

Genetic Heterogeneity of Australian *Candida auris* Isolates: Insights From a Nonoutbreak Setting Using Whole-Genome Sequencing

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Whole-genome sequencing clustered Australian *Candida auris* isolates from sporadic cases within clade III. Case isolates were genomically distinct; however, unexpectedly, those from 1 case comprised 2 groups separated by >60 single nucleotide polymorphisms (SNPs) with no isolate being identical, in contrast to outbreaks where isolates from any 1 individual have differed by <3 SNPs. Multidrug resistance was absent. High within-host genetic heterogeneity should be considered when investigating *C. auris* infections.

Keywords. *Candida auris*; drug resistance; whole-genome sequencing.

Candida auris is a global multidrug-resistant pathogen with a propensity to cause nosocomial outbreaks [1–6]. Next-generation sequencing analyses have shown that strains of

C. auris cluster into 4 predominant, but possibly 5 geographically distinct clades (South Asian [clade I], East Asian [clade II], African [clade III], and South American [clade IV]), with some evidence of phylogeographical mixing of clades [1–3, 7–9]. Short-read whole-genome sequencing (WGS) data demonstrated that there are >10 000 single nucleotide polymorphisms (SNPs) from separate clades, but with lower intraclade diversity (<100 SNP differences) [1]; the low intraclade diversity was most marked in analyses of hospital outbreaks where genomes of outbreak isolates differed by a median of 3 SNPs, and within any 1 patient, by <2 SNPs [1–3, 10]. Whether similar observations emerge in the nonoutbreak setting is uncertain.

Following the first *C. auris* infections in Australia [11, 12], in 2018/2019, Sydney reported 3 additional cases. Here we used WGS to investigate the genetic relationships and markers of drug resistance of Australian isolates from sporadic cases, including from the first report [11]. To place results in the genomic context, we also sequenced several isolates from India and South Africa and included publicly available *C. auris* genomes [1, 3, 4, 8]. Phylogenetic SNP analysis was compared with polymerase chain reaction (PCR) fingerprinting [13]. Unexpectedly, multiple isolates from 1 individual (clade III) had relatively diverse genomes compared with previous observations for this clade [2, 3].

Eighteen clinical isolates, reconfirmed as *C. auris* by internal transcribed spacers (ITS) sequencing, were studied (Table 1). Of 10 Australian isolates, 9 were from 3 separate cases repatriated to Sydney hospitals from South Africa (Cases 1, 2, 3), with Case 4 repatriated to Perth [11]. Two isolates were from South Africa, and 6 were from India, included as “benchmark” isolates from their respective regions. For Case 1, the incident isolate (strain WM_18.177) was cultured during a prior hospitalization in South Africa but was considered an Australian isolate for this study.

Antifungal susceptibility was determined using Sensititre YeastOne YO10 (TREK Diagnostics, Cleveland, OH, USA). MICs were interpreted against the proposed US Centers for Disease Control and Prevention (CDC) breakpoints [14] or proposed *C. auris*-specific Clinical Laboratory Standards Institute epidemiological cutoff values to define wild-type (WT) or non-WT isolates [15]. PCR fingerprinting was also performed [13].

Genomic DNA was extracted using the MasterPure Yeast Kit (Epicentre, Lucigen Corporation, WIS, USA). DNA libraries were prepared (Nextera XT; Illumina, CA, USA) and sequenced on the NextSeq 500 platform (Illumina, CA, USA) [16]. Raw reads were trimmed (Trimmomatic, version 3.6) and taxonomically classified using Kraken, version 1.0.

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Table 1. *Candida auris* Isolates Studied: Geographic Region, Clade, Body Site of Isolation, and in Vitro Susceptibility to 9 Antifungal Agents

Isolate ID	Country	Clade	Body Site	Date of Isolation	Minimum Inhibitory Concentration, mg/L								
					AMB	5-FC	FLC	ITR	VRC	POS	ANI	MIF	CAS
Australian isolates													
Case 1													
WM_18.181	Australia	III	Wound swab PICC site	05.09.2018	1	0.12	256	0.25	2	0.12	0.25	0.12	0.12
WM_18.182	Australia	III	Sputum	05.09.2018	1	0.12	256	0.25	2	0.12	0.25	0.12	0.25
WM_18.187	Australia	III	Axilla swab	06.09.2018	1	0.12	256	0.25	2	0.12	0.5	0.12	0.25
WM_18.188	Australia	III	Sputum	07.09.2018	1	0.25	256	0.5	4	0.25	0.25	0.06	0.12
WM_18.189	Australia	III	Axilla swab	11.09.2018	1	0.12	256	0.25	2	0.12	0.25	0.12	0.25
WM_18.177 ^a	Australia	III	Blood culture	July 2018	1	0.12	256	0.5	2	0.12	0.25	0.12	0.12
WM_18.190	Australia	III	Groin	11.09.2018	1	0.12	256	0.25	2	0.12	0.25	0.12	0.25
Case 2													
WM_18.180	Australia	III	Nose swab	20.09.2018	1	0.12	>256	0.25	2	0.12	0.25	0.12	0.25
Case 3													
WM_18.197	Australia	III	Throat	08.11.2018	1	0.12	256	0.5	2	0.12	0.25	0.12	0.25
Case 4 ^b													
WM_18.176	Australia	III	Sternal bone	2015	1	0.06	>256	1	1	0.12	0.5	0.5	0.5
South African comparison isolates ^c													
WM_18.178	South Africa	III	Blood culture	2017	0.5	0.12	>256	0.5	4	0.25	0.12	0.06	0.06
WM_18.179	South Africa	III	Blood culture	2017	1	0.12	256	0.25	1	0.12	0.25	0.12	0.12
Indian comparison isolates ^d													
WM_18.173	India	I	Blood culture	Unknown	1	<0.06	64	0.25	1	0.03	0.25	0.12	>8
WM_18.174	India	I	Blood culture	Unknown	2	<0.06	64	0.12	0.5	0.03	0.5	0.12	>8
WM_18.175	India	I	Blood culture	Unknown	2	<0.06	128	0.12	0.5	0.03	0.25	0.25	0.5
WM_18.194	India	I	Blood culture	Unknown	2	0.12	32	0.32	0.25	0.008	>8	>8	2
WM_18.195	India	I	Blood culture	Unknown	2	0.12	>256	0.25	1	0.12	0.25	0.12	0.12
WM_18.196	India	I	Blood culture	Unknown	2	0.12	256	0.25	1	0.25	0.25	0.12	0.12

Abbreviations: 5-FC, 5-flucytosine; AMB, amphotericin B; ANI, anidulafungin; CAS, caspofungin; FLC, fluconazole; ITR, itraconazole; MIC, minimum inhibitory concentration; MIF, micafungin; POS, posaconazole; VRC, voriconazole.

^aOriginal (incident) clinical isolate from Case 1, isolated while hospitalized in South Africa.

^bClinical isolate obtained from Heath et al. [11].

^cClinical isolates from South Africa

^dClinical isolates from India.

Single nucleotide polymorphisms (SNPs) were called using 3 pipelines where SNPs were defined as substitutions present in at least 90% of reads with minimum coverage above 30. For the Nullarbor and RedDog pipelines (see below), reads were mapped to *C. auris* strain B11221 (African clade; NCBI GenBank Accession PGLS01000001.1) [17] as the reference. Nullarbor (version e2.0) (<https://github.com/tseemann/nullarbor>) employed FreeBayes (version 1.2.0-dirty) for SNP calling and filtered them using Snippy, version 4.3.5, based on a minimum coverage of 10, a quality of 100, and a read fraction of 0.9 supporting the variant. The analysis was also undertaken using RedDog (<https://github.com/katholt/RedDog>) employing Bowtie2 [18] to map reads to the reference and BCFtools, version 1.3.1 (<https://www.sanger.ac.uk/science/tools/samtools-bcftools-htslib>), for SNP calling. In parallel, sequences were examined using the Oxford pipeline [3], which mapped short reads to the reference sequence T26425 (clade III) using Stampy, version 1.0.23, without BWA pre-mapping, with an expected substitution rate of 0.01. SNPs

were identified with Samtools4, version 0.1.19, mpileup with extended base-alignment quality flag. A consensus of $\geq 75\%$ was necessary to support a SNP, and calls were required to be homozygous under a diploid model. Only SNPs supported by ≥ 5 reads, including 1 in each direction, were accepted. Repetitive regions of the genome were excluded as described by Eyre et al. [3].

Consensus alleles at all SNP variant sites were extracted; SNP sites present in all genomes were concatenated to generate a core SNP alignment for phylogenetic analysis. A maximum likelihood tree was estimated using IQTree, version 1.6.7, at 1000 bootstraps using the SNP matrix of Nullarbor, version 2.0. Sequence reads were deposited in the NCBI Archive (SRA: PRJNA559200). The data set was supplemented with 121 published genome sequences of *C. auris* (SRAs: PRJEB21518, PRJNA328792, PRJNA328792, PRJEB20230) [1, 3, 8]. The number and location of SNPs called by the 3 pipelines were highly concordant (correlation coefficient $r = .999$) (Supplementary Tables 1–4).

Genes known for their role for antifungal drug resistance in *C. auris* and *Candida* spp. (*ERG11*, *MDR1*, *CDR1* [azole resistance], *FKS1* [echinocandin resistance], and *ERG2*, *ERG3*, *ERG5*, *ERG6* [amphotericin B resistance]) [19, 20] were examined for SNPs using the criteria above (CLC Genomics Workbench; Bio, version 7.0, Aarhus, Denmark) [16].

Table 1 summarizes the isolate details including the timeline of Australian isolates. Six of 7 isolates from Case 1 were cultured over a period of 3 weeks during hospitalization. The seventh (incident isolate from South Africa) was forwarded to us for comparative analysis. Isolates cultured from various body sites from Case 1 were available for study.

Phylogenetic analysis of the study isolates identified 2 major isolate clusters assigned to clades I and III (Figure 1A). All Australian isolates clustered within clade III (Table 1), whereas the Indian isolates grouped within clade I, separated from clade III by >40 000 SNPs. Genomes of isolates from Case 1 formed 2 distinct “groups,” separated by 60–70 SNPs (Figure 1B; Supplementary Tables 2–4). Three isolates (strains WM_18.181, WM_18.188, and WM_18.182) were in the same subgroup as the incident isolate (highlighted in yellow). Within each subgroup, the SNP difference between isolates was 6–9 SNPs. For example, for isolates from Case 1, strains WM_18.181 and WM_18.182 were separated by 5–8 SNPs. However, others, also from Case 1, differed by >60 SNPs (eg, WM_18.181 and WM_18.190) (Supplementary Tables 2 and 3).

Isolates from Cases 2, 3, and 4 and 2 other South African isolates (Table 1) were all distinct from each other (>60 SNPs separating their genomes). Of 2 PCR fingerprinting patterns (Figure S1), pattern 1 encompassed the 6 Indian isolates, indistinguishable from each other, and corresponded to genome clade I. Other isolates belonged to pattern 2, also indistinguishable with the exception of 1 strain, and corresponded to clade III.

Isolates had low 5-flucytosine MICs (0.06–0.12 µg/mL) (Table 1). Clade III isolates showed WT MICs to amphotericin B (≤ 1 µg/mL) with no SNPs in *ERG2*, *ERG3*, *ERG5*, and *ERG6*; 5/6 Indian isolates had MICs at the proposed breakpoint of 2 µg/mL [14] and harbored an *ERG2* SNP at nucleotide position A117C (E39D mutation) (Supplementary Table 5). Isolates universally had high fluconazole MICs (32–>256 µg/mL) with varying susceptibility to other azoles. Two SNPs in *ERG11* were present, leading to mutations V125A and F126L in the clade III isolates, while mutations Y132F and K143R were in Indian isolates, as were nonsynonymous SNPs in the *MDR1* (T801G and A1225G) and *CDR1* (A2127T) genes. Australian and South African isolates had echinocandin MICs of ≤ 0.5 µg/mL, but a single pan-echinocandin-resistant isolate (strain WM_18.194) from India contained the *FKS1* mutation, S639F. No *FKS1* mutations was detected in 2 Indian strains with MICs of ≥ 8 µg/mL to caspofungin only (Tables 1; Supplementary Table 5).

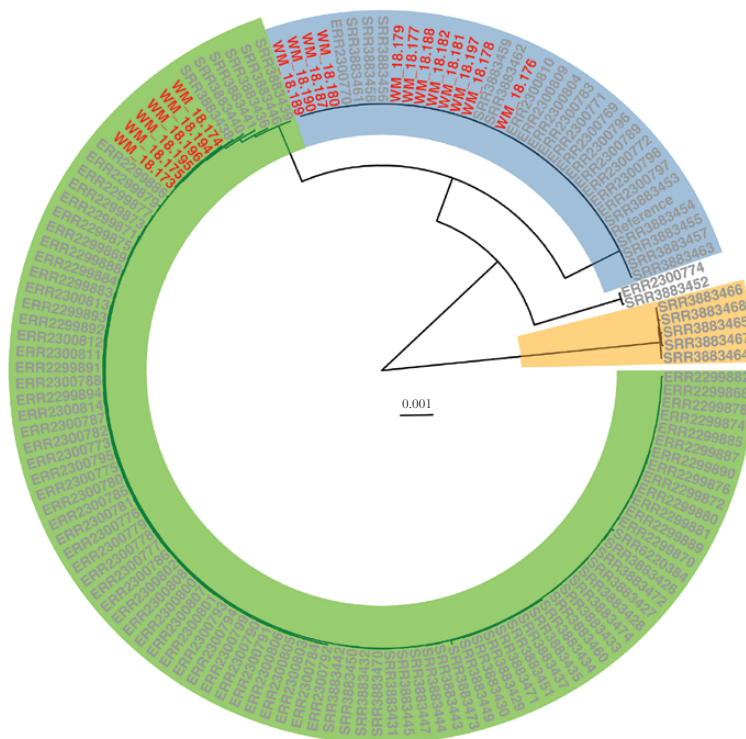
The value of WGS for investigation of nosocomial *C. auris* infections has been documented, yet there are few data on its application to describe the genomic diversity of isolates outside the outbreak setting. Here we present the phylogenetic relatedness based on WGS of 10 Australian isolates from 4 sporadic cases in the wider geographical context. There was no distinct Australian clade as noted for other Australian isolates [11, 12] but rather independent importation events with strains reflecting likely geographic origin of acquisition.

Interestingly, in the nonoutbreak setting herein, whereas isolates from different cases had genomes separated by >60 SNPs, as found elsewhere [1–3] for clade III isolates, those from Case 1 were separated by an unexpectedly higher number of SNPs, with no isolate being identical. This finding contrasts with those from nosocomial *C. auris* outbreaks where intraclade diversity between isolates from any 1 individual was low (<3 SNPs) [1, 2, 21] although 1 study showed less spatiotemporal clustering [4]. One likely explanation for our findings, although unproven, is that at least 2 distinct populations were acquired while in the hospital in South Africa (a group of 4 and another group of 3 isolates) (Figure 1B). Regardless, the results indicate the presence of genetically heterogeneous strain populations colonizing the host simultaneously where such heterogeneity may enhance fitness [4, 22]. The sampling of >1 body site and/or on >1 occasion to detect *C. auris* may assist studies addressing this question. We included 2 de novo South African isolates as a “benchmark” for clade classification. Despite the lower discriminatory power of PCR fingerprinting, it may be useful for rapid clade identification in laboratories without WGS technology, as different clades are associated with different drug resistance patterns, which impact empiric antifungal therapy.

Importantly, despite the absence of interpretive MICs, resistance to ≥ 2 drug classes was not evident among Australian isolates compared with 20%–44% resistance rates elsewhere [1, 6]. Multidrug resistance may be location- or clade-specific. As before, isolates exhibited reduced fluconazole susceptibility and varying susceptibility to other azoles [1–3, 6]. Reassuring, given that echinocandins are first-line agents for treating *C. auris* infections [23], strains had low echinocandin MICs and WT MICs to amphotericin B [2–4, 20]. In parallel, no Australian isolate harbored *FKS1* mutations, for example, involving S639 substitutions [7, 24, 25]. This amino acid is homologous to that at position S645 in *C. albicans*, where amino acid changes confer high-level echinocandin resistance [26]. Conversely, the pan-echinocandin-resistant clade I strain contained the S639F mutation, as have other pan-echinocandin-resistant isolates. The mutations S639P and S639Y, linked to echinocandin resistance, were absent in our isolates [20, 24].

WGS analysis also informed *ERG11* mutations, homologous to those linked to azole resistance in *C. albicans* [1, 26]. That K143R and Y132F substitutions were present in Indian but not Australian and South African isolates is consistent with the

A



B

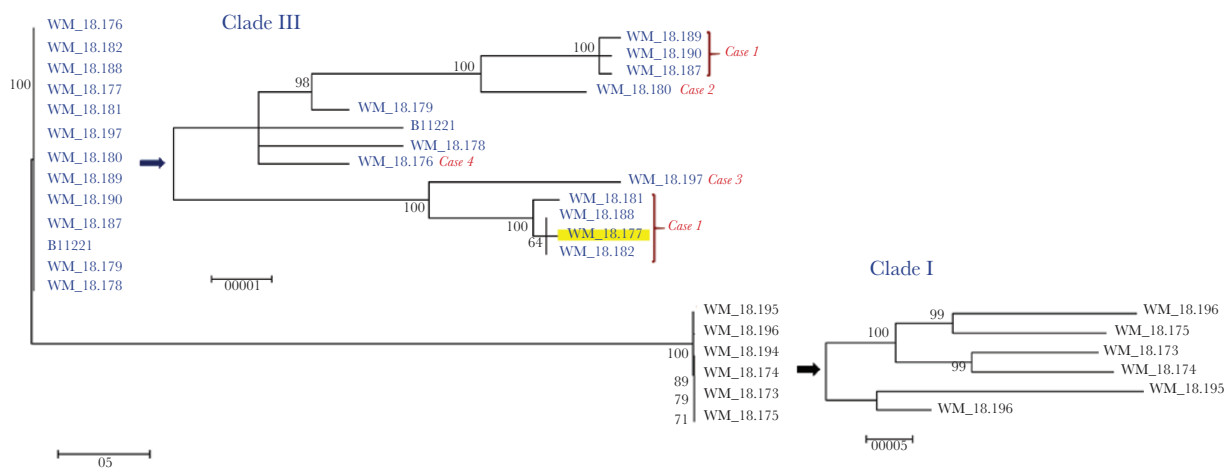


Figure 1. A, Circular maximum likelihood phylogeny by *r* package ggtree of *Candida auris* isolates. Clades are represented by highlighted sections of the tree—clade I (green), clade II (white), clade III (blue), and clade IV (orange)—and generally correspond with geographical regions, namely India/Pakistan, Japan, South Africa, and South America, respectively. “Australian” isolates are depicted by the red tip labels. B, Maximum likelihood phylogeny of *Candida auris* isolates using MEGA, version 6.0, with expanded subtree views with scales and bootstrap values on the righthand side. Isolate identification (tips) and arrows are colored by clade (clade III: blue; clade I: black). The yellow highlighted tip represents the index isolate from case 1 in South Africa before repatriation to Australia (see the text and Table 1 for further details). For the Australian isolates, the case from which the isolate was cultured is shown on the tree tip.

notion these are clade I–specific markers of resistance [1, 17]. Conversely, the F126L and V125A mutations (clade III–specific) [1, 20] were present in Australian and South African isolates only. The association of novel SNPs in *MDR1* and *CDR1* in the Indian isolates with azole resistance is uncertain, as is the link to amphotericin B resistance with the mutation E39D.

The limitations of our study include the small number of Australian isolates due to the sporadic nature of infection, and

in the absence of an outbreak, the environment was not sampled. The unusually high within-host genetic diversity was documented only in 1 case, as multiple isolates from the other patients were not available, limiting wider clinical relevance. Detailed study of patient data and investigation of resistance mechanisms to azoles were outside of the study scope.

In conclusion, analysis of *C. auris* genomes in a nonoutbreak setting demonstrated that WGS added value by differentiating

independent importations from recent transmission events. Within-host diversity of *C. auris* suggests concurrent colonization of the host by heterogeneous populations. These findings improve our understanding of colonization/infection by *C. auris* and assist in interpretation of growing genomic data for this pathogen.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. All authors: no reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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