Thesis progression

Thesis title: Enhanced Detection of Pathogenic Fungi in Eye Infection Using Oxford Nanopore Long-Read Sequencing

Thesis progression title: Secondary Barcoding Sequencing of *Aspergillus* and *Fusarium* Species Using Oxford Nanopore Long-Read Technology

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1. Introduction

Fungal eye infections are a significant cause of ocular morbidity and vision loss worldwide, particularly in tropical and developing regions (Kalkanci & Ozdek, 2011; Mehrandish & Mirzaeei, 2021). Accurate and rapid identification of fungal pathogens is critical for effective treatment; however, conventional diagnostic methods such as direct microscopy, fungal culture, and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) are limited by low sensitivity, prolonged turnaround times, and difficulties in discriminating closely related fungal species (Mendonça et al., 2022; Siller-Ruiz et al., 2017). In Thailand, fungal eye infections remain a substantial clinical burden, with the most reported pathogens belonging to the genera of *Fusarium*, *Aspergillus*, and *Candida* (Czakó et al., 2019; Mahmoudi et al., 2018).

Although the internal transcribed spacer (ITS) region is widely used as the primary fungal DNA barcode, it often lacks sufficient resolution for accurate species-level identification within these clinically important genera, particularly in species complexes of *Fusarium* and *Aspergillus* spp. (Al-Hatmi et al., 2016; Samson et al., 2014; Schoch et al., 2012).

To overcome this limitation, secondary DNA barcoding markers such as translation elongation factor 1-alpha (TEF1α), β-tubulin (TUB2), and calmodulin (CaM) have been increasingly employed to enhance taxonomic resolution and improve discrimination among closely related or cryptic species (Geiser et al., 2007; Raja et al., 2017; Stielow et al., 2015). Integration of these secondary barcodes with long-read sequencing platforms, particularly Oxford Nanopore Technologies (ONT), enables simultaneous multi-gene analysis and represents a critical advancement toward more accurate and rapid diagnosis of fungal eye infections (Ohta et al., 2023; Wang et al., 2021).

In particular, this study emphasizes the use of secondary barcoding markers for *Aspergillus* and *Fusarium* spp., two major causative genera of fungal eye infections in Thailand and other tropical regions. These genera comprise multiple closely related and cryptic species complexes that cannot be reliably differentiated using ITS alone. By incorporating secondary barcodes including TEF1α, β-tubulin (TUB2), and calmodulin (CaM) in combination with long-read ONT, this study aims to improve taxonomic accuracy and strengthen fungal identification beyond primary barcoding, thereby supporting more precise clinical diagnosis and downstream epidemiological analyses.

2. Objective

2.1 Generate high-quality long-read sequences using Oxford Nanopore Technologies (ONT) to support accurate taxonomic classification and downstream bioinformatic analysis.

3. Materials and methods

3.1 Validation of secondary barcoding amplification

Secondary barcoding markers, including TEF1α, β-tubulin (TUB2), and calmodulin (CaM), were validated using genomic DNA extracted from five isolates each of *Aspergillus* and *Fusarium* spp. Previously published primer sets were selected based on amplicon size, reported specificity, and taxonomic resolution. Amplification performance was evaluated based on PCR success rate, specificity, and consistency across isolates. PCR amplification was performed using a gradient annealing temperature, with temperatures set at 68.0 °C, 63.8 °C, and 60.0 °C to optimize primer performance. Finally, the expected amplicon sizes were confirmed by agarose gel electrophoresis.

3.2 Secondary barcoding amplification

PCR amplification of the three fungal housekeeping genes was performed using 3 different primer pair which target three different genes including CF1 and CF4 for Calmodulin (CaM) gene amplification, Ben2f (Peterson et al., 2005), and T22 for beta tubulin (TUB2) (Hubka & Kolarik, 2012), and EF1-983F and EF1-1688R for translation elongation factor 1-α (TEF1α) (Stielow et al., 2015).

PCR amplification was performed in 25 µL reactions containing 12.5 µL of KOD One PCR Master Mix (DM015-R500; TOYOBO, Osaka, Japan), 0.75 µL each of forward and reverse primers, 10 µL of nuclease-free water (Invitrogen, Carlsbad, CA, USA), and 1 µL of genomic DNA. Reactions were conducted on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation at 98°C for 3 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 60.0°C for Cam and TEF1α or 63.8°C for TUB2, and extension at 68°C for 5 s, with a final extension at 68°C for 5 min. Amplicons were verified on a 1.5% agarose gel, producing fragments of approximately 1,200 bp for TUB2 and 600–700 bp for TEF1α and CaM.

3.3 Oxford Nanopore sequencing

PCR products were quantified using a Qubit dsDNA HS Assay Kit (Q32851; Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). For each sample, the three secondary barcode amplicons (TEF1α, β-tubulin (TUB2), and calmodulin (CaM)) were normalized to equal concentrations and pooled prior to PCR barcoding. Barcodes were introduced using the PCR in-housed barcoding expansion. PCR barcoding reactions were performed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation at 98 °C for 2 min, followed by 10 cycles of denaturation at 98 °C for 10 s, annealing at 60.0 °C for 10 s, and extension at 68 °C for 10 s, with a final extension at 68 °C for 5 min. Barcoded PCR products were verified by electrophoresis on a 1.5% agarose gel and subsequently size-selected using 0.5X AMPure XP beads (SK2142024; Beckman Coulter, Brea, CA, USA).

Following purification, the barcoded amplicons were subjected to end repair and dA-tailing using the NEBNext FFPE DNA Library Prep Kit (E6650S; New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. Sequencing libraries were then prepared using the Ligation Sequencing Kit (SQK-LSK114; Oxford Nanopore Technologies) and loaded onto FLO-MIN106D (R10.4.1) flow cells. Sequencing was performed on an ONT MinION Mk1D platform operated with MinKNOW software.

4. Results

Oxford Nanopore sequencing (ONT) of pooled secondary barcode amplicons (TEF1α, β-tubulin (TUB2), and calmodulin (CaM)) generated a sufficient number of high-quality reads

across all *Aspergillus* (A1-A5) and *Fusarium* (F1-F5) isolates which show the detail in Table 1. The total number of reads per sample ranged from 7,273 to 19,931, with an overall average of 12,535 reads across all samples.

Mean read length of each isolate ranged from approximately 758 to 1,143 base pair, while median read length were generally close to the mean values, suggesting relatively balanced barcode representation following pooling and barcoding which ranged from 767 to 1,485 base pair.

The sequencing quality metrics were consistently high across all samples, with mean Phred quality scores ranging from 27.4 to 29.8 and median quality scores ranging from 27.8 to 28.9. The obtained read depth and quality are sufficient for downstream taxonomic classification and comparative analysis of secondary barcode markers.

Table 1. Sequencing statistics of secondary fungal barcoding using ONT-based sequencing.

Sample	Total reads	Mean reads length (bp)	Median reads length (bp)	Mean quality	Median quality
A1	7273	1012.8	767	29.1	28.2
A2	13838	958.4	767	28.3	28.1
A3	12915	1143.6	1485	29.8	28.9
A4	12745	758.4	777	28	27.8
A5	10164	920.5	780	27.8	28
F1	10745	908.9	843	27.6	27.9
F2	16147	844.3	770	29	28.4
F3	9231	1089.8	848	27.4	27.8
F4	19931	1112.1	1407	27.9	28.4
F5	12363	1043.7	1164	28.3	28.7
Total average	12535.2	979.25	960.8	28.3	28.2

5. Conclusion

This study demonstrates the successful amplification and Oxford Nanopore sequencing (ONT) of secondary fungal barcode genes, including TEF1α, β-tubulin (TUB2), and calmodulin (CaM), from clinically relevant *Aspergillus* and *Fusarium* isolates. The PCR amplification protocol showed consistent performance across isolates, producing amplicons of the expected sizes, and enabling efficient pooling and barcoding. ONT-baesd sequemcing generated high-quality long-read data with stable sequencing output across all samples. The obtained read counts and quality scores indicate that the workflow is suitable for reliable sequencing of secondary barcode regions.

Overall, these results support the feasibility of integrating secondary barcoding markers with ONT for improved fungal identification. This approach has the potential to enhance species-level resolution beyond ITS-based identification, particularly for closely related or cryptic species within *Aspergillus* and *Fusarium* genera. The generated sequencing data will be subjected to downstream bioinformatic analysis to further evaluate the effectiveness of secondary barcodes for accurate species-level identification.

6. References

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