



A roadmap for rapid molecular differential characterisation of human pathogenic *Candida* species

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Abstract

The PCR technique is a promising technique used to establish accurate identification methods to gain precise results. The incidence of yeast infections has increased dramatically coinciding with the breakout of HIV infections. A quick and affordable diagnostic procedure is crucial in the quest to tackle such infections. In this study, a detailed diagnostic framework was designed to help in the rapid identification of pathogenic *Candida* species. A sum of 17 *Candida* species were involved in this analysis and a common conserved region of rRNA was detected to design primer pairs for in-silico PCR. Restriction Fragment Length Polymorphism (RFLP) was used for species identification. One universal primer was designed to amplify the conserved region in all species, while two others were specific to particular species. Results showed that the PCR-RFLP technique (by using the universal primer No.1) identified 10 species *Clavispora lusitanae*, *C. Cyberlindnera*, *C. asseri* by unique restriction enzyme *Hinf*I, *C. carpophila* and *C. quercitrusa* using *Hph*I and, *C. glabrata*, *Wickerhamomyces anomalus* using *Pho*I while *P. kudriavzevii* and *C. viswanathii* using *Bgl*I and *Bse*GI respectively. Moreover, *C. albicans* PCR was identified by double restriction enzymes *Stu*I and *Bsp*OI. The PCR-RFLP analysis has identified 7 species using primer pair No.2. These species are *C. parapsilosis* and *Lodderomyces elongisporus* by *Aat*I and *Acv*I respectively. The Cleavage of PCR products using *Bst*4CI has identified four species (*C. glabrata*, *C. lusitanae* and *C. tropicalis*). *C. dubliniensis* was identified by double restriction enzymes (*Dra*I+ *Apo*I). Meanwhile, *C. orthopsilosis* was identified through *Dra*I digestion of virtual PCR product (primer pair No.3). All together, these results showed that PCR-RFLP technique is an efficient and rapid method used to scrutinise accurate results by computational based methods before being applied in the Lab. This study set up for a detailed guideline for an accurate, quick diagnostic procedure without any need for the sequencing step.

Keywords: *Candida* species, in-silico PCR, RFLP-PCR

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INTRODUCTION

DNA amplification is a fascinating invention that alters the scientific research to the level of molecular biology, for instance, investigating gene polymorphism, mutations, studying relatedness among related species, clinical diagnosis or gene manipulation (Solanki 2012). The process is carried out into living cells naturally as a routine cell cycle (Dalton 1998). Later, the biochemist Kary Mullis conducted this process in-vitro by developing the polymerase chain reaction technique (PCR) (Allison 2007, Solanki 2012).

Meanwhile, the bioinformatic field contributes to this process by developing an efficient software that can mimic the *in situ* steps, the in-silico software (Kalendar et al. 2011). A computerised software accomplishes many tasks such as DNA target amplification, examining the annealing of the primer pair against the DNA target, primer specificity and designing the primer (a critical

step to yielding good PCR products) (Kanwal et al. 2017).

Despite the usefulness of the coexistence of *Candida* species in the host, they are seizing the opportunity to weaken the immune system causing mild (superficial) and systemic infections called Candidemia (McCullough et al. 1996, Mcfarland 2000). In recent years, the incidence of yeast infections has increased dramatically after the breakout of HIV infections (Khan et al. 2012). The importance of infections diagnosis is associated with accurate medical management (Mohammadali et al. 2017). Mycologists developed classical and advanced approaches to diagnose yeast; each approach has its own advantages and disadvantages, hence the combination between the two approaches

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presented the best results (Khan et al. 2012, Mohammadali et al. 2017).

The PCR technique is the cornerstone of molecular biology used to differentiate among yeast species. It has shown that there is a conserved region in the yeast genome that can be explored to investigate the relatedness among species. ITS region is the most common region used to differentiate between different yeast species, in addition to the large subunit (26S) of ribosomal DNA of D1\D2 domain (Kurtzman 2011, Kurtzman et al. 2011, Raju and Rajappa 2011).

The yeast identification methods have been developed along with scientific progress. In the beginning, simple and time-consuming techniques were used rely on mycologist expertise, while more advanced and accurate techniques were established to achieve accurate results (Kurtzman et al. 2011, Raju and Rajappa 2011).

The PCR technique is fundamental in molecular biology that is used to differentiate among yeast species (Hiero et al. 2004). Various species of *Candida* were identified as pathogenic, like *Candida albicans* and non-albicans species, such as *C. asseri*, *C. carpophila*, *Clavispora lusitaniae*, *Cyberlindnera fabianii*, *C. dubliniensis*, *C. glabrata*, *Meyerozyma guilliermondii*, *Lodderomyces elongisporus*, *C. metapsilosis*, *C. orthopsilosis*, *C. parapsilosis*, *Pichia kudriavzevii*, *C. quercitrusa*, *C. tropicalis*, *C. viswanathii* and *Wickerhamomyces anomalus* (Ahmad et al. 2018, Mbah et al. 2016, Randhawa et al. 2015, Wengenack and Binnicker 2009).

There are different identification methods that have been established which rely on the PCR technique; for instance Restriction Fragment Length Polymorphism (PCR-RFLP) technique (Al-sweih et al. 2019, Randhawa et al. 2015). The RFLP technique identified *Candida* species and compensated from sequencing in addition to their sensitivity and specificity (Randhawa et al. 2015). Mirhendi et al. (2006) identified five clinical *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. guilliermondii*) following the PCR-RFLP analysis when PCR products were digested with unique restriction enzyme *MspI* (Kim et al. 2017).

This study was set out to design a framework which would help identify pathogenic *Candida* species quickly by using informatics tools. This was carried out by investigating the variation in the conserved region among 17 *Candida* species by which each species could be differentiated. This was done using Computational software, designing primers, scrutinising their binding, amplifying the target DNA fragment and finally choosing a suitable restriction enzyme to produce different sizes of DNA segments among the studied *Candida* species.

Table 1. GenBank accession numbers of rRNA small subunit gene, ITS 1, ITS2 and apart of large subunit rRNA gene 26 of *Candida* species in the NCBI database

<i>Candida</i> species No	<i>Candida</i> species	Accession No.
1	<i>C. albicans</i>	MK394124.1
2	<i>C. asseri</i>	MK394117.1
3	<i>C. carpophila</i>	MK394110.1
4	<i>Clavispora lusitaniae</i>	MK394145.1
5	<i>Cyberlindnera fabianii</i>	MK394133.1
6	<i>C. dubliniensis</i>	MK394123.1
7	<i>C. glabrata</i>	MK394140.1
8	<i>Meyerozyma guilliermondii</i>	MK394108.1
9	<i>Lodderomyces elongisporus</i>	MK394128.1
10	<i>C. metapsilosis</i>	MK394127.1
11	<i>C. orthopsilosis</i>	MK394126.1
12	<i>C. parapsilosis</i>	MK394125.1
13	<i>Pichia kudriavzevii</i>	MK394162.1
14	<i>C. quercitrusa</i>	MK394107.1
15	<i>C. tropicalis</i>	MK394119.1
16	<i>C. viswanathii</i>	MK394122.1
17	<i>Wickerhamomyces anomalus</i>	MK394130.1

MATERIALS AND METHODS

Multiple DNA Sequence Alignments

The DNA sequence of rRNA (small and large subunits of rRNA including the interrupting non-coding regions ITS1 and ITS2) of *Candida* species were recovered from the NCBI database (Table 1). Sequence alignments were carried out by using MUSCLE program implemented in UGENE software version 1.32.0 (<http://ugene.net/>) to identify the conservative region of rRNA.

Oligonucleotide Primer Design and DNA Amplification

Oligonucleotide primers were designed by utilising the web-based software Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The efficiency of the primers was investigated by their annealing to the DNA target and amplifying PCR products virtually in the UGENE software version 1.32.0 (Mirhendi et al. 2006). Other parameters were determined such as primers' length, GC% and primers' Melting Temperature (T_m).

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP)

PCR was carried out virtually in the UGENE software version 1.32.0 (Mirhendi et al. 2006) using the default parameters. PCR products were digested by restriction enzymes. The restriction sites of each sequence were identified by using SnapGene viewer software (version 4.3.8); the restriction enzyme which distinguished single species was selected.

RESULTS AND DISCUSSION

Retrieval of rRNA Sequence of *Candida* Species

Although mycologists established many conventional and advanced identification methods,

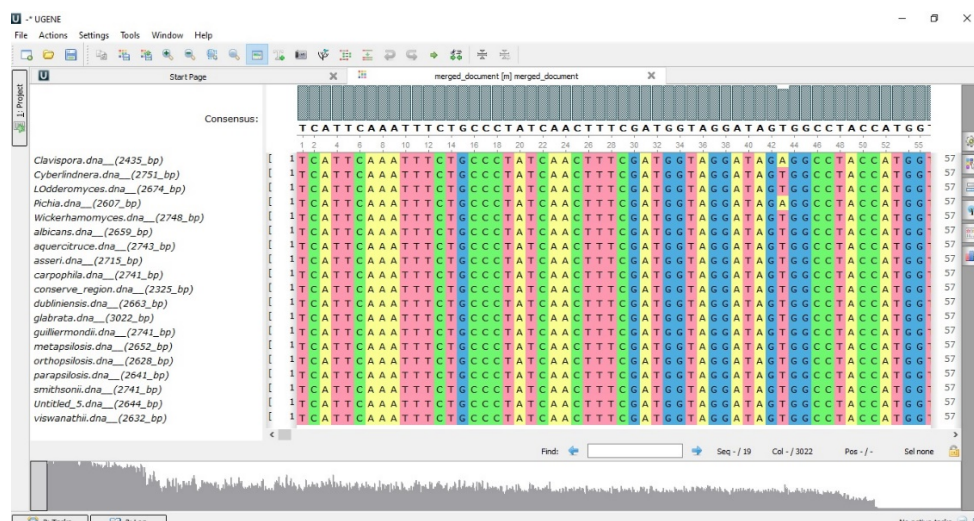


Fig. 1. Nucleotide sequence alignment of small, large subunits of rRNA and intermediate ITS1 and ITS2 regions of 17 *Candida* species including a highly-conserved region
The alignment was carried out using MUSCLE software implemented in the UGENE tools

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T C A T T C A A A T T T C T G C C C T A T C A A C T T T C G A T G G T A G G A T A G T G G C C T A C C A T G G
G C T A C C A C A T C C A A G G A A G G C A G C A G C G C G C A A T T A C C C A A T C C C G A C A C G G G A G G T A G T G A C A A T A A A T A C G A T A C A G G G C C C T T G G T C T T G A A T T G G A A T G A G T
A C A A T G T A A A T A C C T T A A C G A G G A A C A A T T G G A G G C A A G T C T G T G C C A G C A G C C G C G G T A A T T C C A G C T C C A A A G C G T A T A T A A A G T T G T T G C A G T T A A A A G C C T C G T A
G T T G A A C C T T G G G C T T G G T G G C C G T C C T C T T G C G T A C T G A C C A A C G A G C C T T T C C T C T C C T T T T G G C G A A C C A G G A C T T T A C T T T G A A A A A A A T A G A G T G T T C A A A G
A G G C C T T T G C T C G A A T A T T A G C A T G G A A T A A G A A T A G G A C G T G T T C T A T T T G T T G G T T C T A G G A C C A T C G T A A T G A T T A A T A G G A C G T C G G G G C A T C A G T A T
T C A G T T G T C A G A G G T G A A A T T C T T G G A T T A C T G A A G A C T A A C T A C T G C G A A G C A T T G C C A A G G A C G T T T C A T T A A T C A A G A C G A A A G T T A G G G G A T C G A A G A T G A T C A
G A T A C C G T C G T A G T C T T A A C C A A A C T A T G C C G A C T A G G G A T C G G G T G T T G T C T T T A C G C A C T T A C G A G A A A T C A A A G T C T T T G G T T C T G G G G A G A T C A
G G T C G C A A G G C T G A A A C C T T A A A G G A A T T G A C G G A A G G C A C C A C A G G A G T G G A G C T G C G C T T A A T T T G A C T C A A C A C G G G A A A C T C A C C A G G T C C A G A C A C A A T A A
G G A T T G A C A G A T T A G A G C T T T C T T G A T T T G T G G T G T G G T G C A T G C C T T A G T T G G T G A T T G T C T G C T T A A T T G C G A T A A C G A A C C A G A C C T T A A C C T C
T A A A T A G T C T G T A S C A T T T G C T G T T A C A C T T C T T A G A G G A C T A C G A T T C A A G C G A T G C A T G S A A G T T T G A G G C A A T A A C A G G T C T G T A T G C C C T T A G A C T T C T G G C C
G C A C G C G C C T A C A C T G A C G G A G C C A G C G A G A A C C T T G C C G A G A G T C T G G A A A T C T T G T A A A C C C C G T C G G G A T A G A G C A T T G T A A T T G C T C T T C A A
C G A G A A A T C C T A G T A A G C G C A A G T C A T C A G C T T G C G T T G A T A C G T C C C T G C C C T T T G T A C A C C C G C C G T C T A C C G A T T G A A T G G C T T A G T G A G C T T C C G G A T
T G G T T A A G A A G G G G C A A C T C C A T C G A A C C G A A G A G C T A G T A C A A A C T T G G T C A T T T A G A G G A A G T A A A A G T C G T A A C A A G G T T C C G T A G G T G A A C C T C G G A A G G A T
C A T T A C A G A A T T A A A C C C A A A T T T T T C A A A A C T T T C A A C A C A G G A T C T T T G G T T C G C A T C G A T G A A A A C G C A G O S A A A T G C G A T A G T A A T G A A T T G C A G A C G T G A A
T C A T C G A A T C T T G A A C C G C A C A T T G C G C C C T T G G T A T T C A G A G G G C A T G C C T T T T G A G C G T C A T T C C C T C A A A C G G T T T G G T G T T G A C T T G A A A T T A T C C A A C T G T T G
A T A C A A C A T T T G A C C T C A A A T C A G G T A G G A C T A C C C G C T G A A C T T A A G C A T A C A A T A A G C G A G A A A A A A C C A A C A G G A T T G C C T A G T A G C G C G A G T G A A G C G G
C A A A A G C T C A A A T T T G A A A T C T G G C G A G T T G A A T T T G A A G A A G G T T C T T T G G G C T G G T C T T G C T A T G T T C T T G A A C A G G A C G T C A C A G A G G T G A G A A T C C G T G C G A
T G T G C C A G T A A A G T T C C T T C G A A G A G T C G A G T T T T G G A A T G C A G C T C T A A G T G G T G T A A T T C C A T C T A A A G C T A A A T T G G C G A G A G A C C G A T A G C G A A C A A G T A
C A G T G A T G A A A A G T G A A A A G A C T T T G A A A G A G A G T G A A A A G T A C T G A A A T T G T G A A A G G A A G G C T T G A G A T A G A T C A G A T T G G T T A C C G G C C A G A C T C G G T T T G
C G G T A G A A T G G C G T A G G A A T G T G C A C T G C G T G T G T T A T A G C C T T T G T A T A C T G C C A G C T A G A C C G A G G A C T G C G T G C C T A G A T G C T G G C A T A A T G A T C T T A A G
C C S C C C G T C T T G A A A C C G G A C C A A G G A G T C T A A C G T C T A T G C G A G T T T T G G T G T A A A C C C G T A C C G C T A A T G A A A G T G A A
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Fig. 2. The consensus Nucleotide sequence of the conserved region of rRNA of 17 *Candida* species
The conserved region was determined by the alignment of rRNA of 17 *Candida* species. The sequence was used to design primers for further analysis

these approaches have advantages and drawbacks (Raju and Rajappa 2011). Hence the necessity prompts mycologists to develop new quick, easy and cheap methods to be in consent with medical and economical requirements. Therefore, the purpose of this study is to develop a rapid, sensitive and specific identification and discrimination approach to differentiate between human pathogenic *Candida* species. This aim was performed by determining the roadmap of the conservative region of rRNA genes following bioinformatics approaches. The identification was carried out through the PCR-RFLP technique.

Seventeen pathogenic species were included in this study (Table 1). The comparison between available identification approaches showed that molecular genetic identification is a more accurate identification method; however, specialists prefer the combination of conventional and advanced approaches (Kurtzman et al. 2011, Ahmad et al. 2018). Considering the availability of

genomic DNA sequence in the database made the work easier. In this study, the rRNA (small and large subunits of rRNA including the interrupting non-coding regions ITS1 and ITS2) of 17 *Candida* species (Table 1) was restored from NCBI (<https://www.ncbi.nlm.nih.gov/>).

Multiple Sequence Alignment of *Candida* Species

The alignment of restored DNA sequences of the 17 *Candida* species rRNA region was carried out to determine the conserved region and use it in further analysis (Fig. 1). When taking into consideration the chosen nucleotide sequence, there are big regions with many restriction sites, the abundance of many copies of the ribosomal gene increases the opportunity of successful PCR products, and the rRNA gene is characterised by comprising both extremely conserved and variable regions (Mirhendi et al. 2006). The resulted nucleotide sequence alignment showed a conserved region about 2,325 bp in length (Fig. 2).

Table 2. Details of primer pairs sequence parameter and conditions

Primer name	Start (conserved region)	End (conserved region)	Length (pb)	Tm	GC %	Product size (pb)	Sequence 5'-3'	Notes
ITS F1							ATTGGAGGGCAAGTCTGGTG	Universal primer
ITS R1	250	799	20	59.8	55	550	TCAGCCTTGCGACCATACTC	
ITS F2							GGCCGTTCTTAGTTGGTGA	All species amplify PCR products except for <i>W. anomalus</i> , <i>C. Cyberlindnera</i> and <i>P. kudriavzevii</i>
ITS R2	944	1534	20	53.83	55	591	CTGCGTTCTTCATCGATGCG	
ITS F3							CGTGCTGGGGATAGAGCATT	Amplify 16 species except for <i>C. glabrata</i>
ITS R3	1195	1518	20	59.9	55	324	TGCAGAAACCAAGAGATCCG	

*Note: product size differs slightly depending on the *Candida* species rather than product size of the conserved region

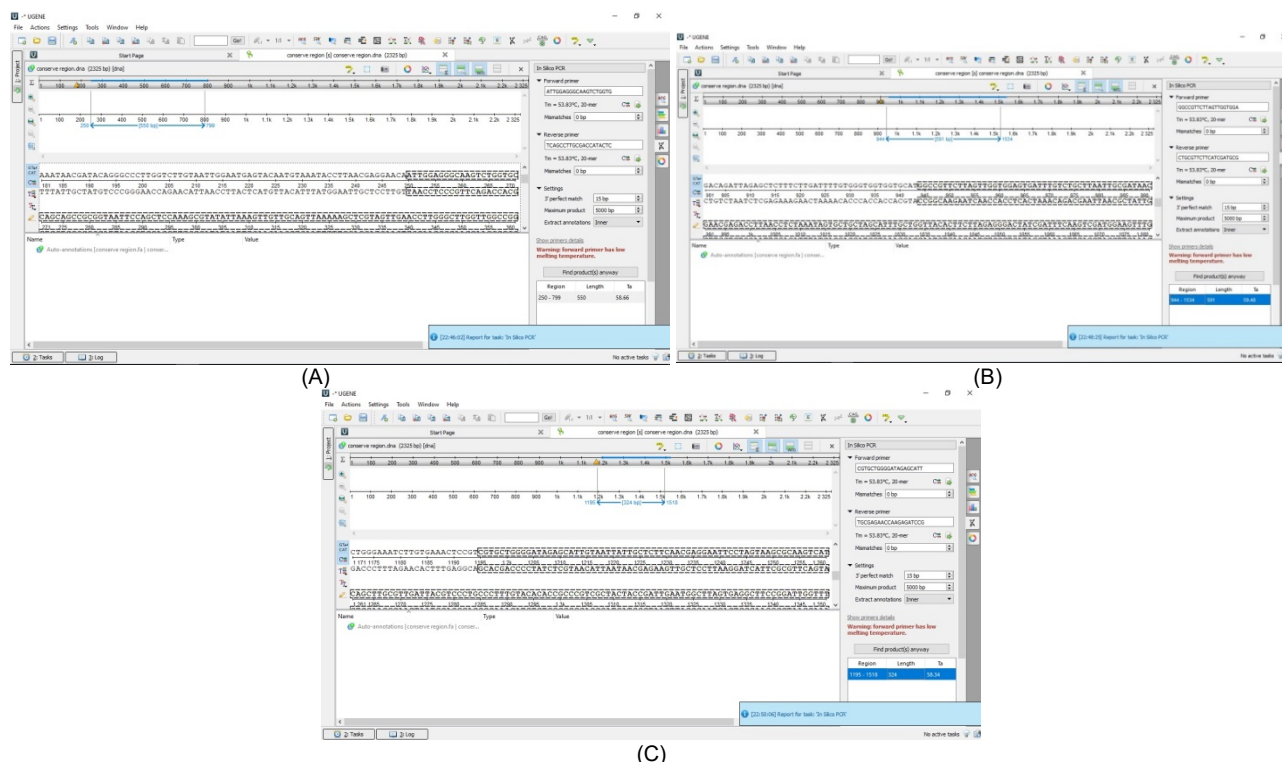


Fig. 3. Photos show In-silico PCR by using UGENE software. (A) rRNA of conserved region fragment was amplified using universal primer No.1., while primer No. 2, 3 and 4 products are shown in figures (B) and (C) respectively

The PCR-RFLP Analysis

The PCR-RFLP technique was used before to identify pathogenic species. This technique identified *Candida* species and compensated from sequencing in addition to their sensitivity and specificity (Kareem et al. 2017). Mirhendi et al. (2006) identified five clinical *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. guilliermondii*) after digestion of ITS conserved region with unique restriction enzyme *MspI* (Kim et al. 2017).

The conserved region (Fig. 2) was exploited for designing an oligonucleotide primer pair to amplify the target sequence. The Primer3Plus software website was used for this purpose. The primers' parameters were calculated in the Primer3Plus website and inspected in the UGENE software (Table 2). Three primer pairs were selected to fit with the requirements of In-Silico PCR amplification. The selected primers were listed in Table 2.

Three primer pairs were designed to amplify different nucleotide sequence loci in the rRNA sequence and digested the products with a restriction enzyme to differentiate among species. In-silico PCR product size differ depending on the primer (Fig. 3).

Primers pair No.1 is the universal primer used to amplify the rRNA of all the strains, PCR product yield ranges between (564-587 bp) (Table 3). Distinguishing between *Candida* species were carried out by digesting the PCR product following the RFLP technique. A unique restriction enzyme was used to identified single species (Table 4). It was shown that *C. asseri*, *C. carpophila*, *Clavispora lusitanae*, and *Pichia kudriavzevii* strains were identified by more than one restriction enzyme; for example *C. asseri* was identified by *HinfI*, *AfiI*, *PhoI* or *SpeI* while *C. quercitrusa*, *C. glabrata*, *C. viswanathii*, *W. anomalus* and *C. Cyberlindnera* were identified only by *HphI*, *PhoI*, *BseGI*, *PhoI* and *HinfI* respectively (Table 4). In Table 4, it could be noticed that cleavage of PCR products of *C. asseri*,

Table 3. Length and position of In-silico PCR products of four primer pairs

Strains		Primer No.1		Primer No.2		Primer No.3	
Sr. No	Candida species	position	Product length (bp)	position	Product length (bp)	position	Product length (bp)
1	<i>C. albicans</i>	253-826	574	972-1684	713	1230-1668	439
2	<i>C. asseri</i>	253-833	581	979-1745	767	1236-1729	494
3	<i>C. carpophila</i>	253-839	587	985-1727	743	1243-1711	469
4	<i>Clavispora lusitaniae</i>	253-817	565	963-1598	636	1217-1582	366
5	<i>Cyberlindnera fabianii</i>	253-833	581	-----	No product	1238-1728	491
6	<i>C. dubliniensis</i>	253-826	574	972-1684	713	1230-1668	439
7	<i>C. glabrata</i>	253-839	587	985-1959	975	-----	No product
8	<i>Meyerozyma guilliermondii</i>	253-829	587	985-1727	743	1243-1711	469
9	<i>Lodderomyces elongisporus</i>	253-825	573	971-1718	748	1229-1702	474
10	<i>C. metapsilosis</i>	253-826	574	972-1702	731	1230-1686	457
11	<i>C. orthopsilosis</i>	253-825	573	971-1684	714	1229-1668	440
12	<i>C. parapsilosis</i>	253-826	574	972-1695	724	1230-1679	450
13	<i>Pichia kudriavzevii</i>	250-813	564	----	No product	1213-1615	403
14	<i>C. quercitrusa</i>	254-839	586	755	985-1739	1243-1723	481
15	<i>C. tropicalis</i>	253-824	572	970-1682	713	1228-1666	439
16	<i>C. viswanathii</i>	253-825	573	971-1678	708	1229-1662	434
17	<i>Wickerhamomyces anomalus</i>	253-832	580	-----	No product	1236-1719	484

Table 4. Fragment sizes for restriction digestion to identify *Candida* species using the RFLP technique

Restriction enzyme	Candida species	Size of the fragments (bp)
<i>HinfI</i>	<i>C. asseri</i>	134, 447
<i>AflI</i>		511, 70
<i>SpeI</i>		162, 419
<i>PhoI</i>		108, 473
<i>TstI</i>	<i>C. carpophila</i>	434, 32, 121
<i>HphI</i>		369, 96, 125
<i>HinfI</i>	<i>C. lusitaniae</i>	221, 344
<i>TstI</i>		89, 32, 444
<i>DdeI</i>		117, 40, 408
<i>HphI</i>	<i>C. quercitrusa</i>	159, 209, 218
<i>PhoI</i>	<i>C. glabrata</i>	99, 9, 37, 442
<i>BseGI</i>	<i>C. viswanathii</i>	130, 443
<i>AflI</i>	<i>Pichia kudriavzevii</i>	221, 343
<i>BglI</i>		504, 60
<i>PhoI</i>	<i>W. anomalus</i>	226, 354
<i>HinfI</i>	<i>Cyberlindnera fabianii</i>	514, 67
<i>BspOI + StuI</i>	<i>C. albicans</i>	219, 355

C. lusitaniae, *C. Cyberlindnera* by *HinfI* produces different fragment sizes while *PhoI* was used to identify *C. asseri*, *C. glabrata* and *W. anomalus*. *C. albicans* was cleaved by double restriction enzymes, *StuI* and *BspOI* producing two fragments (219 and 355) bp.

Primers pair No.2 annealed and amplified the rRNA region in all the species, except for *W. anomalus*, *C. Cyberlindnera* and *Pichia kudriavzevii*. The PCR product size range was 636 to 975 (Table 3).

The PCR products were digested with the restriction enzyme for identification. *C. glabrata*, *C. lusitaniae*, *C. tropicalis*, *C. dubliniensis*, *C. asserise*, *C. parapsilosis* and *L. elongisporus* were identified by digestion with *BanII*, *HincII*, *Bst4CI*, *DraI*, *AatI*, *AcvI* respectively. Table 5 shows the size and number of DNA fragments of identified species resulted from the digestion with restriction enzymes. Each enzyme digests the PCR products producing DNA fragments of different sizes and identified species produce fragments of different sizes compared to those of other species. Using one restriction enzyme, more than one strain could be identified. *Bst4CI* identified four species, *C. glabrata*, *C. lusitaniae* and *C. tropicalis* while *DraI* distinguished

Table 5. Fragment size for restriction digestion to identify *Candida* species using the RFLP technique

Restriction enzyme	Candida species	Size of the fragments (bp)
<i>BanII</i>	<i>C. glabrata</i>	551, 424
<i>HincII</i>		892, 83
<i>Bst4CI</i>		778, 197
<i>DraI</i>		878, 97
<i>Bst4CI</i>	<i>C. lusitaniae</i>	538, 98
<i>HincII</i>		213, 423
<i>Bst4CI</i>	<i>C. tropicalis</i>	634, 79
<i>DraI+ ApoI</i>	<i>C. dubliniensis</i>	301, 322, 90
<i>DraI</i>	<i>C. asserise</i>	413, 181, 173
<i>AatI</i>	<i>C. parapsilosis</i>	601, 123
<i>AcvI</i>	<i>L. elongisporus</i>	573, 175

between *C. glabrata* and *C. asserise*. Double restriction enzymes, *DraI* and *ApoI*, were utilized to identify *C. dubliniensis*.

Primer pair No.3 identified only one strain (*C. orthopsilosis*) using the digestion with single restriction enzyme *DraI*. *DraI* restriction enzyme cleavages PCR product into two fragments 381 and 59 bp. Two species, *M. guilliermondii* and *C. metapsilosis*, showed difficulties in differentiation.

Altogether, this approach was firstly, able to identify 15 pathogenic *Candida* species with no need for sequencing, which saves the time needed for identification. Secondly, compared to a previous work that identified five strains (*C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lusitaniae* species) of total eight selected pathogenic strains (Kim et al. 2017), our work involved and diagnosed more pathogenic *Candida* species. Indeed, the main obstacle for identification is the number of species, selected to be identified in this work, in other words, the increased number of species narrows the variations. A previous study identified *C. parapsilosis* complex species (*C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*) by using double restriction sites (*HhaI* and *Sau96I*) (Mirhendi et al. 2006). The ITS1-5.8S-ITS2 region of the rRNA was selected to amplify and digestion with previous enzymes while Mirhendi et al. (2010) identified *C. parapsilosis* complex

species after digestion with *NlaIII* (Okonechnikov et al. 2012).

Clinical isolates of *C. albicans* were distinguished from *C. glabrata*, *C. tropicalis* and *C. krusei* by *MspI* while *BlnI* was used to digest ITS1-5.8S-ITS2 rRNA regions for differentiation between *C. albicans* and *C. dubliniensis* (Barbedo et al. 2017, Mirhendi et al. 2010, Shokohi et al. 2010). This could explain the reason for the differences in results with other papers related to the amplified locus and differences in designing primers. Shokohi et al. (2010) used different primer sequences than our primers and another position of amplification. Moreover, the comparisons were carried out with different species than the ones in this study.

Importantly, possible species of human pathogens of genus *Candida* with the availability of their sequences on GenBank database were used in this study, providing the researchers and the diagnostic laboratory workers with an easy and rapid guide for pathogenic *Candida* species.

CONCLUSION

The need for rapid and accurate identification approach to yeast infections coincidentally increases

with the highly emerging pathogenic infections within immunocompromised patients. Scientific advances in molecular genetics and bioinformatics software have enhanced the development of a new approach for fast and accurate identification methods. Bioinformatics tools were used to draw the roadmap of the conserved region which is regarded as a barcode to differentiate among closely-related species. The significance of combing old traditional and advanced approaches increases the accuracy of the scientific results. The PCR-RFLP analysis compensates from sending PCR products for sequencing and gets results quickly after digestion with the restriction enzyme. Designing primers for the PCR-RFLP analysis is an acritical step for successful PCR, therefore, scrutiny of primers' efficiency and accuracy by In-silico is a very important step before starting Lab work. It is important for the scientists and mycologists to draw the genetic map and optimise work conditions to save time and costs.

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