Malaysian Journal of Microbiology, Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Analysis of Internal Transcribed Spacer (ITS) region and D1/D2 domain coupled with Random Amplified Polymorphic DNA (RAPD) reveal the inter- and intraspecific relationships of *Diutina rugosa* and *Diutina mesorugosa* isolated from Malaysian patients

Sri Raja Rajeswari Mahalingam[1], Thiba Peremalo[1], Priya Madhavan[2]*, Sharina Hamzah[1], Leslie Thian Lung Than[3], Pei Pei Chong[4], Yoke Kqueen Cheah[5], Jacinta Santhanam[6] and Jasper Elvin James[7]

[1]School of Pharmacy, Faculty of Health and Medical Sciences, Taylor's University, Lakeside Campus, 1 Jalan Taylor's. Subang Jaya, 47500 Selangor, Malaysia.
[2]School of Medicine, Faculty of Health and Medical Sciences, Taylor's University, Lakeside Campus, 1 Jalan Taylor's. Subang Jaya, 47500 Selangor, Malaysia.
[3]Department of Medical Microbiology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia.
[4]School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, Lakeside Campus, 1 Jalan Taylor's. Subang Jaya, 47500 Selangor, Malaysia.
[5]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Selangor, Malaysia.
[6]Biomedical Science Programme, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Selangor, Malaysia.
[7]Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Selangor, Malaysia.
Email: Priya.Madhavan@taylors.edu.my; mpriya0905@gmail.com

Received 14 February 2022; Received in revised form 4 March 2023; Accepted 20 March 2023

ABSTRACT

Aims: This study was aimed to characterise nine clinical isolates in our culture collection that were categorized as *Diutina* species based on their molecular genetic profiles. *D. rugosa* is a species complex comprising four taxa., i.e., *D. rugosa* sensu stricto, *D. pseudorugosa*, *D. neorugosa* and *D. mesorugosa*. The most commonly used phenotypic identification methods for yeasts often lead to the misidentification of this species complex.
Methodology and results: The *Diutina* isolates were received from two local referral hospitals as pure cultures. Species confirmation was performed using conventional phenotypic methods; CHROMagar and RapID Yeast Plus Kit. To study the inter- and intraspecific relationships among the clinical isolates, ITS region, D1/D2 domain and random amplified polymorphic DNA (RAPD) analyses were performed. The results were further validated using the housekeeping gene sequence similarity technique coupled with pairwise sequence alignment. The results from phenotypic methods results were ambiguous and inconclusive. The sequence analyses of ITS regions and D1/D2 domains revealed that the samples consisted of three yeast species; *D. rugosa* complex: *D. rugosa* (n=1), *D. mesorugosa* (n=6), *Candida pararugosa* (n=1) and *Meyerozyma guilliermondii* (n=1). The RAPD analysis with random primers, OPG4, OPG11 and OPA18, demonstrated good banding patterns that could distinguish between the *Diutina* isolates. The pairwise sequence alignment revealed that the *Diutina* isolates were genetically similar to *D. rugosa* ATCC 10571.
Conclusion, significance and impact of study: The molecular methods, D1/D2 domain, ITS1 and ITS4 region, and RAPD analyses have proven helpful for accurately identifying the yeasts, especially closely related species; *D. rugosa* and *D. mesorugosa*.

Keywords: D1/D2 domains, *Diutina rugosa*, *Diutina mesorugosa*, ITS regions, RAPD

INTRODUCTION

Diutina rugosa (*D. rugosa*) is a pathogenic yeast that has been described as an 'emerging' etiological agent of human infectious diseases (Pfaller *et al*., 2006; Padovan *et al*., 2013). *D. rugosa* is known as a species complex that comprises four taxa., i.e., *D. rugosa* sensu stricto, *D. pseudorugosa*, *D. neorugosa* and *D. mesorugosa* (Padovan *et al.*, 2013; Ming *et al.*, 2019). Previously, *D. rugosa* was referred to as *Candida rugosa* and was classified under the genus *Candida. Diutina rugosa* is highly prevalent in fungemia with invasive procedures.

***Corresponding author**

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

The typical clinical features of *D. rugosa* infections are intravenous catheter-associated candidemia and frequent colonizer in burned patients (Eggimann *et al*., 2003; Pfaller *et al*., 2006; Minces *et al*., 2009). Although *D. rugosa* infection is more frequent in immunocompromised individuals, the literature review suggests that critically ill, non-immunocompromised patients are also prone to invasive *D. rugosa* infection. It is noteworthy to recognize the fungal pathogen due to its resistance to antifungal agents - amphotericin B, azole and echinocandin drugs (Pfaller *et al.*, 2006; Diekema *et al*., 2009; Minces *et al*., 2009; Tay *et al*., 2011; Sanchis *et al.*, 2016).

Diutina rugosa infections have been reported worldwide, predominantly in Asia and South America. According to the recent ARTEMIS DISK Antifungal Surveillance Program, a total of 256 882 *Candida* isolates were isolated from 142 medical centers in 41 countries from the Asia-Pacific region, South America, North America, Europe and Africa/Middle East from 1997 to 2007 (Pfaller *et al*., 2010). *Diutina rugosa* was ranked 11th among 31 species of *Candida*, accounting for approximately 0.2% of all isolates (Pfaller *et al*., 2010). Epidemiology of *D. rugosa* infection in Malaysia suggests that *D. rugosa* constitutes about 0.2 to 1.5% of all isolates (Santhanam *et al*., 2013; Ng *et al.*, 2015; Haydar, 2018). However, this data is based on three studies only; as such, the percentage may not represent the whole of Malaysia. The frequency of isolation of *D. rugosa* in Malaysia is above what has been reported worldwide. In a study conducted at the University of Malaya Medical Centre between the years 2000 and 2013, *D. rugosa* was ranked sixth as a commonly isolated fungal species (Ng *et al*., 2015).

The yeast complex still accounts for a relatively small percentage of isolated yeast species. One reason that possibly contributes to this phenomenon is the misidentification of yeasts by relying on phenotypic characteristics (Montoya *et al.*, 2019). The identification systems that are commercially available and used in clinical laboratories are unable to identify uncommon yeasts (Paredes *et al*., 2012). For example, chromogenic agar, CHROMagar, is one of the most widely used phenotypic identification methods for yeasts. Even so, this method is highly unreliable due to its non-specific identification that often leads to misidentification - *D. rugosa* was indistinguishable from those of *C. albicans* and *Candida krusei* (Horvath *et al*., 2003). Another phenotypic method used for yeast identification is the carbohydrate assimilation test (API 20C AUX, ID 32C and Vitek Yeast Biochemical Card). Studies have suggested that these carbohydrate assimilation tests may distinguish the *D. rugosa* complex (Paredes *et al.*, 2012; Padovan *et al*., 2013). However, these studies only used a limited number of strains for the evaluation; thus, the results may not represent the *D. rugosa* complex. On the other hand, *Candida pararugosa* (*C. pararugosa*) belongs to a different taxon than the *D. rugosa* complex; however, this yeast is commonly misidentified as *D. rugosa* when only phenotypic characteristics are analysed (Padovan *et al*., 2013).

Identifying uncommon species is crucial since they are known to have more inherent resistance. Misidentification may lead to overestimating the prevalence of particular species and prejudice against less common species. Also, misidentifications of fungal species by conventional methods that heavily rely on phenotypic identification contribute to unsuccessful clinical management. Therefore, a reliable way that is apt and efficient in identifying a wide and taxonomically diverse array of pathogenic yeasts is crucial for the best clinical management and epidemiological purposes. In this study, we characterise the clinical isolates stored in our culture collection that was categorised under genus *Diutina* obtained from two local hospital laboratories, based on their inter- and intraspecific molecular genetic profiles using an internal transcribed spacer (ITS) region and D1/D2 domain, and RAPD based gene typing analyses to evaluate whether these isolates are appropriately classified according to their species and subspecies. In addition, we have also performed housekeeping gene sequence similarity coupled with pairwise sequence alignment to study the genetic differences of the *Diutina* complex.

MATERIALS AND METHODS

Clinical isolates

We received nine *Diutina* isolates from two tertiary referral hospital laboratories in Malaysia. These isolates were dated from 2007 to 2016 from blood (n=7) and skin (n=2) specimens. The cultures were designated as Cr2745, Cr2672, Cr2692, Cr3715, Cr3114, Cr2610, Cr25103, Cr2014 and Cr2354 and sub-cultured on Sabouraud Dextrose agar (SDA) (Oxoid, Hampshire, UK) prior to confirming the species. As a reference strain, the American Type Culture Collection (ATCC) strain, *Diutina rugosa* (Anderson) Khunnamwong et al. 10571^{TM} (previously known as *D. rugosa* ATCC 10571) was used in this investigation. All isolates and reference strains were maintained on SDA media plates at 4 °C and kept as glycerol stock cultures at -80 °C.

CHROMagar Candida

CHROMagar Candida was prepared following the manufacturer's instructions and incubated at 37 °C for 48 h.

RapID Yeast Plus System

RapID Yeast Plus System (Remel, USA) was performed according to the manufacturer's instructions.

DNA extraction

The DNA extraction of nine *Diutina* isolates and one ATCC reference strain was carried out in accordance with the instructions included with the GeneAll DNA extraction kit (GeneAll Biotechnology, Korea). The purity and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

concentration of the DNA were measured using a nanophotometer (Beckman Coulter, US). The DNA was stored at -20 °C until further application.

PCR conditions of the ITS region amplification

The approach for ITS region sequencing was optimised based on a previously disclosed method with some changes (Isogai *et al*., 2010). The reaction was prepared by using GoTaq® Green Master Mix (GoTaq® Green Master Mix[(a)] is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations) – 1× (Promega: Catalog No. M7122), 10 µM ITS 1 forward primer (5'-TCCGTAGGTGAACCTGCGG-3'), 10 µM ITS 4-reverse primer (5'-TCCTCCGCTTATTGATATGC-3') and sterile ultra-pure water. 100 ng of DNA templates were added to the master mix solution. Negative control was established by substituting sterile ultra-pure water for the DNA templates. The polymerase chain reaction was carried out using MJ Mini Personal Thermal Cycler (Bio-Rad, US). The reaction was started with an initial denaturation at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 35 sec and extension at 72 °C for 1 min followed by a final extension at 72 °C for 8 min.

PCR conditions of the D1/D2 domain amplification

The PCR was optimized for D1/D2 domain sequence analysis based on a previously described method with some modifications (Chaves *et al*., 2013). The reaction was prepared by using GoTaq® Green Master Mix (GoTaq® Green Master Mix[(a)] is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations) – 1× (Promega: Catalog No. M7122), 10 µM ITS 1 forward primer (5'-TCCGTAGGTGAACCTGCGG-3'), 10 µM ITS 4-reverse primer (5'-TCCTCCGCTTATTGATATGC-3') and sterile ultra-pure water. 100 ng of DNA templates were added to the master mix solution. Negative control was established by substituting sterile ultra-pure water for the DNA templates. The reaction consisted of initial denaturation at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 68 °C for 1 min, followed by a final extension at 68 °C for 8 min.

Agarose gel electrophoresis

The agarose gel electrophoresis technique was used to analyse the PCR products. The agarose gel (Vivantis Technologies, Malaysia) was prepared into 1.5% using 1× TBE buffer and stained with SYBR™ Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific). The PCR product (5 µL) was added into the specified wells of the agarose gel together with a 100 bp DNA ladder (New England Biolabs, UK). The electrophoresis was run in 1× TBE buffer at 60 V for 40 min. The gel was visualised using a UV transilluminator and the image was captured digitally using the Quantum ST5 Imaging System (Vilber Lourmat, Germany). The DNA ladder (ThermoFisher Scientific, USA) was used to determine the sizes of the DNA bands. Upon confirming the correct size of the PCR products, the remaining PCR products were purified using a GeneAll DNA purification kit (GeneAll Biotechnology, Korea) and subsequently sent for sequencing using the Sanger Sequencing method (Apical Scientific Sdn. Bhd.).

DNA sequencing

A sequencing service laboratory (Apical Scientific Sdn. Bhd.) performed the DNA sequencing. The DNA fragment was purified and sequenced in both directions, with ITS region analysis using primer pair ITS1 and ITS4, and D1/D2 domain analysis using primer pair NL1 and NL4. A consensus sequence from forward and reverse sequences were aligned using Clustal W and trimmed using BioEdit software (Thompson *et al*., 1994). Primer sequence identification was performed using the Basic Local Alignment Search Tools (BLAST) tool available at the National Center for Biotechnology Information (NCBI) website.

Phylogenetic analysis

The phylogenetic method was applied to study the relationship between the closely related *Diutina* species from seven clinical isolates. ITS region and D1/D2 domain sequences of each *Diutina* isolates were compared with other sequences available in the GenBank database using BLAST. Phylogenetic analysis, which included reference strain sequences obtained from Genbank, was used to determine the species (Table 1). Molecular Evolutionary Genetics Analysis (Mega) software version 7.0 was used to create the phylogenetic tree by employing the Maximum Likelihood method with 1000 bootstrap replicates, Jones-Taylor-Thornton (JTT) substitution model and gaps were dealt with partial deletion (Kumar *et al*., 2016).

PCR conditions of the RAPD amplification

Genetic relatedness among clinically isolated *Diutina* species was analysed using RAPD technique. A previously described method was used for this part of the study (Xu *et al*., 2012). A total of nine random operon primers were chosen, including set A (OPA6, OPA17, OPA18), set E (OPE3, OPE4, OPE18) and set G (OPG4, OPG5, OPG11). The RAPD PCR reactions were prepared by using GoTaq® Green Master Mix (GoTaq® Green Master Mix[(a)] is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations) – 1× (Promega: Catalog No. M7122), 100 µM selected operon primer and sterile ultra-pure water. One hundred ng of DNA templates were added to the master mix solution. The reaction was started with an initial denaturation at 95 °C for 5 min, 45 cycles of denaturation at 94 °C for 1 min, an annealing gradient

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

Table 1: Details on reference strains obtained from GenBank for phylogenetic analysis of ITS region and D1/D2 domain in the present study.

No	Strains	Source/Country	ITS GeneBank Accession No	D1/D2 GeneBank Accession No	References
1	*Diutina rugosa* 10571	ATCC strain.	-	-	-
2	*Diutina rugosa* strain ATCC 10571	ATCC strain.	GU144663	GU144663	(Mota *et al*. 2009) (unpublished)
3	*Diutina rugosa* strain ATCC 10571	ATCC strain.	FJ768914	FJ768915	(Chaves *et al*., 2013)
4	*Diutina rugosa* subtype A	Human blood, Malaysia.	HM641831	HQ412589	(Tay *et al*., 2011)
5	*Diutina rugosa* subtype B	Human blood, Malaysia.	HM641832	HQ412590	(Tay *et al*., 2011)
6	*Diutina mesorugosa* isolate GHA 044	Urine, Ghana.	KM260364	N/A	(Adjapong *et al*., 2016)
7	*Diutina mesorugosa* strain L154	Human blood, Brazil.	FJ768910	FJ768916	(Chaves *et al*., 2013)
8	*Diutina mesorugosa* strain L387A	Human rectal swab, Brazil.	FJ768911	FJ768920	(Chaves *et al*., 2013)
9	*Diutina mesorugosa* strain L412D	Human pericatheter swab, Brazil.	FJ768912	FJ768919	(Chaves *et al*., 2013)
10	*Diutina mesorugosa* strain L2683B	Human blood, Brazil.	FJ768913	FJ768918	(Chaves *et al*., 2013)
11	*Diutina rugosa* strain Zhuan8	Marine yeast, China.	EF197805	N/A	(Guo and Chi, 2006) (unpublished)
12	*Candida pararugosa* strain IDR1000011225	N/A, USA.	JN675331	N/A	(Chaturvedi and Chaturvedi, 2011) (unpublished)
13	*Candida pararugosa* isolate URFM514	Agricultural specimen, France.	KX859684	N/A	(Callon and Verdier-Metz, 2016) (unpublished)
14	*Candida pararugosa* isolate URFM416	Agricultural specimen, France.	KX859779	N/A	(Callon and Verdier-Metz, 2016) (unpublished)
15	*Candida pararugosa* culture CBS:7884	Yeast, Netherlands.	KY102326	KY106684	(Vu *et al*., 2016)
16	*Candida pararugosa* strain MA09-AP	Cheese, Canada.	GQ458032	N/A	(Arteau *et al*., 2010)
17	*Candida pararugosa* UTHSC 08-442	Human urine, USA.	N/A	HE716766	(Paredes *et al*., 2012)
18	*Candida pararugosa* UTHSC 09-2953	Human vaginal, USA.	N/A	HE716765	(Paredes *et al*., 2012)
19	*Meyerozyma guilliermondii* strain CMC_1924	N/A, Italy.	MG367466	N/A	(Colabella *et al*., 2017)
20	*Meyerozyma guilliermondii* isolate M-1	Animal specimen, China.	KT897919	N/A	(Zhou, 2015) (unpublished)
21	*Meyerozyma guilliermondii* strain JY 41	Plant specimen, China.	KP975418	N/A	(Chen *et al*., 2015)
22	*Meyerozyma guilliermondii* isolate CBS 2082	Food specimen, Italy.	KP109736	N/A	(Corte *et al*., 2015)
23	*Meyerozyma guilliermondii* isolate CBS 1909	Food specimen, Italy.	KP109730	N/A	(Corte *et al*., 2015)

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

Table 2: Primers used for the housekeeping genes.

Gene	Primer	Sequence (5'→3')	PCR product size (bp)
ACT1	Act1 forward primer	GATCTGGCACCACACCTTCT	500
	Act1 reverse primer	GCAGGTCTGAACAAGGCTTC	
COX2	Cox2 forward primer	TTTGAACAATCTTCCCAGCA	450
	Cox2 reverse primer	G TTGGCATTAAAGCGTGGTT	
RBP1	Rbp1 forward primer	CATGTGAGCTGGTGTGTATGC	500
	Rbp1 reverse primer	CGAGCTTGAACGTCAAATCA	

within 30 °C to 60 °C for 1 min for each successive cycle and extension at 72 °C for 2 min followed by a final extension at 72 °C for 10 min. The banding patterns were digitally collected and analysed using the Bionumerics software (Version 6.6, Applied Maths) and Alpha Imager Imaging System.

Housekeeping gene sequence similarity

The housekeeping gene sequence similarity was determined using a previously outlined procedure (Chaves *et al*., 2013). The primers used for housekeeping gene analysis were identified as described in the preceding section. In this study, the three housekeeping gene sequences of *D. rugosa* used were selected from the National Center for Biotechnology Information (NCBI) database. The primers for the housekeeping genes and their sequence are listed in Table 2. Using additional genetic markers, the housekeeping genes of *D. rugosa* were chosen to determine the association between the *D. rugosa* strains.

RESULTS AND DISCUSSION

Phenotypic characteristics

A total of nine samples categorised as *D. rugosa*, which was isolated from blood and skin specimen, were obtained from two tertiary referral hospitals in Malaysia from 2007 to 2016. The isolates were identified using conventional and molecular identification methods. Firstly, all nine *Diutina* isolates were phenotypically identified through the use of CHROMagar Candida. Seven isolates yielded brilliant blue colonies with pale borders, which were identified as *D. rugosa*, one isolate yielded pink to purple colonies, which were identified as *Meyerozyma guilliermondii* (*M. guilliermondii*) (Nadeem *et al.*, 2010) and one isolate yielded pale violet colonies, which were identified as *C. pararugosa*. Species-level confirmation was further done using RapID Yeast Plus System for biochemical analysis in which six isolates were identified as *D. rugosa* and two isolates did not show possibility towards any reliable species and one isolate was identified as *M. guilliermondii*. Due to the discrepancies between the results obtained from CHROMagar Candida and RapID Yeast Plus System, all the isolates were further evaluated using molecular methods, sequence analysis of the ITS region and D1/D2 domain was carried out using phylogenetic analyses.

ITS region analysis

Interestingly, findings from ITS region analysis showed that all the nine isolates tested were grouped into three major clades with different phylogenetic species, *D. rugosa* complex (n=7), *C. pararugosa* (n=1) and *M. guilliermondii* (n=1), with 100% bootstrap support (Figure 1). Furthermore, all seven isolates that belong to the *D. rugosa* complex were grouped into two distinct clades of the complex: *D. rugosa* and *D. mesorugosa*. The first clade consisted of six isolates (Cr2745, Cr2672, Cr2692, Cr3715, Cr2610 and Cr37114) from this study that was grouped with six *D. mesorugosa* reference strains isolated from human blood: Malaysia (HM641831), Brazil (FJ768910, FJ768911, FJ768912 and FJ768913) and Ghana (KM260364). As a result, these isolates were characterized as *D. mesorugosa*, a *D. rugosa* complex member. Meanwhile, isolate Cr25103 was grouped into another clade of *D. rugosa* complex - *D. rugosa*, together with reference strains from Malaysia (HM641832) and China (EF197805) isolated from human and marine sources, respectively. The reference strains used for phylogenetic analysis of ITS region and D1/D2 domain of the large subunit ribosomal RNA gene sequence analysis in the present study were retrieved from the GenBank database (Table 1). Although ITS region analysis resolved the identity of all the samples, further analysis of the D1/D2 domain of the large subunit ribosomal RNA sequence was carried out to validate the findings from ITS region analysis of the *Diutina* isolates. *M. guilliermondii* was excluded from the further analysis. This is due to *M. guilliermondii* belonging to the genus *Meyerozyma*. This study focuses on *Diutina* complex isolates; however, we have included *C. pararugosa* in the following analysis to study its relatedness to the *Diutina* complex.

D1/D2 domain analysis

All eight clinical isolates: *D. rugosa* (n=1), *D. mesorugosa* (n=6) and *C. pararugosa* (n=1) were analysed for D1/D2 domain. From the D1/D2 domain phylogenetic tree, all the isolates tested were divided into two major clades; *D. rugosa* complex (n=7) and *C. pararugosa* (n=1) (Figure 2). The network analysis also separated the sequences of *C. pararugosa* from those of *D. rugosa* and *D. mesorugosa* sequences. Strikingly, *C. pararugosa* is phylogenetically diverse from *D. rugosa* and *D. mesorugosa*. The results from D1/D2 domain phylogenetic analysis corroborates with the findings from

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

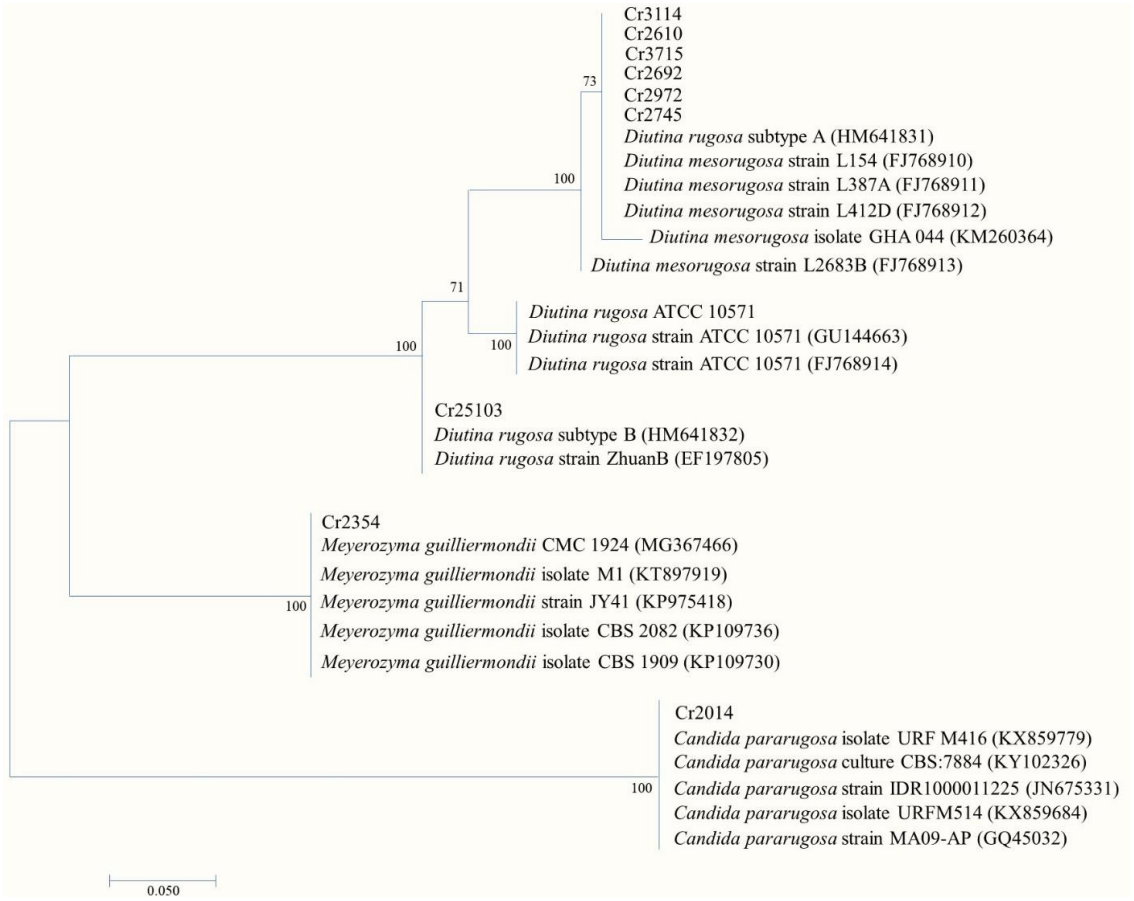


Figure 1: Phylogenetic tree of *D. rugosa* complex: *D. rugosa* and *D. mesorugosa*, *M. guilliermondii*, *C. pararugosa* using the Maximum Likelihood method with sequences from ITS region. The dendrogram shows three major clades of different phylogenetic species with 100% bootstrap support.

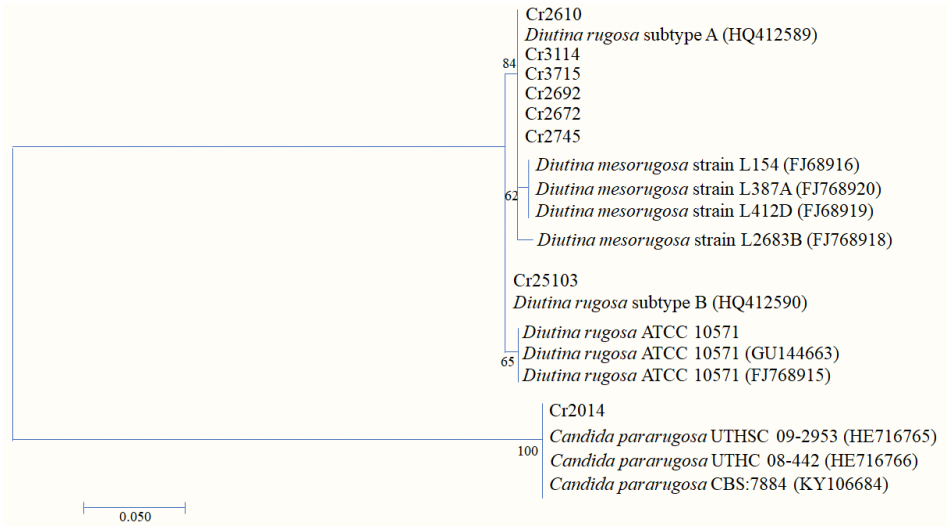


Figure 2: Phylogenetic tree of *D. rugosa*, *D. mesorugosa* and *C. pararugosa* isolate using the Maximum Likelihood method with sequences of D1/D2 domain. The dendrogram shows two major clades of different phylogenetic species with 100% bootstrap support.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

ITS region analysis. The ITS region and D1/D2 domain analyses identified that all the seven *Diutina* isolates as *D. rugosa* complex comprise *D. mesorugosa* (n=6) and *D. rugosa* (n=1). Overall, all *Diutina* isolates were accurately identified molecularly and differentiated to their species level by DNA sequencing of both the ITS region and D1/D2 domain compared to the conventional methods. The traditional techniques were unable to distinguish the *Diutina* species complex. Molecular approaches provide a more objective identification of yeasts, especially involving closely related species such as the *D. rugosa* complex. In the current research, all the *Diutina* isolates were differentiated into their respective species-level classification by ITS region and D1/D2 domain analyses. In addition, we also have further characterised the *Diutina* isolates using the RAPD technique to study if RAPD-PCR can distinguish the *D. rugosa* isolate from *D. mesorugosa* isolates.

RAPD analysis

All the *Diutina isolates* (n=7) were further characterised to study the inter- and intraspecific genetic relationships based on their genotypes using RAPD. In this study, a total of nine Operon primers (OPA, Operon Technologies, Inc, Alameda, California) which include set A (OPA6, OPA17, OPA18), set E (OPE3, OPE4, OPE18), and set G (OPG4, OPG5, OPG11) primers were selected and optimised to compare DNA profiles of all the isolates based on their banding patterns. The RAPD results were consistent with ITS region and D1/D2 domain studies, revealing that all seven *Diutina* isolates belonged to two distinct clades. The band patterns were identical for each primer for all six isolates identified as *D. mesorugosa,* according to the dendrogram from the ITS region and D1/D2 domain analyses. These bands are distinct from those found in *D. rugosa* ATCC 10571, the reference strain. For all of the primers examined, *D. rugosa* isolate has a distinctive banding pattern than *D. mesorugosa* isolates.

The gel electrophoresis images show the fingerprints of the eight primers used in the RAPD analysis (Figure 3). The eight primers used in this study have successfully characterised all the *Diutina* isolates into their respective clusters. The RAPD data analysis revealed that there were two clusters of *D. rugosa* complex species; *D. mesorugosa* (n=6) isolates (Cr2745, Cr2672, Cr2692, Cr3715, Cr3114 and Cr2610) that were grouped under the same cluster, and *D. rugosa* (n=1) isolate (Cr25103) belonged to another cluster. The RAPD results showed that the technique is able to differentiate between these two species. The random primers OPA18, OPG4 and OPG11 have the highest discriminatory power among the nine primers. Our results are in line with several previous studies that have reported that OPG4, OPG5, OPG 18 and OPG11 primers were excellent options to investigate the genetic diversity of *D. rugosa* isolates (Simona *et al.*, 2009; Ślaska *et al.*, 2011; Samaka, 2015). In this study, for all the *D. mesorugosa* isolates (n=6), OPG 4 primer produced seven identical band patterns, which were completely different from the reference strain's banding pattern. The OPG11 primer produced eight fragments with identical band patterns in all the *D. mesorugosa* isolates that were also entirely different from the reference strain. For *D. rugosa* isolate, random primer OPA18 produced an excellent band pattern (Cr25103). As a result, OPG4 and OPG11 primers may be used as appropriate RAPD markers for identifying *D. mesorugosa* strains, while OPA18 can be utilised as an optimal RAPD marker for identifying *D. rugosa* strains.

A RAPD dendrogram cluster analysis was generated using Bionumerics software for genetic comparison of all the *Diutina* isolates (n=7) isolates with *D. rugosa* ATCC 10571 references strain (Figure 4). According to the dendrogram study, Cr25103 displayed 100% similarity with the ATCC 10571 strain. The remaining six isolates showed 99% similarity with *D. rugosa* ATCC strain. There was 89% similarity found among Cr2610, Cr2745 and Cr3114 isolates, whereas 83% similarity were found among Cr2745, Cr3114 and Cr2692 isolates. On the other hand, 84% similarity was found among the Cr3114, Cr2692 and Cr3715 isolates, and 50% similarity among the Cr2692, Cr3715 and Cr2672 isolates. The findings from the dendrogram indicate that all the *D. mesorugosa* isolates (n=6) were genetically similar.

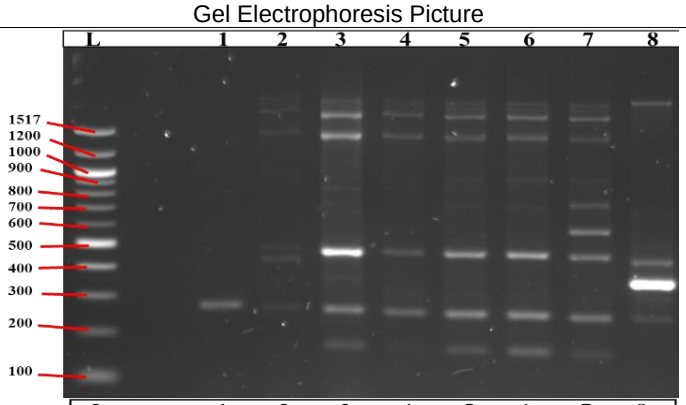
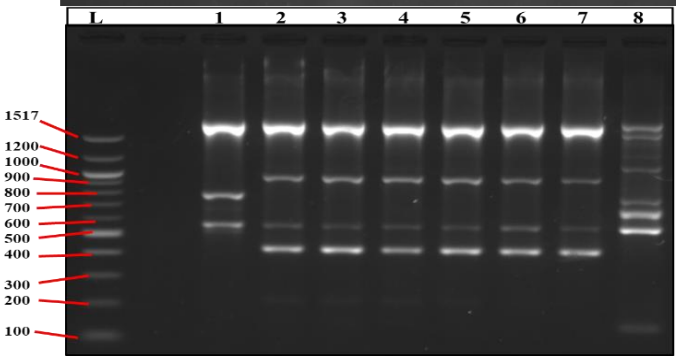
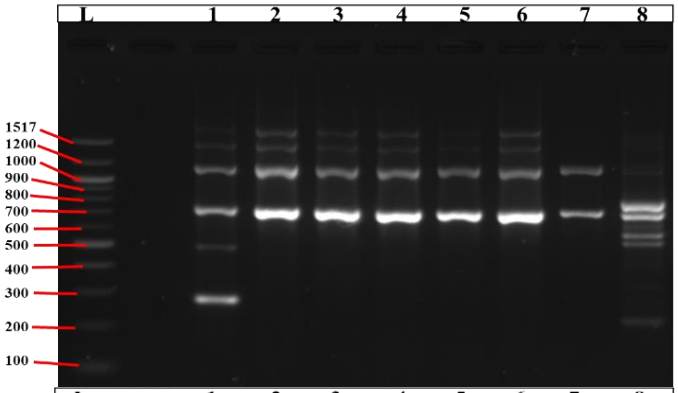
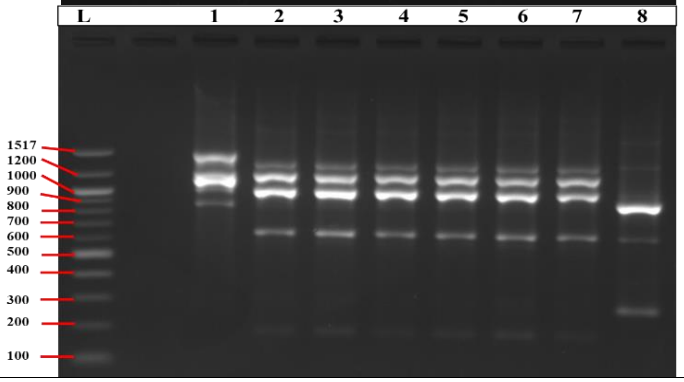
The RAPD-based gene typing is widely used in the genetic relatedness study of various fungal species such as *Candida* and *Aspergillus*. In fact, RAPD typing methods have been used previously to determine the genetic relatedness of *D. rugosa* isolates (Colombo *et al*., 2003; Behera *et al*., 2010; Chaves *et al*., 2013). Several studies have reported the reliability, simplicity, specificity and sensitivity of randomly amplified polymorphic DNA (RAPD) analysis in identifying pathogenic fungal species (Bautista-Muñoz *et al*., 2003; Baires-Varquez *et al*., 2007). Even so, the RAPD is notorious for its lack of reproducibility between laboratories; as such, highly standardised experimental procedures for the polymerase chain reaction (PCR) protocol are required for a successful result. Despite that, Bautista-Muñoz *et al*. (2003) have reported using RAPD for direct, straightforward and relatively rapid identification of nine pathogenic *Candida* species of clinical relevance. The findings from our study show that the RAPD technique is applicable for *D. rugosa* complex identification, mainly to distinguish *D. rugosa* and *D. mesorugosa*.

Housekeeping genes similarity

In addition to the aforementioned analyses, we also attempted to study the inter- and intraspecific genetic differences of the *Diutina* isolates against the reference strain, *D. rugosa* ATCC 10571, using the housekeeping gene similarity coupled with pairwise sequence alignment technique. This method was adapted from a previous study that reported the differences between *D. rugosa* and *D. mesorugosa* by sequencing the additional genetic markers (Chaves *et al*., 2013). To distinguish between *D. rugosa* complex species and *D. rugosa* ATCC 10571 strain, we used three different housekeeping genes:

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

	Primer	Gel Electrophoresis Picture
1	OPA17	
2	OPA18	
3	OPE3	
4	OPE4	

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

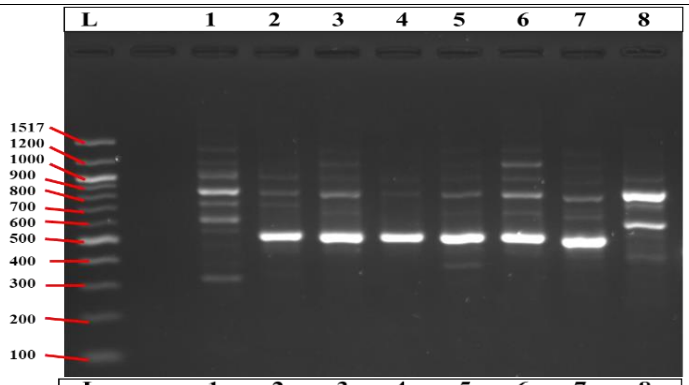
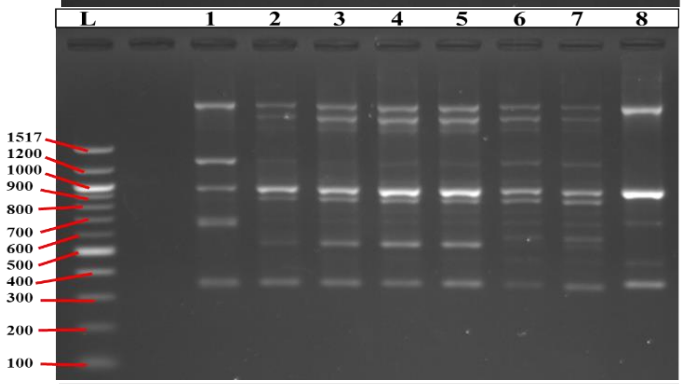
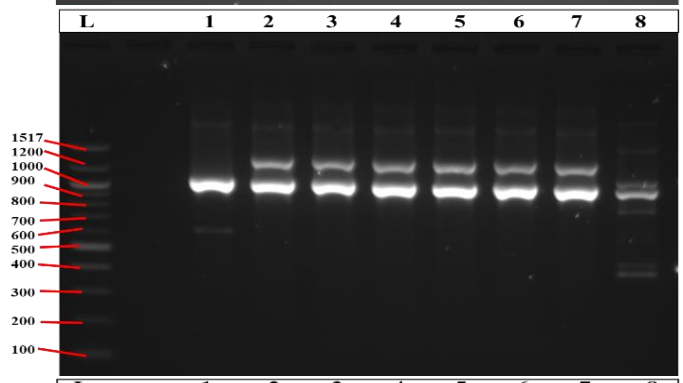
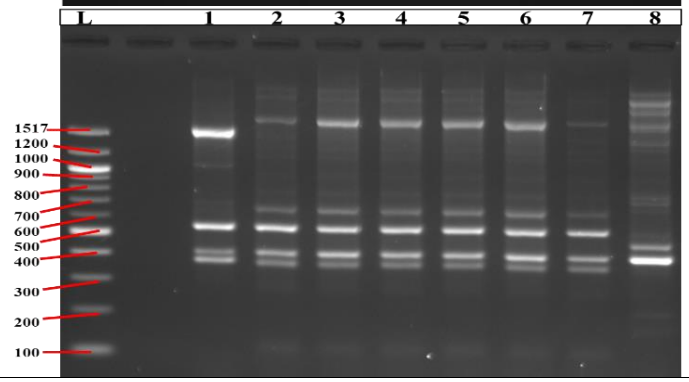
5	OPE18	
6	OPG4	
7	OPG5	
8	OPG11	

Figure 3: RAPD fingerprints of *D. rugosa* and *D. mesorugosa* isolates using various primers. Lane L: 100 bp ladder, Lane 1: Cr10571 ATCC, Lane 2: Cr2745, Lane 3: Cr2672, Lane 4: Cr2692, Lane 5: Cr3715, Lane 6: Cr3114, Lane 7: Cr2610, Lane 8: Cr25103.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

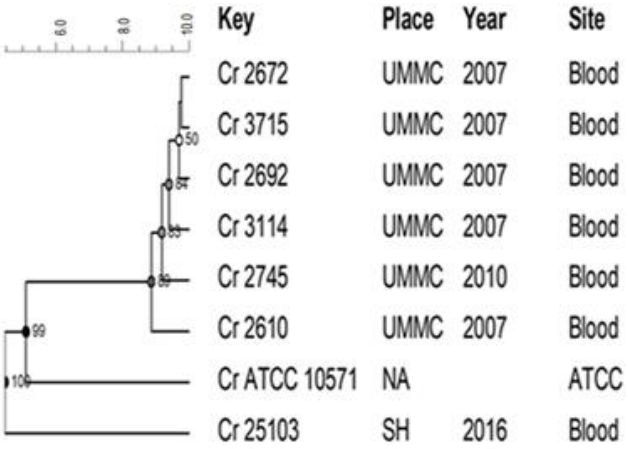


Figure 4: Dendrogram cluster analysis of *D. rugosa* (n=1): Cr25103 and *D. mesorugosa* (n=6): Cr2672, Cr3715, Cr2692, Cr3114, Cr2745 and Cr2610, with *D. rugosa* ATCC 10571. Each strain shows the place of isolation (UMMC = University Malaya Medical Centre, SH = Serdang Hospital, NA = Not applicable), year and site of isolation.

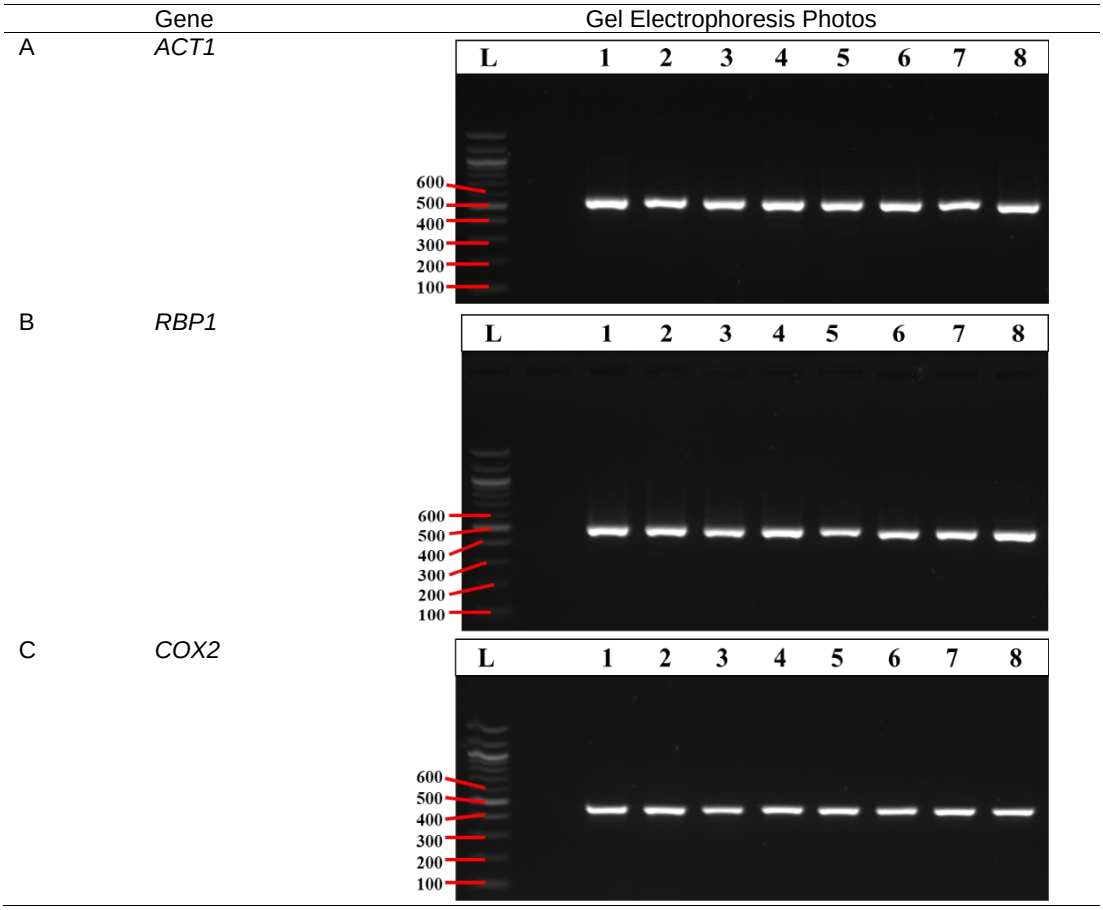


Figure 5: DNA fingerprints of housekeeping genes for all the seven *Diutina* isolates. A - *ACT1* gene, B - *RBP1* gene and C - *COX2* gene. Lane L: 50 bp ladder, Lane 1: Cr10571 ATCC, Lane 2: Cr2745, Lane 3: Cr2672, Lane 4: Cr2692, Lane 5: Cr3715, Lane 6: Cr3114, Lane 7: Cr2610 and Lane 8: Cr25103.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(3) 2023, pp. 261-273
DOI: http://dx.doi.org/10.21161/mjm.221449

Table 3: Percentage of sequence identities of the *Diutina* isolates compared with *D. rugosa* ATCC 10571.

No	Strain ID	*D. rugosa* 10571 *ACT1*(%)	*D. rugosa* 10571 *RBP1* (%)	*D. rugosa* 10571 *COX2* (%)
1	Cr2745	98	98	98
2	Cr2672	98	98	98
3	Cr2692	98	98	98
4	Cr3715	98	98	98
5	Cr3114	98	98	98
6	Cr2610	98	98	98
7	Cr25103	98	97	99

ACT1, RBP1 and COX2. These genes were chosen from previously published studies (Minces *et al.*, 2009; Chaves *et al.*, 2013). The PCR technique was used to amplify all of the housekeeping genes. Figure 5 shows the presence of all three housekeeping genes in all seven *Diutina* isolates after PCR amplification. The Clustal W programme was used to perform a pairwise comparison. The degree of genetic similarity between the *Diutina* isolates and *D. rugosa* ATCC 10571 was shown by pairwise sequence alignment for the ACT1, RBP1 and COX2 genes.

The *Diutina* isolates showed 98% of similarity with *D. rugosa* ATCC 10571 for ACT1 genes. In terms of COX2 genes, *D. mesorugosa* and *D. rugosa* had 98% and 99% of similarity with *D. rugosa* ATCC 10571, respectively. In terms of RBP1 genes, *D. mesorugosa* and *D. rugosa* have 98% and 97% of similarity with *D. rugosa* ATCC 10571, respectively. Table 3 shows the similarities of the *Diutina* isolates' housekeeping genes to *D. rugosa* 10571. The preliminary findings from this study based on the three housekeeping gene sequences suggest that *D. rugosa* and *D. mesorugosa* are genetically similar to *D. rugosa* ATCC 10571. The results from the housekeeping genes similarity technique are also favourable to the results obtained from the ITS region, D1/D2 domain and RAPD analyses. The finding from this study corroborates with previous findings, where there were only minimal differences between the *D. rugosa* and *D. mesorugosa* isolates and *D. rugosa* ATCC 10571 reference strain (Chaves *et al*., 2013). It is tempting to conclude that both the complexes, *D. rugosa* and *D. mesorugosa* are genetically similar to each other. As such, this may be one reason for the misidentification of the species complex. *D. mesorugosa* is often categorised as *D. rugosa*.

Our objective in this work is to investigate the identity of clinical isolates that were classified as *D. rugosa*. Our findings revealed the prevalence of *C. pararugosa* amongst *D. rugosa* isolates present in our culture collection. The misidentification of *C. pararugosa* as *D. rugosa* has been reported in several studies in the past (Chaves *et al*., 2013). Interestingly, our result showed that *M. guilliermondii* was also misidentified as *D. rugosa*. There are many studies that have reported on misidentification of *M. guilliermondii* as other *Candida* species, such as *Candida famata* (Kim *et al*., 2014); however, to our knowledge, this is the first report of misidentification of *M. guilliermondii* as *D. rugosa*. The precise clarification of the taxonomy of yeasts is essential in understanding the epidemiology and its pathogenicity. Our study emphasizes the need to accurately identify the clinical isolates, which can only be achieved by molecular techniques.

CONCLUSION

In summary, the conventional techniques using CHROMagar Candida and RapID Yeast Plus System biochemical kit yielded ambiguous and uncertain results. They were not optimum for the precise identification of the *Diutina* complex. Based on the three techniques tested in this study - ITS region analysis; D1/D2 domain analysis; and RAPD-based gene typing, all nine isolates were grouped into four clusters, *D. mesorugosa* (n=6), *D. rugosa* (n=1), *M. guillermondii* (n=1) and *C pararugosa* (n=1). From the analysis of RAPD-PCR based gene typing, the ideal marker for *D. mesorugosa* identification is OPG4 and OPG11; conversely, OPA18 serves as an optimum RAPD marker for *D. rugosa*. The ITS region analysis, D1/D2 domain analysis and RAPD-based gene typing are rapid, straightforward, reproducible and could be used as an alternative to the conventional yeast identification approach in differentiating closely related species. Although the findings from housekeeping gene sequence similarity support the conclusions from all the other tested methods, limitation in the availability of the whole-genome sequence of *D. rugosa* and *D. mesorugosa* limits the screening of housekeeping genes; thus, results obtained may not be conclusive. Further study is warranted to investigate the whole-genome sequence of *D. rugosa* and *D. mesorugosa*.

ACKNOWLEDGEMENTS

We thank the Ministry of Education Malaysia (FRGS/2/2014/SKK01/TAYLOR/02/1) for funding this research, University Kebangsaan Malaysia and Taylor's University for providing technical support for the research work.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

REFERENCES

Adjapong, G., Bartlett, M., Hale, M. and Garrill, A. (2016). The isolation of *Candida rugosa* and *Candida mesorugosa* from clinical samples in Ghana. *Medical Mycology* **54, 322-326.**

Arteau, M., Labrie, S. and Roy, D. (2010). Terminal-restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis profiling of fungal communities in Camembert cheese. *International Dairy Journal* **20(8), 545-554.**

Baires-Varguez, L., Cruz-García, A., Villa-Tanaka, L., Sánchez-García, S., Gaitán-Cepeda, L. A., Sánchez-Vargas, L. O. *et al.* (2007). Comparison of a randomly amplified polymorphic DNA (RAPD) analysis and ATB ID 32C system for identification of clinical isolates of different *Candida* species. *Revista Iberoamericana de Micologia* **24(2), 148-151.**

Bautista-Muñoz, C., Boldo, X. M., Villa-Tanaca, L. and Hernández-Rodríguez, C. (2003). Identification of *Candida* spp. by randomly amplified polymorphic DNA analysis and differentiation *between Candida albicans* and *Candida dubliniensis* by direct PCR methods. *Journal of Clinical Microbiology* **41(1), 414-420.**

Behera, B., Singh, R. I., Xess, I., Mathur, P., Hasan, F. and Misra, M. C. (2010). *Candida rugosa*: A possible emerging cause of candidaemia in trauma patients. *Infection* **38(5), 387-393.**

Callon, C. and Verdier-Metz, I. (2016). *Wickerhamiella pararugosa* isolate URFM416 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. Available online at https://www.ncbi.nlm.nih.gov/nuccore/KX859684 (Retrieved on 7 February 2022).

Callon, C. and Verdier-Metz, I. (2016). *Wickerhamiella pararugosa* isolate URFM416 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. Available online at https://www.ncbi.nlm.nih.gov/nuccore/KX859779 (Retrieved on 7 February 2022).

Chaturvedi, S. and Chaturvedi, V. (2011). *Wickerhamiella pararugosa* strain IDR1000011225 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. Direct submission. Available online at https://www.ncbi.nlm.nih.gov/nuccore/JN675331 (Retrieved on 7 February 2022).

Chaves, G. M., Terçarioli, G. R., Padovan, A. C. B., Rosas, R. C., Ferreira, R. C., Melo, A. S. A. *et al.* (2013). *Candida mesorugosa* sp. nov., a novel yeast species similar to *Candida rugosa*, isolated from a tertiary hospital in Brazil. *Medical Mycology* **51(3), 231-242.**

Chen, S., Liu, Z., Liu, Y., Lu, Y., He, L. and She, Z. (2015). New depsidones and isoindolinones from the mangrove endophytic fungus *Meyerozyma guilliermondii* HZ-Y_2 isolated from the South China Sea. *Beilstein Journal of Organic Chemistry* **11, 1187-1193.**

Colabella, C., Corte, L., Roscini, L., Shapaval, V., Kohler, A., Tafintseva, V. *et al*. (2017). Merging FT-IR and NGS for simultaneous phenotypic and genotypic identification of pathogenic candida species. *PLoS ONE* **12(12), e0188104.**

Colombo, A. L., Melo, A. S. A., Rosas, R. F. C., Salomão, R., Briones, M., Hollis, R. J. *et al.* (2003). Outbreak of *Candida rugosa* candidemia: An emerging pathogen that may be refractory to amphotericin B therapy. *Diagnostic Microbiology and Infectious Disease* **46, 253-257.**

Diekema, D. J., Messer, S. A., Boyken, L. B., Hollis, R. J., Kroeger, J., Tendolkar, S*. et al.* (2009). *In vitro* activity of seven systemically active antifungal agents against a large global collection of rare *Candida* species as determined by CLSI broth microdilution methods. *Journal of Clinical Microbiology* **47(10), 3170-3177.**

Eggimann, P., Garbino, J. and Pittet, D. (2003). Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *The Lancet Infectious Diseases* **3(11), 685-702.**

Guo, N. and Chi, Z. (2006). Research on diversity of marine yeasts. Available online at https://www.ncbi.nlm.nih.gov/nuccore/EF197805 (Retrieved on 7 February 2022).

Haydar, A. (2018). Epidemiology and outcomes of candidaemia among adult patients admitted at Hospital Universiti Sains Malaysia (HUSM): A 5-year review. *International Medical Journal Malaysia* **17(1), 3-11.**

Horvath, L. L., Hospenthal, D. R., Murray, C. K. and Dooley, D. P. (2003). Direct isolation of *Candida* spp. from blood cultures on the chromogenic medium CHROMagar Candida. *Journal of Clinical Microbiology* **41(6), 2629-2632.**

Isogai, H., Mulu, A., Diro, E., Tekleselassie, H., Kassu, A. and Kimura, K. (2010). Identification of *Candida* species from human immunodeficiency virus-infected patients in Ethiopia by combination of CHROMagar, tobacco agar and PCR of amplified internally transcribed rRNA spacer region. *Journal of Applied Research* **10(1), 1-8.**

Kim, S. H., Shin, J. H., Mok, J. H., Kim, S. Y., Song, S. A., Kim, H. R. *et al.* (2014). Misidentification of *Candida guilliermondii* as *C. famata* among strains isolated from blood cultures by the VITEK 2 system. *BioMed Research International* **2014, Article ID 250408.**

Kumar, S., Stecher. G. and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33(7), 1870-1874.**

Minces, L. R., Ho, K. S., Veldkamp, P. J. and Clancy, C. J. (2009). *Candida rugosa*: A distinctive emerging cause of candidaemia. A case report and review of the

literature. *Scandinavian Journal of Infectious Diseases* **41(11-12), 892-897.**

Ming, C., Huang, J., Wang, Y., Lv, Q., Zhou, B., Liu, T. *et al.* (2019). Revision of the medically relevant species of the yeast genus *Diutina*. *Medical Mycology* **57(2), 226-233.**

Montoya, A. M., Luna-Rodríguez, C. E., Gracia-Robles, G., Rojas, O. C., de J Treviño-Rangel, R. and González, G. M. (2019). *In vitro* virulence determinants, comparative pathogenicity of *Diutina* (*Candida*) *mesorugosa* clinical isolates and literature review of the *D. rugosa* complex. *Mycologia* **111(3), 395-407.**

Mota, A. J., Back-Brito, G. N. and Nobrega, F. G. (2009). Description of a new species of Candida with biochemical and phenotypic pattern similar to *Candida rugosa.* Available online at https://www.ncbi.nlm.nih.gov/nuccore/GU144663 (Retrieved on 7 February 2022).

Nadeem, S. G., Hakim, S. T. and Kazmi, S. U. (2010). Use of CHROMagar *Candida* for the presumptive identification of *Candida* species directly from clinical specimens in resource-limited settings. *Libyan Journal of Medicine* **5(1), 2144.**

Ng, K. P., Kuan, C. S., Kaur, H., Na, S. L., Atiya, N. and Velayuthan, R. D. (2015). *Candida* species epidemiology 2000-2013: A laboratory-based report. *Tropical Medicine and International Health* **20(11), 1447-1453.**

Padovan, A. C. B., Melo, A. S. A. and Colombo, A. L. (2013). Systematic review and new insights into the molecular characterization of the *Candida rugosa* species complex. *Fungal Genetics and Biology* **61, 33-41.**

Paredes, K., Sutton, D. A., Cano, J., Fothergill, A. W., Lawhon, S. D., Zhang, S. *et al*. (2012). Molecular identification and antifungal susceptibility testing of clinical isolates of the *Candida rugosa* species complex and proposal of the new species *Candida neorugosa*. *Journal of Clinical Microbiology* **50(7), 2397-2403.**

Pfaller, M. A., Diekema, D. J., Colombo, A. L., Kibbler, C., Ng, K. P., Gibbs, D. L. *et al*. (2006). *Candida rugosa*, an emerging fungal pathogen with resistance to azoles: Geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program. *Journal of Clinical Microbiology* **44(10), 3578-3582.**

Pfaller, M. A., Diekema, D. J., Gibbs, D. L., Newell, V. A., Ellis, D., Tullio, V. *et al*. (2010). Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: A 10.5-year analysis of susceptibilities of *Candida species* to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *Journal of Clinical Microbiology* **48(4), 1366-1377.**

Samaka, H. M. A. (2015). RAPD-PCR based genetic variation of *Candida albicans* of animal and human origin. *AL-Qadisiya Journal of Veterinary Medicine Sciences* **14(1), 54-57.**

Sanchis, M., Sutton, D. A., Wiederhold, N. P., Guarro, J. and Capilla, J. (2016). Efficacy of echinocandins against murine infections by *Diutina* (*Candida*) *rugosa*. *Diagnostic Microbiology and Infectious Disease* **86(1), 61-65.**

Santanam, J., Yahaya, N. and Aziz, M. N. (2013). Species distribution and antifungal susceptibility patterns of *Candida* species: Is low susceptibility to itraconazole a trend in Malaysia? *Medical Journal of Malaysia* **68(4), 343-347.**

Simona, E. S., Diana, P., Robertina, I., Ionela, A., Ileana, S. and Tatiana, V. D. (2009). Molecular identification of some yeast strains involved in oral candidosis. *Romanian Biotechnology Letters* **14(1), 4180-4186.**

Ślaska, B., Różański, P., Różańska, D. and Nisztuk, S. (2011). Differentiation of *Candida* species and *Candida rugosa* strains with the use of molecular markers in healthy horses. *Annales UMCS Zootechnica* **29(4), 113-124.**

Tay, S. T., Tan, H. W., Na, S. L. and Lim, S. L. (2011). Phenotypic and genotypic characterization of two closely related subgroups of *Candida rugosa* in clinical specimens. *Journal of Medical Microbiology* **60, 1591-1597.**

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22(22), 4673-4680.**

Vu, D., Groenewald, M., Szöke, S., Cardinali, G., Eberhardt, U., Stielow, B., *et al*. (2016). DNA barcoding analysis of more than 9 000 yeast isolates contributes to quantitative thresholds for yeast species and genera delimitation. *Studies in Mycology* **85(1), 91-105.**

Xu, H., Liu, M., Chen, Y., Huang, J., Xu, C. and Lu, L. (2012). Randomly amplified polymorphic deoxyribonucleic acid (DNA) analysis of *Candida albicans* isolates from clinical sources of hospital in south China. *African Journal of Microbiology Research* **6(10), 2552-2558.**

Zhou, Y. Y. (2015) *Meyerozyma guilliermondii* isolate M-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. Available online at https://www.ncbi.nlm.nih.gov/nuccore/KT897919 (Retrieved on 7 February 2022).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538