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นางสาวปิยเนตร์ เกษะโกมล

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ACCURACY OF A TERMINAL-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) METHOD TO CHARACTERIZE ORAL FUNGI

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A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	ACCURACY OF A TERMINAL-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) METHOD TO CHARACTERIZE ORAL FUNGI
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ปิยเนตร์ เกษะโกมล : ความแม่นยำของวิธีทีอาร์แอฟแอลพีเพื่อใช้ในการตรวจกลุ่มเชื้อราในช่องปาก. (ACCURACY OF A TERMINAL-RESTRICTION FRAGMENTN LENGTH POLYMORPHISM (T-RFLP) METHOD TO CHARACTERIZE ORAL FUNGI) อ.ที่ปรึกษาวิทยานิพนธ์หลัก รศ.ทพญ.ดร.อรนาฏ ภาตังคสมบัติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม อ.ทพญ.ดร.อัญชลี วัชรักษะ, 72 หน้า.

Terminal-restriction fragment length polymorphism (T-RFLP) เป็นวิธีที่นิยมใช้ในการศึกษาลักษณะโครงสร้างของกลุ่มจุลชีพ อย่างไรก็ตามมีวิธีข้อจำกัดในการตรวจวัด อีกทั้งยังไม่มีรายงานการศึกษาในกลุ่มเชื้อราในช่องปาก การทดสอบความแม่นยำของวิธีมีความสำคัญก่อนที่จะนำไปประยุกต์ใช้ในการตรวจกลุ่มเชื้อราในช่องปาก เพื่อทดสอบความแม่นยำของวิธี Terminal-restriction fragment length polymorphism (T-RFLP) เพื่อใช้ในการตรวจกลุ่มเชื้อราในช่องปากเปรียบเทียบกับวิธี quantitative PCR (qPCR) เพาะเลี้ยงเชื้อและสกัดดีเอ็นเอจากเชื้อ *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus* และ *Fusarium* spp. ซึ่งเป็นเชื้อราที่มีความสำคัญทางการแพทย์ที่พบได้บ่อยในช่องปาก ทำการสร้างแบบจำลองกลุ่มเชื้อราโดยผสมดีเอ็นเอของ *Cryptococcus*, *Aspergillus* และ *Fusarium* ในอัตราส่วนที่เท่ากัน และผสมกับดีเอ็นเอของ*C.albicans* ที่มีปริมาณแตกต่างกันโดยมีปริมาณลดลงทีละ 10 เท่า จากนั้นทำการทดสอบหาปริมาณเชื้อรา *C.albicans* และปริมาณเชื้อทั้งหมดโดยวิธี T-RFLP และวิธี qPCR โดยวิธี T-RFLP ทำการเพิ่มปริมาณดีเอ็นเอในส่วน ITS โดยใช้คู่ pan-fungal primers ที่มีสายหนึ่งติดฉลากด้วยสารฟลูออเรสเซนต์แล้ววิเคราะห์ผลหลังจากตัดดีเอ็นเอที่ได้ติดฉลาก ด้วยเอนไซม์ตัดจำเพาะ*Msp*I หรือ *Hae*III ส่วนวิธี qPCR ใช้หลักการ SYBR ในการทดสอบ โดยใช้ species-specific primers กับ pan-fungal primers ในการเพิ่มปริมาณดีเอ็นเอ ทำการทดสอบกับแบบจำลองกลุ่มเชื้อราที่มีดีเอ็นเอของเชื้อ*C.albicans* ทั้งหมด 7 อัตราส่วน ระหว่าง 10^0 ถึง 10^6 copies โดยประมาณจากน้ำหนักจีโนมของเชื้อ *C.albicans* วิธี qPCR เป็นวิธีที่มีความแม่นยำสามารถตรวจวัดปริมาณ *C.albicans* ได้ระหว่าง 10^2-10^6 copies ส่วนวิธี T-RFLP สามารถตรวจวัดได้ระหว่าง 10^5-10^6 copies โดยวัดได้ต่ำกว่าค่าจริง 10 เท่า นอกจากนั้น ยังไม่สามารถตรวจพบ *Aspergillus*-specific TRF ได้จากแบบจำลองกลุ่มเชื้อรา ซึ่งอาจเนื่องมาจากข้อจำกัดของวิธี T-RFLP หรือ pan-fungal primers ที่ใช้ในการศึกษานี้ วิธี T-RFLP เป็นวิธีที่สามารถตรวจกลุ่มเชื้อราที่ไม่ทราบชนิด โดยมีความแม่นยำในการตรวจวัดเชื้อที่มีปริมาณมากเพียงพอ แต่อาจมีข้อจำกัดในการตรวจหาเชื้อราบางชนิด ซึ่งในกรณีนี้ต้องอาศัยวิธี qPCR เพื่อยืนยันการระบุและตรวจสอบปริมาณเชื้อราอย่างแม่นยำต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5387833520 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS : Terminal-restriction fragment length polymorphism / T-RFLP / oral fungi / accuracy / *Candida albicans*

PIYANATE KESAKOMOL : ACCURACY OF A TERMINAL-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) METHOD TO CHARACTERIZE ORAL FUNGI. ADVISOR : ASSOC. PROF. ORANART MATANGKASOMBUT, D.D.S., Ph.D., CO-ADVISOR : ANJALEE VACHARAKSA, D.D.S., Ph.D., 72 pp.

Terminal-restriction fragment length polymorphism (T-RFLP) method has been widely used for study profiling of microbial community, however, have some limitations and have never been studies in oral fungi. It is important to evaluate the accuracy of this method as a tool for investigating the oral fungal community. To evaluate accuracy of T-RFLP with quantitative PCR (qPCR) for characterize oral fungi. DNA was harvested from medically important fungi commonly found in the oral cavity: *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Fusarium* spp. cultures. *Cryptococcus*, *Aspergillus* and *Fusarium* DNA were mixed, and 10-fold dilutions of *Candida* specific DNA were added to the DNA mixtures to represent fungal community models with differential *Candida* abundance. Species-specific DNA and total fungal DNA was estimated by T-RFLP and SYBR qPCR. For T-RFLP, mixtures were amplified using pan-fungal fluorescent-labeled primers specific to ITS regions and analyzed after digestion with *Msp*I or *Hae*III. For qPCR, mixtures were amplified using species-specific and pan-fungal primers, and analyzed. Based on the weight of *C.albicans* genomic DNA, seven dilutions of *Candida*-specific targets in fungal community models, corresponding to 10^0 to 10^6 copies, were tested. Detection by qPCR was accurate when the abundance of *Candida*-specific targets was between 10^2 to 10^6 copies, whereas the range for T-RFLP detection was between 10^5 to 10^6 copies. *Candida*-specific T-RF proportion in DNA mixtures appeared to be underestimated by 10-fold. *Aspergilllus*-specific T-RF product was absent from the fungal community model suggesting that the T-RFLP method, or the pan-fungal primers, may have bias against *Aspergillus* detection. T-RFLP is advantageous for the detection of unknown fungal community, with accuracy when targets are highly abundant. However, it may have bias in the detection of some fungi. The species-specific qPCR assay is then required to validate the detection and target abundance.

Field of Study : Medical Microbiology Student's Signature .....

Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

%	Percent
^{o}C	Degree Celsius
ARDRA	Amplified ribosomal DNA restriction analysis
ARISA	Automated ribosomal intergenic spacer analysis
Cat.	Catalog number
DGGE	Denaturing gradient gel electrophoresis
DHPLC	Denaturing high-performance liquid chromatography
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribose nucleic acid
et al.	And others
g	Gram (s)
h	Hour (s)
ITS	Inter transcribed spacer
ml	Milliliter
mM	Millimolar
$MgCl_2$	Magnesium chloride
MTPS	Multitag Pyrosequencing
MPY	MasterPure^{TM} Yeast DNA Purification Kit

ng	Nanogram
µm	Micrometre/Micron
µl	Microliter
PCR	Polymerase chain reaction
REs	Restriction enzymes
S	Second (s)
Spp.	Species
SSCP	Single strand conformation polymorphism
OTUs	Operational taxonomic units
rpm	Round per minute
rRNA	Ribosomal RNA
T-RFLP	Terminal restriction fragment length polymorphism
T-RF	Terminal restriction fragments
U	Unit
UDG	Uracil-DNA Glycosylase

CHAPTER I

INTRODUCTION

The oral mycobiome is the collection of fungal genomes within the microbiota that reside in the oral cavity (1, 2). Most studies in oral microbiota focused on oral bacteria because bacteria are the major component of this community. However, the fungal residents could have influences on oral microbial ecology (3). The shift of oral microbial ecology may be associated with the transitions from health to diseases (4, 5). Due to the limitation of conventional culture-dependent methods, only a few species of fungi could be identified from the oral cavity. The oral fungi in healthy individuals included *Penicillium, Geotrichum, Hormodendrum, Aspergillus, Scopulariopsis* and *Hemispora* (6). Recently, Ghannoum and colleagues used a novel Multitag Pyrosequencing (MTPS) approach to characterize total fungi in the oral cavity of 20 healthy individuals by using the pan-fungal internal transcribed spacer (ITS) primers. They reported that the oral mycobiome was composed of highly diverse fungal species, and a total of 101 species were identified. Some of those species, such as *Candida*, *Cryptococccus*, *Aspergillus* and *Fusarium* spp. are known as human pathogens. *Cryptococccus* and *Fusarium* had never been reported as oral commensals (1).

A number of molecular techniques have been used to characterize fungal communities such as cloning and sequencing, automated ribosomal intergenic spacer

analysis (ARISA) (7), amplified ribosomal DNA restriction analysis (ARDRA) (8), single strand conformation polymorphism (SSCP) (9), terminal-restriction fragment length polymorphism (T-RFLP) (10), denaturing gradient gel electrophoresis (DGGE) (11) and denaturing high-performance liquid chromatography (DHPLC) (12). New technology of pyrosequencing is highly sensitive and large-scale samples can be analyzed. However, its limitations still remain, including high cost and difficulties in data analysis (13). Fingerprinting techniques are less expensive and more feasible than the next generation DNA sequencing. Despite the fact that PCR amplification may be limited to some of the abundant members in the microbial community, DNA fingerprinting is useful for investigating the dominant species and clustering related community members across a large number of samples (14).

T-RFLP is a common technique used for examining changes in the composition of microbial communities (15). For example, the bacterial profile in saliva from healthy individuals and patients with periodontitis were compared using bacterial 16S rRNA-based T-RFLP method (16). The principle of T-RFLP is based on PCR amplification of a target gene using fluorescently labeled primer followed by enzyme digestion (17). The products of each sample are the terminal restriction fragments (T-RF) that vary in lengths and peak heights (15, 18). Advantages of the T-RFLP method include the capability of semi-quantitative analysis, rapidity, reproducibility, sensitivity, and robustness of assessing complex microbial communities (18-20). However, the bias in

DNA extraction or limitation in PCR amplification may reduce the accuracy of T-RFLP method. While amplifying the DNA, the transition from PCR-exponential phase to the plateau phase of the high abundant species occurs earlier than the low abundant species present in the samples. As a result, the efficiency of PCR amplification is inconsistent across the large number of initial templates. This phenomenon can readily be observed using real-time PCR (18). Secondary structure of the incomplete amplicons might lead to faulty T-RF fragments and cause misinterpretation. All of these cause inaccurate estimation for microbial composition and abundance in the samples (21, 22).

T-RFLP method was used for characterizing fungal community and the dynamic changes in the environment (23); however, little evidence was reported for the oral fungi. It is ,therefore, important to evaluate the accuracy of T-RFLP method as a tool for investigating the oral fungal community (24). In this study, we aimed to test the accuracy of the T-RFLP method used for characterizing the simple fungal community model. Accuracy of the T-RFLP for fungal detection and level estimation was compared to the results from using quantitative PCR and the estimation by genomic DNA weight. When optimized, T-RFLP can be an effective diagnostic tool for early detection of fungal pathogens and monitoring of progression of systemic and oral diseases.

CHAPTER II

OBJECTIVES

Hypothesis

T-RFLP method can be used to characterize the medically important oral fungi in simple community model with high accuracy comparable to quantitative PCR method.

Objectives

1. To quantify species-specific and total fungal DNA in the DNA mixture of four oral fungi using terminal restriction fragment polymorphism (T-RFLP) method

2. To quantify species-specific and total fungal DNA in the DNA mixture of four oral fungi using quantitative PCR method

3. To verify the accuracy of TRFLP method for fungal detection as compared with quantitative PCR method

4. To determine the lowest detection limit of T-RFLP and quantitative PCR methods

CHAPTER III

REVIEW LITERATURES

The oral microbial community and their genome (oral microbiome)

The oral microbial community is one of the most complex microbial community in the human body sites that contains a diverse range of bacteria, viruses, archaea, protozoa and fungi (25-27). Every human contains a personal microbiome that essential to maintain equilibrium and homeostasis for health status but able to elicit diseases (4). The oral mycobiome is the collection of fungal genomes in the microbiota that reside in the oral cavity and commonly form biofilms (1). The changes in the oral microbiome has been associated with the transition from health to disease status (5). (Figure1.)

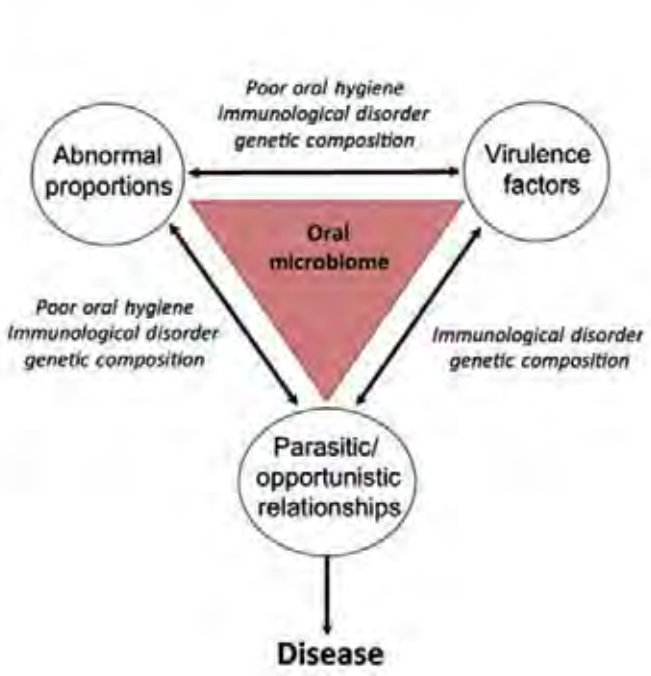

Figure 1. Cycle of etiology cause disease in oral microbiome (4).

Microbiomics and metagenomics study the presence and identity of specific microbiota and understand the nature of fundamentals and specifics of microbiome activity in the human body of health and disease status. The study of the oral microbiome will be beneficial for disease diagnosis and therapy and contributes to the development of personalized dental medicine (4).

In order to attain the long-term benefits of the microbiome research, we must first understand what kinds of microorganisms exist in the body and their activities that affect the health conditions. Moreover, the microbiome patterns of specific diseases should be characterized. Developing proper diagnostic methods and technologies that enable us to identify individual microbial profiles is essential in specific microbes responsible for disease (4).

In the past, microbiologists used conventional culture-dependent methods to try to study the complex oral microbial community. Culture method has limitations because many species are uncultivable (27). For modern era, PCR based high-throughput approaches using small-subunit ribosomal RNA (rRNA), and the 18S rRNA gene sequences for eukaryotes, are used to study microbial community, such as denaturing gradient gel electrophoresis (DGGE) (11, 16) and terminal restriction fragment length polymorphism (T-RFLP)(16). These have been used to study human microbial community, including oral microbial analysis. Metagenomic studies analyze microbial community using shotgun sequencing and pyrosequencing. Although pyrosequencing

is more specific, sensitive, and large-scale than conventional sequencing, it has certain limitations, for example, high cost and difficulties in data analysis (13). Microbiomics requires accurate metagenomics method able to characterize and identify specific microbial composition correlation with health and disease (4).

Genotypic microbial community profiling

The genotypic microbial community profiling methods are culture-independent assays based on polymerase chain reaction (PCR). These methods are often used for rapidly screening and comparing communities, but the identification of community members requires DNA sequencing of individual template (18).

These techniques start by extraction of total DNA from the community. The DNA template is amplified of genetic targets by universal primers from a wide variety of different organisms. Suitable genetic targets should have both conserved and variable regions. The conserved regions served as annealing sites for PCR primers, whereas the variable regions can be used for phylogenetic differentiation (18). PCR products are often in similar sizes but different in the nucleotide composition.

There are a number of genotypic microbial community profiling methods that have been used to characterize fungal communities such as cloning and sequencing, automated ribosomal intergenic spacer analysis (ARISA) (7), amplified ribosomal DNA restriction analysis (ARDRA) (8), single strand conformation polymorphism (SSCP) (9), terminal-restriction fragment length polymorphism (T-RFLP) (10), denaturing gradient gel electrophoresis(DGGE) (11) and denaturing high-performance liquid chromatography (DHPLC) (12).

The terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) both have been frequently used to study oral bacterial communities in health and disease (28, 29). T-RFLP method was found to be more sensitive and simpler for comparison of different runs, and give higher yield number of operational taxonomic units than other PCR fingerprinting methods for assessing the complex microbial communities (18, 30). Even though genotypic techniques are faster and cheaper, but the detection may be limited to some of the most abundant members in the microbial community (14)

Terminal-restriction fragment length polymorphism (T-RFLP)

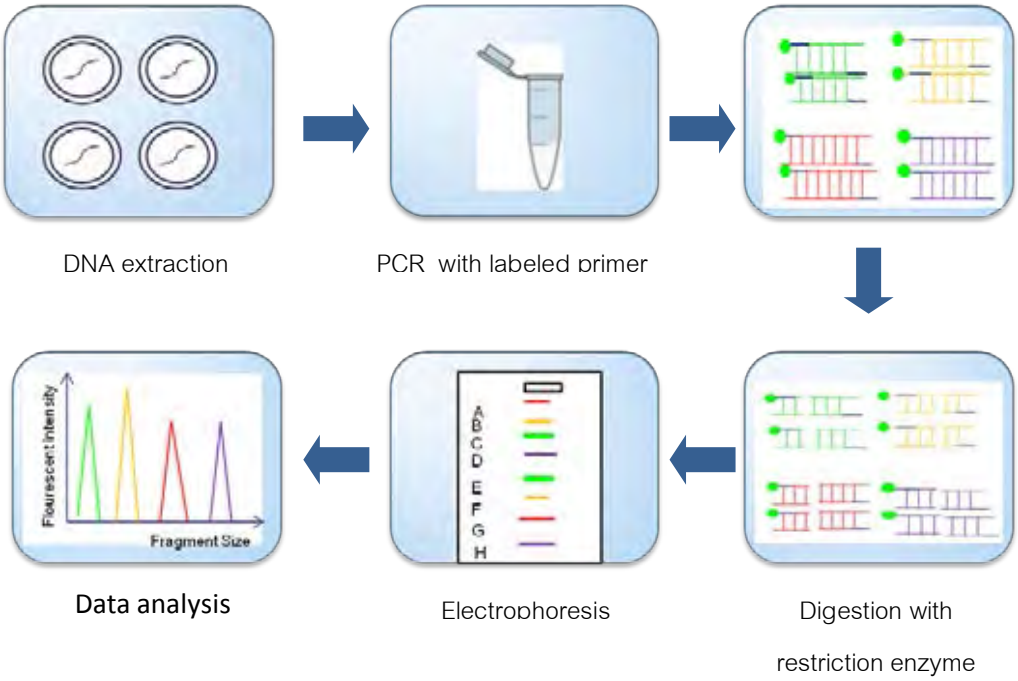

Figure 2. Flow chart of step-by-step procedure of T-RFLP assay.

Terminal-restriction fragment length polymorphism (T-RFLP) method is one of the most widely used methods for analysis of complex microbial communities based on DNA-based fingerprinting (18, 31, 32). T-RFLP method can be used to study structure and dynamics of microbial community such as bacteria, archaea, and fungi from a wide range of environments (19, 20). This approach has been used successfully to identify and assess the dynamics of members within very simple model community. The T-RFLP method was first described by Liu and colleagues and has since been practiced and most widely used for studying microbial communities (17). It has been used to

characterize the bacterial community profiles including the human oral cavity in patients with periodontitis in comparison to healthy subjects using oral rinse samples (16).

This method is a culture-independent, high-throughput, rapid, sensitive, robust, and reproducible method of assessing diversity of complex communities without the need for any genomic sequence information that could generate a greater number of operational taxonomic units (OTUs) than many other PCR-based fingerprinting method (19, 31).

First step of T-RFLP method is to extract the total DNA from the microbial community. There are many methods for extracting nucleic acid. Selected target gene is then amplified by PCR using fluorescently labeled primer, either the forward or the reverse primer. Several different fluorophores including HEX, FAM, and ROX dye chemistries can be used. Flourophores for primer end-labeling should be chosen based on the type of automated sequencer. PCR products are typically passed through PCR-clean up kit (33).

- **Polymerase chain reaction of target genes**

The ribosomal RNA (rRNA) gene complex is a popular molecular target gene for T-RFLP analysis (33). This section includes the 18S, 5.8S and 28S genes, which have conserved nucleotide sequence among fungi (Figure 2.). It also contains the variable DNA sequence areas, the internal transcribed spacer (ITS) regions called ITS1 and ITS2 (34). The ITS regions contains both highly conserved and variable regions which make

them a great potential molecular target for the characterization and identification of human fungal pathogens, both yeasts and moulds (34, 35).

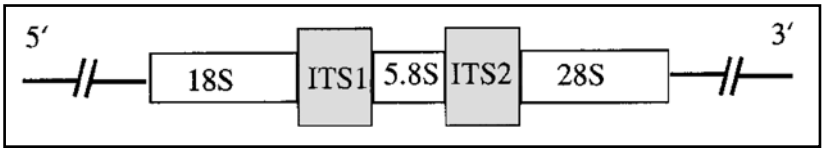

Figure 3. The ribosomal RNA (rRNA) gene complex of fungi (34).

- **Primer choices**

The choice of primers for the analysis of microbial communities requires consideration of two criteria. These are the specificity of primers to the target template and the sufficient recovery of all fungi in a mixed community DNA sample (33). Either the forward or the reverse primer could be labeled with fluorescent moiety, such as HEX, FAM and ROX dye. The kind of fluorescent can be chosen based on the type of automated sequencer (33). Panfungal primers ITS1F and ITS4 (Figure 4.) are widely used for analysis of fungal community such as mycorrhizal fungi and have been adopted by several groups using T-RFLP specifically to amplify fungal templates from mixed community DNA samples (12, 30).

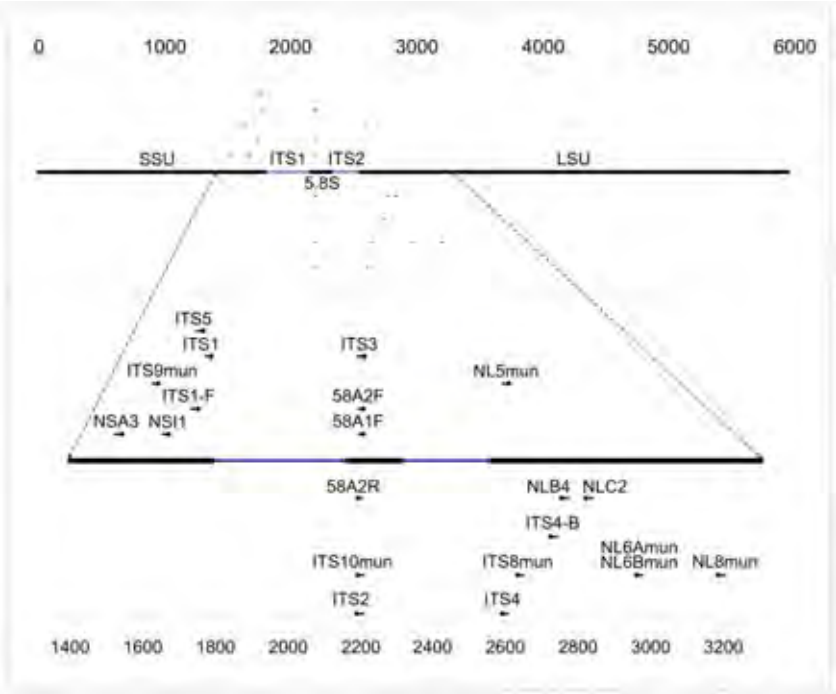

Figure 4. Diagram of primers location in the ribosomal RNA (rRNA) (35).

- **The choice of restriction enzymes**

An appropriate restriction enzyme (RE) must be chosen to allow T-RFLP to distinguish species for estimated diversity and provides the best resolution of the targeted groups (36). Typically, enzymes that have four base-pair recognition sites are used due to the higher frequency of these recognition sites (36). Engebretson and Moyer (2003) test of the restriction endonucleases in microbial populations of varying complexity found *Bst*UI, *Dde*I, *Sau*96I, and *Msp*I had the highest frequency of resolving single populations in their model communities (30).

- **T-RFLP data analysis**

Since the highly conserved region of different organisms are amplified, the size of PCR products will be similar. The PCR products digested by restriction enzyme at different restriction sites will yield terminally labeled fragments of different sizes. Only these labeled terminal restriction fragments (T-RFs) are detected and thus reduced the complexity of the profiles as analyzed by an automated DNA sequencer either gel-based or capillary methods. The T-RFLP analysis using capillary gel electrophoresis is more precise and reproducible than analyses done using polyacrylamide gels (36, 40).

The output from automated DNA sequencers will be in form of an electropherogram; a series of peak (differences in the length fragment) and height (abundance of fluorescently labeled T-RFs). The types of data used for analysis are binary (presence/absence), peak heights, peak areas, relative peak heights or relative peak area (30, 33). T-RFLP data can be analyzed as peak profiles and database T-RFLP.

1) Peak profiles: The technique relies on primers specific to the group of interest. There are no primers that can separate DNA from other fungal DNA. Peak-profile T-RFLP also works on the assumption that a single peak represents a single species. Diversity in peak-profile T-RFLP is calculated as the number of peaks (30).

2) Database T-RFLP: Individual species are identified and analysis focuses on these identified species. The analysis of communities is based on the presence or absence of species, with species presence inferred by matching peaks from community T-RFLP profiles to a database of known T-RFLP patterns. Diversity is measured as the number of species identified (30).

In this study, the capillary electrophoresis used the Applied Biosystem 3130/3730 series DNA sequencer that are fully automated and can generate reproducible profile with run-to-run variation of generally ±1 bp among terminal fragments of the same size. The sizes of T-RFs are estimated by using GeneMapper software.

- **Potential pitfalls and biases in the method**

Although T-RFLP method has many strengths but it has many potential pitfalls. Some of the pitfalls found in all steps that affected methods such as DNA extraction and PCR amplification (37). The simplest approach to distinguish signal from noise is to fix detection threshold that is some arbitrarily chosen value 50 or 100 fluorescence units (FU) (32).

Biases in the DNA extraction step could result from differences in the efficiency of cell lysis between different organisms, especially spores, which can cause different quantitative DNA recovery. A combination of physical (bead beating) and

chemical/enzymatic cell lysis methods most commonly produces the best results (20). Moreover, Avis et al. (2006) reviewed the biases unique for analyzing fungal communities found that spores present the pool of DNA extracted step may be disproportionately represented in the analysis (22)

PCR amplification artifacts could occur at many steps such as differential amplification, primer–template hybrids and amplification of multi-template in DNA mixture. Primer–template PCR could have biases when used in a multi-template for community analysis. Templates which completely match with primer will be preferentially amplified but some templates may not match well with the primers and be underrepresented or missing from the mixture (20).

The copy number of the genomic regions targeted in fungal ecology assumes that the number of copies in different fungal species is similar. It is well known that rRNA genes vary significantly in copy number. Gene copy number could bias estimates of organism abundance as a result. This variation between species complicates the quantification of different fungal species in a mixed DNA pool if the rRNA operon is the target region (12).

The T-RFLP analysis method, like other PCR-fingerprinting approaches, is limited in resolving power and thus diversity is frequently underestimated. The restriction sites where the enzymes digest is not necessarily unique to a particular taxonomic group.

Thus, many sequence types can share the same T-RF length which could lead to an underestimation of community diversity (31).

Moreover, the exponential phase to the plateau phase of the PCR amplification in T-RFLP analysis occurs earlier for the more abundant species in the sample. Therefore, it is not suitable for quantification of pathogens, resulting in distortion of community proportions phenomenon that can be observed using real-time PCR.

There was no significant difference between the TaqMan and SYBR Green in their specificity, quantitativity, and sensitivity. The TaqMan assay required additional manipulation and cost for the probe, while the SYBR Green assay might be suitable for routine clinical examinations. The real-time PCR technique is not suitable for the examination of different species in large numbers of samples (38).

Quantitative PCR (Q-PCR or realtime PCR)

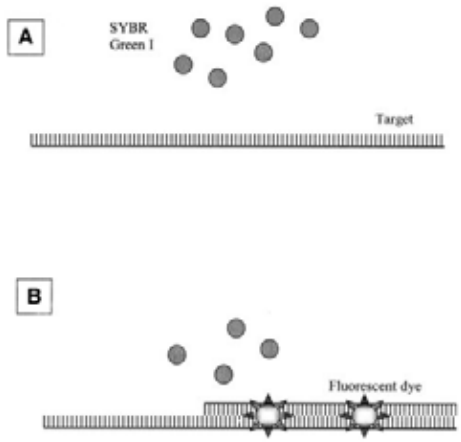

Figure 5. Principles of SYBR Green I technique (39).

Quantitative PCR (Q-PCR or realtime PCR) method is widely used to quantify the abundance in microbial ecology. Q-PCR based on PCR method with fluorescent can detect and quantify very small amount of specific nucleic acid by real time each cycle. It can determine the amount of starting DNA in the sample before the amplification by PCR (40, 41). The SYBR Green I technique is the method using DNA binding dye (SYBR Green I) to incorporate into minor groove of double stranded DNA. When SYBR Green I bound to the double stranded DNA, it will emit fluorescence. However, in free from, SYBR Green I does not emit the fluorescence. Template amplification is measured in each cycle by corresponding increase in fluorescence (42).

CHAPTER IV

MATERIALS AND METHODS

Fungal culture and DNA extraction

The four fungi used for generating fungal community model were *Candida albicans* (ATCC 90029), *Cryptococcus neoformans* (ATCC 34875), *Aspergillus fumigatus* and *Fusarium* spp.. All of these strains, previously identified by phenotypic method, were kindly provided by the Department of Microbiology, Faculty of Medicine, Chulalongkorn University. To maintain cultures, yeasts were cultured on Sabouraud dextrose agar (Oxoid) at 37ºC for 48 h (43) and moulds were cultured on Sabouraud dextrose agar at 25ºC for 7 days (43). To collect cells for DNA extraction, cells from fresh plate of each fungus were separately inoculated in 5 ml Sabouraud dextrose broth (Oxoid) and incubated at 37ºC with shaking incubator at 200 rpm for 48 h. Cells were collected by centrifugation at $\geq$10,000 rpm for 2-5 min. Fungal DNA was extracted from each fungal culture using modified MasterPure™ Yeast (MPY) DNA Purification Kit (Epicentre® Biotechnologies) combined with bead beating cell lysis methods. DNA samples were quantified using NanoDrop 2000 UV-Vis Spectrophotometers (NanoDrop 2000 Thermo Scientific, Wilmington, USA).

The fungal community model

The fungal community model was generated by mixing 25 ng of each genus specific DNA; *Candida*, *Cryptococcus*, *Aspergillus* and *Fusarium* at 1:1:1:1 ratio. Then, differential *Candida* abundance community model were preared by adding a 10-fold serial dilution of *Candida*-specific DNA to *Cryptococcus*, *Aspergillus*, and *Fusarium* DNA mixture.

T-RFLP-PCR

The fungal internal transcribed spacer (ITS) region of the 18rRNA gene was amplified using the fluorescently labeled forward primers ITS1 (6'[FAM]-TCCGTAGGTGAACCTGCGG) (44) or ITS1F (6'[FAM] CTTGGTCATTTAGAGGAAGTAA-3') and the unlabeled reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (45). The PCR reaction contained 2 µl (~100ng) of template DNA of template DNA in 1x TopTaq Master Mix (TopTaq DNA Polymerase; QIAGEN, Cat. no. 200403) 1x PCR Buffer contains 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), 1.25 units TopTaq DNA Polymerase, 0.5 µM concentration of each oligonucleotide primer and the nanopured water was added to total volume of 50 µl. The thermocycler program was set up initial step at 96°C for 5 min, and amplifications was performed for 40 cycles, with denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s, followed by extension at 72°C for 15 min. PCR product was purified using a QIAquick PCR Purification Kits (QIAquick® Spin; QIAGEN, Cat. no. 28104) according to

the manufacturer's instruction. Then PCR products were digested with *Msp*I or *Hae*III restriction endonuclease enzymes in 25 µl reaction mixtures containing 10 µl of PCR product, 1x buffer, 0.1µg μl^{-1} of acetylated bovine serum albumin, and 20 U of restriction enzyme. Reactions were incubated at 37°C for 3 h. DNA fragments were diluted 1:5 in water and submitted to size analysis by DNA Analyzers using the Applied Biosystems 3130 series Genetic Analyzers. T-RFLP profiles were produced using the GeneMapper software V4.0 (46). Fungal-specific peaks were estimated.

Quantitative PCR

To quantify the fungal DNA, the quantitative PCR method using SYBR Green as the detection system was used. The sequences of fungal-specific primers are shown in Table 1. The DNA mixture (50 ng) was added in 10 µl of EXPRESS SYBR® GreenER™ qPCR Supermix Universal (Invitrogen. Cat. No.11784-01K), 0.4 µl of 10 mM each set of forward and reverse primers and DNA template, and distilled water to final volume of 20 µl. The MiniOpticon real-time PCR detection system (BioRad) was set up with the qPCR condition programmed as followed: UDG incubation at 50°C for 2 min and denaturation at 95°C for 2 min; cycling at 95°C for 15 s, and 58°C for 1 min for 40 cycles, and data were recorded at the end of each PCR cycle. A melting curve was created by cooling down at 65ºC for 5 s, then increasing to 95ºC at a rate of 0.5ºC per second while recording fluorescence. The Ct value of *Candida*-specific DNA or total fungal DNA was recorded and the target copy number was estimated based on weight of genomic DNA.

PCR amplicons were verified using 1.5% agarose gel electrophoresis and ethidium bromide staining.

***Aspergilllus*- nucleotide sequencing**

The PCR amplicon was amplified using the forward primer NSI1(5'GATTGAATGGCTTAGTGAGG-3') (35) and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') follow by purified using Gel/PCR DNA Fragments Extraction Kit (Geneaids) for DNA sequencing process. The sequencing step was using ABI PRISM®377 DNA Sequencher.

Table 1. Sequences of primers for quantitative PCR assays

Primer	Sequence	Ref.
***Candida*-specific primers**		
Cand-F	CCTGTTTGAGCGTCRTTT	(47)
ITS-R	TCCTCCGCTTATTGATAT	
***Cryptococcus*-specific primers**		
Cryp I	TCCTCACGGAGTGCACTGTCTTG	(48)
Cryp II	CAGTTGTTGGTCTTCCGTCAA	
***Aspergillus*-specific primers**		
Asp-F	CTGTCCGAGCGTCATTG	(47)
ITS-R	TCCTCCGCTTATTGATAT	
***Fusarium*-specific primers**		
Fsol1	CTCATCAACCCTGTGAACATACC	(49)
Fsol2	ATGCCAGAGCCAAGAGATCC	

CHAPTER V

RESULTS

Primer selection

The oral fungal community model, DNA mixture of *Candida* : *Cryptococcus* : *Aspergillus* : *Fusarium* in 1:1:1:1 ratio was generated. The ITS1/4 or ITS1F/4 panfungal primers or genus-specific primers were tested and PCR products were shown in Figure 6. Each primer set appeared to amplify the correct product when tested with the fungal DNA mixture (Figure 6A). However, PCR bands resulted from the use of two different sets of panfungal primers were inconsistent when genus-specific DNA was amplified separately (Figure 6B). The ITS1/4 panfungal primer pair had less efficiency in detecting moulds; *Aspergillus* and *Fusarium* specific DNA, whereas ITS1F/4 showed constant bands across the four genus-specific DNA used for the fungal community model in this study. Therefore, ITS1F was chosen for further T-RFLP analysis.

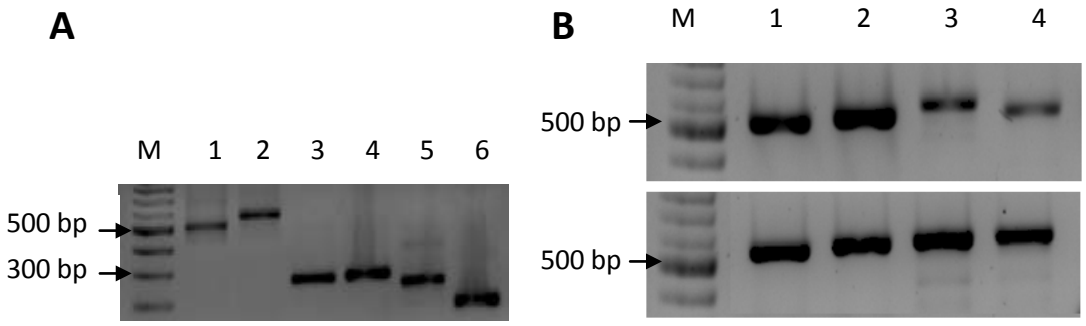

Figure 6. PCR identification of fungal DNA (A) Fungal DNA mixture were amplified using panfungal primers, ITS1/4 (1) or ITS1F/4 (2), and primers specific to *Candida* (3), *Cryptococcus* (4), *Aspergillus* (5), and *Fusarium* (6), respectively. M is DNA size marker (100 bp ladder). **(B)** DNA extracted from *Candida* (1), *Cryptococcus* (2), *Aspergillus* (3) or *Fusarium* cultures (4), were individually amplified using panfungal primers, ITS1/4 (Top) or ITS1F/4 (Bottom).

Restriction Enzyme selection

For T-RFLP, the fluorescent-labeled PCR amplicons of the ITS1F/4 primers were digested by *Msp*I or *Hae*III restriction enzyme. Fungal genus-specific fragments of *Msp*I or *Hae*III digestion were observed in 1.5% agarose (Figure 7A). The fluorescent-labeled terminal-restriction fragments (T-RF), of *Msp*I or *Hae*III digestion, were detected with a DNA analyzer (Figure 7B). The T-RF of *Candida, Cryptococcus, Aspergillus* and *Fusarium* digested by *Msp*I represented 333, 462, 150, and 385 bp, respectively. The T-RF of *Hae*III digestion were 126, 484, and 129 bp for *Candida, Cryptococcus* and *Fusarium*, respectively. However, *Hae*III digestion of *Aspergillus*-specific DNA showed many non-specific peaks. Moreover, *Hae*III digested *Candida* and *Aspergillus*-specific peak sizes are very similar.

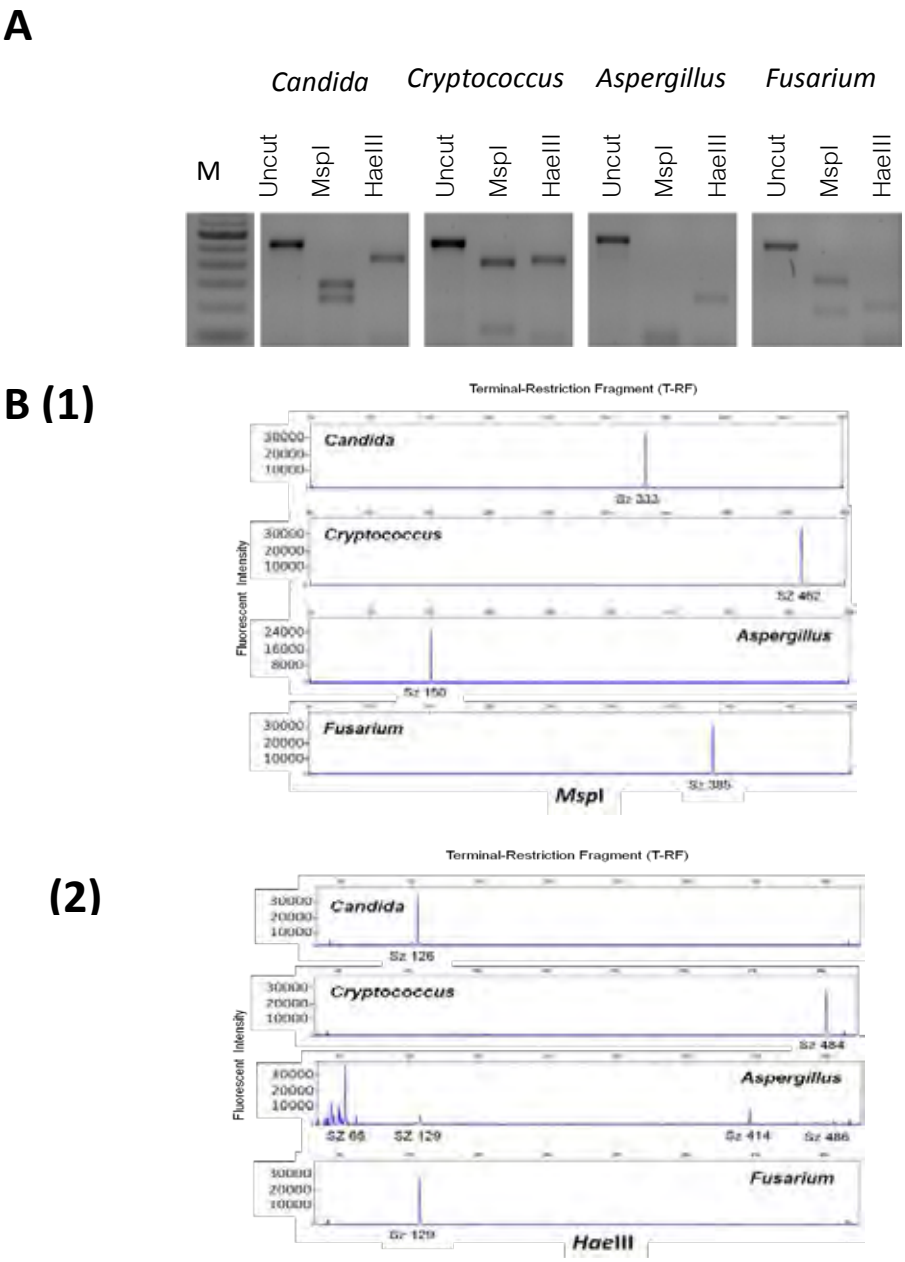

Figure 7. PCR restriction fragment of fungal DNA (A) *Candida* , *Cryptococcus* , *Aspergillus* , or *Fusarium* specific DNA was amplified using ITS1F/4 primers. PCR products (uncut) were digested using endonuclease enzymes *Msp*I or *Hae*III. M is DNA size marker (100-bp ladder). **(B)** Terminal-restriction fragments (T-RF), products of *Msp*I (1) or *Hae*III (2) digestion, specific to *Candida*, *Cryptococcus*, *Aspergillus*, or *Fusarium* were shown.

T-RFLP analysis of fungal community model

In the 1:1:1:1 fungal community model (~25 ng of each specific DNA), DNA was amplified using ITS1F/4 panfungal primers followed by restriction with *Msp*I restriction enzyme. The chromatogram showed T-RF of *Candida, Cryptococcus* and *Fusarium* were 333, 463 and 385 bp (Figure 8). The size of each T-RF was similar with individual analysis (±1bp). However, the fungal community model could not detect T-RF of *Aspergillus*-specific peak.

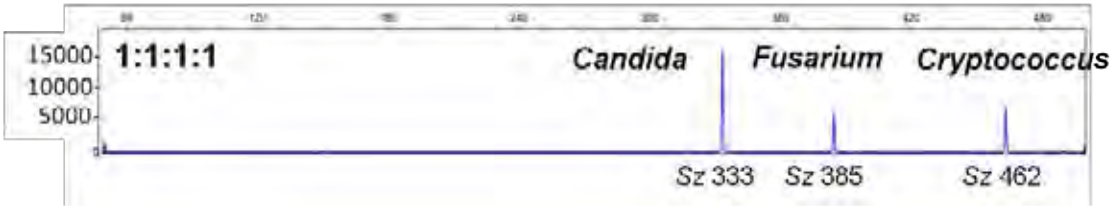

Figure 8. The chromatogram of DNA mixture 1:1:1:1 ratio. The simple fungal community model was amplified using ITS1F/4 primers and digested using *Msp*I restriction enzymes.

Lowest detection limit of T-RFLP for *C.albicans*

The lowest detection limit of the T-RFLP method for detecting *Candida*-specific DNA in fungal DNA mixtures was identified by using ITS1F panfungal primer or *Candida*-specific primers to analyze a series of 10-fold dilutions of *Candida*-specific DNA (Figure 9A). Using T-RFLP, the profile of genus-specific T-RF products was generated (Figure 9B), and peak height represented the relative abundance of each fragment. In the fungal community model with 1:1:1:1 and 0.1:1:1:1 ratio *(*25 and 2.5 ng of *Candida*-specific DNA), the T-RF represented average peak height relative fluorescent unit at 22,147 and 2,838, respectively (Figure 9B). However, when *Candida*-specific DNA alone was amplified using ITS1F/4 panfungal primers followed by restriction with *Msp*I, the T-RFLP assay could detect as low as 0.01ng of *C.albicans* DNA. (Figure 10).

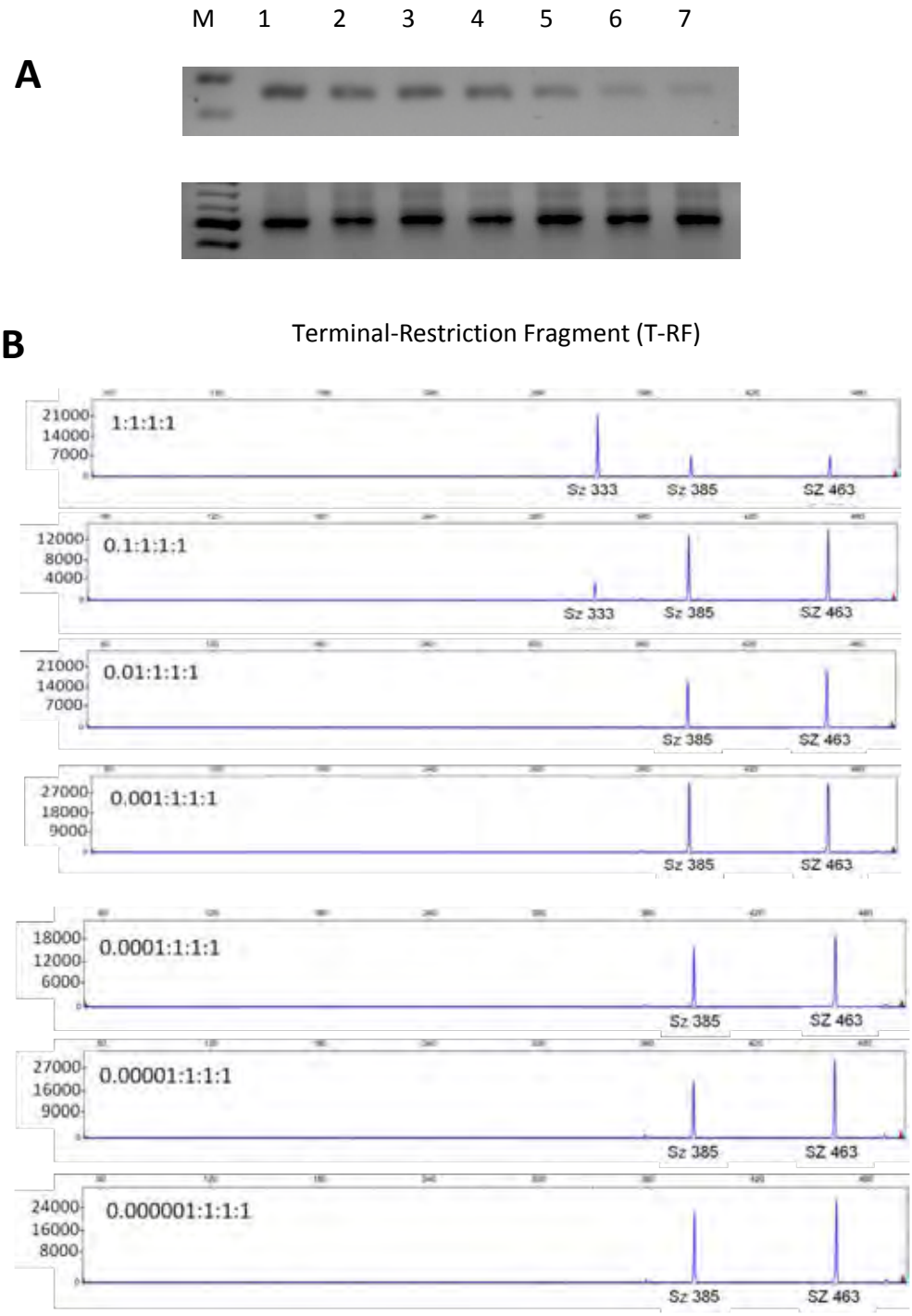

Figure 9. The abundance of *Candida*-specific DNA in fungal DNA mixtures. Ten-fold decrease of *Candida*-specific DNA from 25 to 0.0000025 ng in DNA mixture was shown by endpoint PCR, (M is 100-bp DNA length ladder) (A), and T-RF peaks (B).

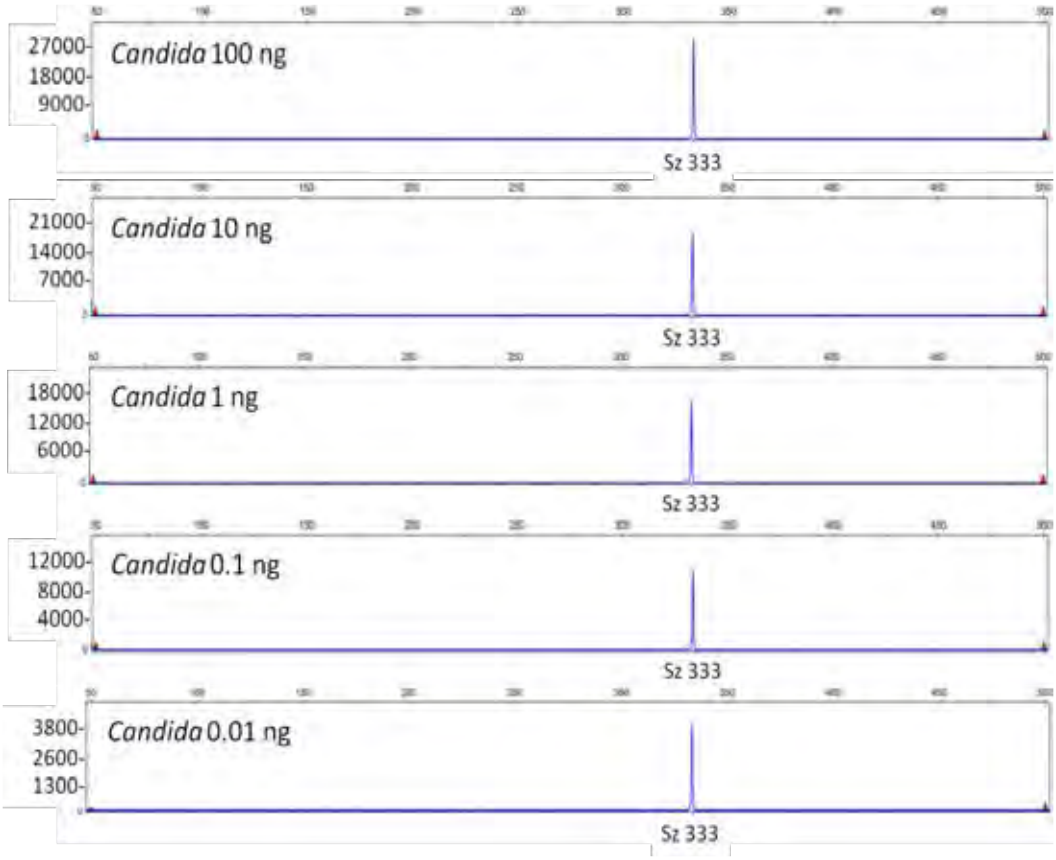

Figure 10. The chromatogram of *Candida*-specific DNA. Ten-fold decrease of *Candida*-specific DNA from 100 to 0.01 ng.

Accuracy of T-RFLP comparing to qPCR and genomic DNA weight

The accuracy of T-RFLP method for fungal detection and level estimation was compared to the results from using quantitative PCR and the estimation by genomic DNA weight. We simulated the detection of varying ratio of *Candida*-specific DNA in the fungal community model because *Candida* is an important component found frequently in the oral cavity (1, 6). *Candida*-specific DNA abundance detected by T-RFLP was shown in comparison to the estimation by genomic DNA weight or by qPCR (Table 2).

Candida-specific targets ranging from 10^5 to 10^6 copies were detected by T-RFLP method, but appeared to be underestimated by 10-fold. In contrast, the detection by qPCR was so highly sensitive that its lowest detect at 1 copy number and could detected between 10^3 to 10^6 copies, but overestimated when the abundance is lower than 10^2 copy number.

The calculation of the *Candida*-specific DNA copy number was estimated based on the genomic DNA weight using amount of ds DNA's weight (ng) / [length of template (bp) x (1.079×10^{-12})](Andrew Staroscik. URI Genomics & Sequencing Center. 2004). Genomic DNA of *Candida* contains 15.6 Mb of DNA (50). The estimated copy number of *Candida*-specific DNA was 1.48×10^6 to 1.48×10^0 copies.

For T-RFLP, the estimate of *Candida* specific DNA copy number in DNA mixture was calculated by average of relative peak height compared with *Candida*-specific DNA at 10^6 copy number (Table 3).

For qPCR, the estimated copy number *of Candida*-specific DNA in fungal community model was calculated using the cycle number (Ct value) of *Candida*-specific DNA in DNA mixture compared with standard curve (Table 4). The standard curve was generated from serial dilution from 10^6 to 10^1 copies number and the cycle number of *Candida*-specific primer in *Candida*-specific DNA (Table 5). The slope of the standard curve was 4.3178 and the correlation coefficient was 0.97 (Figure 11).

Table 2. Estimation of *Candida*-specific DNA copies by genomic DNA, qPCR, or T-RFLP

No.	DNA mixture	*Candida*	qPCR	T-RFLP
1.	1:1:1:1	10^6	10^6	10^5
2.	0.1:1:1:1	10^5	10^5	10^4
3.	0.01:1:1:1	10^4	10^4	Not detected
4.	0.001:1:1:1	10^3	10^3	Not detected
5.	0.0001:1:1:1	10^2	10^3	Not detected
6.	0.00001:1:1:1	10^1	10^2	Not detected
7.	0.000001:1:1:1	10^0	10^2	Not detected

Table 3. T-RFLP result of T-RF *Candida*-specific DNA; peak size, height, average height and estimated copy number in fungal community model.

Sample	*Candida* 100 ng	1:1:1:1	0.1:1:1:1	0.01:1:1:1	0.001:1:1:1	0.0001:1:1:1	0.00001:1:1:1	0.000001:1:1:1
Peak (bp)	333	333	333	Not detected	Not detected	Not detected	Not detected	Not detected
Height	32,332 32,468 29,942	13,920 19,895 32,628	3,461 3,041 2,012	Not detected	Not detected	Not detected	Not detected	Not detected
Average height	31,580	22,147	2,838	Not detected	Not detected	Not detected	Not detected	Not detected
Estimated copy number	10^6	10^5	10^4	Not detected	Not detected	Not detected	Not detected	Not detected

Table 4. The cycle number and copy number of *Candida*-specific DNA in fungal community model.

DNA mixture	1:1:1:1	0.1:1:1:1	0.01:1:1:1	0.001:1:1:1	0.0001:1:1:1	0.00001:1:1:1	0.000001:1:1:1
Cycle number	15.71±1.08	18.75±1.04	22.12±0.95	25.41±0.74	28.28±0.83	30.93±0.91	31.86±1.19
Copy number	1.17 x10^6	1.87 x 10^5	2.65 x 10^4	3.42 x 10^3	4.08 x 10^3	4.70 x 10^2	4.91 x 10^2

Table 5. The cycle number and copy number of *Candida*-specific DNA for generating standard curve

Candida (ng)	50,000	5,000	500	50	5	0.5	0.05
Copy number	2.97x10^6	2.97x 10^5	2.97x 10^4	2.97x 10^3	2.97x 10^2	2.97x 10^1	2.97x 10^0
Cycle number	13.31±0.82	20.51±1.26	23.42±0.83	29.98±1.32	32.25±3.01	35.17±2.06	28.04±0.87

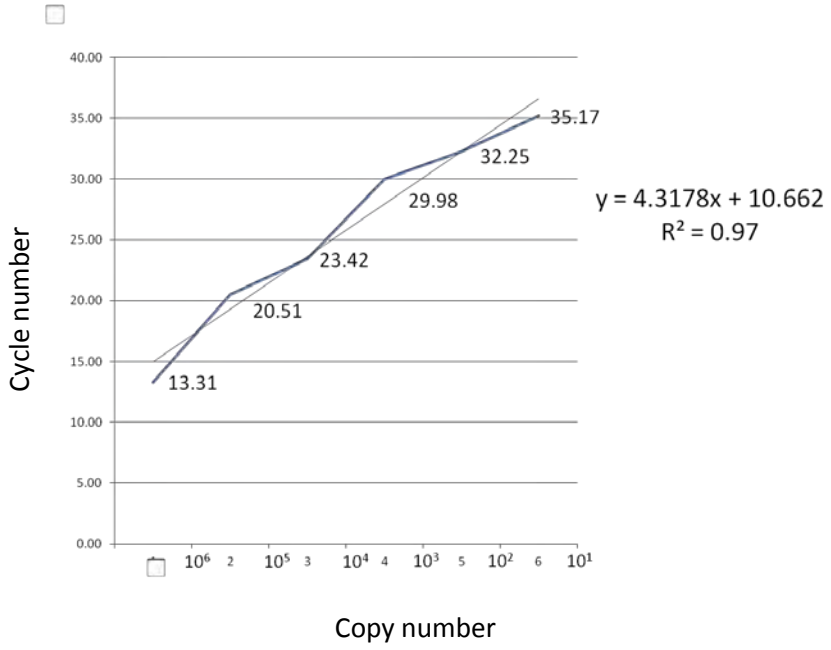

Figure 11. The standard curve of relation between cycle number (Ct value) of *Candida*-specific DNA and copy number

The absence of Aspergillus-specific peak from T-RFLP

When the fungal community model was analyzed by T-RFLP, *Aspergilllus*-specific peak was absent (Figure 8). Although, the panfungal primers ITS1F/4 could amplify *Aspergillus*-specific DNA in isolation (Figure 7), they failed to amplify *Aspergillus*-specific DNA in fungal community for T-RFLP analysis.

One hypothesis why Aspergillus-specific peak was not detected could be due to PCR primer bias. PCR bias from primer–template PCR in T-RFLP had been reported when used in a multi-template for community analysis and some species may be underrepresented or missing from the mixture (20). *Aspergillus*-specific DNA sequencing showed that the sequences of the ITS1F primer are not completely matched to genomic DNA sequences and may amplify *Aspergillus* DNA with lower efficiency (Figure 12). The ITS1F panfungal forward primer mismatched with *Aspergillus*-specific DNA at two positions. The other species-specific DNA sequencing showed that the sequence of the ITS1F primer are mismatched one position at the third base of ITS1F primer, however, completely matched with *Candida* and *Cryptococcus*-specific DNA (Figure 12).

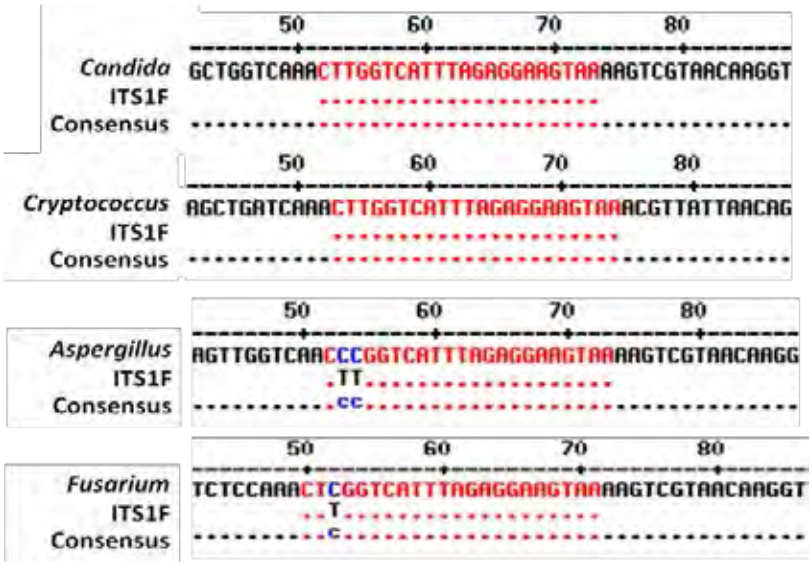

Figure 12. The alignment of each species-specific DNA aligned with ITS1F primer.

Therefore, we selected another panfungal-primer that had previously been used in fungal community analysis, the NSI1 (6'[FAM] GATTGAATGGCTTAGTGAGG) primer (35). However, the NSI1 primer also could not amplify *Aspergillus*-specific DNA in the fungal community model (Figure 13).

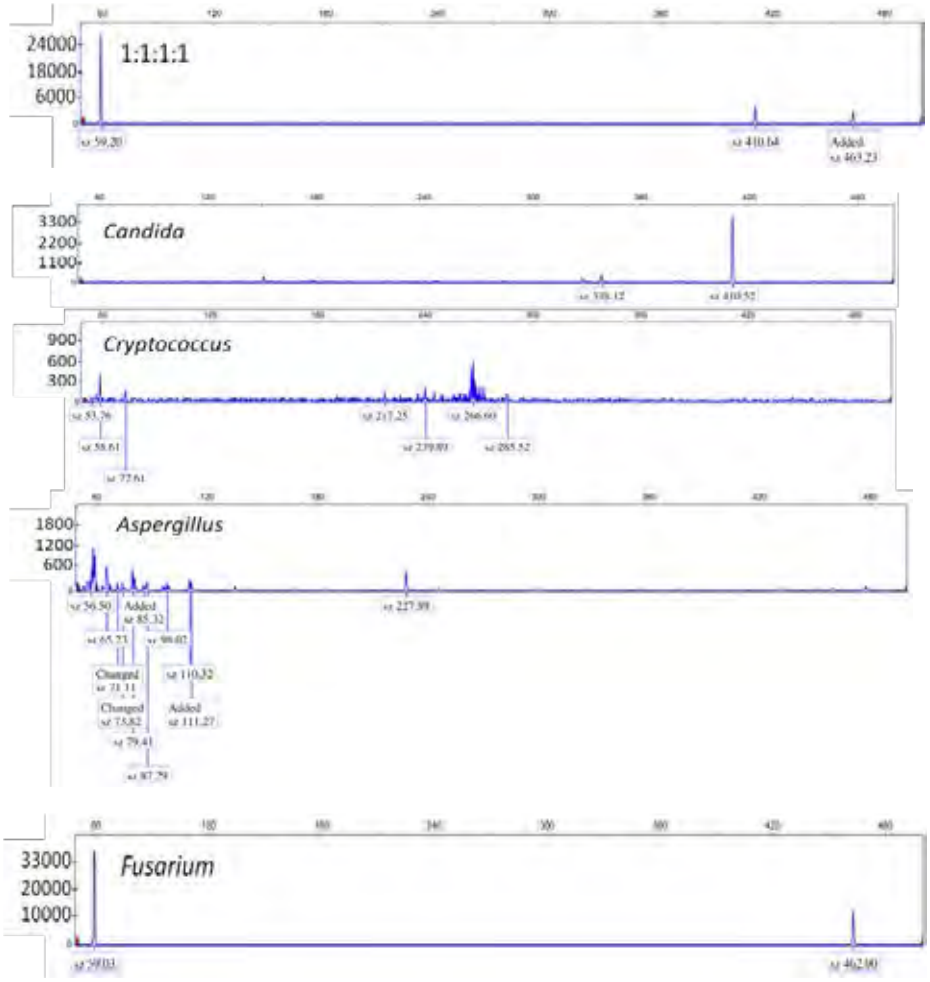

Figure13. The chromatogram of fungal DNA mixture at 1:1:1:1 ratio (A) and *Candida, Cryptococcus, Aspergillus, Fusarium* specific DNA (B) amplified using NSI1/ITS4 primers and digested by *Msp*I.

Nonetheless, the relative abundance of *Aspergillus*-specific fragment using ITS1F panfungal primer and digested by *Msp*I was lower than others by approximately 30% even in the individual analysis (Table 6). The estimation of genomic copy number in fungal ecology assumes that the copy numbers of target gene in different fungal species are similar. However, the copy number of the rRNA operon is known to vary among different fungal species. This could explain the relatively low abundance of *Aspergillus* DNA if the rDNA copy number is lower in the *Aspergillus* genome. Copy number of rDNA in *Aspergillus* varies with strain between 38-91 copies per haploid genome (52), while *C. albicans* has 90 copies (51), *C. neoformans* 55 copies (52) and *Fusarium* 200 copies per haploid genome (53). However, even when *Aspergillus*-specific DNA was increased to 25, 50 and 100 ng in the fungal community, *Aspergillus*-specific peak still could not be detected in fungal community model (Figure 14).

Table 6. Terminal restriction fragments of *Msp*I digestion: size, height, and area.

Sample	Size (bp)	Height	Area
Candida	332.85	32,332	278,745
Cryptococcus	462.79	32,218	399,826
Aspergillus	150.94	23,533	132,697
Fusarium	385.51	32,297	281,126

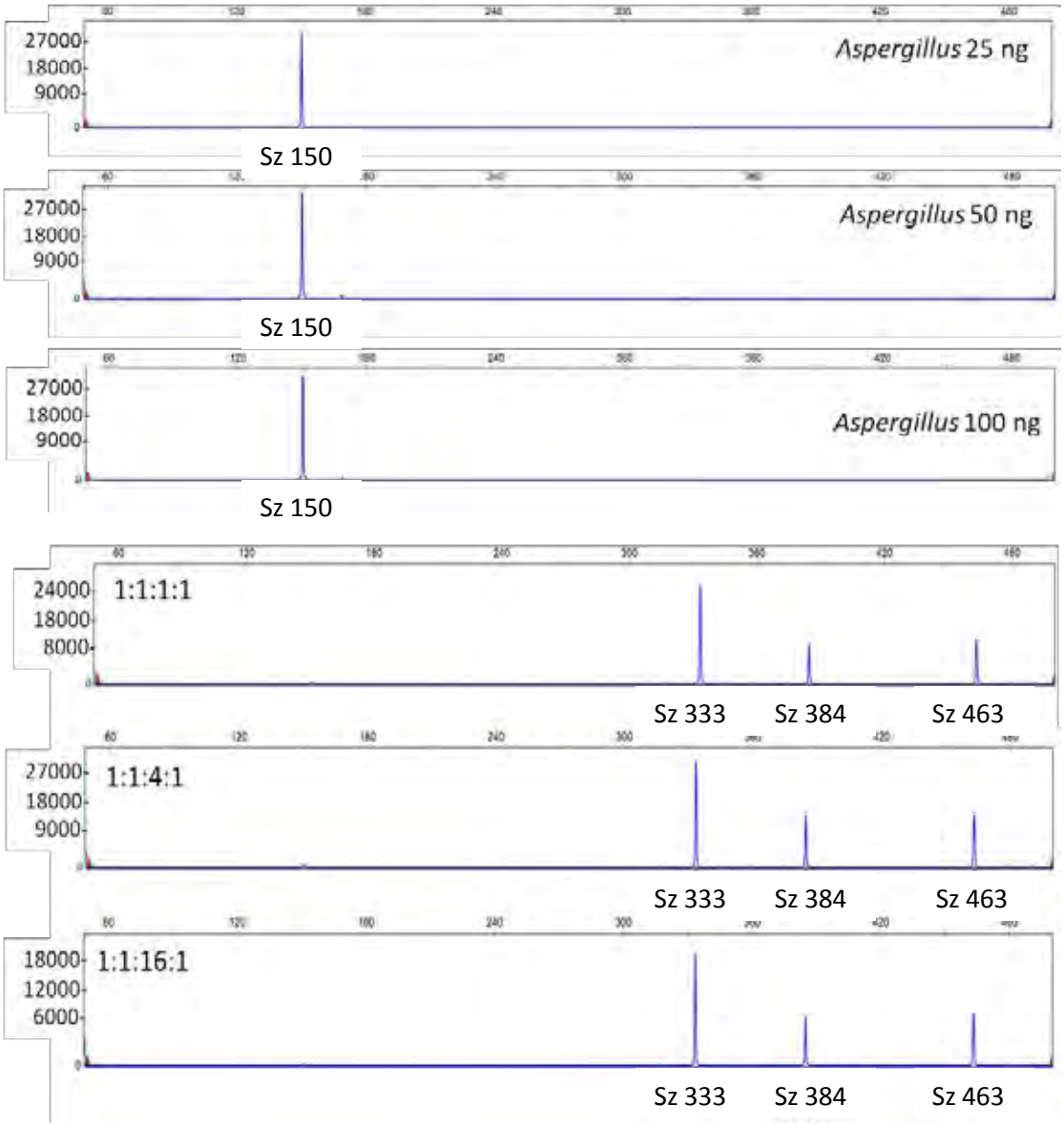

Figure 14. The chromatogram of abundance of individual *Aspergillus* DNA at 25, 50, and 100 ng and increased abundance of *Aspergillus* in DNA mixture at 1:1:1:1, 1:1:4:1 and 1:1:16:1 ratio.

Furthermore,T-RFLP analysis of *Aspergillus* DNA mixed with other DNA in 4:0, 3:1, 2:2 and 1:3 ratio (100:0, 75:25, 50:50 and 25:75 ng) (Figure 15) or when mixed with a single other genus in 3:1 ratio (Figure 16) still could not detect any *Aspergillus*-specific peak.

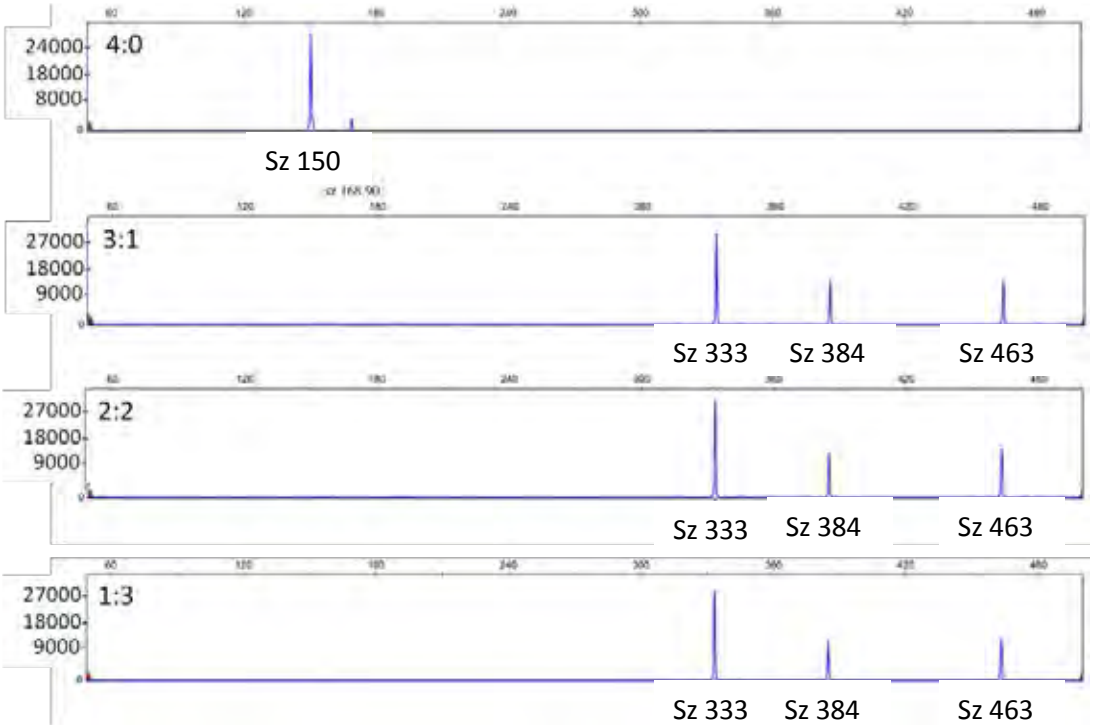

Figure 15. The chromatogram of abundance *Aspergillus* DNA with other genus; *Candida, Cryptococcus* and *Fusarium* at 4:0, 3:1, 2:2 and 1:3

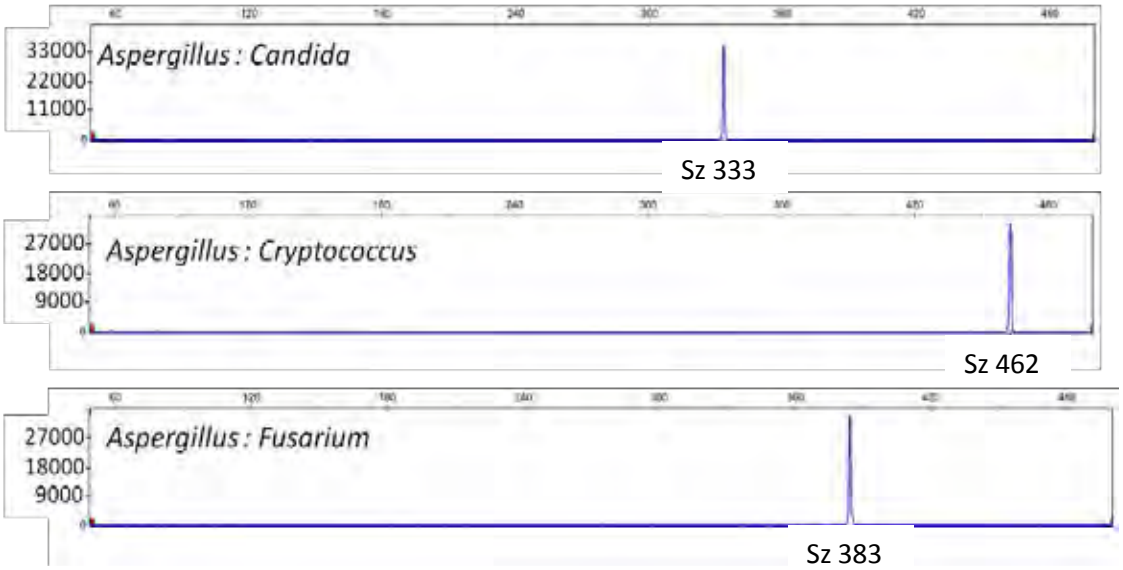

Figure 16. The chromatogram of *Aspergillus* with *Candida, Cryptococcus* or *Fusarium* at 1:3 ratio.

CHAPTER VI

DISCUSSION

The accuracy of T-RFLP analysis for characterize microbial community is important for data interpretation. The two major steps in T-RFLP included DNA extraction procedure and the total fungal PCR amplification. The MasterPure™ (MPY) DNA extraction method was efficient for extracting adequate DNA from yeast cells such as *C.albican*s, but less DNA yielded from *Aspergillus* conidia or hyphae (54). Prior to modeling the fungal community, we analyzed the DNA quantity and quality using NanoDrop 2000 UV-Vis Spectrophotometers (Thermo Scientific, Wilmington, USA). We then adjusted the amount of genomic DNA in the fungal community model as described in materials and methods. The panfungal primers for T-RFLP should be fungal-specific and efficient for targeting most of fungi in the community (15). The ITS1/4 panfungal primers showed less efficiency in detecting *Aspergillus*- and *Fusarium*-specific DNA. In contrast, ITS1F/4 showed bands constantly across all members of the fungal community model in this study (Figure 6B). This result suggested that ITS1/4 panfungal primers might be inadequate for characterizing our fungal community model. The set of ITS1F/4 primers was thus selected for subsequent experiments.

The ITS1F/4 primers were specific for ITS region of ascomycetous, basidiomycetous and zygomycetous fungi. This primer set has been used to amplify

fungi in mixed templates but PCR efficiency have not been examined (55). Terminal fragments were resulted from the selection of restriction enzymes (56). The previous study (57, 58) demonstrated that the restriction sites of *Msp*I and *Hae*III could differentiate the opportunistic fungal pathogens. We showed the fungal-specific T-RF peaks corresponding to *Candida*, *Cryptococcus*, *Aspergillus* or *Fusarium*. We also reported that *Msp*I T-RF products demonstrated the microbial profile better than the products of *Hae*III digestion. Therefore, *Msp*I digestion was used to determine the fungal community model in subsequent experiments.

Importantly, in T-RFLP analysis of the fungal community model, *Aspergilllus*-specific peak was undetectable from the fungal community suggesting that the community approach using T-RFLP might have limitations. This was inconsistent to the results from individual analyses of genus-specific DNA (Figure 6A and 6B). We confirmed that *Aspergilllus*-specific DNA remained in the DNA mixtures using the genus-specific primers, but failed to be amplified by the ITS1F/4 or ITS1/4 panfungal primers when there was other fungal DNA in the background (data not shown). The efficiency of T-RFLP detection might vary among the multiple PCR templates in the mixed fungal community (24).

In the fungal community model, the amount of genomic DNA from each fungus was equally normalized based on the Nanodrop measurement. Therefore, it was assumed that the copy numbers of the targeted regions was similar among four fungal

species. However, the copy number of the rRNA operon can be varied from species to species (12). We reported that the relative abundance of *Aspergillus*-specific fragment was lower than others by approximately 30% even in the individual analysis (Table 6). This variation between species complicates the quantification of different fungal species in a mixed DNA pool if the rRNA operon is the target region (12).

The sequencing of *Aspergilllus*-specific PCR products revealed that the panfungal ITS1F primer has two positions mismatched to *Aspergillus* ITS region (Figure 12). Whether the mismatched sequences were responsible for the disappearance of *Aspergilllus*-specific peak was not yet tested. However, we tested the alternative panfungal primer, NSI1, in detection of *Aspergilllus*-specific product in the fungal community model, but the NSI1 primer still fails to amplify *Aspergillus*-specific DNA in the community. We propose to use ITS2 reverse primer to analyze the fungal community model since this primer has been used in the oral mycobiome study and could detect *Candida, Cryptococcus, Aspergillus* and *Fusarium* in oral rinse (1). Moreover, ITS2 sequences completely match with *Aspergillus* DNA (Figure 17).

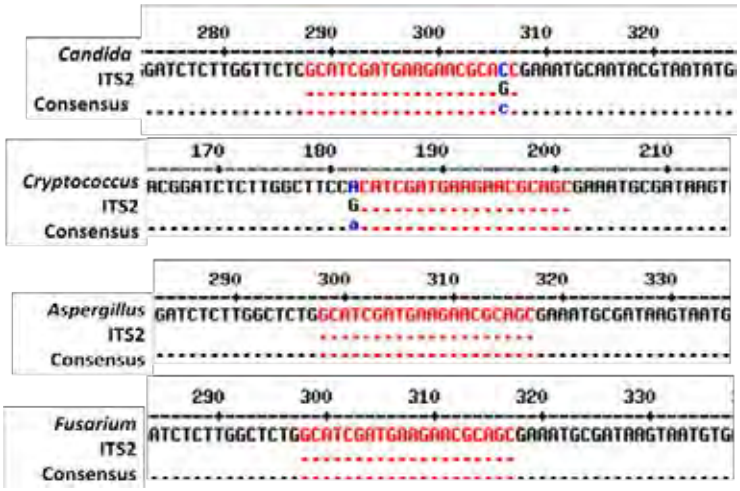

Figure 17. Alignment of ITS2 reverse primer with *Candida, Cryptococcus, Aspergillus* and *Fusarium* DNA sequence.

Candida-specific DNA abundance detected by T-RFLP was shown in comparison to the estimation by genomic DNA weight or by qPCR. The accuracy of target copy number detected by qPCR was quite consistent to the estimation by genomic DNA weight when the abundance of *Candida*-specific targets was ranging from 10^3 to 10^6 copies. However, qPCR might overestimate when target was lower than 10^2 copies. *Candida*-specific targets ranging from 10^5 to 10^6 copies were detected by T-RFLP method, but appeared to be underestimated by 10-fold.

CHAPTER VII

SUMMARY

The T-RFLP method was feasible to be used for detection of unknown fungal species with some limitations. Four representative oral fungi, including *Candida albican*s, *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Fusarium* spp., were used to generate a simple fungal community model *in vitro*. The highly conserved internal transcribed spacer region in the ribosomal DNA was targeted for total fungal amplification. Then, species-specific fragments were digested by *MspI* restriction enzyme. Our results suggested that total-fungal amplification using panfungal primers was the important step of T-RFLP method. Therefore, the choice of panfungal primers needed to be taken into consideration. This method was sufficient for comparison of major differences between fungal communities, however, the use of more than one set of panfungal primers was recommended. Nonetheless, *Aspergillus*-specific DNA in our fungal community model could not be amplified by either ITS1F/ITS4 or NSI1/ITS4 panfungal primers. The optimal panfungal primers for all major oral fungal species needed to be further studied and optimized.

T-RFLP detection of *Candida abicans* abundance in the DNA mixture was evaluated. By comparison T-RFLP analysis to quantitative PCR and the amount of genomic DNA, it appeared that low abundance species in the community could be

underestimated. When fungal species were lower than the limit of detection, a careful interpretation of results should be applied. In addition to T-RFLP, qPCR using specific primers was required to validate the detection and target abundance.

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APPENDICES

Table 7. Terminal restriction fragments of DNA mixture: size, height, and area.

DNA mixture	Fungi	Size	Height	Area
1:1:1:1	*Candida*	333.30	19,895	171,697
	Cryptococcus	463.54	6,600	68,202
	Aspergillus	Not detected	Not detected	Not detected
	Fusarium	385.60	6,626	63,617
0.1:1:1:1	*Candida*	333.36	3,461	29,071
	Cryptococcus	463.46	13,871	145,507
	Aspergillus	Not detected	Not detected	Not detected
	Fusarium	385.72	12,820	127,724
0.01:1:1:1	*Candida*	Not detected	Not detected	Not detected
	Cryptococcus	463.54	19,179	193,741
	Aspergillus	Not detected	Not detected	Not detected
	Fusarium	385.78	15,001	141,165
0.001:1:1:1	*Candida*	Not detected	Not detected	Not detected
	Cryptococcus	463.58	31,431	387,065
	Aspergillus	Not detected	Not detected	Not detected
	Fusarium	385.39	31,668	317,259
0.0001:1:1:1	*Candida*	Not detected	Not detected	Not detected
	Cryptococcus	463.52	18,181	189,545
	Aspergillus	Not detected	Not detected	Not detected
	Fusarium	385.45	15,200	148,288
0.00001:1:1:1	*Candida*	Not detected	Not detected	Not detected
	Cryptococcus	463.33	31,297	325,177
	Aspergillus	Not detected	Not detected	Not detected
	Fusarium	385.72	23,142	212,361
0.000001:1:1:1	*Candida*	Not detected	Not detected	Not detected
	Cryptococcus	463.53	22,221	272,485
	Aspergillus	Not detected	Not detected	Not detected
	Fusarium	385.65	26,208	210,297

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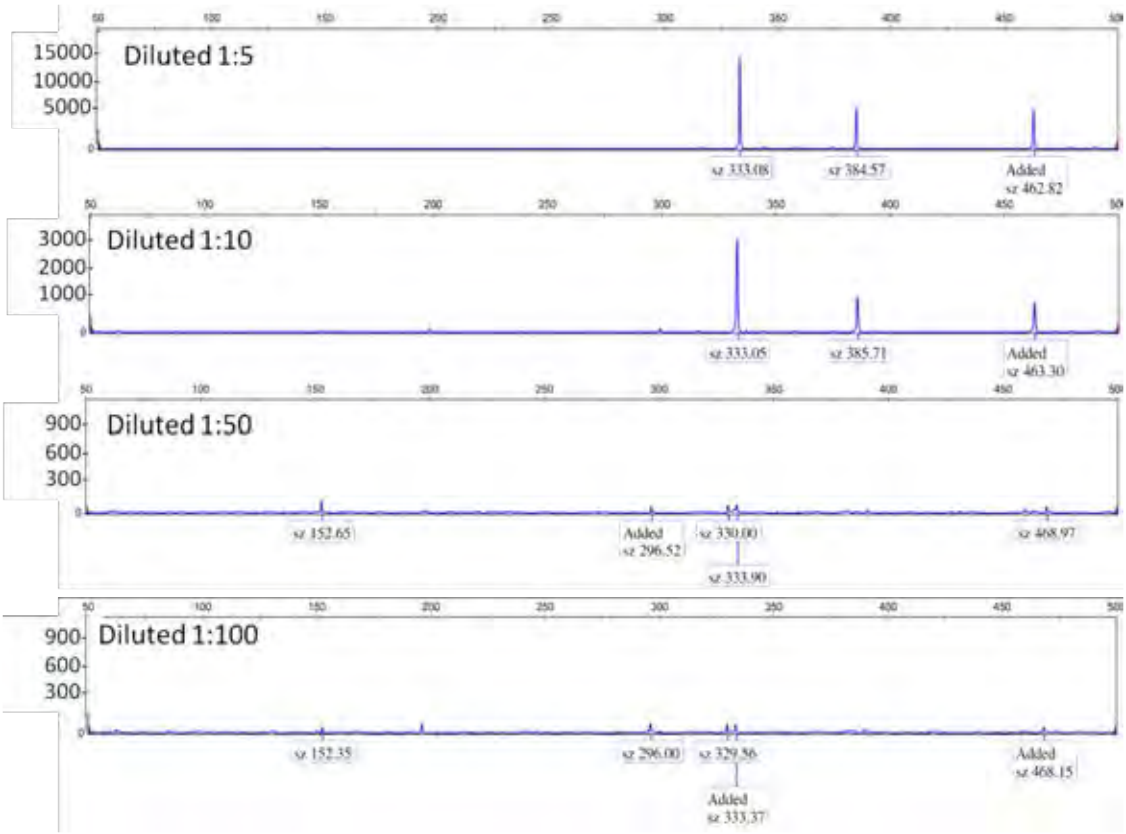

Figure 18. T-RFLP analysis of DNA mixture diluted 1:5, 1:10, 1:50, 1:100.

REAGENTS PREPARATION

1. 10x TAE buffer

Tris base	108	g
Boric acid	55	g
0.5 M EDTA	40	ml

Add DW to final volume of 1 L.

2. 1.5% Agarose gel

Agarose	1.5	g
1x TAE	100	ml

Dissolve by heating in the microwave oven and occasional mix unit no granules of agarose are visible

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