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Research article

Susceptibilities of *Malassezia* strains from pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis to antifungal drugs

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ABSTRACT

The human pathogenic yeast genus *Malassezia* may be an etiological agent of skin disorders and has received considerable attention from dermatologists in recent years. To investigate the different susceptibilities of *Malassezia* species to four antifungal drugs, we isolated a total of 244 *Malassezia* strains and identified six species of *Malassezia* from patients with clinical skin diseases. The minimum inhibitory concentration (MIC) of the four antifungal drugs was obtained by comparing the susceptibility of the isolated *Malassezia* strains to four antifungal drugs (ketoconazole (KTZ), itraconazole (ITZ), fluconazole (FLC) and amphotericin B (Am B)). We demonstrated that *M. furfur, M. sympodialis*, *M. pachydermatis* and *M. globosa* are the most common *Malassezia* species in the three skin diseases. The MICs of KTZ, ITZ, FLC and Am B against *M. furfur, M. sympodialis*, *M. pachydermatis* and *M. globosa* ranged from 0.03 - 16 mg/L, 0.03 - 2.0 mg/L, 0.03 - 8 mg/L, and 13 - 64 mg/L, respectively. The sensitivities of *Malassezia* to the four antifungal drugs from high to low were ITZ $\geq$ KTZ $>$ Am B $>$ FLC. The susceptibilities of the various *Malassezia* species to the four antifungal drugs were different, and the susceptibility of *M. furfur* to KTZ was significantly different from those of the three skin diseases (pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis). Our results suggested that the MIC analysis of the four antifungal drugs would be helpful in preventing drug resistance in the clinical screening of *Malassezia* and choosing better antifungal drugs to treat *Malassezia*-associated skin diseases.

1. Introduction

Malassezia, one of the resident bacteria on the human skin surface [1], is a lipophilic yeast-like fungus; *Malassezia* cultures require lipid supplementation for growth [2, 3]. *Malassezia* can directly infect the skin and cause seborrheic dermatitis, atopic dermatitis, psoriasis, hemorrhoids, onychomycosis, otitis externa and foreskin balanitis [4, 5]. In recent years, reports of systemic infections caused by *Malassezia* have gradually increased, and the skin infection caused by *Malassezia* manifests as many diseases, has a high incidence rate, recurs easy, and has a great impact on the physical and mental health of patients [6, 7, 8, 9, 10, 11, 12]. Commonly isolated *Malassezia* species have differences due to the different regions and countries they are isolated in and the populations they infect, and there are differences between healthy individuals and patients with various skin diseases [13].

There are many studies on the various *Malassezia* species causing diseases, such as pityriasis, Malassezia folliculitis and seborrheic dermatitis, and different species of *Malassezia* have been isolated from various regions [14, 15, 16]. Pityriasis and seborrheic dermatitis are mainly caused by *M. furfur*, followed by *M. globosa*, while Malassezia folliculitis is mainly caused by *M. globosa*; however, the distribution of the strains of these three diseases is different. In China, the highest

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detection rates of *Malassezia* in lesioned skin were observed for *M. globosa*, *M. restricta*, and *M. sympodialis* [17]. The dominant species of pityriasis include *M. furfur* in Indonesia [18] and *M. globosa* in Japan [19], while the dominant strains of pityriasis and seborrheic dermatitis are *M. sympodialis* and *M. globosa* in Canada [20] and *M. furfur* in Brazil [9]. *Malassezia* has been classified into over 14 species, and eight species of *Malassezia* have been isolated from human skin, including *M. dermatis, M. furfur, M. globosa, M. japonica, M. obtusa, M. pachydermatis, M. restricta, M. sympodialis, M. slooffiae,* and *M. yamatoensis* [21]. In the clinic, we have found that *M. globosa, M. furfur* and *M. sympodialis* are the dominant species of *Malassezia* infection in Kunming. *Malassezia* infection usually occurs in young and adult males. Pityriasis versicolor, Malassezia folliculitis, and seborrheic dermatitis are common skin diseases caused by *Malassezia*. *Malassezia* infection in Kunming accounts for 13.8% of superficial fungal infections and is the second highest fungal infection, which may be related to the geographical environment and the consumption of spicy foods in this area. Therefore, the distribution and antifungal drug susceptibilities of *Malassezia* species in pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis need to be analyzed and will be important for guiding clinical treatment.

Although clinical studies have confirmed that fluconazole (FLC), itraconazole (ITZ) and ketoconazole (KTZ) are effective in treating these skin diseases, the long-term safety of antifungal drugs remains unknown. The treatment cycle of fungal infections is generally long, and short-term medication cannot effectively cure the infection, while long-term medication not only is hazardous but also easily increases fungal resistance. In recent years, the systematic use of antifungal drugs, such as amphotericin B (Am B), to treat infections of sensitive *Malassezia* strains has been taken seriously. There are few studies on the susceptibility of *Malassezia* strains isolated from clinical samples to Am B [22, 23]. Álvarez-Pérez S conducted a drug susceptibility test on 60 *Malassezia furfur* strains and showed that the MIC value of Am B among those strains was $\leq$1 mg/L [23]. In this study, the drug susceptibilities of *M. furfur*, *M. pachydermatis*, *M. sympodialis*, and *M. globosa* to four antifungal drugs (KTZ, ITZ, FLC and Am B) were analyzed, and the same species of *Malassezia* were isolated from different skin diseases.

2. Materials and methods

2.1. Malassezia strains and culture

A total of 244 *Malassezia* strains were isolated from patients with pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis, including 85 strains of *M. globosa*, 77 strains of *M. furfur*, 37 strains of *M. sympodialis*, 28 strains of *M. pachydermatis*, 10 strains of *M. restricta*, and 7 strains of *M. slooffiae*. Four standard strains of *Malassezia* (*M. furfur CBS1878*, *M. sympodialis CBS7222*, *M. pachydermatis ATCC4791*, and *M. globosa CBS7990*) were provided by the Medical Fungi Deposit Center of the Institute of Dermatology, Chinese Academy of Medical Sciences. This research was approved by the Ethics Committee of First Affiliated Hospital of Kunming Medical University. Written informed consent was obtained from all patients for the use of their samples and clinical records for our *Malassezia* analysis experiments.

Fatty acid RPMI 1640 medium was used to test the susceptibilities of eight Malassezia species to a new triazole, posaconazole, and to six established antifungal agents by a modified National Clinical Trial Committee for Standardization (NCCLS) M27-A2 microdilution method. Fatty acid RPMI 1640 medium was used to test the susceptibilities of six species of *Malassezia* to the four established antifungal drugs by a modified NCCLS (National Clinical Laboratory Standardization Institute) M27-A2 microdilution method [24]. The strains were cultured twice on Sabouraud's medium containing olive oil at 32 °C for 5 days before the susceptibility test. After the second culture, 1 - 2 colonies with a diameter of $\geq$1 mm were collected; suspensions of the yeasts were made with 1 ml of sterile physiological saline and shaken for 15 s. Then, the concentrations of the yeast suspensions were diluted to 1 - 5 $\times$ 10^3 CFU/ml with *Malassezia* liquid medium. The final concentration of the yeasts was 0.5 - 2.5 $\times$ 10^3 CFU/ml.

2.2. Antifungal drugs

Itraconazole, fluconazole, and ketoconazole were purchased from TCI America (Portland, OR, USA), and amphotericin B was purchased from Vetech Laboratories (Guelph, Canada). Fluconazole was formulated into a 5,120 mg/L drug stock solution by sterilized distilled water; ketoconazole and itraconazole were first dissolved in dimethyl sulfoxide (DMSO), with DMSO concentrations less than 1%, and then diluted with sterile distilled water to prepare a 1,280 mg/L drug stock solution. The drug storage solution was sealed and stored at a temperature of -20 °C. The fluconazole stock solution was diluted 10 times with Malassezia liquid medium to a concentration of 128 mg/L, and this solution was twice the final concentration of the drug in the first well of the drug-sensitive plate (64 mg/L). The raw solutions of itraconazole and ketoconazole were diluted 50 times with Malassezia liquid medium to a concentration of 32 mg/L, which was twice the final concentrations of the two drugs in the first two wells of the drug-sensitive plate (16 mg/L).

2.3. Drug allergy testing

The in vitro susceptibility analysis of *Malassezia* was carried out according to the yeast microdilution method in NCCLS M27-A2. One hundred microliters of the concentrated solution of drugs with 80 μl of *Malassezia* medium was added to each well of a 96-well microplate, and 180 μl of *Malassezia* liquid medium alone was added to a well as a negative control; 20 μl of Alamar Blue was added to each well. Sensitivity determinations of the 4 standard strains were performed simultaneously with each test. Each time, the results showed that the minimum inhibitory concentration (MIC) values were in the same range, and the MIC value of each drug varied by no more than one dilution per measurement. The MIC_{50} value (MIC value of 50% strain inhibition) and MIC_{90} value (MIC value of 90% strain inhibition) were also determined.

2.4. Sanger sequencing, alignment analysis, and system tree building

Genomic DNA was extracted from the *Malassezia* strains using the CTAB method [25]. PCR was performed to amplify DNA, and DNA sequencing primers that targeted the D1/D2 region (NL1: 5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′; NL4: 5′-GGT CCG TGT TTC AAG ACG G-3′), and for ITS1-ITS4 region (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) were used. The DNA sequences of the determined ITS1-ITS4 regions were spliced by Sequencher 5.4.6 software (Ann Arbor, MI, USA). BLAST and the Geneballk database were used to compare similar sequences, and strain were identified based on the highest percentage of bacteria. After the sequences were analyzed by ClustalW2 (Hinxton, Cambridgeshire, United Kingdom), N-J system trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA) software [26].

2.5. Statistical analysis

All statistical analyses were performed using SPSS 17.0 (IBM-SPSS Inc., Chicago, IL, USA). The differences in the distribution of pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis were analyzed by x^2 tests. The MIC value of each drug was log-transformed and converted for one-way analysis of variance; $p < 0.05$ indicated that the difference was statistically significant.

3. Results

3.1. Identification of six Malassezia strains isolated from clinical pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis infections

First, the strains were identified by traditional identification methods (morphological, physiological and biochemical characteristics). Among a total of 342 cases, 106 of the 143 patients with pityriasis versicolor had

culture-positive cases (74.1%), 72 of the 96 patients with Malassezia folliculitis had culture-positive cases (75.0%), and 66 of the 103 patients with seborrheic dermatitis had culture-positive cases (64.1%). In total, 244 clinical strains consisting of six Malassezia species were cultured and isolated (71.3%), including 85 strains of *M. globosa*, 77 strains of *M. furfur*, 37 strains of *M. sympodialis*, 28 strains of *M. pachydermatis*, 10 strains of *M. restricta* and 7 strains of *M. slooffiae*, all of which were identified by traditional identification methods. Among them, *M. globosa* (34.8%), *M. furfur* (31.6%), *M. sympodialis* (15.2%), and *M. pachydermatis* (11.5%) had the highest representation in the three diseases, as shown in Table 1. We also demonstrated that the distribution of strains of the three diseases was significantly different ($x^2 = 33.30$, $p = 0.000$). There was a significant difference between the distribution of pityriasis and Malassezia folliculitis ($x^2 = 30.56$, $p = 0.000$) and between the distribution of Malassezia folliculitis and seborrheic dermatitis ($x^2 = 17.67$, $p = 0.003$). However, there was no significant difference in the distribution of pityriasis versicolor and seborrheic dermatitis ($x^2 = 3.25$, $p = 0.661$). The two main pathogens of pityriasis versicolor and seborrheic dermatitis were *M. furfur* and *M. globosa*. The main pathogen of Malassezia folliculitis was *M. globosa* (59.7%) (Table 1).

3.2. Antifungal effects of four antifungal drugs (ketoconazole, itraconazole, fluconazole and amphotericin B) on Malassezia

Next, we analyzed the antifungal effects of four antifungal drugs (ketoconazole (KTZ), itraconazole (ITZ), fluconazole (FLC) and amphotericin B (Am B)) on 227 strains of *Malassezia* (*M. furfur, M. sympodialis, M. pachydermatis* and *M. globosa*) isolated from clinical samples. The sensitivity of the four *Malassezia* species to KTZ and ITZ was significantly different ($x^2 = 56.06$, $p < 0.05$). The results showed that the MIC range of KTZ against *M. furfur*, *M. sympodialis*, *M. pachydermatis* and *M. globosa* strains 0.03 - 0.5 mg/L, 0.03 - 16 mg/L, 0.03 - 0.5 mg/L, and 0.03 - 16 mg/L, respectively (Table 2). As shown in Figure 1A, the MIC values of KTZ against *M. furfur, M. pachydermatis* and *M. globosa* for all *M. furfur* (77/77) and *M. pachydermatis* strains (28/28) and 89.2% of *M. sympodialis* strains (33/37) were 0.03 mg/L - 0.5 mg/L (Table 3). The MIC ranges of ITZ against *M. furfur, M. sympodialis, M. pachydermatis* and *M. globosa* were 0.03 - 1 mg/L, 0.03 - 0.25 mg/L, 0.03 - 0.25 mg/L, and 0.03 - 0.25 mg/L, respectively (Table 2), although the MIC analysis for ITZ against *M. pachydermatis* showed two peaks at 0.03 mg/L - 0.13 mg/L and 0.25 mg/L (Figure 1B). The sensitivity of the four *Malassezia* strains to FLC was significantly different ($x^2 = 85.47$, $p < 0.05$). The MIC ranges of FLC against *M. furfur*, *M. sympodialis, M. pachydermatis* and *M. globosa* were 0.25 - 64 mg/L, 0.25 - 8 mg/L, 0.5 - 16 mg/L, and 0.13 - 16 mg/L, respectively (Table 2). The MIC analysis of FLC against *M. furfur* showed two peaks at 0.25 - 4 mg/L and 8 - 64 mg/L, at which *M. furfur* was 62.33% (48/77) and 35.06% (27/77), respectively. The MIC analysis of FLC against *M. sympodialis* also showed two peaks at 0.25- 2 mg/L and 2 - 8 mg/L, at which the ratios of *M. sympodialis* were 72.97% (27/37) and 27.03% (10/37), respectively. The MIC analysis of FLC against *M. pachydermatis* showed two peaks, with the first peak at 0.5 - 2 mg/L for 53.57% *M. pachydermatis* (15/28) and the second peak at 4 - 16 mg/L for 46.43% *M. pachydermatis* (13/28). The MIC of FLC against *M. globosa* had 2 ranges, with the main peak at 2 - 16 mg/L for 74.12% *M. globosa* (63/85). The sensitivities of the four Malassezia strains to amphotericin B were significantly different ($x^2 = 124.63$, $p < 0.05$). The MIC ranges of Am B against *M. furfur, M. sympodialis, M. pachydermatis* and *M. globosa* were 0.03–8 mg/L, 0.03 - 4 mg/L, 0.03 - 4 mg/L, and 0.03 - 4 mg/L, respectively. The MIC analysis of Am B against *M. furfur, M. sympodialis, M. pachydermatis*, and *M. globosa* showed two ranges: 0.06 - 1 mg/L and 2 - 8 mg/L. Approximately 70.27% of *M. sympodialis* strains (26/37), 85.71% of *M. pachydermatis* strains (24/28), and 92.94% of *M. globosa* strains (79/85) had an MIC range of 0.06 - 1 mg/L MIC for Am B, while most *M. furfur* (70.12%) had MIC ranges from 2 - 8 mg/L for Am B. All the sensitivities of the *Malassezia* strains to the four antifungal drugs are shown in Figure 1.

The four species of Malassezia were very sensitive to KTZ, but the MIC value of KTZ against *M. sympodialis* was slightly higher than those against the other species. The percentages of *M. furfur, M. sympodialis, M. pachydermatis* and *M. globosa* inhibited by 0.25 mg/L KTZ were 92.21%, 56.76%, 89.29%, and 78.82%, respectively. KTZ activity against these four species of *Malassezia* was in the following order from strong to weak: *M. furfur* > *M. pachydermatis* > *M. sympodialis* > *M. globosa*. The four species of *Malassezia* were very sensitive to ITZ, and the percentages of *M. furfur, M. sympodialis, M. pachydermatis* and *M. globosa* inhibited by 0.13 mg/L ITC were 66.2%, 81.21%, 64.28%, and 81.17%, respectively. The antifungal activity of ITZ against these four species of *Malassezia* ranged from strong to weak in the following order: *M. sympodialis* > *M. globosa* > *M. furfur* > *M. pachydermatis*. The sensitivity of the four species of *Malassezia* to FLC was worse than those of *Malassezia* to the other three drugs. The percentages of *M. furfur, M. sympodialis, M. pachydermatis* and *M. globosa* inhibited by 2 mg/L FLC were 51.94%, 96.42%, 53.57%, and 71.76%, respectively. The bacteriostatic action of FLC against these four strains of *Malassezia* was as follows: *M. sympodialis* > *M. globosa* > *M. pachydermatis* > *M. furfur*. All four species of *Malassezia* were more sensitive to Am B than to FLC and were less sensitive to both KTZ and ITZ. In the presence of 1 mg/L Am B, the *M. furfur, M. sympodialis, M. pachydermatis* and *M. globosa* strains were inhibited by 29.87%, 78.37%, 92.85%, and 95.29%, respectively. The antifungal activity of Am B against these 4 *Malassezia* species ranged from strong to weak in the following order: *M. globosa* > *M. pachydermatis* > *M. sympodialis* > *M. furfur*. The MIC value ranges and the MIC_{50} and MIC_{90} values of the four antifungal drugs against the 244 strains of Malassezia are shown in Table 3. The difference in physiological and biochemical characteristics of the *Malassezia* strains may lead to differences in the activities of antifungal drugs against *Malassezia* in clinics.

3.3. The sensitivity of the same species of Malassezia from the three skin diseases to antifungal drugs

The four antifungal drugs had different MIC values for *M. furfur* strains from the three diseases. The MIC values of KTZ against *M. furfur* strains from the three diseases were significantly different ($p < 0.05$) (Table 4). There was a significant difference in the sensitivity of *M. furfur* strains isolated from Malassezia folliculitis ($p = 0.000$), seborrheic dermatitis ($p = 0.005$), and all three diseases ($p = 0.02$) to KTZ. *M. furfur* from Malassezia folliculitis was the most sensitive to KTZ (0.060 ± 0.011 mg/L). However, there was no significant difference in the MICs of the other three drugs (ITZ, FLC, and Am B) against *M. furfur* from the three skin diseases ($p > 0.05$). In addition, the MIC values of FLC against *M. sympodialis* strains from the three diseases were significantly different ($p = 0.034$), and *M. sympodialis* from Malassezia folliculitis was the most sensitive to FLC (1.000 ± 0.415 mg/L) (Table 4). There was a significant difference in the sensitivity of *M. sympodialis* isolated from pityriasis

Table 1. The species of pityriasis, Malassezia folliculitis, and seborrheic dermatitis (N/%).

Disease	*M. furfur*	*M. sympodialis*	*M. pachydermatis*	*M. globosa*	*M. restricta*	*M. slooffiae*	Total
Pityriasis	41 (38.7)	19 (17.9)	15 (14.1)	22 (20.7)	5 (4.7)	4 (3.7)	106
Malassezia folliculitis	11 (15.2)	9 (12.5)	4 (5.56)	43 (59.7)	4 (5.56)	1 (1.4)	72
Seborrheic dermatitis	25 (37.8)	9 (13.6)	9 (13.6)	20 (30.3)	1 (1.5)	2 (3.0)	66
Total	77 (31.6)	37 (15.2)	28 (11.5)	85 (34.8)	10 (4.1)	7 (2.9)	244

Table 2. MICs (μg/ml) of 7 clinical isolates of Malassezia species to 4 antifungal agents.

Strain	Quantity	KTZ[a]			ITZ			FLC			Am B		
		MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
M. furfur	77	0.03–0.5	0.13	0.25	0.03–2.00	0.13	0.25	0.25–64	2.00	8.00	0.03–8	2.00	4
M. sympodialis	37	0.03–16	0.25	1.00	0.03–0.25	0.13	0.25	0.25–8	1.00	4.00	0.03–4	0.50	4
M. pachydermatis	28	0.03–0.5	0.13	0.50	0.03–0.25	0.06	0.25	0.5–16	2.00	8.00	0.03–4	0.50	1
M. globosa	85	0.03–16	0.13	0.50	0.03–0.25	0.06	0.25	0.13–16	1.00	8.00	0.03–4	0.25	1

[a] KTZ, ketoconazole; ITZ itraconazole; FLC, fluconazole; Am B, amphotericin B ($\mu g \cdot ml^{-1}$).

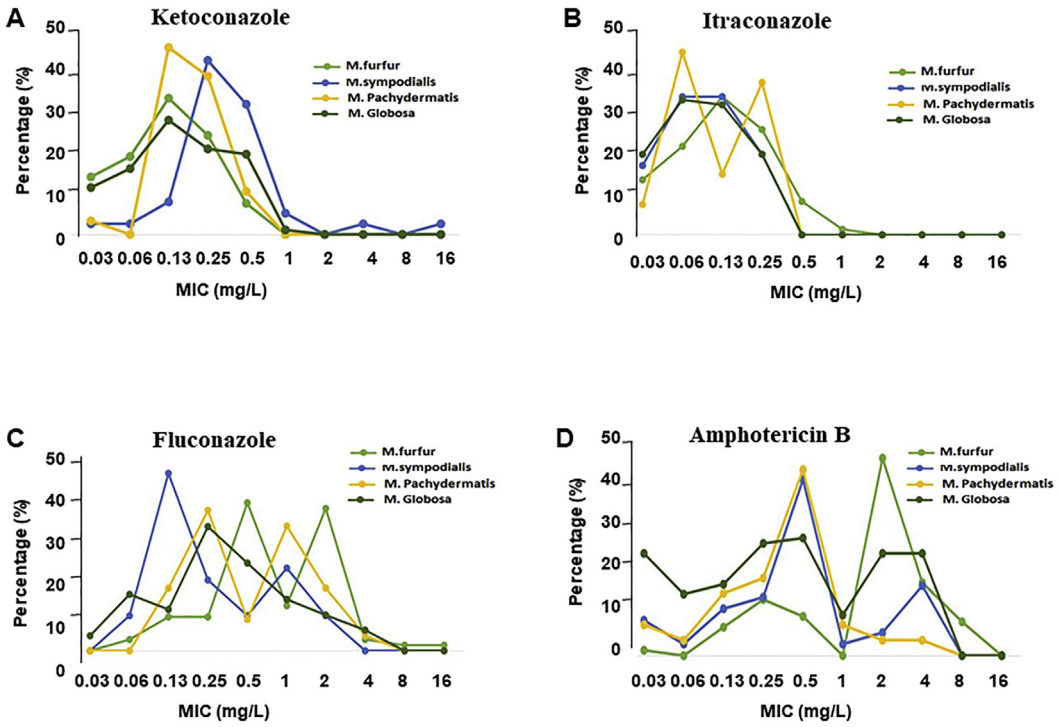

Figure 1. MIC values of four drugs against four *Malassezia* species. A) Ketoconazole (KTZ); B) Itraconazole (ITZ); C) Fluconazole (FLC); D) Amphotericin B (AmB).

versicolor and seborrheic dermatitis ($p = 0.010$). However, there was no significant difference in the MIC values of KTZ, ITZ, and Am B against *M. sympodialis* from the three diseases ($p > 0.05$). There was also no significant difference in the MIC of the four antifungal drugs against *M. pachydermatis* or *M. globosa* isolated from three diseases ($p > 0.05$). Thus, the main treatable species of pityriasis versicolor and seborrheic dermatitis species was *M. furfur*, and the best antifungal agent against *M. furfur* was KTZ. The *Malassezia* strains, depending on the different skin diseases they were isolated from, were cultivated and identified when they were collected so that the same type of *Malassezia* strains were artificially cultivated separately according to different disease sources; this ensures that the same strains from different sources do not contaminate each other during the experiment. The reason why the same strains with different disease origins show different MIC values may be that the strains have different lipase activities [27, 28], suggesting that the formation of lipase activity by the strains may be related to the presence of different sebum concentrations in the skin of the lesion. On another hand, some resistant strains of *Malassezia* were increasingly detected in different disease sources [3, 29], which may also explain the different sensitivities of the same species of *Malassezia* to antifungal drugs among the three skin diseases.

3.4. Analysis of the relationship between drug sensitivity and genotypes of the Malassezia strains

Because the phenotypes of *Malassezia* strains are easily affected by the external environment, traditional identification methods, such as physiological and biochemical methods, can be used to genotype strains and reflect the differences among organisms at the genetic molecular level. We amplified the NL region (B amplification group) to build a phylogenetic tree of Malassezia strains. Among the phylogenetic tree, it can be seen that group 1 (only D17), group 2 (D18, D20, D67, D69, D55, D53, D52, D51, D16, D15, D13, D10, D9, D1, and D7), and group 3 (only D12) formed three relatively independent branches (Figure 2). In addition, the source of strains in group 1 was different from those of the other two groups, and these strains may have evolved from different *Malassezia* species. We also analyzed the relationship between the drug sensitivities and genotypes of the *Malassezia* strains in Table 5. However. There was no significant correlation between genotype and drug sensitivity of *Malassezia* strains from different evolution sources because of the small sample size of analyzed *Malassezia* strains.

4. Discussion

Due to the special physiological characteristics of *Malassezia*, except for *M. furfur*, the growth of other *Malassezia* species is lipid dependent, and these strains are unable to grow in media without lipids. Using the fatty acid medium Elegraki RPMI 1640 medium and the National Clinical Trial Committee for Standardization (NCCLS) M27-A2 microdilution method recommended for antifungal drug sensitivity tests, we measured the sensitivities of four species of Malassezia from three diseases to the antifungal drugs FLC, ITZ, KTZ and Am B to provide a better understanding of Malassezia-related diseases in clinics.

Table 3. Comparison of MIC values of ketoconazole, itraconazole, amphotericin B, and fluconazole against four *Malassezia* species (mg/L).

Strain	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64
KTZ												
M. furfur	11	15	26	19	6	0	0	0	0	0	0	0
M. sympodialis	1	1	3	16	12	2	1	0	0	1	0	0
M. pachydermatis	1	0	13	11	3	0	0	0	0	0	0	0
M. globosa	10	14	25	18	17	1	0	0	0	0	0	0
ITZ												
M. furfur	10	16	25	19	6	1	0	0	0	0	0	0
M. sympodialis	6	12	12	7	0	0	0	0	0	0	0	0
M. pachydermatis	2	12	4	10	0	0	0	0	0	0	0	0
M. globosa	16	27	26	16	0	0	0	0	0	0	0	0
Am B												
M. furfur	1	0	5	10	7	0	35	13	6	0	0	0
M. sympodialis	3	1	4	5	15	1	2	6	0	0	0	0
M. pachydermatis	2	1	4	5	12	2	1	1	0	0	0	0
M. globosa	2	12	14	22	23	8	2	2	0	0	0	0
FLC												
M. furfur	-	-	0	2	6	6	26	8	25	2	1	1
M. sympodialis	-	-	0	3	15	6	3	7	3	0	0	0
M. pachydermatis	-	-	0	0	4	9	2	8	4	1	0	0
M. globosa	-	-	3	11	8	25	17	10	7	4	0	0

Table 4. Drug susceptibility of *M. furfur, M. sympodialis, M. pachydermatis*, and *M. globosa* from different diseases ($\bar{x}\pm$ s (mg/L)).

Antifungal drug	Pityriasis	Malassezia folliculitis	Seborrheic dermatitis	F	p
M. furfur					
KTZ	0.183 ± 0.020	0.060 ± 0.011	0.164 ± 0.025	7.161	**0.020**$^{*\alpha}$
ITZ	0.168 ± 0.021	0.148 ± 0.042	0.226 ± 0.077	0.213	0.808$^{\alpha}$
FLC	5.226 ± 0.869	4.409 ± 1.383	6.350 ± 2.477	0.324	0.724$^{\alpha}$
Am B	2.314 ± 0.318	1.989 ± 0.746	2.396 ± 0.411	0.270	0.765$^{\alpha}$
M. sympodialis					
KTZ	0.559 ± 0.196	0.247 ± 0.557	2.181 ± 1.729	2.151	0.132$^{\alpha}$
ITZ	0.112 ± 0.016	0.117 ± 0.029	0.118 ± 0.028	0.055	0.947$^{\alpha}$
FLC	2.724 ± 0.637	1.000 ± 0.415	1.278 ± 0.374	3.739	**0.034**$^{*\alpha}$
Am B	1.058 ± 0.315	0.868 ± 0.441	1.167 ± 0.538	1.207	0.312$^{\alpha}$
M. pachydermatis					
KTZ	0.210 ± 0.035	0.156 ± 0.031	0.236 ± 0.039	0.641	0.535$^{\alpha}$
ITZ	0.131 ± 0.023	0.172 ± 0.047	0.128 ± 0.031	0.466	0.633$^{\alpha}$
FLC	3.068 ± 0.732	4.125 ± 1.533	3.611 ± 1.628	0.664	0.523$^{\alpha}$
Am B	0.656 ± 0.251	0.406 ± 0.094	0.490 ± 0.197	0.195	0.824$^{\alpha}$
M. globosa					
KTZ	0.217 ± 0.041	0.229 ± 0.026	0.184 ± 0.049	0.655	0.522$^{\alpha}$
ITZ	0.126 ± 0.018	0.102 ± 0.011	0.111 ± 0.019	0.583	0.560$^{\alpha}$
FLC	2.733 ± 0.783	2.384 ± 0.469	3.175 ± 1.072	0.278	0.751$^{\alpha}$
Am B	0.537 ± 0.189	0.494 ± 0.101	0.325 ± 0.061	0.400	0.672$^{\alpha}$

Note: "*" indicates statistical significance; "α" indicates one-way ANOVA p value.

We analyzed the effect of four antifungal drugs on different species of *Malassezia,* and the MIC_{50} and MIC_{90} values of four *Malassezia* species to the four antifungal drugs were investigated in 227 *Malassezia* strains from clinically isolated samples. Among the four antifungal drugs, FLC had the worst antifungal effect against various species of *Malassezia*. Consist with the vitro MICs values, analysis of the efficacy of ITZ and FLC in the treatment of *Malassezia* showed that the total effective rate of ITZ for patients was 90.0% (45/50), better than that of FLC for patients (86. 0%, 43/50) [30]. At the same time, treatment outcomes of 44 patients diagnosed with Malassezia folliculitis during the 5-year period between March 2007 and October 2013 were analyzed in Japan; the study demonstrated that the treatment was "effective" for all patients, and the mean period required for improvement was 27 ± 16 days for 37 patients receiving the topical application of 2.0% KTZ cream and 14 ± 4 days for the 7 patients receiving 100 mg oral ITZ. The results of topical application of 2.0% KTZ cream alone were similar to those of 100 mg oral ITZ, which is recommended for the treatment of systemic fungal infections caused by *Malassezia* [31]. KTZ and ITZ, which were superior to FLC and Am B, are recommended for the treatment of pityriasis versicolor and seborrheic dermatitis and had good antifungal effects on *Malassezia*; however, KTZ can cause toxic necrotizing hepatitis [32]. In a randomized, double-blind, placebo trial that observed both the itraconazole and placebo groups, the itraconazole group showed significantly higher improvement than the placebo group in 57 patients who took

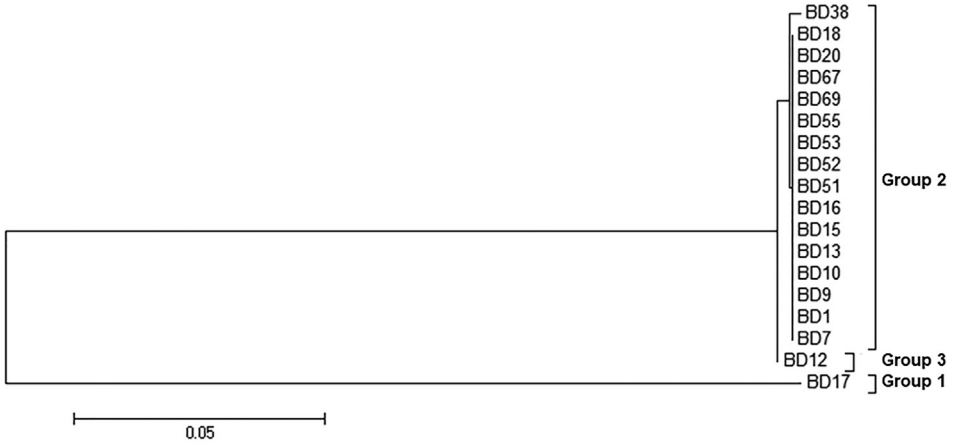

Figure 2. Phylogenetic tree analysis of Malassezia species.

Table 5. MICs (mg/L) values of KTZ, ITZ, FLC, and AmB against *Malassezia* strains, and the molecular identification of the organisms.

Strain No.	KTZ	ITZ	FLC	AmB	NL identification	ITS identification	Traditional identification	Identification Group No.
D20	0.125	0.250	2.000	2.000	*Malassezia furfur*	*Malassezia furfur*	*Malassezia sympodialis*	2
D1	0.125	0.125	2.000	1.000	*Malassezia furfur*	-	*Malassezia globosa*	2
D10	0.031	0.063	1.000	0.031	*Malassezia furfur*	*Malassezia furfur*	*Malassezia pachydermatis*	2
D9	0.125	0.250	8.000	2.000	*Malassezia furfur*	*Malassezia furfur*	*Malassezia furfur*	2
D12	0.250	0.500	8.000	2.000	*Malassezia furfur*	-	*Malassezia furfur*	3
D13	0.125	0.125	1.000	4.000	*Malassezia furfur*	-	*Malassezia furfur*	2
D15	0.500	0.125	0.500	8.000	*Malassezia furfur*	-	*Malassezia furfur*	2
D16	0.250	0.500	8.000	2.000	*Malassezia furfur*	*Malassezia furfur*	*Malassezia furfur*	2
D18	0.250	0.250	16.000	0.500	*Malassezia furfur*	*Malassezia furfur*	*Malassezia furfur*	2
D38	0.500	0.250	4.000	0.500	*Malassezia furfur*	*Malassezia furfur*	*Malassezia globosa*	2
D51	0.031	0.063	1.000	0.063	*Malassezia furfur*	*Malassezia furfur*	*Malassezia restricta*	2
D52	0.031	0.125	2.000	0.063	*Malassezia furfur*	*Malassezia furfur*	*Malassezia globosa*	2
D53	0.500	0.063	2.000	1.000	*Malassezia furfur*	*Malassezia furfur*	*Malassezia globosa*	2
D55	0.031	0.063	1.000	0.063	*Malassezia furfur*	*Malassezia furfur*	*Malassezia globosa*	2
D69	0.250	0.500	8.000	2.000	*Malassezia furfur*	*Malassezia furfur*	*Malassezia furfur*	2
D7	0.125	0.250	8.000	2.000	*Malassezia furfur*	*Malassezia furfur*	*Malassezia furfur*	2
D67	0.250	0.125	1.000	2.000	*Malassezia furfur*	*Malassezia furfur*	*Malassezia furfur*	2
D17	0.250	0.0625	0.250	0.500	*Malassezia furfur*	-	*Malassezia furfur*	1

itraconazole at 200 mg/daily or a placebo for one week and then the first two days of every month for the following three months [33]. Further studies in patients who responded to these antifungal drugs are essential for correlating in vitro results and investigating whether such clinical outcomes fit with the in vitro MIC values of these drugs. The physiological and biochemical characteristics of the species of *Malassezia* revealed differences in the sensitivity of the four species of *Malassezia* to the same antifungal drug.

Among the four species, *M. sympodialis* and *M. globosa* were more sensitive to ITZ, while *M. furfur* and *M. pachydermatis* were more sensitive to KTZ; such data are similar to the results reported by Hammer [34, 35], although these species have been isolated from different geographical regions. We also demonstrated that all of the *Malassezia* strains were resistant to FLC in vitro, which is consistent with the results reported by Margarita [36]. In summary, the sensitivity of the *Malassezia* strains to the four antifungal drugs was as follows in decreasing order: ITZ, KTZ, Am B, and FLC. We further investigated the sensitivities of *Malassezia* strains isolated from different skin diseases to the four antifungal drugs. The sensitivities of *M. furfur* isolated from three skin diseases to KTZ were significantly different. *M. furfur* derived from Malassezia folliculitis was the most sensitive to KTZ and showed no difference in sensitivity to the remaining drugs. *Malassezia* is the main treatable species for pityriasis versicolor and seborrheic dermatitis. As a commonly used drug for treating diseases related to *Malassezia*, KTZ not only is used to treat various scalp seborrheic dermatitis but also has been incorporated in an adapted vehicle, further promoting its efficacy [37]. The treatment of pityriasis versicolor and seborrheic dermatitis with KTZ in the clinic may also be the reason why *M. furfur* derived from Malassezia folliculitis is more sensitive to KTZ than that derived from the other two diseases.

Finally, due to the different growth rates of different species of *Malassezia*, we found that *M. furfur* and *M. pachydermatis* grew faster. It was likely that *M. furfur* and *M. pachydermatis* competitively inhibited the growth of *M. sympodialis* and *M. globosa*, so the strains of *M. furfur* and *M. pachydermatis* isolated from the culture might not be all pathogenic or the main pathogens of these diseases when using traditional culture methods. In recent years, some laboratories have applied molecular biological methods to identify the mycelium DNA in samples taken from the skin lesions of patients to avoid traditional culture methods, and these methods can be used to quickly and comprehensively determine the mycelium distribution of *Malassezia*-related diseases.

In summary, *M. furfur*, *M. sympodialis*, *M. pachydermatis* and *M. globosa* are the dominant strains causing *Malassezia* infection in our area. The dominant *Malassezia* species of Malassezia folliculitis is *M. globosa,* and the dominant *Malassezia* species of pityriasis versicolor and seborrheic dermatitis is *M. furfur*. There are differences in the distribution of dominant species in each disease. The sensitivities of *Malassezia* to the four

antifungal drugs were ITZ > KTZ > Am B > FLC. *M. furfur* from three diseases had a different sensitivity to KTZ. Our results showed that KTZ and ITZ had the best antifungal activity against Malassezia, but KTZ can cause toxic necrotizing hepatitis. Therefore, it is recommended that for the treatment of *Malassezia* in the clinic, ITZ is preferred as the drug of choice. In the future, the identification and drug susceptibility testing of various species of *Malassezia* should be performed, which will improve the treatment of these skin diseases in the clinic.

Declarations

Author contribution statement

J. Wang and Y. Li: Contributed reagents, materials, analysis tools or data.

K. Wang: Performed the experiments; Wrote the paper.

L. Cheng and W. Li: Performed the experiments.

H. Jiang: and X. Zhang: Analyzed and interpreted the data; Wrote the paper.

S. Liu, Y. Huang, Q. Mingye and T. Dong: Analyzed and interpreted the data.

S. Feng: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

H. Li: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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