Whole genome sequencing of emerging multidrug resistant *Candida auris* isolates in India demonstrates low genetic variation

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Abstract

Candida auris is an emerging multidrug resistant yeast that causes nosocomial fungaemia and deep-seated infections. Notably, the emergence of this yeast is alarming as it exhibits resistance to azoles, amphotericin B and caspofungin, which may lead to clinical failure in patients. The multigene phylogeny and amplified fragment length polymorphism typing methods report the *C. auris* population as clonal. Here, using whole genome sequencing analysis, we decipher for the first time that *C. auris* strains from four Indian hospitals were highly related, suggesting clonal transmission. Further, all *C. auris* isolates originated from cases of fungaemia and were resistant to fluconazole (MIC >64 mg/L).

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Introduction

Candida auris is a multidrug-resistant yeast first described in 2009 as a species closely resembling *Candida haemulonii* [1–3]. The emergence of *C. auris* as an agent of nosocomial fungaemia and deep-tissue infections is alarming as this yeast is notorious for multidrug resistance [4–6]. Antifungal susceptibility patterns in various studies showed resistance to azoles, amphotericin B and caspofungin that may lead to clinical failure in patients [3,7]. Many laboratories worldwide rely on phenotypic commercial automated systems for routine identification of yeasts, which may lead to misidentification of *C. auris* as *C. haemulonii*, so underestimating the spread of this yeast [7]. Recently, *C. auris* genetic and proteomic diversity analyses using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), amplified fragment length polymorphism and multi-gene phylogeny in Indian, South African, Japanese, Korean and Brazilian isolates demonstrated geographically specific clustering [8,9]. In this study we confirm for the first time using whole genome sequencing that *C. auris* strains exhibit high clonality in different hospitals in India.

Materials and methods

Candida auris isolates

Five *C. auris* strains from candidaemia patients in four hospitals in India were sequenced for whole genome analysis [3,8]. Their origin and clinical details are presented in Table 1. The isolates were identified by MALDI-TOF and sequencing of internal transcribed spacer and D1/D2 regions.

IlluminaMiSeq sequence determination

Genomic DNA was extracted using a column-based method with a QIAamp DNA minikit (Qiagen, Hilden, Germany) and processed using the Nextera XT DNA protocol (Illumina, Inc., San Diego, CA, USA) to generate sequencing-ready libraries. The genome was sequenced using an Illumina MiSeq platform with MiSeq v3 protocol (Paired-End, 300 × 2). Equal volumes of normalized libraries were combined, diluted and heat denatured and the resulting FASTQ files were imported into CLC Genomics Workbench for analysis. The genomes were assembled by the combination of Velvet v1.2.0 [10], SSPACE 2.0 and Gap-Filler v1.10 [11,12]. Repetitive sequences were masked using Repeat-Masker v4.0.5 (http://www.repeatmasker.org), followed by *ab initio* gene prediction using GeneMark-ES 2.0 [13]. Next, rRNA and tRNA were predicted with RNAmmer and tRNAscan-SE v1.21 respectively [14,15]. Single-nucleotide polymorphism was

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TABLE 1. Clinical details and *in vitro* antifungal susceptibility profile of *Candida auris* ($n = 5$) isolates

Isolate	Specimen/ year of isolation	Hospital	Age /Sex	Diagnosis	Risk factors	Therapy	Outcome	Drugs[a] (MIC, mg/L)									
								ITC	VRC	ISA	POS	AMB	CFG	MFG	AFG	FLU	FC
VPCI 669/P/12	Blood/ 2012	Hospital 1, New Delhi	74/M	Jejunal perforation, peritonitis, septicaemia, MODS, DM	CVC, Broad-spectrum antibiotics, Surgery within 30 days, Intensive care, Antifungals within 30 days, Indwelling urinary catheter	CFG (loading dose of 70 mg, then 50 mg daily) for 2 weeks	Died 14 days after presentation	0.125	0.125	0.125	0.06	0.5	0.25	0.125	0.5	64	0.125
VPCI 692/P/12	Blood/ 2012	Hospital 1, New Delhi	3 days/F	PT, TEF, ICH sepsis	Neutropenia, Broad-spectrum antibiotics, Intensive care, Indwelling urinary catheter	CFG (loading dose of 70 mg, then 50 mg daily) for 5 days	Died 8 days after admission	0.125	0.25	0.06	0.125	1	0.25	0.125	0.125	64	0.125
VPCI 479/P/13	Blood/ 2013	Hospital 2, Kochi	48/F	RHD	CVC, Broad-spectrum antibiotics, Parenteral nutrition, Intensive care, Antifungals within 30 days, Indwelling urinary catheter	FLU (400 mg once daily) for 7 days	Died 1 month after admission	0.125	0.5	0.125	0.06	0.25	0.5	0.06	0.125	>64	0.5
VPCI 510/P/14	Blood/ 2014	Hospital 3, New Delhi	37/M	Chronic liver disease with decompensation	Broad-spectrum antibiotics, Antifungals within 30 days.	FLU(400 mg once daily) for 7 days	Not followed	0.5	16	0.25	0.25	4	0.5	0.125	0.5	64	0.25
VPCI 550/P/14	Blood/ 2014	Hospital 4, New Delhi	62/M	Hepato biliary carcinoma	Broad-spectrum antibiotics, Antifungal within 30 days, Urinary catheter, Intensive care, Chemotherapy	FLU	Died	0.25	2	0.25	$\leq$0.015	1	8	>8	>8	>64	>64

MODS, multi organ dysfunction syndrome; DM, diabetes mellitus; PT, pre-term; TEF, tracheo-oesophageal fistula; ICH, intracranial haemorrhage; RHD, rheumatic heart disease; CVC, central venous catheter.
[a]ITC, itraconazole; VRC, voriconazole; ISA, isavuconazole; POSA, posaconazole; AMB, amphotericin B; CFG, caspofungin; MFG, micafungin; AFG, anidulafungin; FLU, fluconazole; FC, flucytosine.

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detected using UNIFIED GENOTYPER from the Genome Analysis Toolkit package [16]. The functional annotation was performed using the Clusters of Orthologous Groups of proteins (COGs) database [17]. To determine that *C. auris* belongs to the CUG *Candida* clade, a webserver Bagheera [18] was used to identify CUG codon usage in *C. auris* genomes.

Average nucleotide identity and phylogenetic analysis of *C. auris*

Average nucleotide identity (ANI) was calculated using the JSPECIES package [19] using MUMMER (ANIm). Five *Candida auris* genomes (contigs) were compared with other publically available *Candida* species genomes ($n = 8$) detailed in Table 2 by aligning the sequences using progressive MAUVE with the default settings [20]. The strip Subset 'Locally Collinear Blocks' script was used to extract core blocks, creating core alignments longer than 500 bp including all 13 genomes. A core genome alignment in XMFA format was obtained which was converted into FASTA format using a PerlScript (https://github.com/lskatz/lskScripts). A phylogenetic network was constructed using SPLITSTREE based on concatenated alignment of the core genes [21].

Antifungal susceptibility testing and antifungal resistance genes analysis

Antifungal susceptibility testing of *C. auris* ($n = 5$) was carried out using the CLSI broth microdilution method, following the M27-A3 guidelines [22]. In addition, a control group of 20 *Candida albicans* isolates originating from invasive and superficial candidiasis and stocked in the culture collection of Vallabhbhai Patel Chest Institute (VPCI) were subjected to antifungal susceptibility testing. Newly revised epidemiological cut-off MIC values for *C. albicans* for fluconazole (>0.5 mg/L), itraconazole (>0.12 mg/L), voriconazole (>0.03 mg/L), posaconazole (>0.06 mg/L), amphotericin B (>2 mg/L), flucytosine (>0.5 mg/L), anidulafungin (>0.12 mg/L), caspofungin (>0.12 mg/L) and micafungin (>0.03 mg/L) were used for comparison of *C. auris* MIC data [23]. The contigs from all the five *C. auris* strains were aligned and screened for the presence of *ERG* (*ERG3* and *ERG11*) and *FKS* (*FKS1*, *FKS2* and *FKS3*) genes and compared with the *C. albicans* and *Candida glabrata ERG* and *FKS* genes. Also, a solitary echinocandin-resistant *C. auris* isolate (VPCI 550/P/15, Table 1) was subjected to amplification and sequencing of the *FKS1* and *FKS2* genes to analyse the mutations similar to those reported in *C. glabrata* [7].

Results

The internal transcribed spacer and D1/D2 regions of the ribosomal DNA sequences of *C. auris* isolates showed 99%

TABLE 2. Results of average nucleotide identity analysis giving percentage similarity between *Candida auris* ($n = 5$) and other *Candida* species ($n = 8$)

Strains	VPCI 479/P/13	VPCI 669/P/12	VPCI 692/P/12	VPCI 510/P/14	VPCI 550/P/14	*Candida dubliniensis* (CD36)	*Candida albicans* (WO-1 and SC 5314)	*Candida guillermondii* (ATCC6260)	*Candida lusitaniae* (ATCC42720)	*Candida tropicalis* (MYA-3404)	*Saccharomyces cerevisiae* (S288C)	*Candida parapsilosis* (317)	*Candida glabrata* (CBS 138)
VPCI 479/P/13	100	99.84	99.85	99.83	99.85	84.31	83.89	85.77	86.34	83.41	83.95	84.18	84.14
VPCI 669/P/12	99.85	100	99.84	99.81	99.83	85.24	84.72	86.35	86.38	84.43	84.21	85.54	84.83
VPCI 692/P/12	99.84	99.83	100	99.81	99.81	84.23	83.45	85.83	86.36	83.51	84.83	84.38	84.44
VPCI 510/P/14	99.84	99.82	99.83	100	99.84	84.16	83.73	85.83	86.3	83.47	84.35	84.04	84.1
VPCI 550/P/14	99.85	99.83	99.81	99.84	100	84.26	83.50	85.58	86.42	84.43	84.85	85.23	84.27
C. dubliniensis (CD36)	84.07	85.11	84.37	84.07	84.26	100	87.84	85.53	85.16	85.33	84.54	84.96	84.45
C. albicans (WO-1 and SC 5314)	83.61	86.77	83.89	83.89	83.50	88.29	100	83.89	81.98	85.74	83.21	84.76	88.37
C. guillermondii (ATCC6260)	85.8	86.31	85.93	85.9	85.58	85.33	86.05	100	86.2	85.87	84.36	85.78	86.01
C. lusitaniae (ATCC42720)	85.95	86.07	86.07	86	86.42	84.68	84.96	86.1	100	84.47	84.87	84.76	85.58
C. tropicalis (MYA-3404)	83.52	83.51	83.54	83.35	84.43	85.18	85.39	84.31	84.14	100	83.85	84.62	83.1
S. cerevisiae (S288C)	84.86	85.13	85.08	84.95	84.85	84.3	84.85	83.82	85.09	84.18	100	84.5	86.62
C. parapsilosis (317)	84.07	85.01	84.03	84.07	85.23	84.86	84.91	85.45	84.95	84.91	84.05	100	84.95
C. glabrata (CBS138)	84.25	84.97	84.43	84.24	84.27	84.87	84.81	86.43	85.8	84.44	86.8	85.62	100

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homology with the type *C. auris* isolates in GenBank. In all candidaemia cases the most common risk factor was the concomitant use of broad-spectrum antibiotics, intensive care unit stay and presence of indwelling urinary catheter. Other risk factors included parenteral nutrition and use of antifungals in three patients (Table 1). Four patients who developed breakthrough fungaemia had a fatal outcome.

Phylogenetic analysis

The assembled *C. auris* genome is diploid, comprising 12.3 Mb with a G+C content of 44.8%. A total of 6675 coding sequences among all the isolates were found with one 5.8S rRNA, 184 tRNA and 3262 repetitive elements. A phylogeny based on the concatenated sequences of 136 conserved core genes of the *C. auris* genomes ($n = 5$) and other *Candida* species ($n = 8$) confirms that the *C. auris* isolates are more similar to each other than to other *Candida* species analysed (Fig. 1a). Remarkably, the genome of all *C. auris* isolates analysed was highly related with only 0.2% variation observed among their genomes (Table 2). Among all sequenced *C. auris* isolates, single-nucleotide polymorphism differences ranging from 3048 to 4330 were observed. ANI calculated for the *C. auris* isolates is consistently above 99% (Table 2). In contrast the low ANI value (<95%) among *C. auris* and other *Candida* species suggested its divergence from the *Candida* genomes listed (Fig. 1a, Table 2). Among the eight *Candida* species aligned with *C. auris*, the highest ANI value (85.9%–86.4%) was observed for *Candida lusitaniae*. Also, the functional annotation data suggest 40% of the *C. auris* proteins to be orthologous to *C. lusitaniae* with most of them assigned as hypothetical or functionally uncharacterized.

Functional annotation of *C. auris*

Although a major proportion of the *C. auris* genome remains uncharacterized, a large number of proteins possessed transporter along with binding and catalytic activity involved in cellular and metabolic processes followed by proteins involved in signal transduction (Fig. 1b). *Candida auris* shares virulence traits common to *C. albicans*. The presence of orthologues including ion transporters, amino acid and metabolite transporters, oligopeptide transporters, secreted proteinases, lipases, phospholipases, adhesins, secreted aspartyl proteases, mannosyl transferases, MADS-box (for Minichromosome Maintenance1, Agamous, Deficiens and Serum Response Factor) and STE (Serine/threonine enzyme)-related proteins in *C. auris* genome were observed when compared with *C. albicans* as a reference genome (Fig. 1b). The above-mentioned gene classes may contribute to virulence

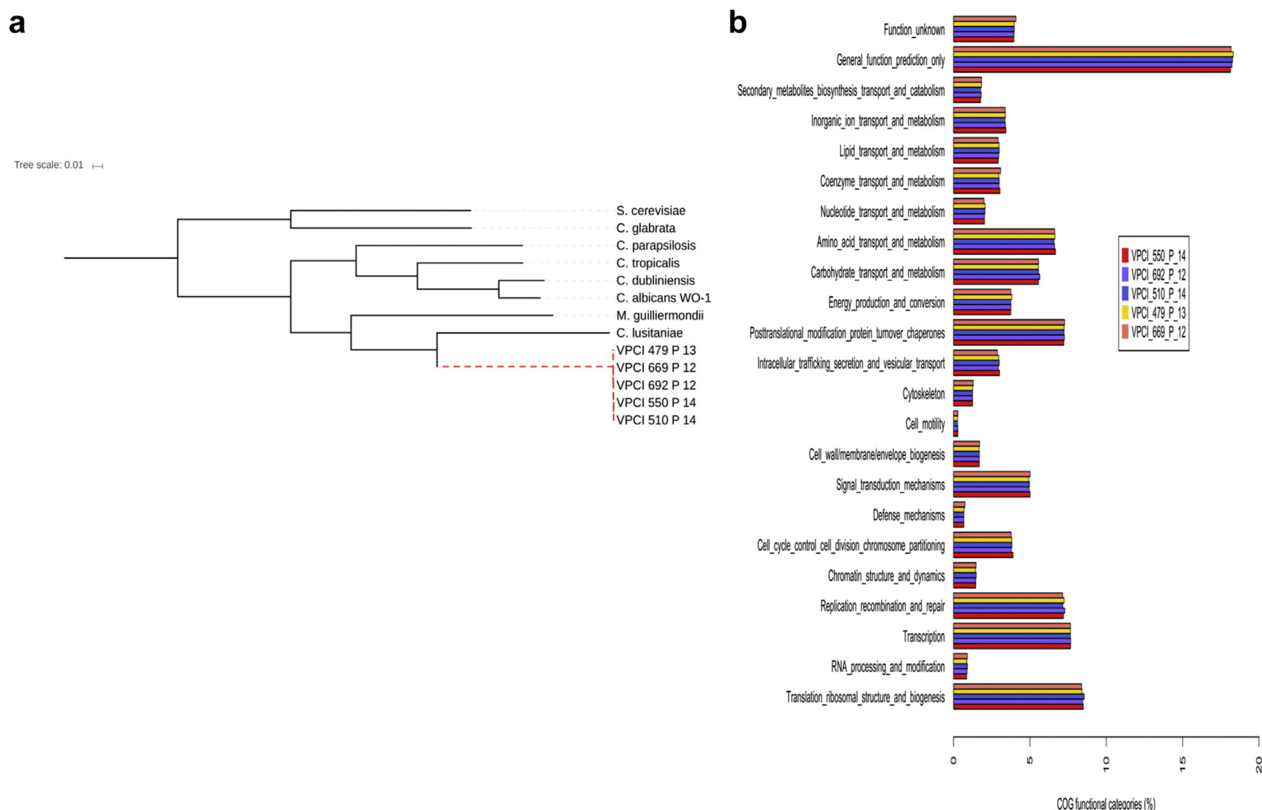

FIG. 1. (a) Phylogenetic tree based on 136 core/conserved genes of the *Candida auris* isolates and other *Candida* species. (b) Functional annotation of *Candida auris* genome based on Clusters of Orthologous Groups of proteins (COG) database classification.

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acquisition in *C. auris* isolates. The codon usage analysis in *C. auris* appears to be more similar and overlapping with *C. lusitaniae* than with any other *Candida* species, indicating that *C. auris* is part of the CUG clade. This similarity has been supported by the phylogenetic and functional data.

Antifungal susceptibility patterns and analysis of antifungal resistance genes

The *in vitro* susceptibility data of *C. auris* ($n = 5$) is presented in Table 1. All isolates were resistant to fluconazole, one each was resistant to voriconazole and amphotericin B and the other isolate was resistant to voriconazole, flucytosine and echinocandins. The PCR targeting *FKS1* and *FKS2* genes of a solitary echinocandin-resistant *C. auris* isolate (VPCI 550/P/15) generated amplicons of 391 bp and 460 bp, respectively. Echinocandin-resistant *C. auris* did not reveal any mutation in the hot spot regions previously reported for caspofungin-resistant *C. glabrata*. In contrast to *C. auris*, all *C. albicans* isolates were found to be highly susceptible to all azoles including itraconazole (MIC_{50}, 0.03 mg/L; MIC_{90}, 0.03 mg/L); voriconazole (MIC_{50}, 0.03 mg/L; MIC_{90}, 0.03 mg/L); posaconazole (MIC_{50}, 0.015 mg/L; MIC_{90}, 0.015 mg/L) and amphotericin B (MIC_{50}, 0.5 mg/L; MIC_{90}, 0.5 mg/L). The alignment of the *C. auris* contigs with *C. albicans* and *C. glabrata ERG* and *FKS* genes revealed that *ERG3*, *ERG11*, *FKS1*, *FKS2* and *FKS3* genes were present as a single copy in the *C. auris* genome. *ERG* and *FKS* genes of *C. auris* exhibited 78%–85% similarity with those of *C. albicans* and *C. glabrata*.

Furthermore, the *C. auris* genome when analysed with *C. albicans* and *Saccharomyces cerevisiae* revealed that a significant portion of its genome encodes ABC and MFS transporter family along with drug transporters. A number of zinc cluster transcription factor orthologues such as *TAC1* (29% similarity with *C. albicans*) and protein kinases, which may contribute to the acquisition of drug resistance were observed (Fig. 1b). The protein kinases such as protein kinase A, *HOG1* have been reported to be activated on perceiving stress thereby regulating the stress signalling pathways to enhance tolerance of pathogenic fungi to various fungicides [24].

Mating locus analysis

Mining of the *de novo* assembly of *C. auris* sequence reads the presence of *MATα* locus, similar to *Naumovozyma castellii* CBS 4309, in all the *C. auris* strains investigated. However, the *MATa* gene was absent in all the isolates in the present study.

Discussion

The application of whole genome sequencing confirms for the first time that *C. auris* strains in Indian settings are highly clonal, suggesting the possibility of a common source of origin or a phenomenon of their recent differentiation. It is noteworthy to mention that ANI analysis showed *C. auris* strains exhibiting a highly divergent relationship with other clinically significant *Candida* species namely *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata*. However, the genome of *C. auris* may be more closely related to *C. haemulonii* with whom *C. auris* is commonly misidentified using phenotypic methods but whose genome is not yet sequenced. Future studies analysing the whole genome sequencing of other related *Candida* species might provide more insight about their similarity with *C. auris*. The lack of information on the ecological niche of *C. auris* hampers the understanding of its divergence from other *Candida* species. Further, all the strains of *C. auris* analysed in the present study were recovered from five individual patients in four hospitals located 50–2000 km apart and at a different time period. However, irrespective of the variability in the above parameters all strains exhibited low genetic diversity. Also, all the *C. auris* strains exhibited *MATα* mating locus and its presence was also validated by gene-specific PCR. Further experiments analysing the mating locus in a large number of *C. auris* isolates will determine the true sexual status of this pathogen. Furthermore, the antifungal resistance profile of the isolates was variable but uniformly showed the presence of ABC and MFS transporters, which may explain the multidrug resistance. It is also possible that the undiscerning use of antifungals has resulted in its emergence as a successful multidrug-resistant pathogen. Overall our study provides the first comparative analysis of *C. auris* genomes using whole genome sequencing and highlights clonal expansion of *C. auris* isolates in India.

Conflict of interest

JFM received grants from Astellas, Basilea and Merck. He has been a consultant to Astellas, Basilea and Merck and received speaker's fees from Merck, United Medical and Gilead. All other authors have no potential conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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