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Optimization and Validation of *Candida auris* Short Tandem Repeat Analysis

Theun de Groot,[a,b] Bram Spruijtenburg,[a,b] Lindsay A. Parnell,[c] Nancy A. Chow,[c] Jacques F. Meis[a,b]

[a]Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands
[b]Centre of Expertise in Mycology, Radboud University Medical Center/Canisius Wilhelmina Hospital, Nijmegen, The Netherlands
[c]Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Theun de Groot and Bram Spruijtenburg contributed equally to this work. Author order was determined by reverse seniority.

ABSTRACT *Candida auris* is an easily transmissible yeast with resistance to different antifungal compounds. Outbreaks of *C. auris* are mostly observed in intensive care units. To take adequate measures during an outbreak, it is essential to understand the transmission route, which requires isolate genotyping. In 2019, a short tandem repeat (STR) genotyping analysis was developed for *C. auris*. To determine the discriminatory power of this method, we performed STR analysis of 171 isolates with known whole-genome sequencing (WGS) data using Illumina reads, and we compared their resolutions. We found that STR analysis separated the 171 isolates into four clades (clades I to IV), as was also seen with WGS analysis. Then, to improve the separation of isolates in clade IV, the STR assay was optimized by the addition of 2 STR markers. With this improved STR assay, a total of 32 different genotypes were identified, while all isolates with differences of >50 single-nucleotide polymorphisms (SNPs) were separated by at least 1 STR marker. Altogether, we optimized and validated the *C. auris* STR panel for clades I to IV and established its discriminatory power, compared to WGS SNP analysis using Illumina reads.

IMPORTANCE The emerging fungal pathogen *Candida auris* poses a threat to public health, mainly causing outbreaks in intensive care units. Genotyping is essential for investigating potential outbreaks and preventing further spread. Previously, we developed a STR genotyping scheme for rapid and high-resolution genotyping, and WGS SNP outcomes for some isolates were compared to STR data. Here, we compared WGS SNP and STR outcomes for a larger sample cohort. Also, we optimized the resolution of this typing scheme with the addition of 2 STR markers. Altogether, we validated and optimized this rapid, reliable, and high-resolution typing scheme for *C. auris*.

KEYWORDS *Candida*, genetics, genotypic identification, phylogenetic analysis

*C*andida auris is a transmissible, pathogenic yeast that can cause invasive infections with high mortality rates and outbreaks in health care facilities. Once present in the health care environment, *C. auris* can be extremely difficult to eradicate (1, 2). When *C. auris* has been identified in more than one patient in a health care facility, it is essential to understand whether transmission took place within the hospital and, if so, to localize the source of colonization or infection.

Genotyping is a useful method for understanding transmission and introduction of pathogens into a health care facility. There are various methods to genotype microorganisms, each with its own benefits regarding turnaround time, costs, broad implementation, and ability to discriminate samples. For the latter, whole-genome sequencing (WGS) is the gold standard because it allows genotyping based on the whole genome (3). As for any method, there are various parameters affecting its outcome, including input sequence quality, coverage, and the choice of sequence assembly tool, read mapping tool, and single-nucleotide

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polymorphism (SNP) pipeline (4, 5). In 2021, a WGS benchmark data set was published, allowing standardized comparisons of phylogenomic pipelines (6). The disadvantages of WGS analysis include long turnaround time, high costs, and required bioinformatic expertise. An alternative to high-resolution genotyping is short tandem repeat (STR) analysis, which is still used in forensics (7). This method uses the highly variable repeat number of STRs between strains and must be set up for each species separately. STR typing is fast, sensitive, reliable, and inexpensive, making it feasible to screen large cohorts of samples in a short time (8).

Due to these advantages, we previously established a microsatellite analysis for *C. auris*, in which four panels of 3 STRs were included (9, 10). The assay was then tested on 444 isolates, of which 25 isolates had been analyzed previously by WGS. Comparing the results, we found that isolates that differed by <20 SNPs, as determined by WGS, often were not differentiated by STR analysis, while most isolates that differed by >30 SNPs were differentiated by STR analysis at ≥1 STR marker. Because that comparison included only 25 isolates, we now analyze 171 isolates that were previously studied by WGS SNP analysis by the Centers for Disease Control and Prevention to validate the future use of STR analysis and to understand its potential and limits.

RESULTS

Optimization of *C. auris* STR analysis. STR analysis was performed on 171 isolates that had been genotyped previously via WGS analysis (11). After analysis of all isolates, identical STR profiles were found (data not shown) for isolates from Colombia ($n = 23$) and Panama ($n = 12$), while earlier WGS analysis of these isolates identified a maximal difference of 143 SNPs (11). To differentiate these "subclades" with STR analysis, Illumina reads from different Colombian and Panamanian isolates were used to search for differentiating STRs. This analysis identified 2 STRs with a repeat length of 3 nucleotides with variable copy numbers, for which an additional M3-III STR panel was developed (Table 1). The addition of this extra panel to the original *C. auris* STR panels differentiated the Colombian and Panamanian isolates, leading to a total number of 32 genotypes for all isolates (Fig. 1). The distribution of isolates among these four clades was identical to that found previously with WGS analysis (Fig. 1) (11).

Validation of the *C. auris* STR assay. Next, the discriminatory potential of the STR analysis was investigated by comparing WGS and STR genotyping within each clade. For clade I, we found that isolates B12779 and B8441, which were separated by >500 SNPs from the other isolates and by 134 SNPs from each other, were separated by STR analysis with 4 markers from each other and with ≥3 markers from the other isolates in clade I (Fig. 2). The remaining isolates in clade I consisted of two major subgroups of isolates from the United States and Kenya. Isolates from the two countries were differentiated with 38 to 64 SNPs and with >1 STR marker, except for isolate B13343 from the United States, which was separated by 42 to 49 SNPs from the other U.S. isolates but demonstrated the same STR profile. The other U.S. isolates were separated by a maximum of 23 SNPs, and all except B13520 showed the same STR profile. The group of 10 Kenyan isolates was separated by a maximum of 10 SNPs and showed the same STR profile with the exception of B16487. Finally, isolates B13464 from Canada, B11105 from Pakistan, and B13520 from the United States were differentiated by at least 10, 16, and 10 SNPs, respectively, from other clade I isolates and were also distinguished by STR analysis.

Isolates from clade II, which were separated by at least 54 SNPs, all demonstrated different STR profiles (Fig. 2). Clade III, which was divided by WGS into a large subpopulation of 43 isolates and 2 separated isolates, showed a similar division by STR analysis. Isolates B12037 from Canada and B11230 from South Africa, which were differentiated by >1,028 and >66 SNPs, respectively, from other isolates, were separated by ≥5 and ≥1 STR markers, respectively, from the other isolates in clade III (Fig. 2). The remaining 43 isolates differed by at most 35 SNPs, with a maximum of 31 SNPs between isolates with the same STR genotype (genotype 15). Among these 43 isolates, five different STR genotypes were found.

Finally, clade IV isolates were divided by WGS into four major subgroups, which differed by 100 to 164 SNPs, with each subgroup showing a different STR genotype (Fig. 2). Isolates within the subgroups were directly related to the country of origin. Within the Panamanian

TABLE 1 Overview of PCR primers and STR characteristics for additional M3-III panel

PCR panel and primer name	Primer sequence (5′–3′) for:		Conc (µM)[b]	No. of bases of primer-flanking sequence	Repeat unit	No. of repeats[c]		No. of genotypes	D value[d]	Intragenic/locus protein coding gene[e]
	Forward primer[a]	Reverse primer				Range	Ref			
M3-IIIa	FAM-TGATGCAATCACTTAGCCAAC	GTTTGGATTGAGGCAAATGAG	5	116	CAG	24–27	25	4	0,75	Intragenic
M3-IIIb	JOE-GTTGGTGATTTTGTTGATTTTG	AACACTACCAATTCCTCTAACACC	5	117	TGT	10–26	26	4	0,74	Intragenic

[a]FAM, 6-carboxyfluorescein; JOE, 4′,5′-dichloro-2′,7′-dimethoxyfluorescein.
[b]The concentrations of the forward and reverse primers were identical.
[c]Ref, the reference strain is B12342 (GenBank accession number GCA_016772155.1).
[d]The discriminatory power of the STR assay, as determined via the Simpson index of diversity.
[e]The reference strain is B12342.

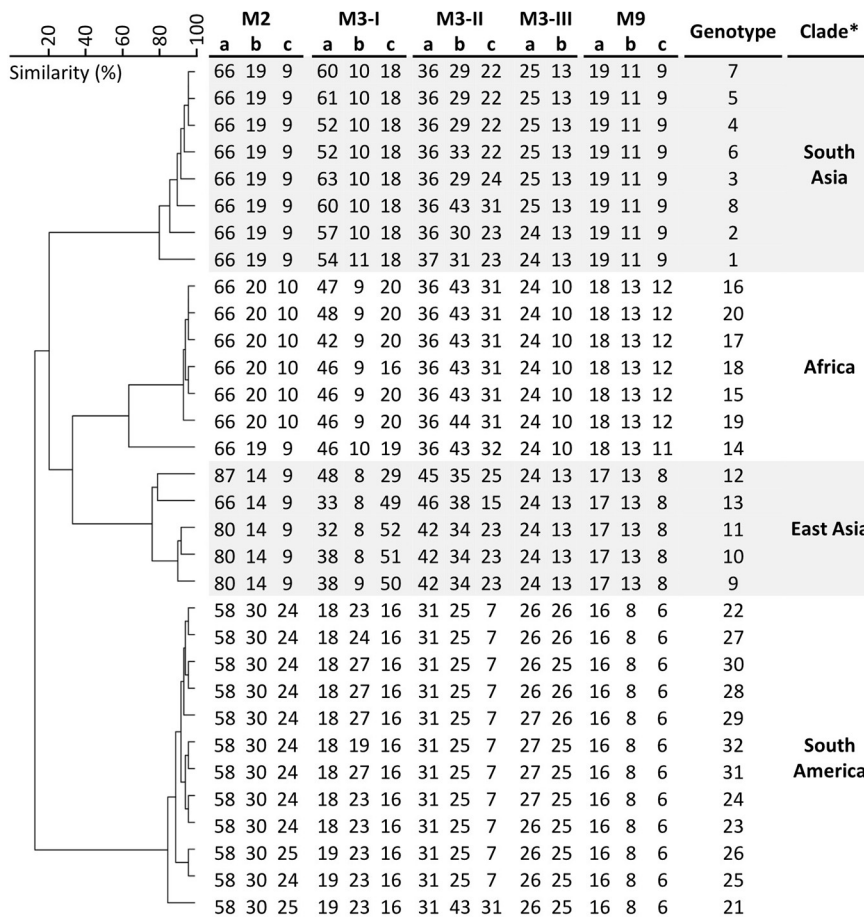|	M2			M3-I			M3-II			M3-III		M9			Genotype	Clade*
	a	b	c	a	b	c	a	b	c	a	b	a	b	c		
	66	19	9	60	10	18	36	29	22	25	13	19	11	9	7	
	66	19	9	61	10	18	36	29	22	25	13	19	11	9	5	
	66	19	9	52	10	18	36	29	22	25	13	19	11	9	4	
	66	19	9	52	10	18	36	33	22	25	13	19	11	9	6	South Asia
	66	19	9	63	10	18	36	29	24	25	13	19	11	9	3	
	66	19	9	60	10	18	36	43	31	25	13	19	11	9	8	
	66	19	9	57	10	18	36	30	23	24	13	19	11	9	2	
	66	19	9	54	11	18	37	31	23	24	13	19	11	9	1	
	66	20	10	47	9	20	36	43	31	24	10	18	13	12	16	
	66	20	10	48	9	20	36	43	31	24	10	18	13	12	20	
	66	20	10	42	9	20	36	43	31	24	10	18	13	12	17	
	66	20	10	46	9	16	36	43	31	24	10	18	13	12	18	Africa
	66	20	10	46	9	20	36	43	31	24	10	18	13	12	15	
	66	20	10	46	9	20	36	44	31	24	10	18	13	12	19	
	66	19	9	46	10	19	36	43	32	24	10	18	13	11	14	
	87	14	9	48	8	29	45	35	25	24	13	17	13	8	12	
	66	14	9	33	8	49	46	38	15	24	13	17	13	8	13	
	80	14	9	32	8	52	42	34	23	24	13	17	13	8	11	East Asia
	80	14	9	38	8	51	42	34	23	24	13	17	13	8	10	
	80	14	9	38	9	50	42	34	23	24	13	17	13	8	9	
	58	30	24	18	23	16	31	25	7	26	26	16	8	6	22	
	58	30	24	18	24	16	31	25	7	26	26	16	8	6	27	
	58	30	24	18	27	16	31	25	7	26	25	16	8	6	30	
	58	30	24	18	27	16	31	25	7	26	26	16	8	6	28	
	58	30	24	18	27	16	31	25	7	27	26	16	8	6	29	
	58	30	24	18	19	16	31	25	7	27	25	16	8	6	32	South America
	58	30	24	18	27	16	31	25	7	27	25	16	8	6	31	
	58	30	24	18	23	16	31	25	7	27	25	16	8	6	24	
	58	30	24	18	23	16	31	25	7	26	25	16	8	6	23	
	58	30	25	19	23	16	31	25	7	26	25	16	8	6	26	
	58	30	24	19	23	16	31	25	7	26	25	16	8	6	25	
	58	30	25	19	23	16	31	43	31	26	25	16	8	6	21	

FIG 1 STR genotypes of 32 *C. auris* isolates typed with five multiplex PCRs, i.e., M2, M3-I, M3-II, M3-III, and M9, which amplify 14 STR targets with repeat sizes of 2, 3, or 9 nucleotides. Cluster analysis showed that the four clades formed distinct clusters based on STR profiles. The UPGMA dendrogram was generated with BioNumerics. *, According to WGS SNP analysis.

subgroup, isolates that were separated by WGS with >12 SNPs were also separated by STR analysis, while isolates from Colombia were separated by a maximum of 27 SNPs, which coincided with two STR genotypes. Among the 3 Israeli isolates, B11896, which was separated by at least 26 SNPs from the other 2 isolates, also demonstrated a different STR profile, while the other isolates, with differences of only 5 SNPs, had identical STR profiles. The 48 isolates from Venezuela, which demonstrated a maximal difference of 24 SNPs, were subdivided into four different STR genotypes, which did not coincide with specific subgroups based on SNP analysis.

DISCUSSION

When there is a potential *C. auris* outbreak, it is useful to determine the isolates' genotype to potentially understand the transmission route and trace the source. In this study, we compared two genotyping methods, STR and WGS analyses, and conclude that these two methods generate concordant results. The two methods referred the isolates to the same clade, while isolates that differed by >50 SNPs were also differentiated by STR genotyping.

There are various methods to genotype *C. auris* isolates, each with its own advantages and disadvantages regarding turnaround time, reproducibility, complexity, and resolution. The turnaround time, reproducibility, and application of STR analysis are ideal for laboratories that do not have WGS capacity and bioinformatic expertise; however, its resolution is generally lower than that of WGS SNP analysis (12–14). Implementation of the *C. auris* STR analysis in outbreak settings has already demonstrated its value in an epidemiological context for understanding potential relationships between isolates (12, 15, 16). Previously, we compared
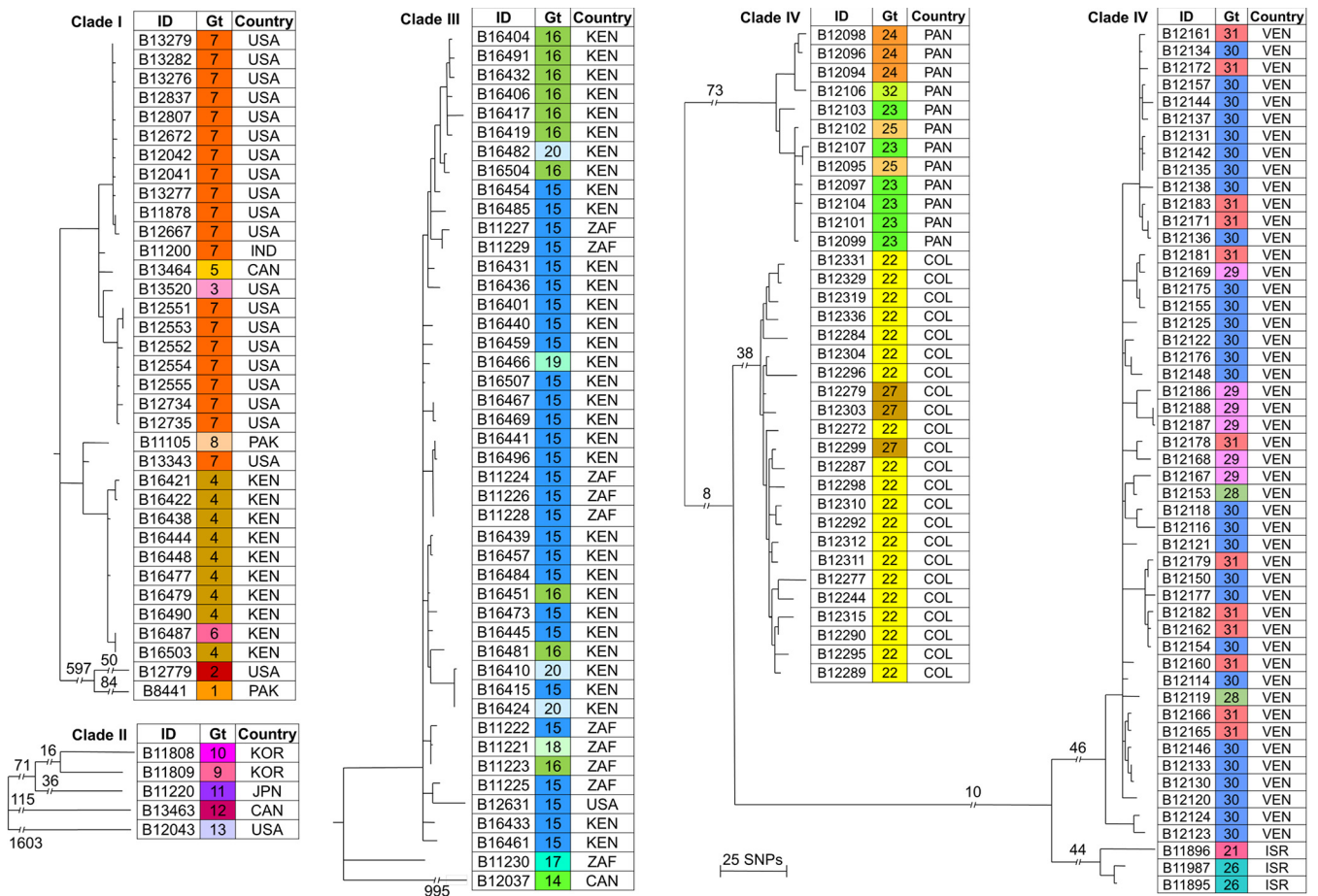
FIG 2 Comparison of genotypic differences of *C. auris* isolates determined via STR and WGS analyses. The trees on the left show the genetic relatedness of isolates based on SNPs, which was compared with the STR genotype. Numbers above the branches indicate SNPs. Gt, genotype according to STR analysis; USA, United States; IND, India; CAN, Canada; PAK, Pakistan; KEN, Kenya; KOR, South Korea; JPN, Japan; ZAF, South Africa; PAN, Panama; COL, Colombia; VEN, Venezuela; ISR, Israel.

WGS SNP outcomes for 25 *C. auris* isolates with STR genotyping and found that isolates that differed by >30 SNPs were also differentiated by STR analysis (9). In the present study, we compared WGS SNP analysis with STR genotyping for 171 *C. auris* isolates and found that all isolates with differences of >50 SNPs were separated by ≥1 STR marker. Previously, we analyzed 10 *Candida krusei* isolates by WGS SNP and STR analyses and found that *C. krusei* isolates that differed by >19 SNPs were differentiated by STR analysis (17). In contrast, comparison of *Mycobacterium avium* subsp. *paratuberculosis* isolates with both tandem repeat analysis and WGS SNP typing demonstrated that the repeat genotyping even failed to identify distantly related isolates, limiting the applicability of this typing scheme to the study of mycobacteria (18). WGS SNP genotyping of *Mycobacterium tuberculosis* isolates from a single STR cluster improved the understanding of the spread of these isolates (19). Similarly, WGS SNP analysis of *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolates with identical STR genotypes revealed distinct outbreaks (20), illustrating the power of WGS over STR analysis in bacteriology. Because STR assays must be developed for each pathogen separately, it is important to determine the resolution of each STR assay for valid interpretation of its results. For most STR assays, clusters with identical genotypes should be further investigated with WGS SNP analysis to determine their precise relatedness.

Having investigated the ability of STR analysis to separate isolates differentiated by SNP analysis, we then compared other outcome parameters, including the overall distance between isolates as determined via SNP and STR analyses. In this study, *C. auris* isolates B12779 (clade I), B8441 (clade I), B12043 (clade II), and B12037 (clade III), which were separated by >600 SNPs from other isolates in the same clade, were differentiated by ≥3 STR

markers. In contrast, isolates that differed from each other by around 20 to 120 SNPs usually differed by only 1 or 2 STR markers, suggesting that the number of differences in STR markers gives a rough indication of the genetic distance between isolates. Furthermore, some isolates, such as B16487 and B16503 from clade I and B16415 and B16424 from clade III, did not show any differences in SNP analysis but were differentiated by STR analysis at 1 STR marker, with copy numbers of 4 and 2, respectively. This discrepancy between the two genotyping methods is likely caused by the higher mutation rate of repeat regions, which are not included in WGS SNP analysis, in comparison with nonrepeating sequences (21). Finally, the addition of the M3-III STR marker panel increased the number of isolates differentiated within clade IV. No copy number variations within clades II and III were found for the new STR panel. In clade I, a copy number variation in 1 marker was found, but this did not lead to additional genotypes. Therefore, the use of this new STR marker panel seems especially useful for clade IV isolates.

This study has some limitations. The discriminatory power of the STR assay was determined by comparison with WGS SNP analysis. The number of SNPs between isolates, however, depends on the overall number and relatedness of isolates included in the WGS analysis, which complicates the use of 50 SNPs as an absolute value to estimate the discriminatory power of this STR assay. Furthermore, included isolates were only from clades I to IV. The discriminatory power of STR analysis for clade V isolates remains to be established.

Altogether, we validated the earlier published STR assay for *C. auris* using a panel of 171 isolates with available WGS SNP data (9). The STR assay distinguished isolates with >50 SNPs, suggesting that this is an excellent method to quickly screen large cohorts. For isolates with identical STR genotypes, follow-up WGS SNP analysis might be required to determine the precise relatedness between isolates and to better understand their epidemiology.

MATERIALS AND METHODS

STR assay. Microsatellite analysis of *C. auris* isolates was performed by STR typing, as described previously (9). Briefly, *C. auris* DNA was extracted using the MagNA Pure 96 DNA and viral nucleic acid (NA) small-volume kit, the Pathogen 200SV protocol, and a MagNA Pure 96 instrument (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. Subsequently, multiplex PCR assays, amplifying 14 STR targets with repeat sizes of 2, 3, or 9 nucleotides, were performed and corresponding copy numbers were determined using GeneMapper software (Applied Biosystems, Foster City, CA, USA). After the relative contribution of the M9 panel was increased 5-fold, the relatedness between isolates was analyzed with BioNumerics v7.6.1 software (Applied Maths, Kortrijk, Belgium) by employing the unweighted pair group method with arithmetic means (UPGMA) using the multistate categorical similarity coefficient as described previously (22).

Selection and design of new STR markers. The Colombian *C. auris* clade IV reference genome B12342 (GenBank accession number GCA_016772155.1) was downloaded from the NCBI database and uploaded to Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html) using the advanced search option (alignment parameter, 2,7,7; minimum alignment score to report repeat, 80; maximum period size, 3; maximum tandem repeat array size, 2) (23). Resulting STRs were screened, and repeats that contained deletions or insertions, exhibited a match score of <90%, or had a copy number of <20 were excluded. Paired-end reads for B12094 (GenBank accession number SRR10461252), B12097 (GenBank accession number SRR10461249), B12098 (GenBank accession number SRR10461248), B12101 (GenBank accession number SRR10461183), B12277 (GenBank accession number SRR10461190), and B12304 (GenBank accession number SRR7140044) were aligned against the reference genome B12342 using BWA-MEM (24). PCR duplicates were removed with RmDup, local realignment was performed using BamLeftAlign, and unpaired reads were removed with BAM filter. Mapped reads with mapping quality (MAPQ) scores of <60 were removed. Alignments around tandem repeats were visually inspected using JBrowse v1.16.11 (25). Primers were designed with Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using the default settings except for primer size (minimum, 19; optimum, 21; maximum, 24), primer melting temperature (T_m) (minimum, 57.0; optimum, 59.0; maximum, 62.0), maximum poly-X of 3, and CG clamp of 1 (26). Primers that formed no self- or cross-dimers with ≥5 nucleotides from the last 7 nucleotides of the 3' end of a primer, according to the multiple-primer analyzer from Thermo Fisher Scientific, were ordered via Eurogentec (Seraing, Belgium).

Phylogenetic analysis and visualizations. The original WGS sample collection comprised the publicly available sequences generated previously and represented four clades (11). Paired-end reads from this collection were quality controlled and aligned to the assembly strain B8441, and variants were identified as described previously (11). In this study, phylogenetic analysis was performed using the variant calls from a subset of the original sample collection from each clade (clade I, $n = 35$; clade II, $n = 5$; clade III, $n = 45$; clade IV, $n = 86$). Neighbor-joining phylogenetic trees were constructed for each clade using MEGA X v10.0.5 (27). Gaps and missing data were treated as complete deletions. The resulting neighbor-joining trees were visualized using MicroReact, with SNPs displayed as branch lengths (28).

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