MATERIALS AND METHODS

Patients and fungal isolates. This study was approved by the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster and Peking Union Medical College Hospital. As described previously, the National China Hospital Invasive Fungal Surveillance Net (CHIF-NET) study was a prospective, laboratory-based, multicenter study of invasive yeast infections which started on 1st August, 2009 (1). A total of 22 "rank-A tertiary" hospitals with 900 to 4,300 beds per hospital, serviced by a regional reference mycology laboratory, in fourteen major cities in China were involved in the study. These included four hospitals from Hangzhou, three hospitals from Beijing, two hospitals each from Guangzhou, Harbin, and Shenyang, and one each from Chengdu, Qingdao, Fuzhou, Yinchuan, Nanjing, Shanghai, Tianjin, Changsha, and Wuhan. The present study included all the blood culture *Y. lipolytica* isolates identified in the CHIF-NET study from 1st August, 2009 to 31st July, 2012. For each episode of yeast isolation, the clinical and microbiological data were entered into a predesigned database. All yeast isolates were identified by a previously described protocol (2). Briefly, Vitek 2 YST ID Card system (bioMérieux, France) was used for identification for all yeast isolates in the CHIF-NET study. For isolates that had a single identification with a confidence value of <99.9%, multiple identifications, or no identification, internal transcribed spacer (ITS) region sequencing was used for confirmation. The isolates which were identified as *Y. lipolytica* by this protocol were included in the present study.

Mycological studies and comparison with API-20C AUX system for identification of *Y. lipolytica*. The *Y. lipolytica* isolates were inoculated onto Sabouraud dextrose agar (SDA) (Oxoid, the United Kingdom) and incubated for 24 to 48 h at 35 ^{o}C for subsequent analysis. The

biochemical profiles of the isolates were characterized using the API 20C AUX system (bioMérieux), according to the manufacturer's instructions.

MALDI-TOF MS. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was performed by the formic acid extraction method according to the manufacturer's instruction and our previous publications with slight modifications (3), and all the chemical reagents used were of liquid chromatography/mass spectrometry (LC/MS) grade. Briefly, for each *Y. lipolytica* isolate cells on SDA were harvested after 24 to 48 h of incubation at 30 °C with a sterile cotton swab and suspended in 500 µl of water (Sigma-Aldrich, St. Louis, MO, USA), followed by washing with 1 mL of water. After centrifugation at 16,100 rcf for 2 min, the pellet was resuspended in 700 µl of water and 300 µl of absolute ethanol (Merck, Germany). The mixture was then vortexed and centrifuged at 16,100 rcf for 2 min. The supernatant was then removed, and the pellet was air-dried for 30 min. Subsequently, the pellet was resuspended in 30 µl of 70% formic acid (Merck) and an equal volume of acetonitrile (Fluka, Switzerland), followed by centrifugation at 16,100 rcf for 2 min. Using the IVD Bacterial Test Standard (BTS) (Bruker Daltonics, Germany) as a control, one microliter of the supernatant was transferred to an individual spot on the MSP 96 polished steel BC targets plate (Bruker Daltonics), and was air-dried. Each isolate was analyzed on four different spots. Each spot was further overlaid with the α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Sigma-Aldrich), and was air-dried. The target plate was then analyzed by the microflex LT system (Bruker Daltonics), where each spot was read six times, 40 shots each. For each isolate, 24 mass spectra with m/z values of 2,000 to 20,000 were generated. Fungal identification was achieved using FlexControl 3.4 (Bruker Daltonics). Moreover, for each isolate the mass spectra generated were integrated to give a sum spectrum using MALDI Biotyper 3.1 (Bruker Daltonics), and the sum spectra of all

the isolates obtained were processed and analyzed for generation of dendrogram by hierarchical cluster analysis using MALDI Biotyper 3.1.

DNA extraction, PCR and direct PCR product sequencing. DNA extraction, PCR amplification and DNA sequencing of the ITS region for the six isolates were performed according to our previous publications (3-10) using the primer pairs ITS1/ITS4 (11) for the ITS and NL1/NL4 (12) for the 25S nrDNA. Briefly, all yeast cells on SDA were harvested with a sterile cotton swab and suspended in 1 ml of autoclaved distilled water. DNA was then extracted from the yeast cells using the DNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The extracted DNA was eluted in 40 µl of distilled water, and 1 µl of the extracted DNA was used for PCR. For PCR, each 20 µl-PCR reaction mixture contained diethylpyrocarbonate (DEPC)-treated water (Invitrogen, Carlsbad, CA, USA), fungal DNA, PCR buffer (10 mM of Tris-HCl [pH 8.3], 50 mM of KCl and 3.125 mM of $MgCl_2$) (Applied Biosystem, Foster City, CA, USA), 1 mM of each primer (Invitrogen, Carlsbad, CA, USA), 200 µM of each dNTP (Roche Diagnostics, Switzerland) and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystem, Foster City, CA, USA). The PCR reaction mixtures were first heated at 95°C for 10 min, then heated in 45 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and finally incubated at 72°C for 10 min in the GeneAmp PCR System 9700 automated thermal cycler (Applied Biosystem, Foster City, CA, USA). Autoclaved distilled water was used as the negative control in each run of PCR.

Ten microliters of each amplified product was electrophoresed in 1.5% (w/v) agarose gel (SeaKem LE Agarose) (Lonza, Switzerland), and the PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol. Both strands of the PCR products were sequenced twice with an ABI Prism 3700 DNA Analyzer (Applied

Biosystems, Foster City, CA, USA), using the respective PCR primers. The sequencing electropherograms obtained were viewed using Chromas Lite 2.1.1 (Technelysium, Australia). Preparation of PCR master mix, addition of DNA samples into the reaction tubes and post-PCR steps, including gel electrophoresis, purification of PCR products and DNA sequencing, were performed in separated rooms to avoid possible contamination.

Cloning and sequencing. Cloning of the PCR products into plasmids and subsequent DNA sequencing were performed. Freshly-prepared gel-purified PCR products were cloned into pCRII-TOPO vector using TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. The TA-ligated plasmids were then transformed into *Escherichia coli* DH5α by electroporation. The electroporated cells were then grown on LB agar, Lennox (Difco, BD Diagnostic Systems, Sparks, MD, USA) with kanamycin (50 µg/mL) (Sigma-Aldrich) for the selection of positive transfomants, as well as isopropyl β-D-1-thiogalactopyranoside (IPTG) (40 µg/mL) (Sigma-Aldrich) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (100 µg/mL) (Promega, Madison, WI, USA) for blue-white screening. White colonies were then selected and grown in LB broth, Lennox (Difco, BD Diagnostic Systems) with kanamycin (50 µg/mL) overnight and the plasmids in the bacterial cells were extracted using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's protocol. The purified plasmids were then sequenced using the PCR primers directly. The sequencing electropherograms obtained were viewed using Chromas Lite 2.1.1.

Comparative sequence identity analyses and phylogenetic analyses. The sequences of the PCR products were compared with sequences of closely related species from the DDBJ/ENA/GenBank databases by multiple sequence alignment using MUSCLE 3.8 (13) and were then end-trimmed. The aligned, end-trimmed sequences of the PCR products were

comparatively analyzed by pairwise alignment with the optimal GLOBAL alignment parameters using BioEdit 7.2.0 (14). Poorly aligned or divergent regions of the aligned, end-trimmed DNA sequences were then removed using Gblocks 0.91b (15, 16) with relaxed parameters. Tests for substitution models and phylogenetic tree construction by the maximum likelihood method were performed using MEGA 6.06 (17).

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