**Introduction**

The rapid and accurate classification of microbial communities is a crucial tool in many different fields. In medicine, there is a need for metagenomic classification tools to detect novel viruses that could be transmitted via blood transfusion (Sauvage & Eloit 2016). Additionally, some new clinical diagnosis tools utilize metagenomics to help identify diseases (Govender et al. 2021). In public health, cases of COVID-19 have been identified using metagenomic classification (Carbo et al. 2020). Food safety has begun to rely on these tools too, utilizing them to ensure the quality of various products (Billington et al. 2022). Regardless of the context, it is clear that timely and precise metagenomic classifiers would be an advantage to its various applications.

Initially, most metagenomic identification was accomplished with marker-based techniques (Ye et al. 2019). This remains a quick and effective method, as its reference databases are made from a small subset of highly conserved genes known as DNA barcode regions (Badotti et al. 2017, Edgar 2018). For prokaryotes, the most commonly used gene is 16S, but other types of organisms like fungi or eukaryotes use ITS (Ye et al. 2019, Badotti et al. 2017). While marker-based techniques can be used for abundance estimations, their results can be biased if the organisms present possess differing concentrations of the barcode genes (Edgar 2018). This may make it more challenging to accurately assess the composition of a diverse community. Promising full-genome approaches have been developed that help eliminate this bias, making them the new go-to tool for metagenomic classification.

DNA-based and protein-based metagenomic classifiers have become far more prevalent over the past few years. These tools are similar in concept to NCBI’s renowned Basic Local Alignment Tool (BLAST), of which DNA-based approaches are most similar to BLASTn, while protein-based ones are to BLASTx (Ye et al. 2019). As the original BLAST tools are too sensitive to feasibly run on a mass scale, these metagenomic classifiers utilize their own algorithms that trade sensitivity for speed and performance (Ye et al. 2019). At first, DNA-based tools were designed around the classification of raw short-read shotgun sequences. But recent work has shown that raw long-read classification tends to be more accurate, despite longer run times and more intense resource use (Ye et al. 2019, Portik et al. 2022). The assembly of shorter reads into longer contigs before feeding them through a classifier also appears to have a positive impact on results (Tran & Phan 2020). Although protein-based tools have been shown to be more sensitive than DNA-based ones, they are exponentially more demanding, owing to the necessary translation of nucleotides into proteins (Ye et al. 2019). Such techniques are best used in specific circumstances where high precision is absolutely necessary, such as to distinguish the difference between variants of the same sequence.

The central objective of this project is to determine the most accurate methods of DNA-based species classification, so as to facilitate the creation of an optimized end-to-end metagenomic pipeline. My hypothesis is that raw long-read classification will prove the most accurate, as they are at the forefront of recent developments. Contig classification will probably be a close second, as this method has also been shown to perform well. Raw short-read classification is expected to have the worst performance. These classification methods will be tested with different classifier tools to establish which individual programs work the best. Performance metrics such as precision, recall, F1 score, and abundance will be analyzed to enable comparison. Higher performance metric scores shall indicate whether a certain method or tool is superior to another. My hypothesis will be evaluated based on a comparison of these performance metrics.

**Specific Aims**

The first specific aim of this proposal is to determine whether a raw long-read, contig, or raw short-read classification approach is most accurate. This will be accomplished with assembled contigs from a colleague and raw read data provided by CIAN Diagnostics. Contigs are expected to be in a FASTA format and raw reads in FASTQ. Data will be run through four selected classifiers, all of which accept either format and produce a CSV of identification results (Wood 2020, Kim 2021, Marcelino 2022, BugSeq 2023). Because the true composition of each dataset is known, accuracy can be gauged by comparing performance metrics like F1 score and abundance. Although most classifier review papers rely on raw reads, past research has shown that assemblers are an effective way to improve classifier performance (Tran & Phan 2020). I expect raw long-reads to be superior, if only marginally, as this is the direction in which most recent research has been trending. Raw short-reads, although anticipated to perform the worst, are included as a baseline against which to judge the other approaches. To keep things simple, only DNA-based classifiers have been considered for this study. It should be noted, however, that protein-based classifiers are a promising alternative (Ye et al. 2019). MMseqs2, a protein-based classifier, has proved to be quite accurate, though it does have comparatively demanding requirements (Mirdita et al. 2021). In the event that something goes wrong with the DNA-based approach or one wishes to explore further, this would be a valuable avenue to investigate.

The second specific aim is to determine which selected classifier tools perform the best: Kraken 2, CCMetagen, Centrifuge, or BugSeq. This is also to be accomplished via a comparison of the performance metrics, such as F1 score and abundance. Raw short-reads will be run through Kraken 2, CC Metagen, and Centrifuge, while raw long-reads go through BugSeq. Contigs can be tested with all four classifiers. Since Kraken 2 and Centrifuge are widely utilized as benchmarks, Kraken 2 for being ultra-fast and Centrifuge for being able to run on a desktop computer, they seemed like obvious choices to include (Wood & Salzberg 2014, Kim et al. 2016). CCMetagen is a more recent tool that aims to enhance the identification of eukaryotic and fungal species without significantly compromising the identification of prokaryotic ones (Marcelino et al. 2020). Although it claims to be superior to Kraken 2 and Centrifuge, I will determine whether that holds true within the context of this study (Marcelino et al. 2020). BugSeq is a new cloud-based tool for long-read classification that is remarkably simple to use and excels at species-level classification with highly-accurate data (Fan et al. 2021, Portik et al. 2022). I anticipate that BugSeq will perform the best, followed by CCMetagen, Kraken 2, and Centrifuge. Other classifiers are available for both short-read and long-read classification, such as CLARK, ProPhyle, and MEGAN-LR (Ounit et al. 2015, Brinda et al. 2017, Huson et al. 2018). In the event that the selected tools do not work as hoped, these may be viable substitutes.

The third specific aim is to determine how results are affected with or without a quality control step. FastQC is the quality control tool of choice. It takes a FASTQ input and produces a ZIP with the relevant analysis data (Andrews 2019). This is a relatively popular tool that I have some limited experience with, so it seemed like a good choice for this project (Sprang et al. 2021). While it stands to reason that quality-controlled data will perform better across the board, there is still value in assessing the discrepancy. Some tools may do particularly poorly with bad data, while others less so. Noting such details may assist researchers in choosing the programs that will work best with the data they have. I anticipate that the quality-controlled data will perform better by a large margin, on account of how crucial this step is always hailed. Another popular quality control tool is FastP (Sprang et al. 2021). In the event that something goes wrong with FastQC, it would be a good alternative.

As a whole, I anticipate that the long-read classification with BugSeq will have the best accuracy, followed by contig classification with CCMetagen. The F1 scores and abundance ratios should reflect this or the hypothesis will be rejected. There are numerous alternative programs that can be used in place of the ones I have selected. CLARK and ProPhyle are potential alternatives for Kraken 2 and Centrifuge respectively (Ye et al. 2019). CLARK was designed to compete with Kraken, and ProPhyle can run on a desktop similar to Centrifuge (Ounit et al. 2015, Brinda et al. 2017). MEGAN-LR could work in place of BugSeq, as it is also designed specifically for long-read classification (Huson et al. 2018). A protein-based approach could also be adopted too, ideally with MMseqs2 (Mirdita et al. 2021). For quality control alternatives, FastP is another popular tool that could be utilized (Sprang et al. 2021). I anticipate that the project will work as intended, but it is reassuring to have viable alternative tools at our disposal.

**Citations**

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