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Research Article

PCR Primer Design for *In-Silico* Rapid Detection of Ocular Infection Caused by *Candida* Species in Humans

Abstract

Background: Computational analyses have shown great potentials for providing tools for the rapid detection and identification of fungi for medical, scientific and commercial purposes. Various bioinformatics tools have been developed for finding the specific regions within the ribosomal RNA (rRNA) gene complex. *Candida* is a genus of yeast that includes about 150 different species and is the most common cause of human ocular infections. In the present study, rapid detection method of *Candida*, based on specific regions (18S, 5.8S and 28S) of ribosomal RNA (rRNA) genes of eight (8) species e.g. *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and *C. tropicalis* has been developed. Rapid diagnosis and early identification of causative agent through computational based methods with high accuracy will result in effective treatment.

Objective: Development of rapid detection method and assay for *Candida* species based on bioinformatics tools.

Methodology: Ribosomal RNA (18S, 5.8S and 28S) sequences of eight *Candida* species were retrieved from GenBank/EMBL databases. A set of unique primers were designed based on the conserved region in the given yeast species. To verify the *in-silico* specificity of the designed primers, the NCBI-BLAST program was employed to search the primers in short, near exact sequences. The primers were further analyzed by the AmplifX tool to determine their specificity and sensitivity against *Candida* species.

Conclusions: The study resulted in the development of rapid and reproducible detection strategy of *Candida* species on the basis of computational PCR that will be very helpful for the doctors/practitioners to prescribe targeted medicine against *Candida* and related causative agents.

Introduction

Yeasts are the microorganisms commonly found in nature [1], among them *Candida* is famous genera containing a wide range of species and sub species. Although among *Candida* species, few are harmless endosymbionts for hosts such as humans. However, many species that are otherwise harmless but if present in improper place can cause disorders. Out of about 200 species of *Candida*; *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. dubliniensis*, *C. kefyr* and *C. lusitaniae* are known to cause most human ocular infections [2]. A warm, moist climate and a rural agricultural environment may influence the sensitivity of healthy eyes to fungi and fungal infections [3].

To detect fungal species that can cause infections, specific computational polymerase chain reaction was developed that was effective and enabled scientists to know the root cause of fungal eye infections. Conserved regions of 18S ribosomal RNA genes were used to design specific primers to amplify the targeted regions of desired fungi, ultimately to diagnose *Candida* and infections developed by *Candida*. Because effective treatment of any disease can be done only when we know the root cause of disease and we are able to identify and detect the disease causing agents. In this sense computational polymerase chain reaction is more effective way for detection other than conventional microbiological techniques. Because in computational polymerase chain reaction, time saving is main advantage and accuracy of results is more than other techniques [4,5]. Genome of many *Candida* species is being sequenced, so polymerase chain reaction can specify them by using specific probes with 100% efficacy, sensitivity and specificity. Genome includes ribosomal RNA in this section for development of polymerase chain reaction methods to detect human fungal pathogens by focusing on 18S ribosomal RNA genes, 5.8S and the 5' end of 28S RNA gene in most of the studies conducted [6-8].

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Due to many problems in traditional diagnosis methods for detection of fungal systematics and fungal infections, now it has become very necessary to develop rapid detection methods that should be specific and sensitive [9]. The manual assortment of optimum PCR oligonucleotide primer sets can be quite dull and thus offers itself very naturally for computational analysis. The basic cause which can affect function of the oligonucleotides and their melting temperatures as well as possible homology among primers are well defined and straightforward tasks that are easily encoded in computer software. Software provides a minimum number of candidate set of primers, so that the primers can be easily selected with the help of softwares. Scientists are taking benefits of accurate computed calculations and using all the versions of primer's placements, length, co-relation with other primers to find out efficient one that meet all the conditions given by the user. Among a wide range of primer pairs examined by computational methods, software can select only those that are appropriate for the experiment. So, by this method over all excellent quality primers can be selected [10,11]. Hundreds of programs have been designed to select and make primer's sets having variations in specifications. Primers are also available commercially and primer designing software are also available that provides enhanced efficacy in results [12].

Materials and Methods

Retrieval of nucleotide sequences and their alignment:

The rRNA (18S, 5.8S and 28S) nucleotide sequences of eight (8) ocular infection causing *Candida* species; *Candida albicans*, *Candida kefyr*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida lusitaniae*, *Candida glabrata*, and *Candida guilliermondii* were retrieved from NCBI (www.ncbi.nlm.nih.gov) and there accession No. are listed in the Table 1. The selected sequences were aligned by using ClustalW (www.genome.jp/tools/clustalw/) to determine the conserved regions. The templates of conserved regions (18S, 5.8S & 28S) were predicted with their corresponding *Candida* species along with sequence and product size ranges from 110-111 and 190-194 bp [13].

Designing of universal primers against conserved regions

For the sake of designing primers, conserved regions (18S, 5.8S & 28S) were used into the Geneious (version 10.0.9) tool (www.geneious.com/). Two primer sets were designed with the size of 19bp (ACGGGGAAACTCACCAGGTCCA), (TCCCAGCACGACGGAGTTT) and 22bp (GTGATGCCCTTAGACGTTCTGG), (GGGCAGGGACGTAATCAACGCA) respectively.

Primers were then improved and updated primers were then re-analyzed with the help of AmplifX (1.7.0 version) tool (www.amplifx.software.informer.com/1.7/). The modified primers were checked by using parameters such as oligocalc [14] and to make sure that primer have good quality, (T_m (melting temperature), Length of primer, GC content, 3' end stability, hairpins and Poly X tail parameters were determined.

Table 1: Fungal *Candida Species* with their accession numbers.

Sr. No	Species	Accession No
1.	*Candida albicans*	M60302.1
2.	*Candida tropicalis*	M60308.1
3.	*Candida parapsilosis*	M60307.1
4.	*Candida kefyr*	M60303.1
5.	*Candida krusei*	M60305.1
6.	*Candida guilliermondii*	M60304.1
7.	*Candida lusitaniae*	M60306.1
8.	*Candida glabrata*	M60311.1

Analysis and selection of restriction site for *Candida* species

With the help of NEBcutter (V 2.0) tool (www.nc2.neb.com/NEBcutter2/) the eight *Candida* species sequences were subjected to restriction digestion using the restriction endonucleases type-II, listed in the REBASE database (www.rebase.neb.com/) that select the enzymes to cut the sequences differently at not more than 5 cleavage sites [15].

Results and Discussion

In newborns, candidal retinitis is the most common intra ocular fungal infection [16,17]. Endogenous candidal chorioretinitis causes pain and decrease in vision due to associated anterior uveitis [18]. The full length sequences of eight *Candida* species namely *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and *C. tropicalis* were retrieved from NCBI and all these 8 *Candida* species were then subjected to alignment by using online tool clustalW. The 18S, 5.8S and 28S rRNA nucleotides were chosen as the target regions for this study [19]. Figures 1,2 shows the positions of the primers sequences obtained from "GENEIOUS" software.

Template

The 18S, 5.8S and 28S regions of *Candida species* were generated with eight templates given in Tables 2,3 respectively.

Template of 18S, 5.8S and 28S rRNA gene

The sequence and size of 18S, 5.8S, and 28S region of template is given below of size ranges from 110 to 111 bp.

Template of 18S, 5.8S and 28S rRNA gene

The sequence and size of 18S, 5.8S and 28S regions of template is given below of size ranges from 190 to 194 bp.

The product sizes of each pair of primers were determined by the help of *Candida* primer annealing map, as listed in (Table 4).

Primer improvement

Prior to further process, primers were checked by AmplifX tool as shown in Figure 3. The *in-silico* primers were designed by using Geneious tool, which is freely available on Just Bio website (www.justbio.com). The results of redesigned primer's

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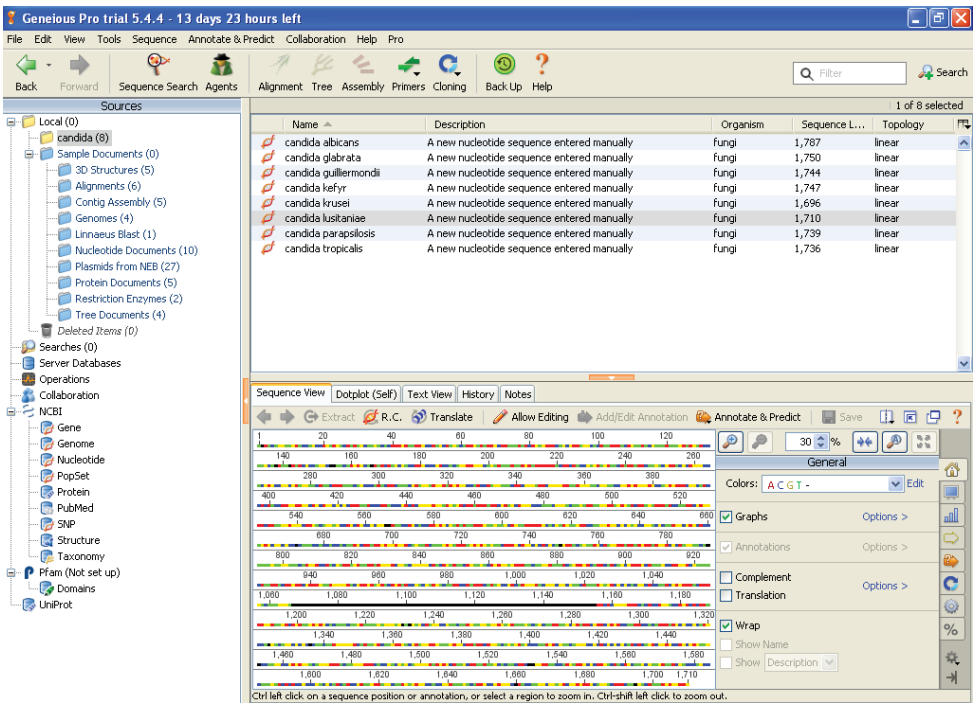

Figure 1: Shows the alignment of eight *Candida* species.

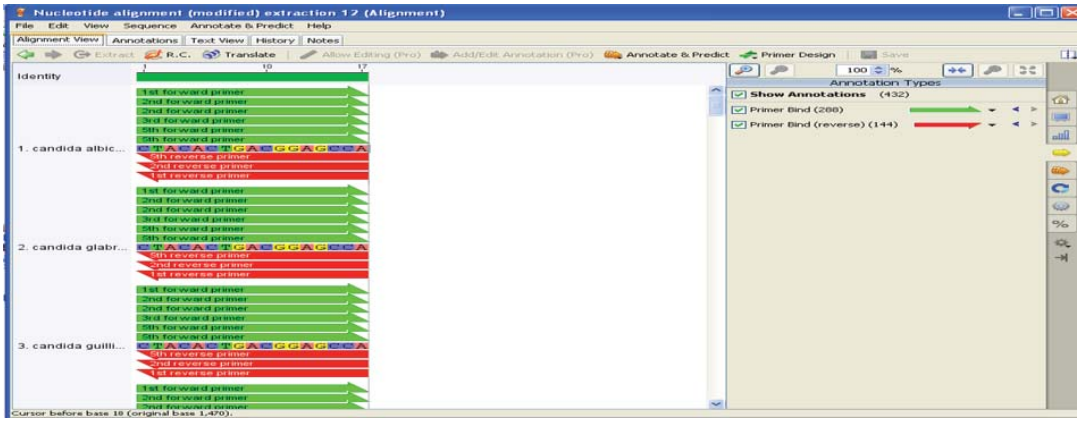

Figure 2: Shows the "GENEIOUS" output of primers that were designed against *Candida* species.

Table 2: 18S, 5.8S and 28S rRNA gene template.

Organism	Template Sequence	Length (bp)
Candida albicans	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTATAAGCCTTGGCCGAGAGGTCT GGGAAATCTTGTGAAACTCCGTCGTGCTGGGG	111
Candida glabrata	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTCTAACCTTGGCCGAGAGGTCTT GGTAATCTTGTGAAACTCCGTCGTGCTGGGG	110
Candida guilliermondii	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTATTAACCTTGGCCGAGAGGTCT GGGAAATCTTGTGAAACTCCGTCGTGCTGGGG	111
Candida krusei	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCAAGTCCAACCTTGGTCGAGAGGCCCG GGTAATCTCGTGAAACTCCGTCGTGCTGGGG	110
Candida kefyr	GTGATGCCCTTAGACGTTCTGGGCGCACGCGCGCTACACTGACGGAGCCAGCGAGTACAACCTTGGCCGAGAGGTCTGG GTAATCTTGTGAAACTCCGTCGTGCTGGGG	109
Candida lusitaniae	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTTGCCTTGGCCGAGAGGTCTGG GAAATCTTGGGAAACTCCGTCGTGCTGGGG	108
Candida parapsilosis	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTATAAACCTTGGCCGAGAGGTCT GGGAAATCTTGTGAAACTCCGTCGTGCTGGGG	111
Candida tropicalis	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTATAAACCTTGGCCGAGAGGTCT GGGAAATCTTGTGAAACTCCGTCGTGCTGGGG	111

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parameters with the help of AmplifX tool are shown in Table 2. These parameters confirmed that new primers were of good quality. Primer amplification efficiencies are given in Table 5 [20,21].

AmplifX was used to seek in a collection of primers, it was used to amplify a fragment into a target sequence. The information was automatically computed by AmplifX (like T_m, Quality, length) associated with each primer.

The selected species were differentiated by using the restriction enzyme digestion of the PCR products. *Candida* speciation would be an important aid to effective patient treatment, facilitating the application of species-specific antifungal therapy, thereby avoiding problems of drug resistance.

For finding the genotype of a particular *Candida* species and identification of gene, software NEB Cutter was used. This cutter was used for the linear DNA analysis and the restriction enzymes were used to cleave the DNA without need for expensive gene sequencing [22]. In similar manner restriction enzymes were used to digest genomic DNA.

After finalization, the results of each *Candida* species, the number of restriction sites, nucleotide position of each cut, list of enzymes and specificity of common and unique enzymes were separated manually as shown in Tables 6,7. The enzyme

Table 3: 18S, 5.8S and 28S rRNA gene template.

Organism	Template Sequence	Length (bp)
Candida albicans	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTATAAGCCTTGGCCGAGAGGTCTGGGA AATCTTGTGAAACTCCGTCGTGCTGGGGATAGAGCATTGTAATTGTTGCTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTCATCA GCTTGCGTTGATTACGTCCCTGCCC	194
Candida glabrata	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTCTAACCTTGGCCGAGAGGTCTTGGTA ATCTTGTGAAACTCCGTCGTGCTGGGGATAGAGCATTGTAATTATTGCTCTTCAACAGGAATTCCTAGTAAGCGCAAGTCATCAGC TTGCGTTGATTACGTCCCTGCCC	192
Candida guilliermondii	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTATTAACCTTGGCCGAGAGGTCTGGGA AATCTTGTGAAACTCCGTCGTGCTGGGGATAGAGCATTGTAATTATTGCTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTCATCA GCTTGCGTTGATTACGTCCCTGCCC	194
Candida krusei	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCAAGTCCAACCTTGGTCGAGAGGCCCGGGT AATCTCGTGAAACTCCGTCGTGCTGGGGATAGAGCATTGTAATTTTTGCTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTCATCA GCTTGCGTTGATTACGTCCCTGCCC	193
Candida kefyr	GTGATGCCCTTAGACGTTCTGGGCGCACGCGCGCTACACTGACGGAGCCAGCGAGTACAACCTTGGCCGAGAGGTCTGGGTAA TCTTGTGAAACTCCGTCGTGCTGGGGATAGAGCATTGTAATTATTGCTCTTCAACAGGAATTCCTAGTAAGCGCAAGTCATCAGCT TGCGTTGATTACGTCCCTGCCC	191
Candida lusitaniae	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTTGCCTTGGCCGAGAGGTCTGGGAAAT CTTGGGAAACTCCGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGGAATTCCTAGTAAGCGCAAGTCATCAGCTT GCGTTGATTACGTCCCTGCCC	189
Candida parapsilosis	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTATAAACCTTGGCCGAGAGGTCTGGGA AATCTTGTGAAACTCCGTCGTGCTGGGGATAGAGCATTGTAATTATTGCTCTTCAACGAGGAATTCTAGTAAGCGCAAGTCATCAG CTTGCGTTGATTACGTCCCTGCCC	194
Candida tropicalis	GTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCAGCGAGTATAAACCTTGGCCGAGAGGTCTGGGA AATCTTGTGAAACTCCGTCGTGCTGGGGATAGAGCATTGTAATTGTTGCTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTC ATCAGCTTGCGTTGATTACGTCCCTGCCC	194

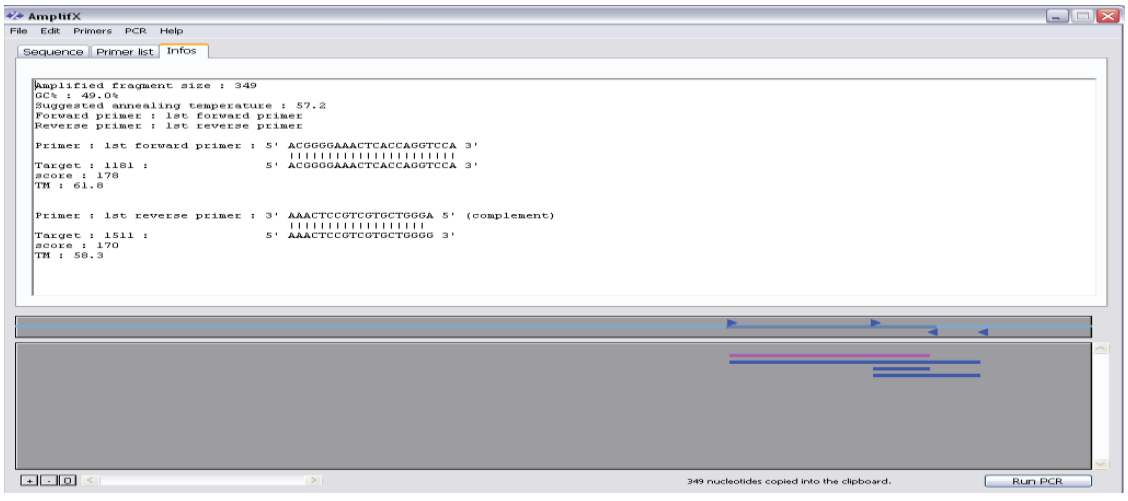

Figure 3: AmplifX design of new primers.

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TspRI was found as a common restriction enzyme present in all eight species.

While five enzymes were unique; HinFI, MseI, CviQI, TaqI and BsrDI that would subsequently allow identification of *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lusitanae* species. These five unique restriction enzymes provide greatest level of species discrimination.

Table 4: Detail of Primers Designed against Selected *Candida* species.

Primer Name	Sequence of Primers (5′ → 3′)	Primer Length (bp)
1st reverse primer	TCCCAGCACGACGGAGTTT	19
3rd reverse primer	GGGCAGGGACGTAATCAACGCA	22
1st forward primer	ACGGGGAAACTCACCAGGTCCA	22
3rd forward primer	GTGATGCCCTTAGACGTTCTGG	22

Table 5: AmplifX output to show the parameters of newly designed primers.

Region	Parameters for (new) forward primers			Parameters for (new) reverse primers		
18S, 5.8S and 28S	**3rd forward primer**			**3rd reverse primer**		
	TM	57.4	Good	TM	61.7	Good
	GC percent	54	Good	GC percent	59	Good
	3′ end stability	3	Good	3′ end stability	3	Good
	polyX	0	Good	polyX	0	Good
	Self Dimer	12	Good	Self Dimer	14	Good
	Self End Dimer	0	Good	Self End Dimer	0	Good
	1st forward primer			**1st reverse primer**		
	TM	61.8	Good	TM	58.3	Good
	GC percent	59	Good	GC percent	57	Good
	3′ end stability	4	Bad	3′ end stability	2	Good
	polyX	0	Good	polyX	0	Good
	Self Dimer	16	Good	Self Dimer	12	Good
	Self End Dimer	0	Good	Self End Dimer	0	Good

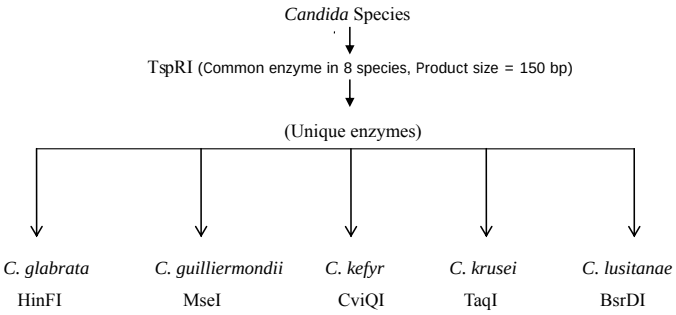
Table 6: Common enzymes present in eight species.

Enzyme	Specificity	Cut Positions	Product
Alu	AGCT	170	24
ApoI	RAATTY	144/148	50
BfaI	CTAG	150/152	44
BseYI	CCCAGC	105/109	89
BsmFI	GGGAC(N)10NNNN	*170/174	24
BspQI	GCTCTTCNNNN	138/141	56
BssHII	GCGCGC	*30/34	164
DdeI	CTNAG	9/12	185
EarI	CTCTTCNNNN	138/141	56
EcoRI	GAATTC	144/148	50
Hpy99I	CGWCG	*103/98	91
MboII	GAAGA(N)7N	125/124	69
NlaIV	GGNNCC	*47	147
SapI	GCTCTTCNNNN	138/141	56
StyI	CCWWGG	63/67	131
TspRI	NNCASTGNN	44/35	150

Table 7: Unique enzymes present in species.

Unique Enzymes in sp.	Enzyme	Specificity	Cut Positions	Product
glabrata	HinFI	GANTC	*54/57	140
	MlyI	GAGTC(N)5	63	131
	PleI	GAGTCNNNNN	62/63	132
guilliermondii	MseI	TTAA	59/61	135
kefyr	CviQI	GTAC	55/57	139
	HaeIII	GGCC	66	128
	PhoI	GGCC	66	128
krusei	AvaI	CYCGRG	*76/80	118
	BsoBI	CYCGRG	76/80	118
	BssSI	CACGAG	86/90	108
	HpaII	CCGG	*77/79	117
	MmeI	TCCRAC(N)18NN	82/80	112
	MspI	CCGG	77/79	117
	SmaI	CCCGGG	*78	116
	TaqI	TCGA	68/70	126
	TspMI	CCCGGG	*76/80	118
	XmaI	CCCGGG	*76/80	118
lusitaniae	BsrDI	GCAATGNN	114/112	80
	BstAPI	GCANNNNNTGC	125/122	69
	HpyCH4V	TGCA	119	75

Identification Strategy



Conclusion

We found that rapid identification of *Candida* species has become more important because of an increase in ocular infections. An advantage of genotypic identification of *Candida* species is its rapidity and therefore it will be very helpful for the doctors to detect the specific species and help them to prescribe relevant medicine. Furthermore, traditional methods which were used for the identification of *Candida* species including morphological and biochemical analysis, and serotyping are based on phenotypic expression, which make them unreliable. Traditional tests are also time consuming. However, computational techniques make identification of *Candida* species very rapid. In limited medical facilities, the prediction of *Candida sp.* involved in ocular infection will be a valuable addition of information in the field of medicine.

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