Polyphasic identification of clinical *Candida albicans* in the Philippines

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Abstract
       Candida albicans is opportunistic pathogen causing invasive and noninvasive diseases. This yeast is not well-studied in the Philippines. Considering that identification to species level is necessary for empirical treatment of diseases it causes, and the difficulty in its identification using phenotypic methods, use of polyphasic assays is explored. Increasing yeast infections and drug resistance worldwide necessitate isolation and identification of *C. albicans* in its surveillance in the country. Study identified clinical isolates from two tertiary hospitals in Metro Manila using phenotypic and genotypic methods. Twenty-six clinical isolates were identified phenotypically using germ tube and chlamydospore production and carbon assimilation of glucose, maltose, galactose, and sucrose. Genotypic identification was done by determining internal transcribed space (ITS) sequences and construction of neighbor-joining tree using ITS sequences. Twelve isolates were phenotypically identified as *C. albicans,* while one isolate was identified as *C. parapsilosis*. These identities were confirmed at the ITS level. Yeast isolates that could not be identified phenotypically were identified as *C. albicans* following genotypic analysis. Genotypic identification is the gold standard for identification of *C. albicans*. A polyphasic approach in fungal identification provides a plethora of phenotypic and genotypic properties, which creates robust and accurate results.

Key words – *Candida* spp. – Chlamydospore – ITS sequencing – Yeast identification

Introduction
       Over the last 20 years, there has been a significant increase in the incidence rates of fungal diseases. Among these is candidiasis, which is one of the major nosocomial fungal infections reported worldwide (Mishra et al. 2007). Organisms under the genus *Candida* cause this fungal infection, yet they can thrive as commensals in the mouth and gastrointestinal tract of humans (Kim & Sudbery 2011). It is notable that the development of candidiasis depends on the balance between the yeast's and the host's immune defense status. Changes in this delicate host-fungus balance can lead to levels of patient mortality (Charles et al. 2003). Among the *Candida* spp., *C. albicans* is the predominant causative agent of invasive fungal infections, and may pose significant challenges to the public health due to increase in costs of care and duration of hospitalization (Lai et al. 2012).

Candidiasis infection encompasses secondary or opportunistic infections ranging from acute to chronic and even life-threatening mycoses. A number of *Candida* species are reported to cause candidiasis which infect sites such as the vaginal tract, skin, fingers, oral cavity and intestinal tracts, although sometimes, these infections become systemic as candidemia, endocarditis, and meningitis (Khan et al. 2010). Reported species are: *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, and *C. krusei* (Rappleye & Goldman 2006). *Candida glabrata* is found to be a recurrent pathogen due to an increase in the use of immune suppressive agent. *Candida parapsilosis* is commonly isolated from blood cultures, while *C. tropicalis* is reported from patients with leukemia (Hasan et al. 2009). On the other hand, *C. dubliniensis* is also present in acquired immunodeficiency syndrome (AIDS) patients (Khan et al. 2012).

The identification of *Candida* to the species level is crucial before any antifungal drug can be prescribed to the patient (Page & Kurtzman 2005). The conventional method for the identification of yeasts isolated from clinical samples involves relatively time consuming and laborious morphological and physiological characterization. Several selective or differential media have been developed for culturing *Candida* spp. (Gaschet et al. 2008). Initial identification of *Candida* is performed by observing distinct morphological features of yeast under the microscope, while commercial kits like API20C and API32C have also been developed to detect different species of *Candida* on the basis of their ability to assimilate different carbohydrate sources (Xu et al. 2002). The use of phenotypic methods in identifying *Candida* species tends to become problematic in some cases, since morphological and physiological traits are unstable and sometimes variable in yeasts. The variability and inconsistency of phenotypic results are then inconclusive for the identification of *Candida* species (Latouche et al. 1997). Moreover, yeast species might demonstrate similar phenotypic profiles but harbor highly diverse genetic make-up, which adds to the problem in delineating species during the identification process. Current genotypic methods for the identification of yeast species include the amplification of the internal transcribed space (ITS) regions using universal nucleotide primers for fungi and their subsequent sequencing (Taira et al. 2014).

In the Philippines, studies on *Candida* spp. are very limited. A literature search showed that there is a lack of recent studies on clinical *C. albicans* in the Philippines. Bulmer et al. (1999) conducted a study to identify yeasts isolates in 16 medical centers throughout the country, while Handog & Dayrit (2005) reported on the prevalence of fungal infections. The tropical climate of the Philippines, coupled with different environmental factors suggest the prevalence of *Candida* among Filipinos. However, clinical *Candida* spp. are still among the poorly studied fungal groups in the country. This gap implies the need for isolating and identifying *Candida* in clinical samples, which presently are not part of the routine protocols in the country's hospital setting and diagnostic laboratories. This study aimed to conduct a polyphasic approach which integrated both phenotypic and genotypic characterization in the identification of *C. albicans* isolates from clinical samples in the Philippines.

Materials & Methods

Collection of Yeast isolates

Twenty-six yeast cultures from different clinical samples were collected from November 2016 to January 2017 in two tertiary hospitals in Metro Manila, Philippines. These included skin scrapings, throat swab, sputum, and colonoscopy drain. The isolates were cultured and purified using Sabouraud dextrose agar (SDA, Merck). Gram-staining was done to confirm the isolates as Gram-positive unicellular budding yeast cells. Suspected *Candida* isolates were all maintained and stored in the presence of 10% glycerol at -80 °C.

Germ tube test

Yeast isolates were subjected to germ tube test for initial identification (Barlow 1974). The isolates were inoculated on SDA plates and incubated at 37 °C for 24 hrs. A small portion of an

isolated yeast colony was inoculated into 5 mL bovine serum (DIFCO) and incubated at 37 °C for 90 minutes. Following incubation, a drop of the culture was placed on a clean glass slide overlaid with a cover slip. Slides containing the yeast cells were examined microscopically for the presence of germ tubes, a characteristic of *C. albicans* and *C. dubliensis*. Positive germ tube result is indicated by the presence of a germ-tube like extension from the parent cell with no constriction at the point of attachment to yeast cell.

Dalmau-plate culture method for chlamydospore production

Yeast isolates were inoculated in a zigzag manner on cornmeal agar plates containing 1% Tween 80. A sterile cover slip was placed on each plate, covering the portion of inoculated streaks, and all plates were incubated in a dark room at 25 °C for five days. All plates were examined microscopically for the presence of chlamydospores, which are seen as large, highly refractile thick walled cells (Beheshti et al. 1975).

Carbon assimilation

The ability of the yeast isolates to assimilate and utilize different carbohydrate sources was determined by growing the cultures on 10 ml Yeast Nitrogen base (YNB) medium with 1% carbohydrate (Wickerham & Burton 1948). Cell density was adjusted to 0.5 McFarland standard prior to inoculation. Carbon sources used in the study were: glucose, maltose, galactose, and sucrose. Inoculated tubes were incubated at 37°C, and results were read after 14 days. All species of *Candida* are able to utilize glucose as sole carbon source (Martin & Schneidau 1970). Thus, negative glucose utilization indicates that the isolate does not belong to the genus *Candida*.

Extraction of genomic DNA

DNA extraction was done using InstaGene matrix solution (Bio-Rad). Yeasts were cultured on SDA plates and incubated at 37°C for 48 hours. Two to three colonies from each plate were suspended in one ml of sterile distilled water in a microfuge tube and centrifuged for one minute at 4,480 x g. The supernatant was removed and 100 µl of InstaGene matrix solution were added to the pellet. All tubes were incubated at 56 °C for 30 minutes and vortexed for 10 seconds. The tubes were then placed in boiling water bath for 10 minutes. All tubes containing DNA extracts were stored at -20°C until use.

Polymerase chain reaction amplification and agarose gel electrophoresis

Polymerase chain reaction (PCR) amplification mixtures were prepared containing the following: 200 ng template DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 200mM dNTP, 0.5 µM each of forward and reverse primers and 0.05 units/ µl of DNA polymerase. Fungi-specific universal primer combinations for the internal transcribed spacer region with the following sequences were used: forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al.1990). PCR reaction was carried out as follows: initial denaturation at 94 °C for 2 min and 35 cycles at 94 °C for 15 sec, 55 °C for 30 sec and 72 °C for 1 min, followed by final extension at 72°C for 5 min. Each PCR reaction was run with *C. albicans* ATCC 1023 as positive control and a negative (no DNA) control. The amplicons were viewed after electrophoresis in 1.8% agarose gel with GelRed for 3 hours at 50 V. PCR products were sent to First Base Laboratories, Malaysia for sample purification and sequencing.

Alignment and construction of phylogenetic tree

Internal transcribed space (ITS) sequences of related fungal taxa and type strains were downloaded from the GenBank (ncbi.nlm.nih.gov/GenBank) and aligned with the gene sequences of the test isolates using CLUSTAL W alignment. Phylogenetic analysis using MEGA 5.0 was done using Kimura-2-Parameter algorithm and bootstrap of 1000 replicates (Tamura et al. 2011). A neighborhood-joining tree was created using *Sporidiobolus salmonicolor* as outgroup. The ITS nucleotide sequences of the samples were deposited in the GenBank with the following accession

numbers: MF797623, MF797624, MF797625, MF797626, MF797627, MF797628, MF797629, MF797630, MF797631, MF797632, MF797633, MF797634, MF797635, MF797636 and MF797637. The aligned sequences and phylogenetic tree were deposited at TreeBase (Http://treebase.org) with reviewer access url of http://purl.org/phylo/treebase/phylows/study/TB2:S21733?x-access-code=f8acdb3a894a2e6a5ac9e87965eaf0f1&format=html

Results

Phenotypic identification

Table 1 shows the results of the phenotypic identification of the isolates. Twelve of the 26 yeast isolates or 46.15% were identified as *C. albicans.* The isolates produced colonies that were off-white to cream in color, opaque, with dry surface, circular in shape with entire margins, were Gram positive unicellular budding yeast cells, produced germ tubes when incubated in the presence of a protein rich bovine serum medium, produced chlamydospores existing singly at ends of the hyphae or pseudohyphae (Fig. 1) and utilized all four carbon sources, namely: glucose, maltose, sucrose, and galactose. Six (23.08%) yeast strains were positive for germ tubes and chlamydospores, but demonstrated variability in terms of carbon assimilation abilities (JRP41, JRP53, JRP72, CGP21, CGP22 and CGP41). Strains JRP41 (galactose negative), and strains JRP72, CGP21, and CGP41 (sucrose negative) failed to assimilate one carbohydrate source, while strains JRP53, and CGP22 were not able to utilize maltose and sucrose. Six (23.07%) of the strains, JRP62, CGP23, CGP62, CGP73, CGP101 and CGP103 produced germ tubes and assimilated all carbon sources, but failed to exhibit chlamydospores. Strains JRP52, JRP53, and CGP22 showed similar results in germ tube test and carbon assimilation tests. Difference in these three, was observed in chlamydospore production where strain JRP52 was not able to produce chlamydospores. One strain (JRP31) that was negative for all tests except glucose assimilation was identified as *C. parapsilosis*. Due to inconsistencies in the phenotypic characteristics that resulted in difficulty in identifying the isolates, the isolates were further identified using the ITS nucleotide sequences.

Table 1 Phenotypic characterization and identification of yeast isolates in the study.

ISOLATE CODE	GERM TUBE	CHLAMYDOSPORE	CARBON ASSIMILATION				PHENOTYPIC IDENTIFICATION
			GLU	MAL	GAL	SUC	
JRP11	+	+	+	+	+	+	*Candida albicans*
JRP31	-	-	+	-	-	-	*C. parapsilosis*
JRP41	+	+	+	+	-	+	UI
JRP51	+	+	+	+	+	+	*C. albicans*
JRP52	+	-	+	-	+	-	UI
JRP53	+	+	+	-	+	-	UI
JRP61	+	+	+	+	+	+	*C. albicans*
JRP62	+	-	+	+	+	+	UI
JRP72	+	+	+	+	+	-	UI
JRP91	+	+	+	+	+	+	*C. albicans*
CGP21	+	+	+	+	+	-	UI
CGP22	+	+	+	-	+	-	UI
CGP23	+	-	+	+	+	+	UI
CGP41	+	+	+	+	+	-	UI
CGP62	+	-	+	+	+	+	UI
CGP73	+	-	+	+	+	+	UI
CGP74	+	+	+	+	+	+	*C. albicans*
CGP81	+	+	+	+	+	+	*C. albicans*
CGP82	+	+	+	+	+	+	*C. albicans*
CGP83	+	+	+	+	+	+	*C. albicans*
CGP101	+	-	+	+	+	+	UI

Table 1 Continued.

ISOLATE CODE	GERM TUBE	CHLAMYDOSPORE	CARBON ASSIMILATION				PHENOTYPIC IDENTIFICATION
			GLU	MAL	GAL	SUC	
CGP102	+	+	+	+	+	+	*C. albicans*
CGP103	+	-	+	+	+	+	UI
CGP104	+	+	+	+	+	+	*C. albicans*
CGP121	+	+	+	+	+	+	*C. albicans*
CGP122	+	+	+	+	+	+	*C. albicans*

Legend: (+) presence of germ tube, chlamydospores and growth on carbon sources; (-): negative results; UI: unidentified.
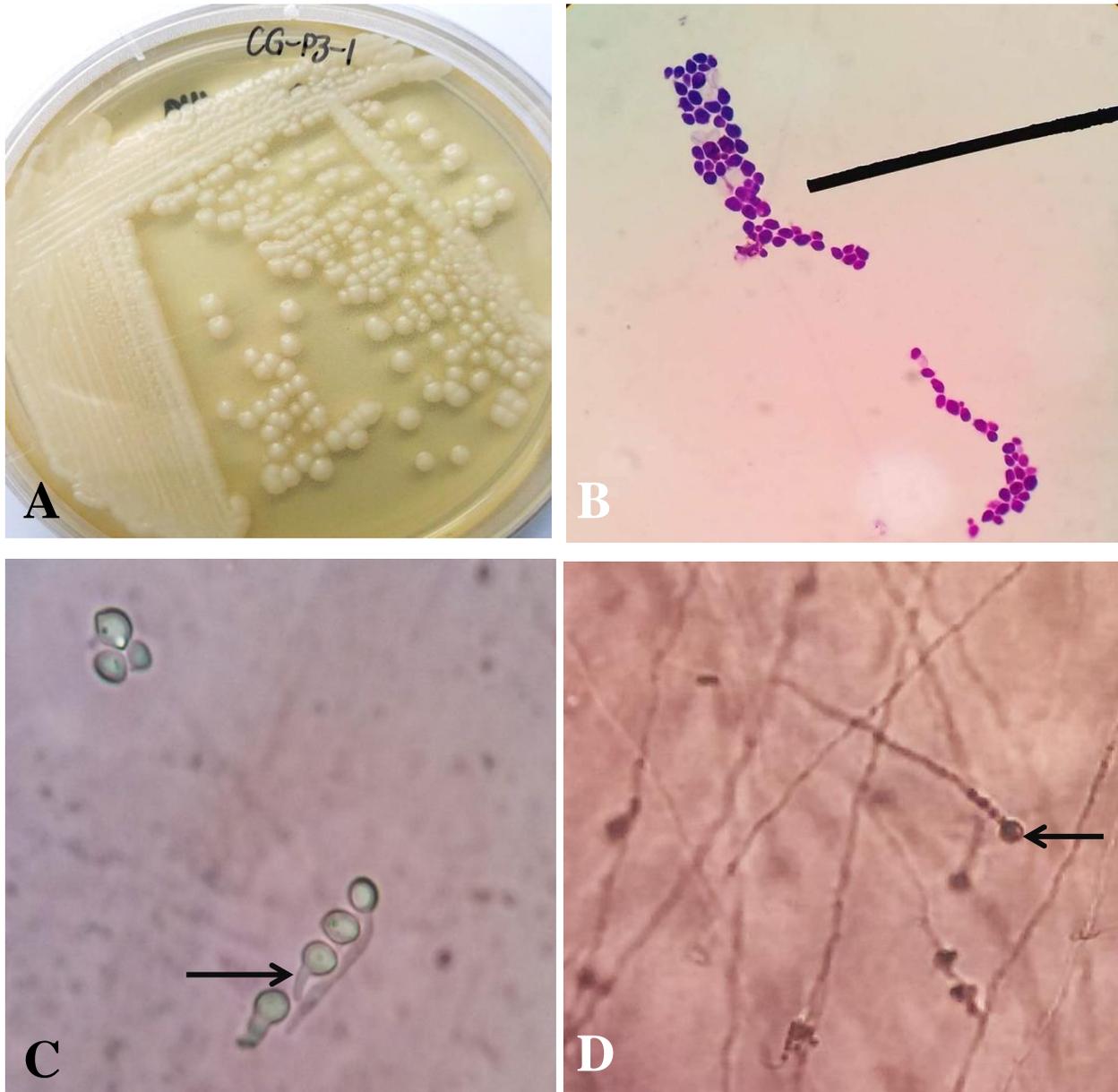
Fig. 1 − A Cultural morphology of *Candida* cultured on Sabouraud dextrose agar medium at 37 °C for 48 hours. B Gram-positive unicellular budding yeast cells (1000x). C Germ tubes produced by yeast isolate (1000x). D Morphology on cornmeal agar +1% Tween 80 showing presence of chlamydospores (100x).

Genotypic identification

Using the universal fungi- specific ITS1 and ITS4 primers, the ITS regions of the yeast isolates were successfully amplified (Fig. 2). The sequences of these isolates were compared with the available sequences in the GenBank database. All of the isolates showed ≥98% homology with other sequences deposited and available in the database. Phylogenetic analysis (Fig. 3) confirmed the species of the isolates. Five representative strains (JRP51, JRP61, JRP91, CGP74 and CGP81) identified as *C. albicans* phenotypically, showed similar results using the ITS sequencing as confirmed by their taxonomic position in the phylogenetic tree. The identity of the isolates was confirmed as *C. albicans,* which was supported with a high bootstrap support of 100 based on 1000 replicates. Strains JRP41, JRP53, JRP72, CGP22 and CGP41, which were unidentified using phenotypic methods due to inconsistent results in the carbon assimilation tests, were also identified to be belonging to *C. albicans* level. In addition, isolates CGP62, CGP73, and JRP52, JRP62, which were chlamydospore negative, strikingly were identified as *C. albicans* following molecular and phylogenetic analysis. Moreover, isolate JRP31 that was phenotypically identified as *C. parapsilosis* was confirmed as such using the ITS sequences.
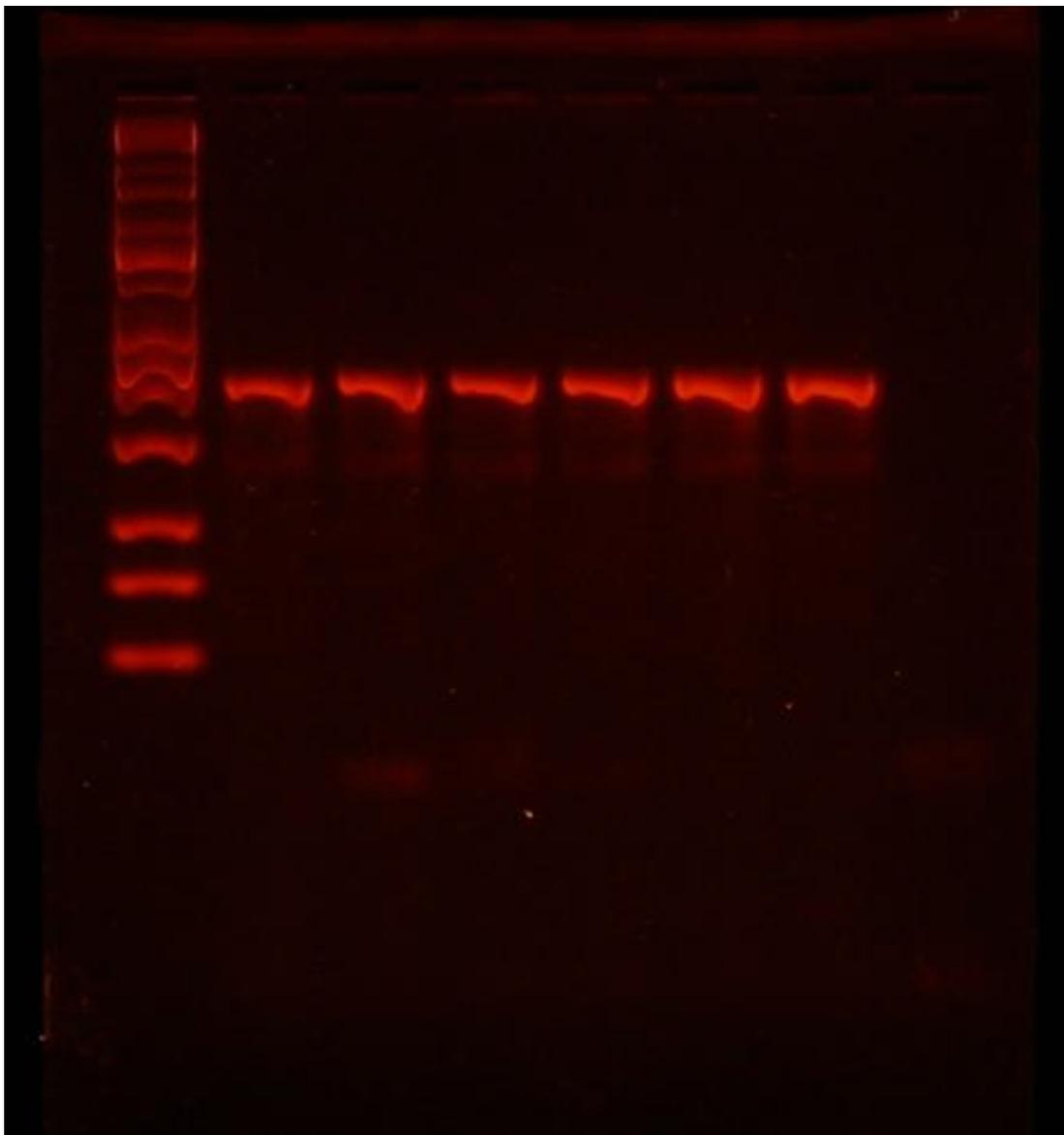

Fig. 2 – DNA amplicons following PCR amplification using ITS1 (f) and ITS4 (r) primers. Lane 1 - DNA ladder (KAPA Universal ladder); lane 2 - positive control (*C. albicans* ATCC 14053 strain); lane 3-7 test isolates used in the study (535 bp), lane 8: negative control (DEPC water).
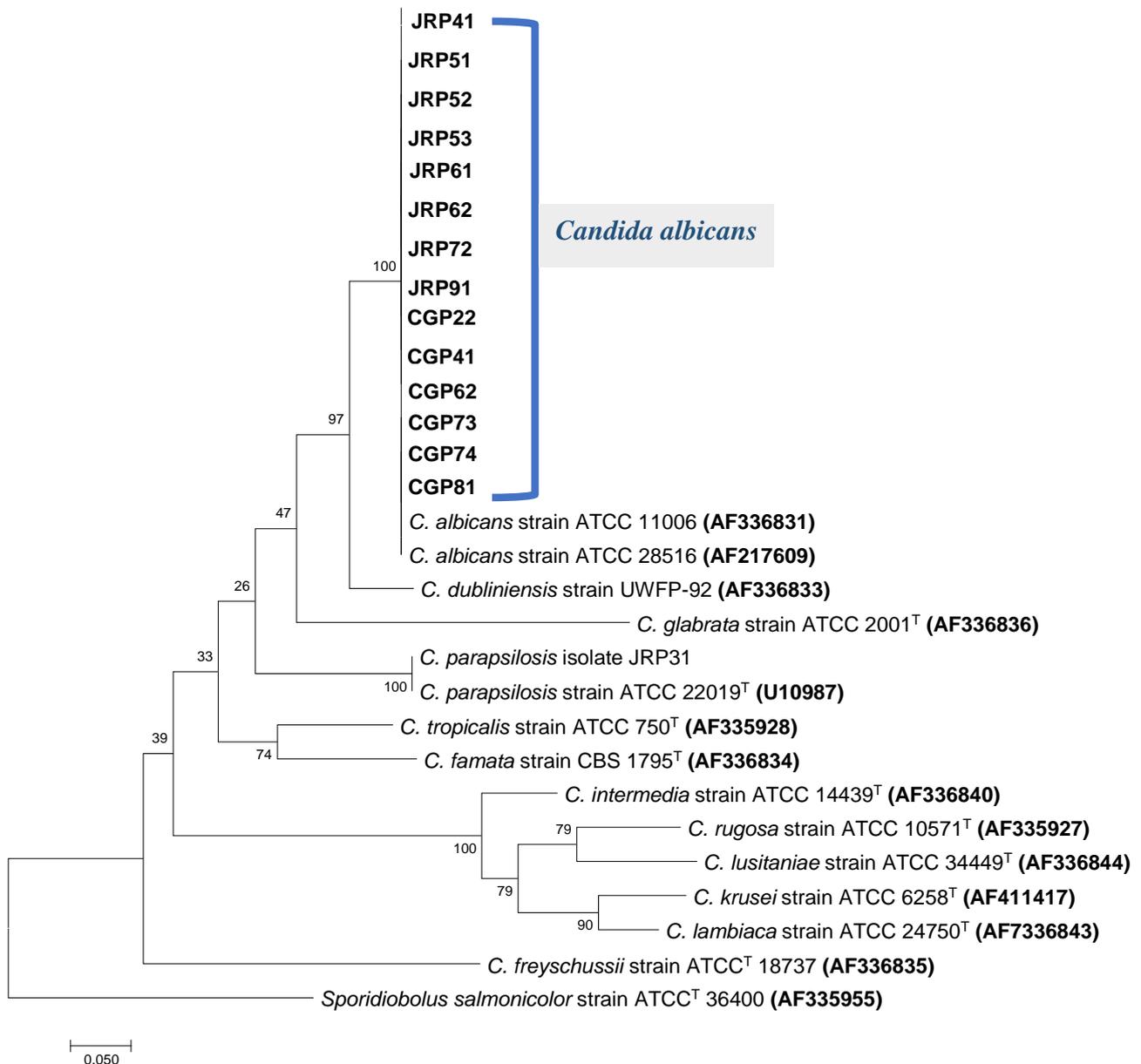
Fig. 3 – Neighborhood-joining tree of the *C. albicans* isolates showing relationships with related taxa and other reported sequences based on ITS sequencing.

Discussion

The study of *C. albicans* at a clinical level in the Philippines is not yet well-exploited, in contrast to neighboring countries and other countries worldwide (Dagi et al. 2016, Patel et al. 2017). In most cases, this fungal group is greatly overlooked in clinical specimens and remains unidentified and uncharacterized. In our study, of the total 26 isolates studied, phenotypic results identified only twelve isolates as *C. albicans*, and one isolate as *C. parapsilosis*. Thirteen isolates could not be identified using the different phenotypic tests. However, ITS sequence analysis identified these as *C. albicans*.

It is noted that all *Candida* isolates that were identified as *C. albicans* using ITS sequencing were positive for the germ tube test. The germ-tube test is fast and economical presumptive test for *C. albicans* used for the differentiation of *C. albicans* from other species. However, this test requires training and expertise on the part of the clinical professional in differentiating germ-tube structures from pseudohyphae to avoid subjective interpretation of results.

The limitation of our phenotypic identification can be attributed to the lack of other protocols commonly done for identifying *C. albicans*: growth on media with chromogenic substances (Madhavan et al. 2011), and the use of API 20C AUX and APIC *Candida* (bioMerieux) (Liguor et al. 2010), which can provide comprehensive results on the biochemical profiles of yeasts. The results of the chlamydospore test were not conclusive and provided indeterminate data in identifying the strains. Isolates that gave negative chlamydospore results were identified as *C. albicans* using the genotypic method. Also, one limitation of the chlamydospore test is that *C. albicans* might lose its physiological ability to produce the chlamydospore structures under low nutrient and oxygen-deprived medium following repeated streakings. Carbon assimilation profiles presented in some strains of this study were also incoherent and were not decisive to yeast identification. Phenotypic tests, though relatively inexpensive, produced low capability in distinguishing *Candida* species. In addition, assimilation results are dependent on temperature, methodology and duration of incubation period (Marinho et al. 2010, Syverson 1981). Our results are in accordance with the reports of Mähnß et al. (2005) and Livério et al. (2017), where phenotypic tests alone are not enough for the definitive identification of *C. albicans*. Hence, genotypic methods are still requisite and indispensable for a conclusive identification. Phenotypic tests in the study provided a suggestive identification in some isolates that needed to be confirmed at the molecular level.

Molecular identification using ITS sequence provided a fast and correct identification of *C. albicans* in our study. Genotypic method addressed the ambiguous results shown in the phenotypic methods. The use of ITS sequences in fungal identification has been exploited in defining species level of clinical *Candida* (Kumar et al. 2016, Sampath et al. 2017). Other studies have also dealt on the use of other molecular methods (Chen et al. 2005, McEwen et al. 2008, Ying et al. 2016) in confirming identities of clinical *Candida* spp.

The polyphasic approach performed provides a better way in minimizing and eliminating limitations brought by each method, phenotypic and genotypic. The importance of reduced time to examine clinical specimens is vital in choosing the appropriate antifungal drug against resistant strains. Hence, we considered the use of genotypic method for the identification of *C. albicans* for fast and precise results. To the best of our knowledge, this is the first report in using a polyphasic method in the identification of clinical *C. albicans* in the Philippines.

Acknowledgement

References

Barlow AJE, Aldersley T, Chattaway FW. 1974 – Factors present in serum and seminal plasma which promote germ-tube formation and mycelial growth of *Candida albicans*. Journal of General Microbiology 82, 261-272.

Beheshti F, Smith A, Krause G. 1975 – Germ tube and chlamydospore formation by *Candida albicans* on a newmedium. Journal of Clinical Microbiology 2, 345-348.

Bulmer GS, Marquez ML, Co-Barcelona L, Fromtling R. 1999 – Yeasts and fluconazole susceptibility in the Philippines. Mycopathologia, 146, 117-120.

Charles PE, Doise JM, Quenot JP, Haube H et al. 2003 – Candidemia in critically ill patients: difference of outcome between medical and surgical patients. Intensive Care Medicine 29, 2162-2169.

Chen KW, Lo HJ, Lin YH, Li SY. 2005 – Comparison of four molecular typing methods to assess genetic relatedness of *Candida albicans* clinical isolates in Taiwan. Journal of Medical Microbiology 54, 249-258.

Dagi HT, Findik D, Senkeles C, Arslan U. 2016 – Identification and antifungal susceptibility of *Candida* species isolated from bloodstream infections in Konya, Turkey. Annals of Clinical Microbiology and Antimicrobials 15, 1-5.

Gaschet A, L'Olliver C, Laplanche A, Vagner O et al. 2008 – Evaluation of CandiSelect4, a new chromogenic medium for isolation and presumptive identification of *Candida* species from clinical specimens. Medical Mycology 18, 89-95.

Handog E, Dayrit J. 2005 – Mycology in the Philippines, Revisited. Japanese Journal of Medical Mycology 46, 71-76.

Hasan F, Xess I, Wang X, Jain N et al. 2009 – Biofilm formation in clinical *Candida* isolates and its association with virulence. Microbes and Infection 11, 753-61.

Khan MSA, Ahmad I, Aqil F, Owais M et al. 2010 – Virulence and pathogenicity of fungal pathogens. In: Ahmad, I, Owais, M, Shahid M, Aqil F, editors. Combating fungal infections: problems and remedies, 21-45.

Khan Z, Ahmad S, Joseph L, Chandy R. 2012 – *Candida dubliniensis*: an appraisal of its clinical significance as a bloodstream pathogen. PLoS ONE 7, 32952.

Kim J, Sudbery P. 2011 – *Candida albicans*, a major human fungal pathogen. The Journal of Microbiology 49, 171-77.

Kumar D, Banerjee T, Chakravarty J, Singh SK et al. 2016 – Identification, antifungal resistance profile, *in vitro* biofilm formation and ultrastructural characteristics of *Candida* species isolated from diabetic foot patients in Northern India. Indian Journal of Medical Microbiology 34, 308-314.

Lai CC, Wang CY, Liu WL, Huang YT, Hsueh PR. 2012 – Time to positivity of blood cultures of different *Candida* species causing fungaemia. Journal of Medical Microbiology 61, 701-704.

Latouche GN, Daniel HM, Lee OC, Mitchell TG et al. 1997 – Comparison of use of phenotypic and genotypic char- acteristics for identification of species of the anamorph genus *Candida* and related teleomorph yeast species. Journal of Clinical Microbiology 35, 3171-180.

Livério HO, da Silva Ruiz L, de Freitas RS, Nishikaku A et al. 2017 – Phenotypic and genotypic detection of *Candida albicans* and *Candida dubliniensis* strains isolated from oral mucosa of AIDS pediatric patients. Revista do Instituto de Medicina Tropical de São Paulo 59, e15.

Madhavan P, Jamal F, Chong PP, Ng KP. 2011 – Identification of local clinical *Candida* isolates using CHROMagar Candida as a primary identification method for various *Candida* species. Tropical Biomedicine 28, 269–74.

Mähnß B, Stehr F, Schäfer W, Neuber K. 2005 –Comparison of standard phenotypic assays with a PCR method to discriminate *Candida albicans* and *C. dubliniensis*. Mycoses 48, 55-61.

Marinho SA, Teixeira AB, Santos OS, Cazanova RF et al. 2010 –Identification of *Candida* spp. by phenotypic tests and PCR. Brazilian Journal of Microbiology 41, 286-294

Martin MV, Schneidau JD. 1970 – A simple and reliabile assimilation test for the identification of *Candida* species. American Journal of Clinical Pathology 53, 875-79.

McEwen JG, Taylor JW, Carter D, Xu J et al. 2008 – Molecular typing of pathogenic fungi. Medical Mycology 38, 189-97.

Mishra NN, Prasad T, Sharma N, Payasi A et al. 2007 – Pathogenicity and drug resistance in *Candida albicans* and other yeasts species. A review. Acta Microbiologica et Immunologica Hungarica 54, 201-35.

Page BT, Kurtzman CP. 2005 – Rapid identification of *Candida* species and other clinically important yeast species by flow cytometry. Journal of Clinical Microbiology 43, 4507-4514.

Patel PN, Sah P, Chandrashekar C, Vidyasagar S et al. 2017 – Oral candida speciation, virulence and antifungal susceptibility in type 2 diabetes mellitus. Diabetes Research and Clinical Practice I25, I0-I9.

Rappleye CA, Goldman WE. 2006 – Defining virulence genes in the dimorphic fungi. Annual Review of Microbiology 60, 281-303.

Sampath A, Weerasekera M, Gunasekara C, Dilhari A et al. 2017 – A sensitive and a rapid multiplex polymerase chain reaction for the identification of *Candida* species in concentrated oral rinse specimens in patients with diabetes. Acta Odontologica Scandinavica 75, 113-22.

Syverson RE. 1981 – Variable assimilation of carbon compounds by *Candida albicans*. Journal of Clinical Microbiology 13, 163-66.

Taira CL, Okay TS, Delgado AF, Ceccon MEJR et al. 2014 – A multiplex nested PCR for the detection and identification of *Candida* species in blood samples of critically ill paediatric patients. BMC Infectious Diseases 14, 406.

Tamura K, Peterson D, Peterson N, Stecher G et al. 2011 – MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731-2739.

Wickerham LJ and Burton KA. 1948 – Carbon assimilation tests for the classification of yeasts. Journal of Bacteriology 56, 363-371.

Xu J, Millar BC, Moore JE, McClurg R et al. 2002 – Comparison of API20C with molecular identification of *Candida* spp. isolated from bloodstream infections. Journal of Clinical Pathology 55, 774–777.

Ying C, Zhang H, Tang Z, Chen H et al. 2016 – Antifungal susceptibility and molecular typing of *Candida albicans* isolates obtained from vulvovaginal candidiasis patients in 3 Shanghai maternity hospitals. Medical Mycology 54, 394-399.