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ORIGINAL ARTICLE

Genotypic diversity of Iranian *Cryptococcus neoformans* using multilocus sequence typing (MLST) and susceptibility to antifungals

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Abstract

Cryptococcus species is an opportunistic yeast pathogen and classified into different molecular types according to typing techniques including multilocus sequence typing (MLST). The study aimed to investigate the genotypes of environmental *Cryptococcus* isolates using MLST and the relationship between the in vitro antifungal susceptibility and sequence types of isolates. Genotyping *Cryptococcus* isolates was performed by the MLST method at seven nuclear loci. Antifungal susceptibility was determined by using CLSI broth micro-dilution method for amphotericin B, fluconazole, itraconazole, voriconazole, flucytosine, and luliconazole. Seven sequence types (ST) were detected using MLST analysis, with the most frequent (50%) ST77, followed by ST4 (16.7%) among 30 *C. neoformans* isolates. All antifungals demonstrated excellent activity against isolates, except for itraconazole and amphotericin B that were non-wild type against 53.3% and 10% of isolates, respectively. Although seven sequence types belonging to *C. neoformans* isolates were detected, ST77 was the main sequence type in Ahvaz. Also, non-wild type isolates were only found against itraconazole and amphotericin B.

Keywords *Cryptococcus neoformans* · Multilocus sequence type · Antifungal susceptibility

Introduction

The genus *Cryptococcus* contains haploid encapsulated yeasts that belong to the division Basidiomycota, and they can cause life-threatening infections in immunocompromised individuals [1–3]. This genus was initially classified based on capsular epitopes and serological tests into four serotypes (A to D) [1], two species (*Cryptococcus neoformans* and *C. gattii*), and three varieties, including *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), *C. gattii* var. *gattii* (serotype B and C) [4]. According to the revised taxonomy in 2015, this genus has been now categorized into seven species include *C. neoformans*, *C. deneoformans*, *C. gattii*, *C. deuterogattii*, *C. bacillisporus*, *C. tetragattii*, and *C. decagattii* [5]. Out of them, *C. neoformans* (previously *C. neoformans* var. *grubii*) is the most common species and has a worldwide geographic distribution. It is frequently isolated from pigeon excreta, and less commonly from other sources such as soil, tree, and woody debris [6–8].

Since 2009, the multi-locus sequence typing (MLST) technique has been adopted by the International Society for Human and Animal Mycology (ISHAM) working group as a standard method for studying fungal genetic diversity, molecular epidemiology, and ancestral evolution. MLST and PCR-based typing techniques caused extensive and essential changes in the classification of *C. neoformans* [9], so that *C. neoformans* includes three molecular types VNI, VNII, and VNB for *C. neoformans* and VN IV for *C. deneoformans* [5, 10].

Both amphotericin B and 5-flucytosine (5FC) are considered as the gold standard for treating cryptococcal infections. In addition, azoles, especially fluconazole, are also used in the disease's maintenance and prevention phase [11]. However, resistance to azoles and 5FC has been reported both in vitro and in vivo studies, which can be due to clinical resistance and differences in molecular types [12, 13]. In the present study, we analyzed *C. neoformans* isolates by MLST using seven genetic loci and our genotypes' association with

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other countries' genotypes was evaluated. The antifungal susceptibility of isolates was evaluated against six antifungals. Due to the lack of information about *C. neoformans* sensitivity to luliconazole, its sensitivity results were compared with other routine drugs.

Materials and methods

Strains of *Cryptococcus neoformans*

In this study, 30 isolates of *C. neoformans* (*C. neoformans* var. *grubii*) with guano pigeon origin were investigated. All isolates were previously isolated using niger seed agar from pigeon droppings samples from private houses and pet-shops in different areas in Ahvaz (a city in the southwest of Iran, capital of Khuzestan province) [14]. Isolates were identified using molecular methods and sequences data deposited in GenBank (Accession numbers; LC535977-80, LC535983-90, LC535993-5, LC536010, LC536012-3, LC536015-6, LC537134, LC537136-9, LC537153, LC545844-5, LC536002, LC536004) [14]. All isolates were kept in distilled water at room conditions in the department of Medical Mycology, Ahvaz Jundishapur University of medical sciences.

DNA extraction

DNA extraction of isolates was performed by the described method of Makimura et al., with some modification [15]. Briefly, isolates were subcultured on Sabouraud dextrose agar (SDA) (Biolife, Italia) at 35 °C overnight. Yeast colonies were collected in the sterile microtubes contain 50 mg glass beads (Sigma—Aldrich, USA) and 300 µL lysis buffer and stored at − 20 °C for 24 h. Then microtubes contents were homogenized by a SpeedMill PLUS Homogenizer (Analytikjena, Germany). Supernatants were removed and DNAs extracted using phenol-chloroform-isoamyl alcohol (Sigma—Aldrich, Germany) and kept at − 20 °C.

MLST genotyping

For MLST genotyping, six unlinked genes including *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and the *IGS1* non-coding region were used, as described by the ISHAM consensus MLST scheme for the *C. neoformans* species complexes (http://mlst.mycologylab.org/). The PCR conditions for *CAP59*, *GPD1*, *SOD1*, *URA5* and the *IGS1* loci were performed as described by Meyer et al. [16]. The PCR conditions for the *LAC1* locus were modified, as 94 °C 4 min; 30 cycles: 94 °C 30 s, 61.5 °C 30 s, 72 °C 1 min. Also, the conditions of 12 cycles: 65 °C 30 s with a 2 °C step-down every two cycles 95 °C 2 min; 95 °C 30 s, 65 °C 30 s, 72 °C 1 min; followed by 20 cycles: 95 °C 30 s, 65 °C 30 s, 72 °C 1 min were used for *PLB1* locus. Amplification was performed using Red master mix (Amplicon, Denmark) and PCR products sequenced using the primers presented by Meyer et al. [16]. All sequencing results from each gene were aligned using the Clustal W algorithm at MEGA V.6.06 software available at http://megasoftware.net after being manually edited using the software Chromas v. 2.6.6 available at http://technelysium.com.au/ChromasPro.html.

All 210 sequences (7 loci for 30 isolates) were deposited at GenBank and accession numbers were obtained, accession numbers, LC598352–LC598381 for *CAP59*; accession numbers, LC598535–LC598564 for *URA5*; accession numbers, LC598565–LC598594 for *SOD1*; accession numbers, LC598595–LC598624 for *PLB1*; accession numbers, LC598625–LC598654 for *LAC1*; accession numbers, LC598655–LC598684 for *IGS1*; accession numbers LC598685–LC598714 for *GPD1*. Then, allele type (AT) and sequence types (ST) were identified by loading and comparing sequences on the *C. neoformans* MLST database, a global database of sequence information from *C. neoformans* available at http://mlst.mycologylab.org/.

Phylogenetic analysis

The UPGMA algorithm was implemented in the BioNumeric program to confirm evolutionary relationships in all sequenced types, show allelic profiles, and compare allelic profiles' drug sensitivity. The allelic profiles of the isolates were used to generate a minimum spanning tree including isolate from China, Thailand, Japan, Brazil, India, Hong Kong, Indonesia, Kuwait, Qatar and Italy. Data for these countries were extracted from published papers [9, 17–19].

Nucleotide diversity

In this study several indexes such as number of polymorphic sites (S), nucleotide diversity (p), number of haplotypes (h), haplotype diversity (Hd), and average number of nucleotide differences (k) were calculated by DNAsp 6.12.03 software available at (http://www.ub.edu/dnasp/) to assess the extent of DNA polymorphisms. Also, three tests, Tajima's D, Fu & Li's D, and Fu & Li's F for neutrality were calculated using this software. A negative or positive D value suggests purifying selection/population size expansion or balancing selection/a decrease in population size, respectively. The phenomenon of recombination inside the *C. neoformans* isolates was calculated using Watterson's estimate per sequence (Θs) at DNAsp 6.12.03 and genetic disequilibrium method by Fstat software available at https://www2.unil.ch/popgen/softwares/fstat.htm.

Antifungal susceptibility

Antifungal susceptibility tests were performed against amphotericin B (Sigma—Aldrich, Germany), fluconazole (Serva, USA), itraconazole (Sigma—Aldrich, Germany(, voriconazole (Sigma—Aldrich, Germany), 5FC (Sigma—Aldrich, Germany), and luliconazole (APIChem Technology, China) based on broth microdilution of clinical and laboratory standards institute (CLSI) M27 4th protocol [20]. Antifungals were prepared in dimethyl sulfoxide (DMSO, Merck, Germany) in a two-fold dilution series including 0.00781–16 µg/ml for amphotericin B, 0.01562–32 µg/ml for fluconazole, 0.00195–4 µg/ml for itraconazole and flucytosine, 0.00097–2 µg/ml for voriconazole, and 0.000244–0.5 µg/ml for luliconazole.

A yeast suspension equivalent to 0.5 McFarland in sterile saline solution (0.85%) was prepared from a 48 h culture of *C. neoformans*. Then, a concentration of $1–5 \times 10^3$ cells/mL was prepared from each yeast suspension in RPMI 1640 (Gibco, UK). 100 µL of yeast suspension and 100 µL of serial dilutions of each antifungal were added into each microplate well. A drug-free well (positive control) and yeast-free (negative control) were included in the test. Microplates were incubated at 35 °C for 48–72 h and then the minimum inhibitory concentrations (MIC) of each antifungal were detected for isolates. The MIC that inhibited 50% (MIC_{50}) and 90% (MIC_{90}), and $MIC_{Geometric\ (GM)}$ were also calculated. There are not defined breakpoints for used antifungals against *Cryptococcus* species. As a result, epidemiological cutoff values (ECVs) were calculated by M59 guideline as follows: ≥ 0.5 µg/ml for amphotericin B, ≥ 8 µg/ml for fluconazole and flucytosine, and ≥ 0.25 µg/ml for itraconazole and voriconazole [21].

Results

Multilocus sequence type

Using the MLST analysis, seven sequence types including ST77, ST4, ST31, ST55, ST57, ST58, and ST324 were detected among 30 *C. neoformans* isolates. The most prevalent genotype was ST77 in 15 (50%) isolates, followed by ST4 in 5 (16.7%) isolates. The allelic profile of all sequence types is shown in Fig. 1. All sequence types were previously found in other countries such as China (ST31, ST57), India (ST31, ST4, ST77), Thailand (ST31, ST4), Brazil (ST31, ST77), German (ST77, ST58), Japan (ST31, ST4), Italy (ST55) and Indonesia and Hong Kong (ST4) [9, 18, 19].

Phylogenetic analysis

According to UPGMA phylogenetic algorithm, two separate clusters were detected among our *C. neoformans* VNI isolates based on similarity in allelic profiles. The first cluster consisted of 2 sequence types and three isolates, whereas the second cluster supported five sequence types and 27 isolates. There was also a 46.8% similarity between the two clusters. The highest similarity (85.7%) was seen between ST77 and ST31 which differ only in two nucleotides at the *IGS1* locus. In contrast, ST324 in cluster 1 had the highest divergence compared to other sequence types (Fig. 1).

The MStree analysis shows that most of our sequence types are similar to Chinese, Italian, and Brazilian sequence types. However, they are genetically descended from India and, to a lesser extent, from Kuwait. Sequence type 324 and sequence type 58 are ancestrally close to sequence types from Italy with a distant genetic affinity with ancestral sequence types in India (Fig. 2).

Nucleotide diversity

In this study, 18 allele types for seven MLST gene regions were detected based on the MLST database. These allele types are as follows; 5 allele types for *LAC1*, 3 allele types for *PLB1*, 3 allele types for *IGS1*, 3 allele types for *URA5*, 2 allele types for *CAP59*, 1 allele type for *GPD1*, and 1 allele type for *SOD1* loci (Fig. 1). The highest polymorphism was observed in *LAC1* (46 sites polymorphisms), whereas two loci, *GPD1* and *SOD1*, were monomorphic. According to three indexes h, Hd, and π, the highest and lowest genetic diversity was observed in the *LAC1* gene region (h = 7, Hd = 0.680, π = 0.01329) and *CAP59* (h = 2, Hd = 0.129, π = 0.00023) respectively (Table 1).

According to Tajima's D test, D value in all loci except *GPD1* and *IGS1* was negative and significant (≤ 0.05). It is evidence of purifying selection or population size expansion in these five loci. In comparison, evidence of balancing selection or a decrease in population size was observed in two other loci. The recombination phenomenon was analyzed using three tests, theta Watterson (Θs), Pairwise Homoplasy Index (PHI), and the minimum number of recombination events (Rm) (Table 1). Based on these tests, three recombinations have occurred within our study population with the most recombinant, and mutation in the LAC1 locus (Rm = 1 and Θs = 8.330). In contrast, recombination in the concatenated dataset (intergenic) was not observed in our population (PHI, P = 1). Furthermore, the linkage disequilibrium test between pairs of loci did not show a statistical significance for any loci except *LAC1* and *IGS1*. These results could be evidence of recombination within our isolates and rejection of the null hypothesis (Table 1).

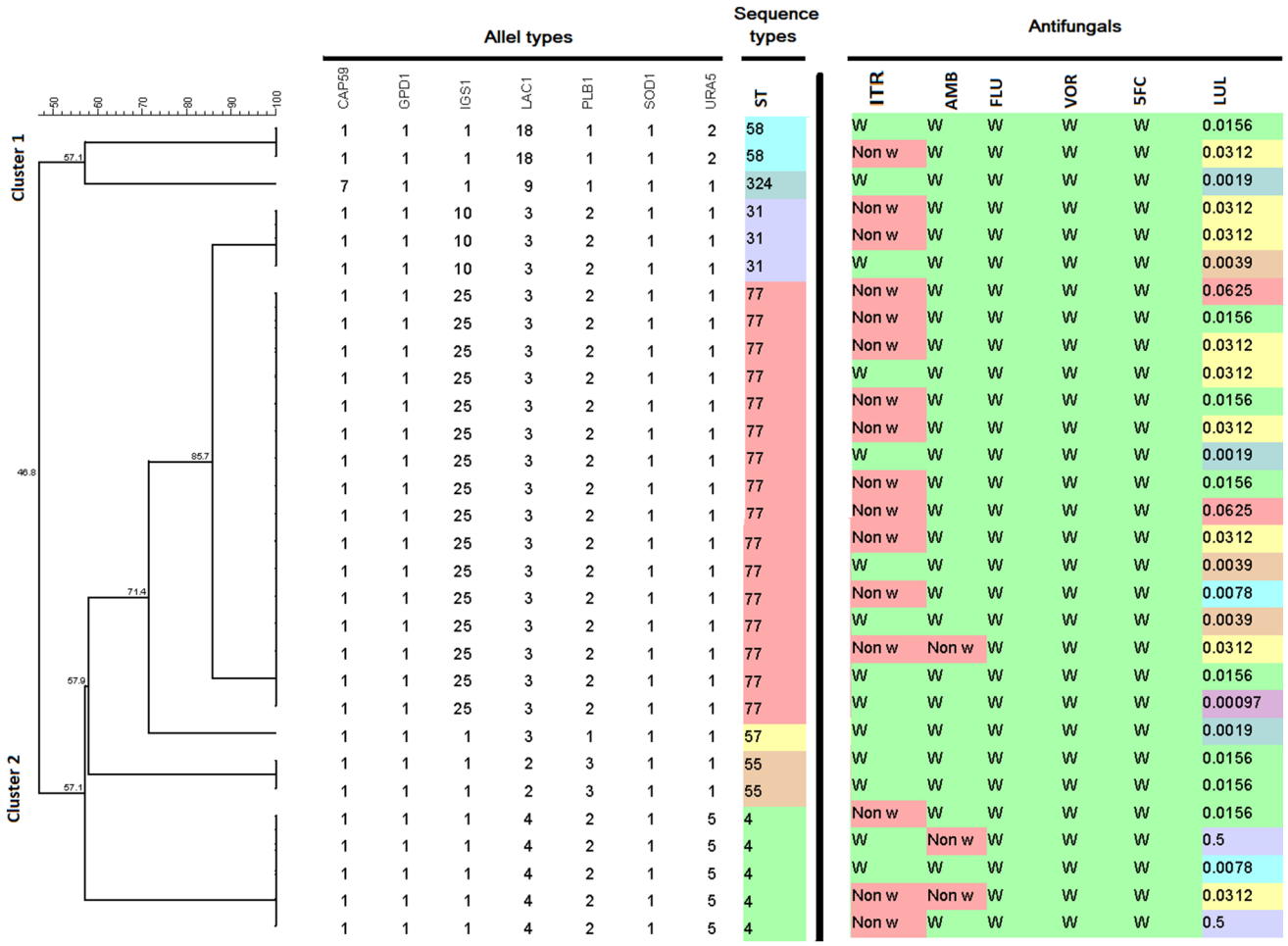

Fig. 1 Allele type number, sequence type, and antifungals susceptibility for 30 environmental isolates of *Cryptococcus neoformans* by UPGMA algorithm. *ITR* itraconazole, *AMB* amphotericin B, *FLU* fluconazole, *VOR* voriconazole, *5FC* 5-flucytosine, *LUL* luliconazole, *W* wild type, *Non W* NON-wild type

Antifungal susceptibility testing

The susceptibility of 30 *C. neoformans* isolates to six antifungal agents is presented in Table 2. Based on defined ECVs by CLSI M59 guidelines, all isolates were found to be wild-type phenotype (WT) to fluconazole, voriconazole, and flucytosine. It is found that 53.3% and 10% isolates were non-wild type phenotype (non-WT) to itraconazole and amphotericin B, respectively. Although there was no relationship between sequence types and the non-WT phenotype to itraconazole and amphotericin B, it is important to note that the non-WT phenotypes were observed in four sequence types, ST58, ST31, ST77, and ST4 (Fig. 1).

Discussion

Unfortunately, despite the development of genotypic methods, very limited studies have been conducted on the genotypic diversity of *Cryptococcus* spp. in clinical and environmental isolates in some geographical regions. Most clinical available reports in Iran are as case reports [2, 22, 23], and environmental epidemiological studies are limited to formal identification and/or consequent PCR and sequencing of the ITS or *IGS1* regions or PCR–RFLP [24, 25], and AFLP method by Pakshir et al. [26]. Molecular techniques have shown that there is no geographical difference in the distribution of *Cryptococcus* complex species, however, VNI and

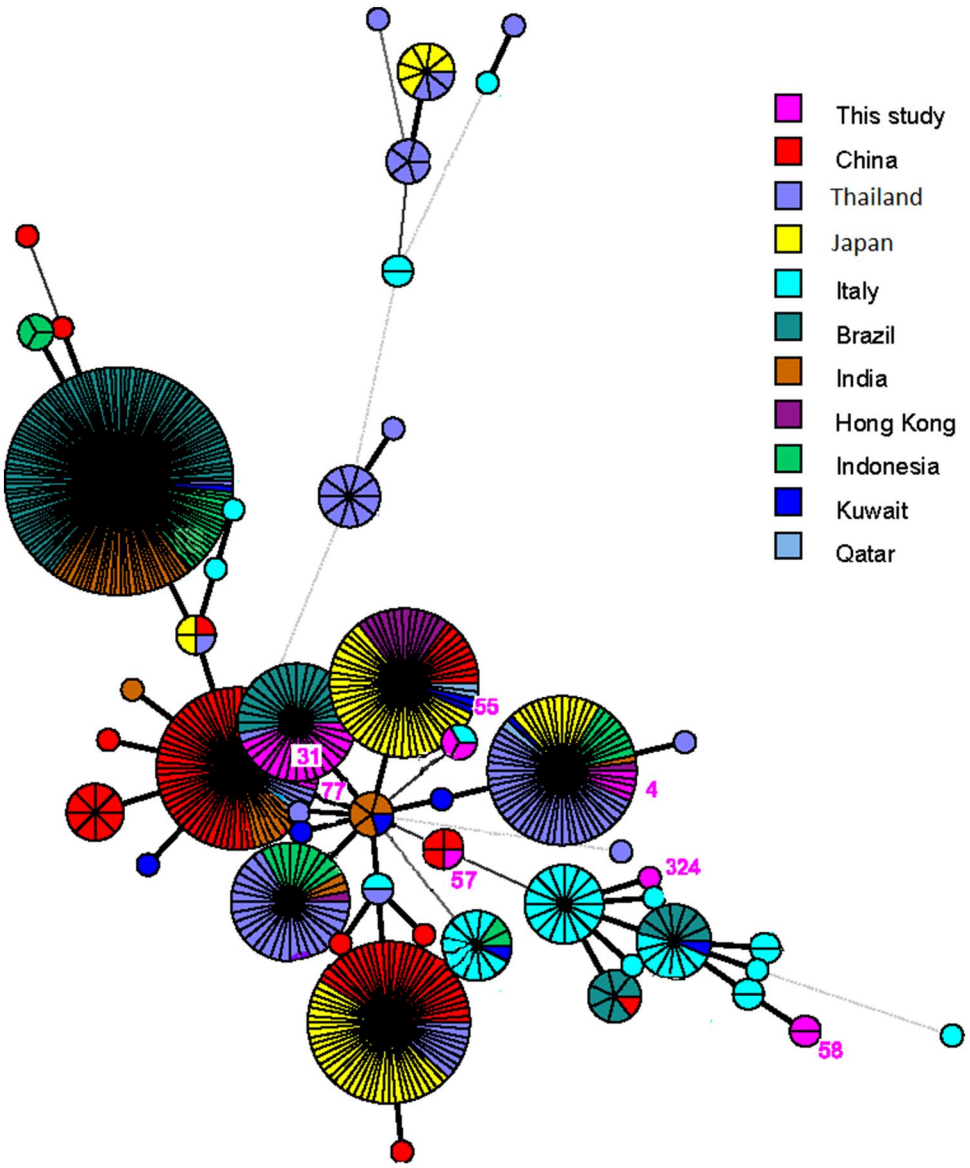

Fig. 2 Minimum spanning trees showing sequence type relationships among the different geographic regions and our data *Cryptococcus neoformans* isolates. The circle size and the branch thickness indicate the number of isolates and the evolutionary relationship between the isolates, respectively. Different color represents different geographic areas. (The type of STs and the ST numbers added in supplementary file)

VGI are the predominant molecular types for *C. neoformans* and *C. gattii*, respectively [27]. Therefore, in the present study, we analyzed the MLST characteristics of 30 *C. neoformans* isolates by the ISHAM-MLST consensus scheme.

Our results showed moderate genetic variation by the existence of seven sequence types from 30 isolates in Ahvaz, Iran, with the dominance of ST77. ST77 is widespread in other geographical regions of the world. Most environmental isolates of *C. neoformans* in the Andrade–Silva study belonging to ST77 [28]. Prakash et al. in molecular epidemiology in India, reported ST77 as the second most common sequence type [3]. Also, this sequence type was included in half of the environmental isolates from Brazil [6], reflecting the fact that ST77 has adapted to many geographic niches, both environmental and clinical and can cause infection due to recovering this sequence type from environmental and clinical samples. ST4 was the second most frequent sequence type in our study. This sequence type was reported in Thailand, Japan, India, Hong Kong, Indonesia, Kuwait, Qatar [19]. Although no sequence type from Iran has been reported so far, a study showed the presence of ST4, ST5, ST23, ST69, ST93, ST174, ST175, ST185, and ST192 in Kuwait and ST4, ST5, ST31 in Qatar (neighbor countries of Iran) [17]. Only two of these sequence types (ST4 and ST31) overlap with what was obtained in this study. The most common sequence types (ST31, ST77, ST55, and ST57) were directly originated from the Indian isolates. ST324 and ST58 have a genetic distance from other isolates, and originated from Italian isolates. ST4 is similar to Kuwait, Qatari, Japanese, and Indonesian isolates. A glance at these limited data suggests that there is likely abundant genetic diversity of *C. neoformans* in Iran and all isolates have Indian ancestor.

Table 1 Genetic parameters describing in each MLST locus and concatenated sequences of *Cryptococcus neoformans* (VNI) isolates in this study

Locus	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Concatenated
Length	565	544	724	478	534	536	637	4018
GAP	5	0	4	11	1	0	1	33
Invariable sites	559	544	708	434	529	534	630	3926
SVS	0	0	0	12	0	2	3	15
PIS	1	0	12	34	4	0	3	44
π	0.00023	0.000	0.00833	0.01329	0.00156	0.00025	0.0014	0.00359
k	0.129	0	5.995	6.205	0.832	0.133	0.903	4.313
h	2	1	3	7	3	3	5	14
Hd	0.129	0	0.570	0.680	0.393	0.130	0.602	0.867
Θs	0.252	0.00	3.029	8.330	1.010	0.505	1.515	14.893
D	− 0.7637	0.00	3.19550	− 0.92829	− 0.45137	− 1.50738	− 1.15136	− 0.14628
FD	0.59448	0.00	1.46957	− 0.07781	1.05802	− 2.28108	− 1.11084	0.04883
FF	0.25254	0.00	2.34917	− 0.41743	0.71674	− 2.38176	− 1.30777	− 0.01843
RM	0	0	0	1	0	0	0	3

SVS singleton variable sites, *PIS* parsimony informative sites, *π* nucleotide diversity, *k* average number of nucleotide differences per sequence, *h* number of haplotypes, *Hd* haplotype diversity, *Θs* Watterson's Θ per sequence, *D* Tajima's D, *FD* Fu and Li's D, *FF* Fu, and Li's F

Table 2 In vitro antifungal susceptibility results for 30 environmental *Cryptococcus neoformans* isolates

Antifungals	Minimum inhibitory concentration (MIC µg/mL)				% ECV			
					Wild type		Non-wild type	
	MIC range	MIC_{50}	MIC_{90}	MIC_{GM}	No	%	No	%
Amphotericin B	0.0312–4	0.0625	0.25	0.1139	27	90	3	10
Fluconazole	0.0312–8	1	4	0.9169	30	100	0	0
Itraconazole	0.0156–4	0.25	1	0.2574	14	46.7	16	53.3
Voriconazole	0.0019–2	0.0156	0.0312	0.0183	30	100	0	0
5-Flucytosine	0.0039–1	0.25	0.5	0.1165	30	100	0	0
Luliconazole	0.00097–0.5	0.0156	0.0625	0.0163	ND	ND	ND	ND

ECV epidemiological cut-off values, *ND* not defined

Three recombination events were found in concatenated sequences using Rm analysis and a pairwise genotypic disequilibrium test detected recombination phenomenon among *LAC1* and *IGS1* loci. The nucleotide diversity in the *LAC1* locus can be attributed to the adaptation of the fungus to environmental conditions (cold and heat) [9]. Similarly, Cogliati et al. illustrated that environmental isolates of *Cryptococcus* have a higher nucleotide diversity at the *LAC1* locus than clinical isolates [9]. The results of the PHI test also did not show a significant difference, which refers to the clonal nature of our *C. neoformans* population structure. Finally, taken together, these results may indicate recombination within our isolates and/or suggest non-meiotic reproduction [19].

From 2018, ECVs have been described for several antifungals drug against *C. neoformans* in the CLSI guideline. According to this guideline, all our isolates (100%) were found to be WT (susceptible) to fluconazole, voriconazole, and flucytosine. Moreover, 53.3% and 10% of isolates were non-WT (resistant) to itraconazole and amphotericin B, respectively. Although our results were consistent with Rocha et al. in amphotericin B and fluconazole susceptibility [29], in contrast, we demonstrated resistance to itraconazole in 53.3% isolates. Furthermore, Chen et al. found similar results with us for the three antifungals fluconazole, voriconazole, and 5FC, but they found that all isolates were sensitive to amphotericin B and itraconazole in contrast with our findings that 10% and 53.3% of isolates were non-WT to amphotericin B and itraconazole, respectively [18].

Conclusions

Overall, the differences in ECVs were not associated with sequence types. However, except for ST77 and ST4, most sequence types were limited to one or two isolates, and the possibility of correct evaluation and correlation of sequence type with drug profile was weak. Luliconazole is a new imidazole, and no ECVs or breakpoints have been reported in either the global CLSI or EUCAST protocols. Although the MIC range of luliconazole was considerably lower than other studied azoles, the MICs obtained for *C. neoformans* were much higher than the MICs obtained for others studied filamentous fungi and *Candida* spp., so far [30–32].

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06433-7.

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Author contributions Study concept and design, AZM and MF; sampling, isolation, conducting the experiments, MM; data analysis, MM, and NK; drafting of the manuscript, MM; Critical editing, AZM.

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Declarations

Conflict of interest The authors declare that they have no potential conflicts of interest.

Ethical approval The ethical committee approved this project of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1398.648).

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