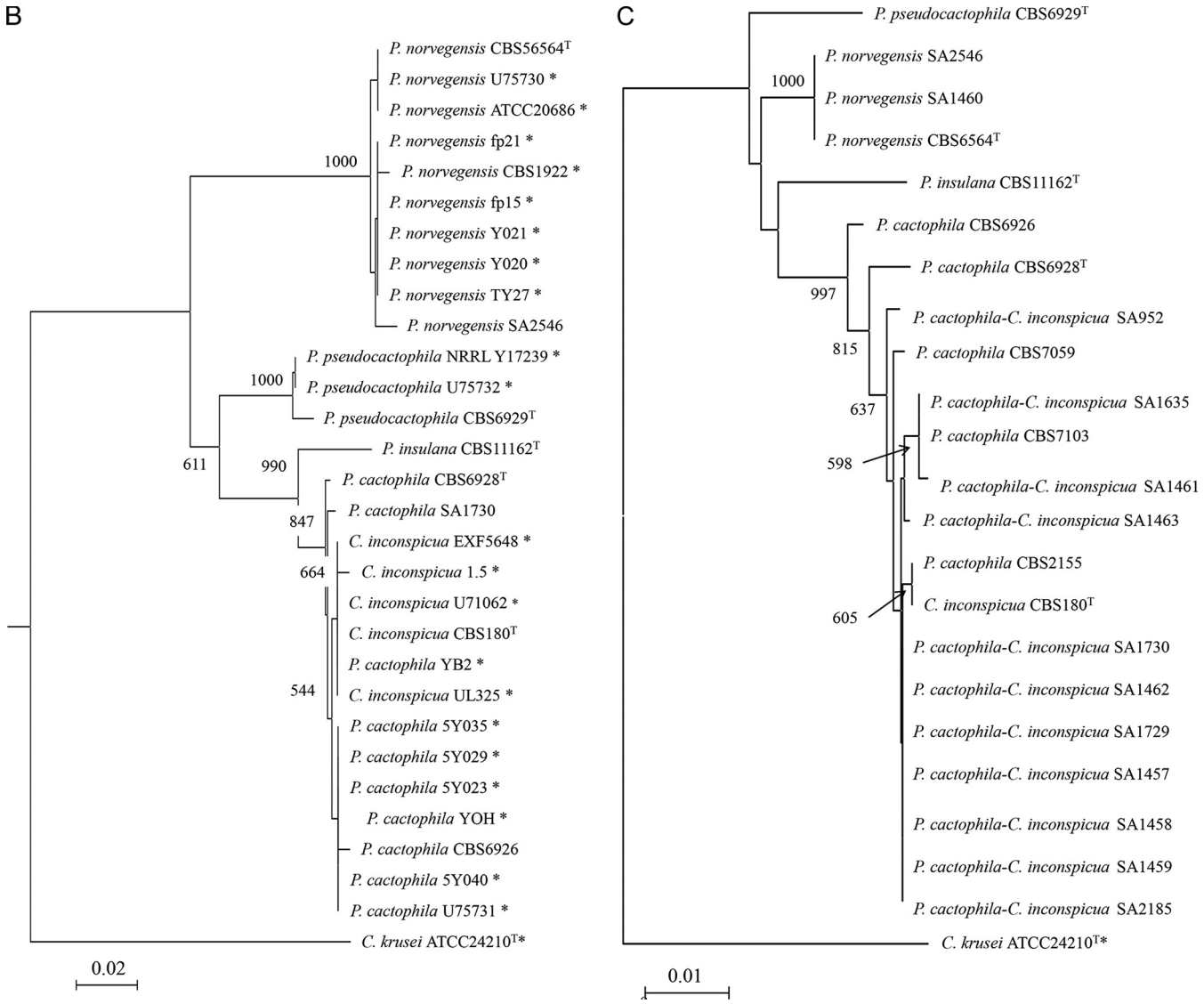

FIG 1 continued

cal, proteomic (MALDI-TOF), and molecular (direct sequencing) approaches for definitive identification.

From our results, it appears that sequencing the EF-1α locus is the most suitable approach for the identification of these species. Indeed, chromatograms are consistently easy to read, and the sequences are divergent enough to distinguish between all the different species of the clade, with the notable exception of *P. cactophila* and *C. inconspicua* (see below). Taking these results as the reference, we confirmed that identification based on ID32C may be not the most reliable approach since a misidentification between *C. inconspicua* and *C. norvegensis* occurred in two cases and one *C. inconspicua* strain was not identified. In contrast, these data confirmed the usefulness of MALDI-TOF analysis as a powerful tool to differentiate closely related species such as *C. norvegensis* and *C. inconspicua*. The lack of discrimination between *P. cactophila* and *C. inconspicua* seen using MALDI-TOF analysis, which has been shown to be able to discriminate between some cryptic species (16), was in agreement with the results of molecular methods and the hypothesis that *C. inconspicua* is the anamorph of *P. cactophila* (see below).

Analysis of ribosomal DNA sequences, notably, the D1/D2 domain of the large subunit and the ITS loci, is considered a method of choice for the identification of ascomycetous yeast species (17, 18). The rDNA of eukaryotes consists of a tandem repeat of a region that includes 4 genes, the 18S, 5.8S, 28S, and 5S units. In fungal genomes, there are generally a few dozen to a few thousand repeats that are arranged either in a single large cluster or in multiple tandem arrays found on one or a few chromosomes (19). It is considered that polymorphism between rDNA repeat units is rare, supporting the idea of concerted evolution for these regions. However, after cloning was performed, we found, in this study, the existence of multiple (as many as 3) alleles of the ITS region for some strains, including in some of the reference strains. This feature appeared to be a distinctive trait of the clade, since strains from all five species tested exhibit such a profile, while *C. krusei* (tel. *I. orientalis*), one of the most closely related species, differs on

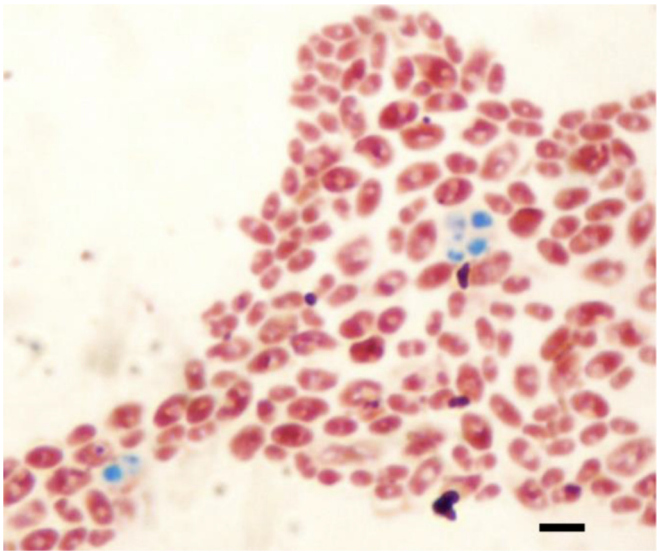

FIG 2 Example of sexual reproduction obtained with the *C. inconspicua* CBS180 strain. Strains were spotted on potassium acetate agar, and Wirtz staining was used to reveal the presence of ascospores (in green) and blastospores (in pink). The black bar represents 5 μm.

this point. Since rDNA alleles are usually similar in diploid yeast cells, this supports the idea of the existence of multiple nonhomologous loci of rDNA and/or a diversity of sequence among the tandem repeats of rDNA and/or the presence of hybrids in the clade. The latter hypothesis seems less plausible, as hybrids often lose one of the parental rDNA copies (20). The former has been demonstrated for members of the subphylum *Pezizomycotina*, which contained multiple dispersed copies of the 5S regions with multiple different types (21). This is also the case for other eukaryotes such as those belonging to the *Apicomplexa* phylum. For example, *Plasmodium*, *Eimeria*, and *Toxoplasma* spp. (22–24) are known to contain dispersed and highly divergent 18S rDNAs in their genome. Diversity of sequence among the repeats of rDNA has been shown for flatworms that may have a few distinct rRNA types maintained within the genome (25). Among the hemiascomycete yeasts, it is worth noting that *C. glabrata* possesses two clusters of rDNA but with identical sequences (19). On the other hand, *Yarrowia* (*Candida*) *lipolytica* harbors divergent rDNA sequences with extended microheterogeneity among the repeats and differences in the number of repeats (26). Additional studies are required to specify the molecular mechanisms involved in this phenomenon in the *P. cactophila* clade.

Several arguments led us to postulate that *C. inconspicua* and *P. cactophila* are the anamorph and teleomorph of the same species. First, it is noticeable that while MALDI-TOF analysis is well known for its ability in differentiating closely related species, even within species complexes, this method failed to distinguish between *C. inconspicua* and *P. cactophila*. Also, we found very limited nucleotide divergence between these 2 species in the loci tested, with values of variability at a level more in accordance with intraspecies variability. Finally, phylogenetic analyses using any of the three loci tested in this study failed to distinguish these 2 species but rather support the idea of a single cluster gathering *C. inconspicua* and *P. cactophila* strains. This is in agreement with a previous study by Kurtzman et al., who concluded that *C. inconspicua* and *P. cactophila* are conspecific (9). Indeed, in their study, the type strains of *C. inconspicua* and of *P. cactophila*, subjected to sequencing at 4 loci (D1/D2 domain, small subunit of the rDNA, EF-1α, and mitochondrial rDNA small subunit), were found to differ by only a single nucleotide insertion/deletion plus a nucleotide substitution in the D1/D2 domain.

The hypothesis that *P. cactophila* is the teleomorph of *C. inconspicua* was further supported by the evidence indicating that *C. inconspicua* does undergo sexual reproduction. Indeed, we showed that all our clinical strains of *C. inconspicua-P. cactophila* as well as the *C. inconspicua* reference strain were able to form asci. Within these structures, ascospores were very similar to those described for other *Pichia* species: evanescent asci containing 1 to 4 ascospores (27). Analysis of sequence chromatograms of the EF-1α locus that contained features suggestive of heterozygous point mutations supports the idea of the diploid nature of *P. cactophila* that has been previously reported (28). Those cells may convert to ascospores, but the heterothallic or homothallic pattern of sexuality has not been yet determined.

In conclusion, our report adds evidence that mass spectrometry MALDI-TOF analysis is a powerful tool for the identification of yeast species, including closely related ones that are difficult to distinguish using biochemical profiles. Our study also demonstrated that, in contrast to the vast majority of fungal species, the use of ITS or D1/D2 loci should be avoided for *C. inconspicua-C. norvegensis* identification due to the existence of multiple divergent alleles containing an insertion or deletion within a given strain. Finally, on the basis of molecular and spectrometric analyses and a study of the sexual reproduction, we strongly hypothesize that *C. inconspicua* is the anamorph stage of *P. cactophila*.

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