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Original Article

Biological and genomic analyses of *Clavispora sputum sp. nov.*, a novel potential fungal pathogen closely related to *Clavispora lusitaniae* (syn. *Candida lusitaniae*) and *Candida auris*

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ABSTRACT

Several human fungal pathogens, including drug-resistant *Candida auris* and species of the *Candida haemulonii* complex, have emerged over the past two decades, posing new threats to human health. In this study, we report the isolation and identification of a novel species belonging to the genus *Clavispora*, herein named as *Clavispora sputum*, from a clinical sputum sample of a COVID-19 patient. *Cl. sputum* is phylogenetically closely related to fungal pathogens *Clavispora lusitaniae* (syn. *Candida lusitaniae*) and *C. auris*. When grown on CHROMagar *Candida* Plus medium, *Cl. sputum* exhibited a similar coloration to *C. auris* strain CBS12372. *Cl. sputum* was able to develop weak filaments on CM medium. Although *Cl. sputum* and *Cl. lusitaniae* are phylogenetically closely related, comparative genomic and synteny analyses indicated significant chromosomal rearrangements between the two species. Although *Cl. sputum* could not grow at 37 °C under regular culture condition, an increased fungal burden in the lung tissue of a mouse systemic infection model implies that it could be a potential opportunistic pathogenic yeast in humans.

1. Introduction

Emerging fungal pathogens are becoming a new threat to public health worldwide [1]. Multiple factors could contribute to the increased emergence of novel fungal pathogens in clinical settings, such as the increasing use of antifungal chemicals in hospitals (such as azoles or echinocandins) and in agriculture (such as trizoles), global warming, and ecological disturbances [2–4]. It has been suggested that the emergence of the superfungus *Candida auris* could be a result of global warming [4]. Over the past two decades, several drug-resistant fungal species related to *C. auris* (e.g., *Candida haemulonii* species complex and *Candida vulturna*) have emerged in clinical settings [5–7].

C. auris was first described in 2009, having been isolated from the ear discharge of a female Japanese patient [8]. Different genetic clades of this fungal pathogen then emerged on different continents almost simultaneously and rapidly spread worldwide [9–11]. Due to its high mortality rate from bloodstream infections, multidrug resistance, and ease of transmission, *C. auris* has attracted considerable attention from both clinical and basic research scientists [9]. The opportunistic pathogens *Candida haemulonii* and *Clavispora lusitaniae* are phylogenetically related to *C. auris* [9]. Several novel species of the *C. haemulonii* complex were identified in the past decade [12,13]. A common characteristic of these species is their intrinsic resistance to multiple antifungal drugs, especially azoles [14–16]. Infections caused by these drug-resistant

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pathogenic fungi have increasingly occurred in recent years [13,16].

In this study, we isolated and identified a novel species of the *Clavispora* genus from a patient with COVID-19. This species, named *Clavispora sputum sp. nov.*, is phylogenetically closely related to *Cl. lusitaniae* and *C. auris*. To characterize this species, we performed comparative biological and genomic analyses among *Cl. sputum* and several other phylogenetically related species. Our findings indicate that *Cl. sputum* could represent a novel emerging human fungal pathogen.

2. Material and methods

2.1. Strains isolation and DNA sequencing

Three strains were isolated from the sputum samples of a patient with COVID-19 in a pulmonary hospital (Shanghai, China) using single-colony isolation assay on CHROMagar *Candida* Plus medium (CHROMagar, Pairs, France). The holotype of new species was deposited in China General Microbiological Culture Collection Center (CGMCC). Taxonomic nomenclature and descriptions were deposited in Fungal Names [17].

Two loci, including the D1/D2 domain of the large subunit (LSU) ribosomal RNA gene, and ITS regions were amplified and sequenced. Amplification of the LSU regions were performed using primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′). The ITS regions were amplified with primers ITS1 (5′-GTCGTAACAAGGTTTCCGTAGGTG-3′) and NL-4.

2.2. Phenotypic characterization

The following strains were used in this assay: *Clavispora sputum* (FK184101), *Clavispora lusitaniae* (FK702, FK969-2), and *Candida auris* (clade I: BJCA001; clade II: CBS12372; clade III: C1; clade IV: SA0386). Cells of *Cl. sputum*, *Cl. lusitaniae*, and *C. auris* were initially plated on YPD solid medium and incubated for 2 days at 30 °C. Activated cells were then used for subsequent assay.

Given the close phylogenetic relationship among theses strains, cells were streaked onto CHROMagar *Candida* Plus plates and incubated at 30 °C for 5 days to differentiate the new species from other yeasts. The general phenotypes of colonies and cells were observed on YPD, YP (2 % peptone, 1 % yeast extract, 2 % agar), and Lee's Glucose media. Approximately 100 cells were plated on these media and cultured at 25 °C or 30 °C for 5 days. The ability to form true hyphae and pseudohyphae was investigated on both solid cornmeal (CM) and yeast extract-malt extract (YM; 0.3 % yeast extract, 0.5 % peptone, 1 % glucose, 0.3 % malt extract, pH 5.0) mediums.

For stress-resistance assay, YPD solid media containing various concentrations of NaCl or SDS were used. Each strain was adjusted to 2.5×10^7 cells/mL, and then 10-fold serial dilutions of cells were spotted onto different plates, with each spot containing initial cell counts: 5×10^4, 5×10^3, 500, 50, and 5.

2.3. Phylogenetic analyses

The MEGA v7 software was used to obtain consensus sequences [18], and MAFFT online v7 was used to align different locus [19]. Maximum-Likelihood (ML) and Bayesian Inference (BI) methods were used to conduct phylogenetic analyses through the CIPRES Science Gateway portal [20].

The ML analyses were conducted under the GTRGAMMA + I model and 1,000 replicated in RAxML-HPC BlackBox v8.2.12 [21]. The Bayesian analyses were performed using MrBayes v3.2.7a [22,23], incorporating the best evolutionary models for each gene as determined by MrModelTest v2.4 [24]. Four simultaneous Markov Chain Monte Carlo chains were used for Bayesian analyses computation, for the first dataset, running for 10 million generations with a sampling frequency of every 1,000 generations, and the sampling frequency was set to 100 generations for the other two datasets.

A clade is considered supported if the RAxML Bootstrap support value was ≥70 % and the Bayesian PP value was ≥0.95. The resulting trees were visualized using FigTree v1.4.2. All sequences generated in this study were deposited in GenBank (Table S1), and alignments used were uploaded in TreeBASE (submission ID 31589).

2.4. Whole genome sequencing and analysis

Single colonies of *Clavispora sputum* strains were suspended into 20 mL YPD liquid medium and incubated at 30 °C and 200 rpm in a shaking incubator for 24 h. *Cl. sputum* cells were collected and washed with ddH_2O, then TIANamp Yeast DNA Kit (TianGen Biotech, Beijing, China) was used to extract the genomic DNA. Single-molecule real-time (SMRT) sequencing was performed by Beijing Novogene Bioinformatics Technology Co., Ltd. using the PacBio Sequel SMRT sequencing system (Pacific Biosciences, Menlo Park, CA, USA). The sequence data generated from the DNBseq platform were used to correct and refine the PacBio assembly sequence. After error-correcting and trimming, the PacBio data were assembled and scaffolded using Canu v1.8 software, guided by an estimated genome size of 12.5 Mb [25]. The quality of the sixteen newly assembled genomes was assessed using Quast v5.0.2 [26]. Genome synteny analysis was performed with Mauve 10c [27]. Protein coding genes were predicted using AUGUSTUS v3.2.1 [28]. Putative open reading frames (ORFs) were searched against the NR database of NCBI and the *Candida* genome database. All genomic raw data were deposited in NCBI BioProject PRJNA1133981 (accession no. SRR29767006 and no. SRR29767007).

2.5. Phylogenomic tree and gene family analysis

Gene family analyses were conducted across *Cl. sputum* and 12 other species within the subphylum *Saccharomycotina*. The assembly and protein sequences for all species were achieved from the NCBI, including *Candida albicans* (GCA_000182965), *Candida auris* (GCA_002759435), *Candida tropicalis* (GCA_000006335), *Candida glabrata* (GCA_000002545), *Candida parapsilosis* (GCA_000182765), *Candida dubliniensis* (GCA_000026945), *Candida orthopsilosis* (GCA_000315875), *Clavispora lusitaniae* (GCA_000003835), *Candida haemulonii* (GCA_002926055), *Candida pseudohaemulonii* (GCA_003013735), *Candida duobushaemuli* (GCA_002926085), and *Saccharomyces cerevisiae* (GCA_002057635). Protein orthogroups and orthologs were analyzed using OrthoFinder v2.5.5 [29]. Phylogenomic tree and specific protein tree were constructed using Raxml-ng v1.2.1 [30], with the best model identified using Modeltest-ng v0.1.7 [31].

To compare gene families bewteen *Cl. sputum* and *Cl. lusitaniae*, the protein sequences of *Cl. sputum* and the other 12 species were searched for PFAM domains using InterProScan v5.31 [32]. The protein counts for different gene families were used to construct a clustered heat map using pheatmap package in R.

2.6. Antifungal susceptibility testing

Testing to nine antifungal drugs was performed according to the NCCLS document M27 [33] and a previous investigation [34]. Approximately 1000 cells of each strain were suspended in 200 μL of liquid RPMI 1640 medium in a 96-well U-bottom microplate and incubated at 35 °C for 24 h. Three biological replicates were performed. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 served as quality control strains.

2.7. Animal experiments

All animal experiments were performed according to the guidelines approved by the Fudan University Animal Care and Use Committe. Four female BALB/c mice (6-week-old) were used for systemic infection with

each strain. The mice were housed in a P2 laboratory for 7 days of acclimation before infection. Each strain was injected into the lateral tail vein of the mice at a dose of 2×10^7 cells in 250 μL 1 × PBS. The mice were euthanised after 24 h post-infection. Organs were collected, weighed, ground, and plated on YPD solid medium with chloramphenicol (final concentration, 34 μg/mL) for fungal burden detection.

3. Results

3.1. Case report

A 73-year-old female patient was admitted to a pulmonary hospital (Shanghai, China) due to COVID-19 infection and severe pneumonia. Before admission, the patient tested positive for COVID-19 persisted for as long as 18 days and had a fever over 40 °C along with a cough producing with white sputum. Chest computed tomography (CT) examination revealed multiple ground-glass nodular shadows in both lungs. In addition, the patient had both hypertension and nephrotic syndrome. Considering her severe pneumonia caused by Gram-negative bacilli, she was treated with 500 mg nemonoxiacin per day by intravenous injection combined with oxygen therapy. After 3 days of treatment, the patient tested negative for COVID-19. Her sputum samples were collected and subcultured onto CHROMagar *Candida* Plus culture medium. Three yeast-like strains which formed light pink colonies were isolated from the medium. Preliminary analysis of ITS sequence indicated that the strains belonged to the *Clavispora* genus. After a total of 7 days of anti-infective treatment, the patient got better and was discharged.

3.2. Clavispora sputum is phylogenetically closely related to Clavispora lusitaniae and Candida auris

A high-confidence phylogenetic tree was constructed using 45 strains from 37 species, with *Hyphopichia wangnamkhiaoensis* (CBS 11695) as an outgroup (Fig. 1). The multi-locus alignment covered 942 bases (including gaps) across both gene regions. The optimal nucleotide substitution model for the ITS was GTR + I + G, whereas SYM + I + G was chosen for the LSU. The topology of multi-locus phylogenetic trees obtained from ML and BI analyses was identical. Our analysis indicated that the three strains isolated in this study formed a strongly supported genealogically distinct clade, which belongs to the *Clavispora s.str.* clade but cannot be assigned to any known species. Moreover, this new species exhibited a close relationship with several MDR (Multi-drug resistance)

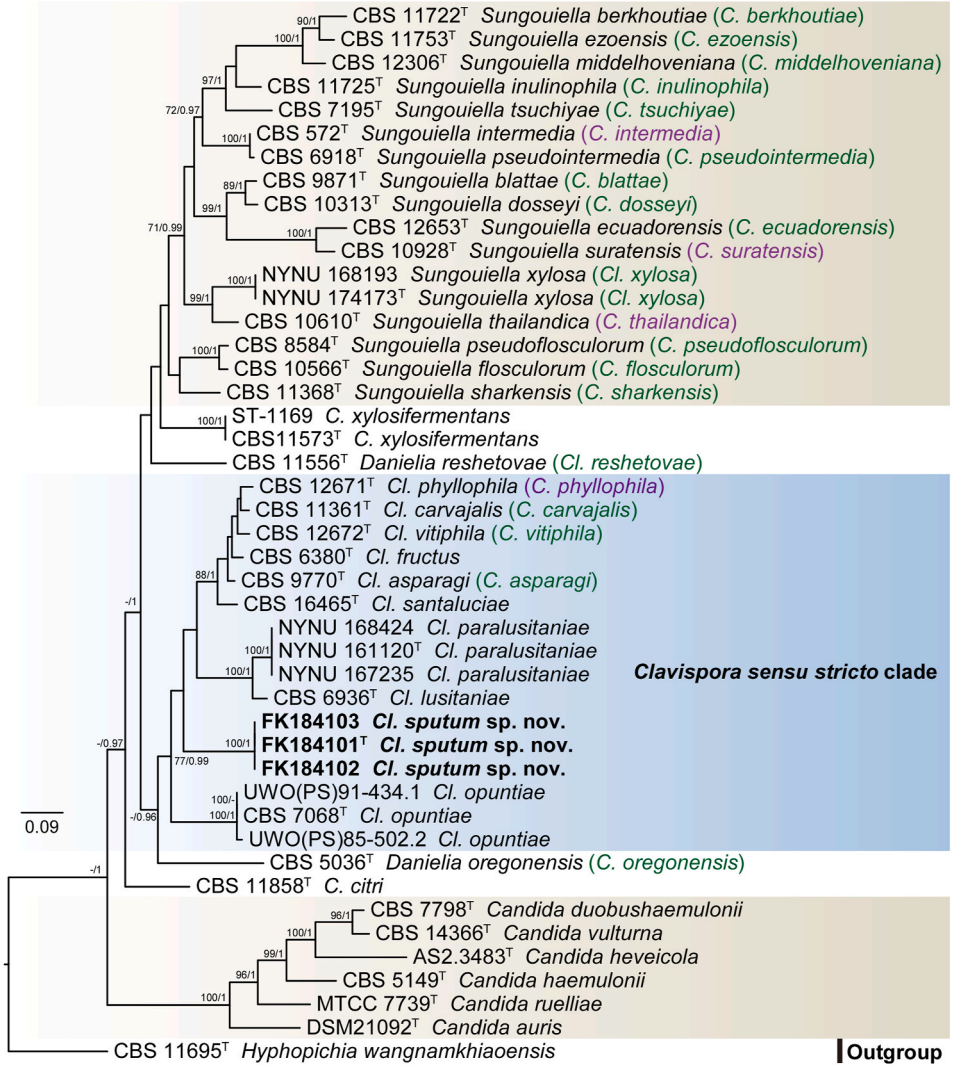

Fig. 1. Phylogenic tree of species from *Clavispora* and allied genera based on combined ITS-LSU regions. The phylogenic tree was constructed using combined ITS and LSU ribosomal RNA gene regions. *Hyphopichia wangnamkhiaoensis* (CBS 11695) was used as the outgroup. The RAxML Bootstrap support values (ML-BS ≥ 70 %) and Bayesian posterior probabilities (BI-PP ≥ 0.95) are displayed at the nodes (ML-BS/BI-PP). Type strains are indicated with a superscript "T". Strains of *Cl. sputum* isolated in this study are highlighted in bold. Basionyms are highlighted in green, while synonyms are highlighted in purple. *Cl.*, *Clavispora*; *C.*, *Candida*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fungal pathogens, including *Cl. lusitaniae* and *C. auris* (Fig. 1).

3.3. Taxonomy

Clavispora sputum S.L. Han, L. Cai, Q.S. Zheng & G.H. Huang *sp. nov*. Fungal name. FN 571973; Fig. 2.

Etymology. Sputum (Lat., noun) – spittle; named after the substrate, sputum, from which the fungus was isolated.

Material examined. China, Shanghai Municipality (E121.5, N31.3), from human sputum samples, 20 Oct. 2023, *Q.S. Zheng*, CGMCC 2.8528 (= FK184101, holotype designated here, preserved in a metabolically inactive state); *ibid.*, FK184102; *ibid.*, FK184103.

Description. On YM medium after 5 days at 25 °C or 30 °C, colonies are white, soft, and round. Cells are ovoid to ellipsoidal (1.5–5.1 × 1.7–6.2 μm in diameter, Fig. 2A). On CM medium after 5 days at 25 °C or 30 °C, colonies are creamy-white, soft, and round. Yeast-form cells are ellipsoidal (1.6–5.2 × 2.6–6.2 μm in diameter) and occur singly. Pseudohyphae-like cells are formed on CM medium after 5 days at 25 °C, but no filamentous cells are observed at 30 °C (Fig. 2B). Growth is not observed both on YM and CM media at 37 °C. In addition, *Cl. sputum* grew well on standard media used for culturing other pathogenic *Candida* species, such as YPD, YP, and Lee's glucose media (Fig. S1B). On YPD medium after 5 days at 25 °C and 30 °C, *Cl. sputum* cells appeared ellipsoidal (1.8-3.8 × 2.7–4.7 μm in diameter). On YP medium (in the absence of glucose, Fig. S1B), a portion of *Cl. sputum* cells were elongated (2.6–3.0 × 9.7–12.0 μm in diameter) when cultured at 25 °C.

Note. *Cl. sputum* shows at least 14 % divergence in the LSU D1/D2 domains and 24 % divergence in the ITS regions compared to other *Clavispora* species. Morphologically, *Cl. sputum* formed pastel yellow colonies on CHROMagar *Candida* Plus medium, similar to those produced by the *C. auris* strain CBS12372 at 30 °C (Fig. S1A). In contrast, *Cl. lusitaniae* formed blue colonies on the same medium. Furthermore, this novel lineage cannot grow at 37 °C compared with *Cl. lusitaniae* and *C. auris*.

3.4. Genomic characteristics of Clavispora sputum

To explore the genomic features of *Cl. Sputum,* we performed deep whole-genome sequencing (WGS) and generated a complete genome assembly for *Cl. sputum* strain FK184101. A detailed comparative description of the genomic sequences of *Cl. sputum* and *Cl. lusitaniae* P1 (ASM949805v1) is presented in Table 1. The size of the *Cl. sputum* genome was 11.59 Mb, consisting of 8 chromosomes, with the largest chromosome being 2.16 Mb. A total of 5,504 genes and 890 introns were predicted in *Cl. sputum*. The G + C content of *Cl. sputum* was 49.11 %, which is much higher than that of *Cl. lusitaniae*. Genome-wide synteny analysis implied that there were many large chromosomal rearrangements between strains *Cl. sputum* (FK184101) and *Cl. lusitaniae* (P1). About half of the orthologous LCBs were found in the reverse orientation (Fig. S2).

3.5. Analysis of virulence-associated genes in Cl. sputum based on genomic sequences

Since *Cl. Sputum* exhibited a close relationship with MDR fungal pathogens, we next compared the virulence-associated factors across these species. The closely phylogenetic relationship among *Cl. sputum*, *Cl. lusitaniae* and *C. auris* was further supported by phylogenomic analysis using a concatenated alignment of 2184 single-copy core orthologous protein sequences (Fig. S3A). *Cl. sputum* possessed all the common virulence factors previously identified in pathogenic *Candida/Clavispora* species (Fig. S3B).

Similar to *Cl. lusitaniae*, *Cl. sputum* has fewer proteins in several gene families, including ferric reductases, secretory lipase, cytochrome P450s, OPT transporters, compared to other *Candida/Clavispora* species. Notably, *Cl. sputum* had the fewest copies of aspartyl protease (8 proteins) and lysophospholipase (3 proteins). Interestingly, *Cl. sputum* had more genes encoding EPA family proteins (19 proteins) compared to *Cl. lusitaniae*. EPA proteins are often associated with adhesion in pathogenic fungi, and *C. glabrata* has the largest number of EPA proteins (27) [35, 36]. Both *Cl. sputum* and *Cl. lusitaniae* contained four proteins with the Flo11 domain, which is essential for surface adhesion, invasion, and pseudohyphae development [37]. This is significantly more than 0–2 proteins found in other analyzed yeast species. These findings suggest that *Cl. sputum* could be potentially be a human fungal pathogen.

3.6. Growth of Cl. sputum under NaCl and SDS stresses

Interestingly, we found that the optimal temperature for *Cl. sputum* was 30 °C, and it was unable to grow at 37 °C, the physiological temperature of humans, under regular culture conditions. Since *Cl. sputum* was initially isolated from sputum samples, we next investigated how salt stresses affected its growth. As shown in Fig. 3, although *Cl. sputum* did not grow at 37 °C on YPD medium, it did grow at 37 °C on the same medium when supplemented with 0.5 M or 1 M NaCl, with a particularly noticeable growth enhancement at 0.5 M NaCl. We then assessed whether SDS, which induces osmotic stress similar to NaCl, affected *Cl. sputum* growth. Unlike NaCl, SDS did not promoted *Cl. sputum* growth at high temperatures and actually suppressed cell growth at 25 °C (Fig. 3). Notably, *Cl. lusitaniae* and *C. auris* exhibited different growth patterns under these conditions.

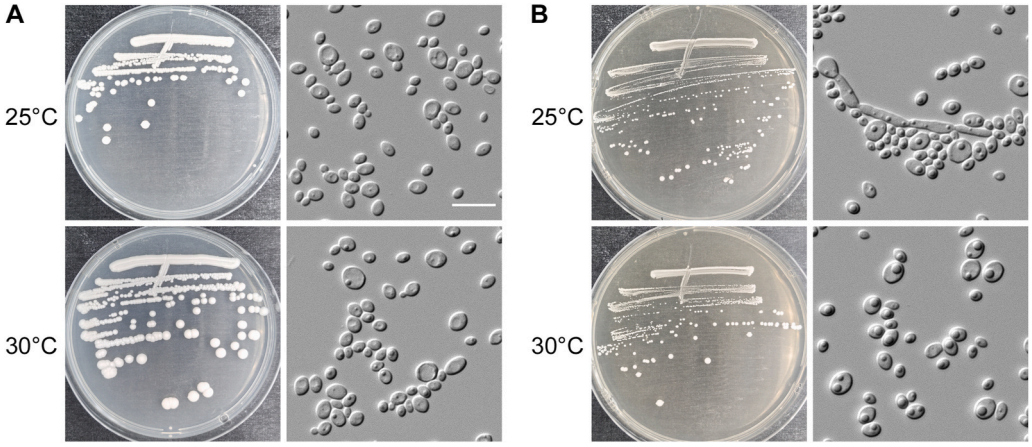

Fig. 2. Colony and cellular morphologies of *Cl. sputum* on YM and CM media. *Cl. sputum* cells were initially grown on YPD medium at 30 °C for 2 days. Cells were then streaked onto YM medium agar (A) or CM medium agar (B) and incubated at 25 °C or 30 °C for 5 days. Scale bar for cells, 10 μm.

Table 1
Genome properties for *Clavispora sputum* and *Clavispora lusitaniae*.

Species	*Clavispora sputum*	*Clavispora lusitaniae*				
Isolates	FK184101	P1	P2	P3	P4	P5
Total assembly size (Mb)	11.59	12.08	12.09	12.10	12.10	12.09
Chromosome counts	8	8	8	8	8	8
Largest chromosome (Mb)	2.16	2.45	2.45	2.45	2.45	2.45
N50 (Mb)	1.82	2.01	2.01	2.01	2.01	2.01
N90 (Mb)	0.77	0.74	0.74	0.74	0.74	0.74
GC%	49.11	44.54	44.54	44.54	44.54	44.54
Gene counts (bp)	5,504	5,882	5,886	5,869	5,883	5,892
Ave. gene length (bp)	1539.6	1414.9	1416.5	1414.4	1416.7	1415.4
Intron counts (bp)	890	231	229	229	229	228
Ave. intron size (bp)	157.3	166.2	167.3	167.3	167.3	167.2
Ave. intergenic space distance (bp)	556.5	658.0	658.1	664.7	657.2	658.3

Assembly accessions: ASM949805v1 (P1), ASM949807v1 (P2), ASM949809v1 (P3), ASM949813v1 (P4), ASM949811v1 (P1).

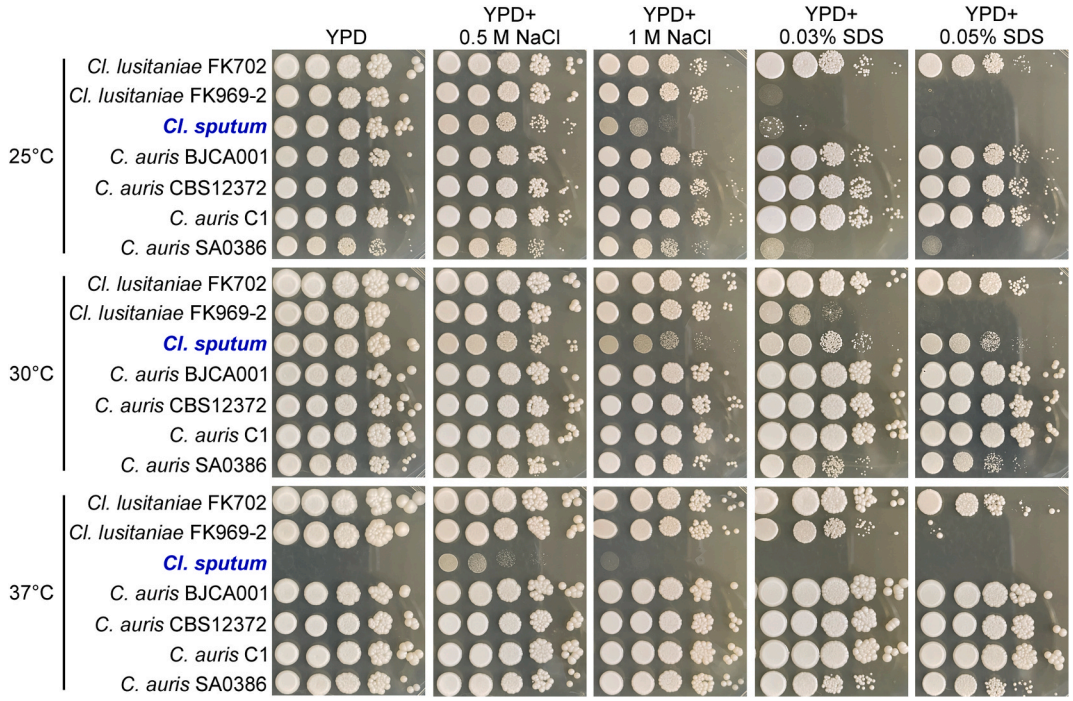

Fig. 3. Growth of *Cl. sputum*, *Cl. lusitaniae*, and *C. auris* on different media in the absence or presence of NaCl and SDS. *Cl. lusitaniae* (strains FK702 and FK969-2), *Cl. Sputum* (strain FK184101), and *C. auris* (strains BJCA001, CBS12372, C1, and SA0386) were used. Cells of each strains were initially grown on YPD medium for 2 days at 30 °C. Fungal cells were then collected, and adjusted to serial dilutions (5 x 10^4, 5 x 10^3, 500, 50, or 5 cells in 2 μL ddH_2O), and spotted onto YPD medium or YPD medium containing NaCl (0.5 M or 1 M) or SDS (0.03 % and 0.05 %). Plates were incubated at 25 °C, 30 °C, or 37 °C for 2 days.

Table 2
Antifungal susceptibility testing of *Cl. sputum*, *Cl. lusitaniae*, and *C. auris*.

	FLC	POC	ITC	VOC	AmB	CAS	AFG	MFG	5-FC
Clavispora sputum	2	0.5	0.5	0.03125	1	0.5	0.5	0.5	0.008
Clvaspora lusitaniae FK702	0.25	0.5	0.25	<0.008	2	0.5	0.5	0.5	0.02
Clvaspora lusitaniae FK969-2	0.5	0.5	0.25	<0.008	1	0.5	0.25	0.5	0.02
Candida auris BJCA001	>32	0.5	1	1	2	0.25	0.125	0.5	0.0625
Candida auris CBS12373	>32	0.5	1	0.5	1	0.0625	0.0625	0.125	0.125
Candida auris C1	>32	0.5	0.5	0.5	2	0.5	1	1	0.125
Candida auris SA0386	>32	0.5	1	4	4	0.5	0.5	1	0.125

FLC, fluconazole; POC, posaconazole; ITC, itraconazole; VOC, voriconazole; AmB, amphotericin B; CAS, caspofungin; AFG, anidulafungin; MFG, micafungin; 5-FC, 5-fluorocytosine. Minimal inhibitory concentration (MIC, μg/mL) were shown in table.

3.7. Antifungal susceptibilities of Clavispora sputum

Given that *Cl. sputum* is closely related to drug-resistant species, such as *Cl. lusitaniae* and *C. auris*, we performed antifungal susceptibility assays on *Cl. sputum* using the CLSI method. We tested the minimum inhibitory concentrations (MICs) of nine antifungals: fluconazole (FLC), posaconazole (POC), itraconazole (ITC); voriconazole (VOC), amphotericin B (AmB), caspofungin (CAS), anidulafungin (AFG), micafungin (MFG), and 5-fluorocytosine (5-FC). *Cl. lusitaniae* and *C. auris* strains served as reference strains. As shown in Table 2, the MIC values for FLC in *Cl. sputum* were 4–8 folds higher than those of *Cl. lusitaniae* but much lower than those for *C. auris*. All three species exhibited low MICs for POC, ITC, and VOC. The *Cl. sputum* strains showed higher MIC values for CAS and AFG and a lower MIC value for 5-FC compared to *C. auris*. In addition, all strains had similar MIC values for AmB (1–2 μg/mL) and MFG (0.125–0.5 μg/mL). These findings indicate that *Cl. sputum* exhibits a generally similar antifungal susceptibility pattern to *Cl. lusitaniae* and *C. auris*.

3.8. Clavispora sputum exhibits spleen and lung tropism during systemic infections

We next evaluated the virulence of *Cl. sputum* using a mouse systemic infection model, with *Cl. lusitaniae* and *C. auris* strains serving as references. As shown in Fig. 4, approximately 2×10^7 cells of each strain were injected into the mice via the tail vein. After 24 h of infection, the fungal burdens of *Cl. sputum* were significantly lower than those of *Cl. lusitaniae* and *C. auris* in the animal tissues. However, the tissue tropisms differed between *Cl. sputum* (spleen > lung > liver > kidney > brain) and *Cl. lusitaniae* or *C. auris* (spleen > liver > kidney > lung > brain). *Cl. sputum* cells showed a notable tropism for the lung and spleen. Given that *Cl. sputum* was original isolation from sputum samples, further investigation is needed to determine whether this lung tropism in humans is related to its tissue tropism.

4. Discussion

The emergence of pathogenic fungi represents a significant public health threat [38]. A recent example is the global rise of the multidrug-resistant superbug fungus *C. auris* [9]. In this study, we report the isolation and identification of a novel species, *Clavispora sputum*, from sputum samples. *Cl. sputum* is phylogenetically closely related to the opportunistic fungal pathogens *Cl. lusitaniae* and *C. auris*. The multidrug-resistant feature of these fungi often results in treatment failures. The COVID-19 pandemic has exacerbated the risk for fungal infections, further complicating the situation [39]. Notably, we isolated *Cl. sputum* strains from a patient who was positive for COVID-19. Viral lung infections or damages can create environments conducive to the colonization of emerging pathogenic fungi, increasing the risk of fungal infections, particularly in critically ill patients.

Cl. lusitaniae, originally assigned to the teleomorphic state of *Candida lusitaniae,* belongs to the genus *Candida/Clavispora* within the family *Metschnikowiaceae* and the order *Saccharomycetales* [40]. Recent multi-locus phylogenetic analyses have shown that some species within the *Clavispora* clade are also conspecific with names previously classified under the anamorph-typified genus *Candida* [41]. The 'one fungus, one

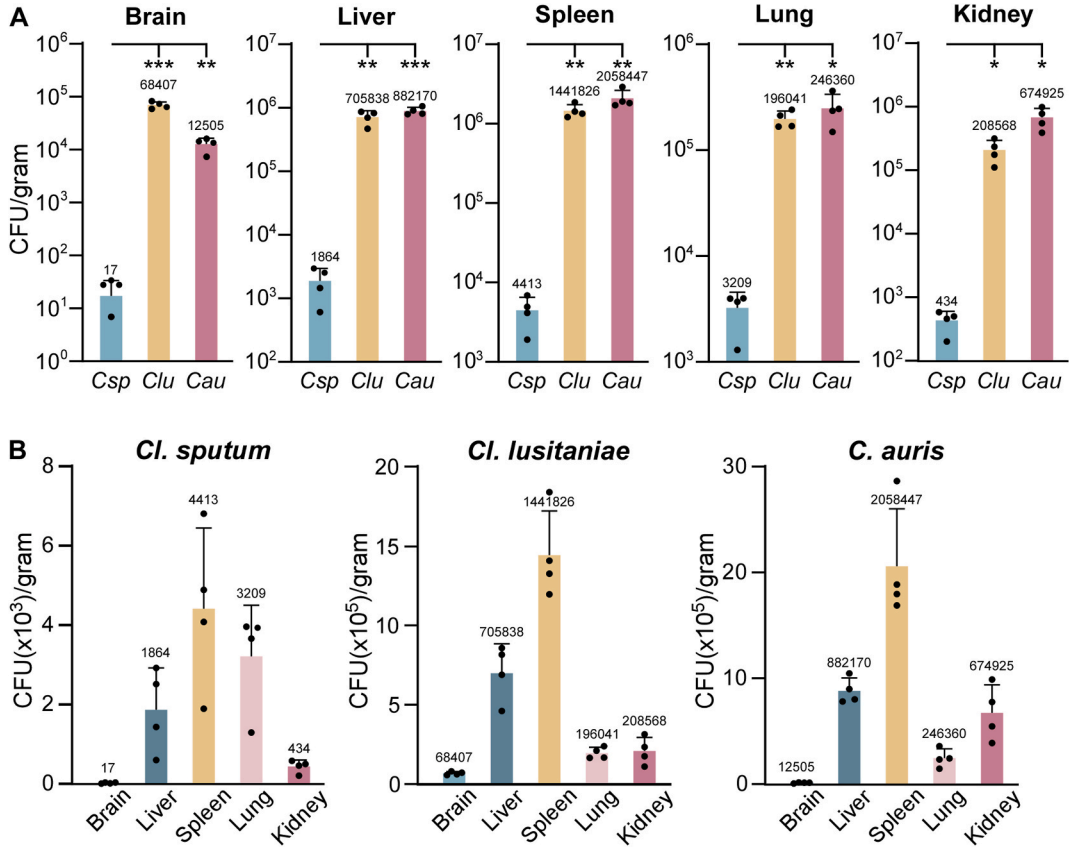

Fig. 4. Fungal burdens of *Cl. sputum*, *Cl. lusitaniae*, and *C. auris* in a mouse systemic infection model. *Cl. sputum* (strain FK184101), *Cl. lusitaniae* (strain FK702), and *C. auris* (strain BJCA001) were tested. Approximately 2×10^7 fungal cells of each strain were injected via the tail vein into four mice per strain. Mice were euthanised and fungal burdens in the indicated five organs were analyzed after 24 h of infection. (A) Fungal burdens of the three species in different organs. Statistical significance between *Cl. sputum* and the other two species is indicted (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t*-test, two-tailed). (B) Comparison of fungal burdens of each species in different organs. Black dots represent CFU data points for each mouse, and the numbers indicate the mean values of the four CFUs. Error bars represent standard deviations.

name' scheme presents challenges for species traditionally classified within these two genera [42–46]. Recently, Liu et al. performed phylogenomic and comparative genomic analyses of this group, redefining *Clavispora* in a narrower sense to include only the *Clavispora s.str.* clade, which currently contains nine species: *Cl. asparagi*, *Cl. carvajalis*, *Cl. fructus*, *Cl. lusitaniae*, *Cl. opuntiae*, *Cl. paralusitaniae*, *Cl. phyllophila*, *Cl. santaluciae* and *Cl. vitiphila* [47]. Meanwhile *Cl. Xylosa* [46] and *Cl. reshetovae* [48] were transferred to the newly established genera *Sungouiella* and *Danielia*, respectively [47]. Based on the close phylogenetic relationship between *Cl. lusitaniae* and the novel species identified in this study, as well as the original isolation sources, we have named this novel species *Clavispora sputum*.

Antifungal susceptibility assays revealed that *Cl. sputum* exhibits similar MICs for nine antifungal drugs compared to the *Cl. lusitaniae* strain (Table 2). In contrast to *C. auris*, *Cl. sputum* was generally susceptible to all tested antifungals. It is noteworthy that some clinical isolates of *C. auris* are also susceptible to certain antifungal drugs, such as azoles and AmB [49]. Antifungal resistance can evolve due to exposure of fungal cells to antifungal drugs, and rapid development of resistance has been observed in both *Cl. lusitaniae* and *C. auris* [34,50]. Therefore, it is crucial to monitor the emergence of antifungal resistance in *Cl. sputum* in future studies.

Morphological analysis indicates that *Cl. sputum* cells are similar to those of *Cl. lusitaniae* and *C. auris* (Fig. S1). However, *Cl. sputum* was unable to grow at 37 °C under standard *in vitro* culture conditions. The addition of a moderate level of NaCl to the medium promoted *Cl. sputum* cell growth at 37 °C, possibly mimicking the conditions of sputum samples. Genomic sequencing analysis reveals that *Cl. sputum* has an increased number of genes encoding EPA family-associated proteins compared to *Cl. lusitaniae* and *C. auris*. Fungal burden assays show that *Cl. Sputum* cells exhibit a tissue tropism for lung and spleen tissues, a characteristic not observed in *Cl. lusitaniae* and *C. auris* (Fig. 4). Taken together, our findings suggest that *Cl. sputum* possesses key features of human fungal pathogens in terms of virulence genes and tissues colonization during systemic infections.

In summary, *Cl. sputum* may represent a novel potential fungal pathogen of humans. Given its close phylogenetic relationship with *Cl. lusitaniae* and *C. auris*, future surveillance of *Cl. sputum* in clinical settings will be essential. Over the past two decades, at least six novel species within the *Candida/Clavispora* clade have been identified in clinical settings [5,6,8,51–53]. The underlying reasons for the emergence of species within this clade should also be investigated in future research.

CRediT authorship contribution statement

Qiushi Zheng: Writing – original draft, Visualization, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Jian Bing:** Visualization, Software, Funding acquisition, Formal analysis, Data curation. **Shiling Han:** Writing – original draft, Software, Formal analysis, Data curation. **Shuyun Guan:** Methodology, Investigation. **Tianren Hu:** Resources, Investigation. **Lei Cai:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Haiqing Chu:** Writing – review & editing, Supervision, Project administration. **Guanghua Huang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability

All raw data of genomic sequences generated in this study have been deposited into the NCBI Bioproject PRJNA1133981. The SRA files can be found uner accession on. SRR29767006 and no. SRR29767007.

Ethics statement

All animal experiments were performed according to the guidelines approved by the Animal Care and Use Committee of Fudan University. Deidentified health records were analyzed with approval from the hospital ethics committee.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2024.101506.

References

[1] Wiederhold NP. Emerging fungal infections: new species, new names, and antifungal resistance. Clin Chem 2021;68(1):83–90. https://doi.org/10.1093/clinchem/hvab217.
[2] Revie NM, Iyer KR, Robbins N, Cowen LE. Antifungal drug resistance: evolution, mechanisms and impact. Curr Opin Microbiol 2018;45:70–6. https://doi.org/10.1016/j.mib.2018.02.005.
[3] Garcia-Bustos V, Cabañero-Navalon MD, Ruiz-Gaitán A, Salavert M, Tormo-Mas M, Pemán J. Climate change, animals, and Candida auris: insights into the ecological niche of a new species from a One Health approach. Clin Microbiol Infect 2023;29(7):858–62. https://doi.org/10.1016/j.cmi.2023.03.016.
[4] Casadevall A, Kontoyiannis DP, Robert V. Environmental Candida auris and the global warming emergence hypothesis. mBio 2021;12(2). https://doi.org/10.1128/mBio.00360-21.
[5] Sugita T, Takashima M, Poonwan N, Mekha N. Candida pseudohaemulonii Sp. Nov., an amphotericin B-and azole-resistant yeast species, isolated from the blood of a patient from Thailand. Microbiol Immunol 2006;50(6):469–73. https://doi.org/10.1111/j.1348-0421.2006.tb03816.x.
[6] Sipiczki M, Tap RM. Candida vulturna pro tempore sp. nov., a dimorphic yeast species related to the Candida haemulonis species complex isolated from flowers and clinical sample. Int J Syst Evol Microbiol 2016;66(10):4009–15. https://doi.org/10.1099/ijsem.0.001302.
[7] Du H, Bing J, Xu X, Zheng Q, Hu T, Hao Y, et al. Candida vulturna outbreak caused by cluster of multidrug-resistant strains, China. Emerg Infect Dis 2023;29(7):1425–8. https://doi.org/10.3201/eid2907.230254.
[8] Satoh K, Makimura K, Hasumi Y, Nishiyama Y, Uchida K, Yamaguchi H. Candida auris sp. nov., a novel ascomycetous yeast isolated from the external ear canal of an inpatient in a Japanese hospital. Microbiol Immunol 2009;53(1):41–4. https://doi.org/10.1111/j.1348-0421.2008.00083.x.
[9] Du H, Bing J, Hu T, Ennis CL, Nobile CJ, Huang G. Candida auris: epidemiology, biology, antifungal resistance, and virulence. PLoS Pathog 2020;16(10):e1008921. https://doi.org/10.1371/journal.ppat.1008921.
[10] Spruijtenburg B, Badali H, Abastabar M, Mirhendi H, Khodavaisy S, Sharifisooraki J, et al. Confirmation of fifth Candida auris clade by whole genome sequencing. Emerg Microb Infect 2022;11(1):2405–11. https://doi.org/10.1080/22221751.2022.2125349.
[11] Suphavilai C, Ko KKK, Lim KM, Tan MG, Boonsimma P, Chu JJK, et al. Discovery of the sixth Candida auris clade in Singapore. Infectious Diseases (except HIV/AIDS) 2023. https://doi.org/10.1101/2023.08.01.23293435 [Internet].
[12] Cendejas-Bueno E, Kolecka A, Alastruey-Izquierdo A, Theelen B, Groenewald M, Kostrzewa M, et al. Reclassification of the Candida haemulonii complex as Candida haemulonii (C. haemulonii group I), C. duobushaemulonii sp. nov. (C. haemulonii group II), and C. haemulonii var. vulnera var. nov.: three multiresistant human pathogenic yeasts. J Clin Microbiol 2012;50(11):3641–51. https://doi.org/10.1128/jcm.02248-12.
[13] Gómez-Gaviria M, Martínez-Álvarez JA, Chávez-Santiago JO, Mora-Montes HM. Candida haemulonii complex and Candida auris: biology, virulence factors, immune response, and multidrug resistance. Infect Drug Resist 2023;16:1455–70. https://doi.org/10.2147/idr.S402754.
[14] Françoise U, Desnos-Ollivier M, Le Govic Y, Sitbon K, Valentino R, Peugny S, et al. Candida haemulonii complex, an emerging threat from tropical regions? PLoS Neglected Trop Dis 2023;17(7):e0011453. https://doi.org/10.1371/journal.pntd.0011453.

[15] Deng Y, Li S, Bing J, Liao W, Tao L. Phenotypic switching and filamentation in Candida haemulonii, an emerging opportunistic pathogen of humans. Microbiol Spectr 2021;9(3):e0077921. https://doi.org/10.1128/Spectrum.00779-21.
[16] Mendoza-Reyes DF, Gómez-Gaviria M, Mora-Montes HM. Candida lusitaniae: biology, pathogenicity, virulence factors, diagnosis, and treatment. Infect Drug Resist 2022;15:5121–35. https://doi.org/10.2147/idr.S383785.
[17] Wang F, Wang K, Cai L, Zhao M, Kirk PM, Fan G, et al. Fungal names: a comprehensive nomenclatural repository and knowledge base for fungal taxonomy. Nucleic Acids Res 2023;51(D1):D708–d716. https://doi.org/10.1093/nar/gkac926.
[18] Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016;33(7):1870–4. https://doi.org/10.1093/molbev/msw054.
[19] Katoh K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings Bioinf 2019;20(4):1160–6. https://doi.org/10.1093/bib/bbx108.
[20] Miller MA, Pfeiffer W, Schwartz T. Creating the CIPRES science gateway for inference of large phylogenetic trees. In: 2010 Gateway computing environments workshop (GCE). New Orleans, LA, USA: IEEE; 2010. p. 1–8. https://doi.org/10.1109/GCE.2010.5676129 [Internet].
[21] Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 2014;30(9):1312–3. https://doi.org/10.1093/bioinformatics/btu033.
[22] Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003;19(12):1572–4. https://doi.org/10.1093/bioinformatics/btg180.
[23] Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 2012;61(3):539–42. https://doi.org/10.1093/sysbio/sys029.
[24] Nylander JA, Wilgenbusch JC, Warren DL, Swofford DL. AWTY (are we there yet?): a system for graphical exploration of MCMC convergence in Bayesian phylogenetics. Bioinformatics 2008;24(4):581–3. https://doi.org/10.1093/bioinformatics/btm388.
[25] Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res 2017;27(5):722–36. https://doi.org/10.1101/gr.215087.116.
[26] Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. Bioinformatics 2013;29(8):1072–5. https://doi.org/10.1093/bioinformatics/btt086.
[27] Darling AC, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res 2004;14(7):1394–403. https://doi.org/10.1101/gr.2289704.
[28] Stanke M, Steinkamp R, Waack S, Morgenstern B. AUGUSTUS: a web server for gene finding in eukaryotes. Nucleic Acids Res 2004;32(Web Server issue):W309–12. https://doi.org/10.1093/nar/gkh379.
[29] Emms DM, Kelly S. OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol 2019;20(1):238. https://doi.org/10.1186/s13059-019-1832-y.
[30] Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics 2019;35(21):4453–5. https://doi.org/10.1093/bioinformatics/btz305.
[31] Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T. ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. Mol Biol Evol 2020;37(1):291–4. https://doi.org/10.1093/molbev/msz189.
[32] Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, et al. InterProScan 5: genome-scale protein function classification. Bioinformatics 2014;30(9):1236–40. https://doi.org/10.1093/bioinformatics/btu031.
[33] NCCLS. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2. second ed. Villanova, PA: NCCLS; 2017.
[34] Bing J, Hu T, Zheng Q, Muñoz JF, Cuomo CA, Huang G. Experimental evolution identifies adaptive aneuploidy as a mechanism of fluconazole resistance in Candida auris. Antimicrob Agents Chemother 2020;65(1). https://doi.org/10.1128/aac.01466-20.
[35] Sundstrom P. Adhesion in Candida spp. Cell Microbiol 2002;4(8):461–9. https://doi.org/10.1046/j.1462-5822.2002.00206.x.
[36] Valotteau C, Prystopiuk V, Cormack BP, Dufrêne YF. Atomic force microscopy demonstrates that Candida glabrata uses three epa proteins to mediate adhesion to abiotic surfaces. mSphere 2019;4(3). https://doi.org/10.1128/mSphere.00277-19.
[37] Bouyx C, Schiavone M, François JM. FLO11, a developmental gene conferring impressive adaptive plasticity to the yeast Saccharomyces cerevisiae. Pathogens 2021;10(11). https://doi.org/10.3390/pathogens10111509.
[38] Lockhart SR, Chowdhary A, Gold JAW. The rapid emergence of antifungal-resistant human-pathogenic fungi. Nat Rev Microbiol 2023;21(12):818–32. https://doi.org/10.1038/s41579-023-00960-9.
[39] Baten N, Wajed S, Talukder A, Masum MHU, Rahman MM. Coinfection of fungi with SARS-CoV-2 is a detrimental health risk for COVID-19 patients. Beni Suef Univ J Basic Appl Sci 2022;11(1):64. https://doi.org/10.1186/s43088-022-00245-9.
[40] Rodrigues de Miranda L. Clavispora, a new yeast genus of the Saccharomycetales. Antonie Leeuwenhoek 1979;45(3):479–83. https://doi.org/10.1007/bf00443285.
[41] Limtong S, Kaewwichian R. Candida phyllophila sp. nov. and Candida vitiphila sp. nov., two novel yeast species from grape phylloplane in Thailand. J Gen Appl Microbiol 2013;59(3):191–7. https://doi.org/10.2323/jgam.59.191.
[42] Daniel HM, Lachance MA, Kurtzman CP. On the reclassification of species assigned to Candida and other anamorphic ascomycetous yeast genera based on phylogenetic circumscription. Antonie Leeuwenhoek 2014;106(1):67–84. https://doi.org/10.1007/s10482-014-0170-z.
[43] Kurtzman CP, Robnett CJ, Basehoar E, Ward TJ. Four new species of Metschnikowia and the transfer of seven Candida species to Metschnikowia and Clavispora as new combinations. Antonie Leeuwenhoek 2018;111(11):2017–35. https://doi.org/10.1007/s10482-018-1095-8.
[44] Kaewwichian R, Khunnamwong P, Am-In S, Jindamorakot S, Groenewald M, Limtong S. Candida xylosifermentans sp. nov., a d-xylose-fermenting yeast species isolated in Thailand. Int J Syst Evol Microbiol 2019;69(9):2674–80. https://doi.org/10.1099/ijsem.0.003505.
[45] Drumonde-Neves J, Čadež N, Reyes-Domínguez Y, Gallmetzer A, Schuller D, Lima T, et al. Clavispora santaluciae f.a., sp. nov., a novel ascomycetous yeast species isolated from grapes. Int J Syst Evol Microbiol 2020;70(12):6307–12. https://doi.org/10.1099/ijsem.0.004531.
[46] Chai CY, Li Y, Yan ZL, Hui FL. Phylogenetic and genomic analyses of two new species of Clavispora (Metschnikowiaceae, Saccharomycetales) from Central China. Front Microbiol 2022;13:1019599. https://doi.org/10.3389/fmicb.2022.1019599.
[47] Liu F, Hu ZD, Zhao XM, Zhao WN, Feng ZX, Yurkov A, et al. Phylogenomic analysis of the *Candida auris-Candida haemuli* clade and related taxa in the *Metschnikowiaceae*, and proposal of thirteen new genera, fifty-five new combinations and nine new species. Persoonia 2024;52:22–43. https://doi.org/10.3767/persoonia.2024.52.02.
[48] Yurkov A, Schäfer A, Begerow D. Clavispora reshetovae. Persoonia 2009;23:182–3.
[49] Wang X, Bing J, Zheng Q, Zhang F, Liu J, Yue H, et al. The first isolate of Candida auris in China: clinical and biological aspects. Emerg Microb Infect 2018;7(1):93. https://doi.org/10.1038/s41426-018-0095-0.
[50] Pappagianis D, Collins MS, Hector R, Remington J. Development of resistance to amphotericin B in Candida lusitaniae infecting a human. Antimicrob Agents Chemother 1979;16(2):123–6. https://doi.org/10.1128/aac.16.2.123.
[51] de Jong AW, Al-Obaid K, Mohd Tap R, Gerrits van den Ende B, Groenewald M, Joseph L, et al. Candida khanbhai sp. nov., a new clinically relevant yeast within the Candida haemulonii species complex. Med Mycol 2023;61(2). https://doi.org/10.1093/mmy/myad009.
[52] Kabtani J, Boulanouar F, Militello M, Cassagne C, Ranque S. Candida massiliensis sp. nov. Isolated from a Clinical Sample. Mycopathologia 2023;188(6):957–71. https://doi.org/10.1007/s11046-023-00792-4.
[53] Chaves GM, Terçarioli GR, Padovan AC, Rosas RC, Ferreira RC, Melo AS, Colombo AL. Candida mesorugosa sp. nov., a novel yeast species similar to Candida rugosa, isolated from a tertiary hospital in Brazil. Med Mycol 2013;51(3):231–42. https://doi.org/10.3109/13693786.2012.710345.