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From genomics to metagenomics

Narayan Desai[1], Dion Antonopoulos[2,3], Jack A Gilbert[2,4], Elizabeth M Glass[1] and Folker Meyer[1,2]

Next-generation sequencing has changed metagenomics. However, sequencing DNA is no longer the bottleneck, rather, the bottleneck is computational analysis and also interpretation. Computational cost is the obvious issue, as is tool limitations, considering most of the tools we routinely use have been built for clonal genomics or are being adapted to microbial communities. The current trend in metagenomics analysis is toward reducing computational costs through improved algorithms and through analysis strategies. Data sharing and interoperability between tools are critical, since computation for metagenomic datasets is very high.

Addresses

[1] Mathematics and Computer Science Division, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA
[2] Institute for Genomics and Systems Biology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA
[3] Biosciences Division, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA
[4] Department of Ecology and Evolution, University of Chicago, 5640 South Ellis Avenue, Chicago, IL 60637, USA

Corresponding author: Meyer, Folker (folker@anl.gov)

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Metagenomics: a brief history and current trends

Metagenomics has changed in the past 13 years from sequencing of cloned DNA fragments using Sanger technology [1–4] to direct sequencing of DNA without heterologous cloning [5,6]. Studies like the Global Ocean Survey (GOS) led to the creation of novel tools like fragment recruitment [2]. The vast majority of the tools, however, were developed in the context of 'clonal genomics' projects. Currently, technical aspects limit metagenomics. Consider, for example, the inability of current tools to reliably assemble longer contigs from multistrain, multispecies mixtures. Therefore, metagenomics cannot be viewed simply as an extension of genomics; instead, it requires a different paradigm of bioinformatics tool design. Just as human genome analysis is moving from one to many sequenced representative genomes, bioinformaticians dealing with microbial community sequence data need to consider metagenomes as complex mixtures of (pan)genomes [7].

The cost of DNA sequencing has plummeted [8] in recent years, making this technology accessible to more researchers. This has resulted in the generation of large numbers of massive datasets. The primary factor driving metagenomic research in next-generation sequencing technologies is financial. Short-read sequencing platforms provide an inexpensive means to deeply sample a microbial community. However, these sequencing technologies are less than ideal since they were driven by the human genome resequencing effort, which does not necessarily require long reads. Hence, metagenomics researchers have found themselves analyzing large, short-read datasets using tools built for long reads and, most important, for clonal datasets.

Data analysis is the key limiting factor in metagenomic studies. DNA sequence data production is no longer the bottleneck in microbial studies; instead, increased data volumes are posing significant challenges to the existing analysis tools and indeed to the community providing analysis systems. Even simple computing of sequence similarity results is becoming a limiting factor in metagenome analysis. This growth in dataset size, in conjunction with computational complexity of analysis, has left the metagenomics community in an unsustainable position, in terms of both financial cost and feasibility of analysis itself.

From genomics to metagenomics

Many metagenomics projects have been using a version of the standard genomics analysis workflow: sequence as deeply as possible, assemble reads into consensus 'contigs,' and annotate these contigs [9•,10,11••]; however, this is a costly strategy when using any of the current sequencing platforms. It is also important that each analysis strategy match the scientific goals of the study. For example, assembling into contigs, deleting singleton reads, and subsequently performing an analysis of the microbial communities will result in significant biases.

Challenges of metagenomic data

Metagenomic sequencing data from a complex community often represents only a minute fraction of the community. For example, to produce a dataset representing onefold coverage of a microbial community from a gram of soil would require more than 6000 HiSeq2000 runs at a cost of $267 million. And this is no guarantee, since unknown community composition and relative abundances limit our ability to calculate the coverage required to 'sequence to extinction' *robustly*.[1] Metagenomics currently lacks the tools to determine whether sufficient coverage is available for the type of analysis planned or whether one can interpret data of a certain depth for a community of a given complexity the way that is being planned. Therefore, the standard low coverage in metagenomic studies generates a dataset that reflects a random subsampling of the genomic content of the individual community members. If money was no object and complete coverage of all individuals was achieved, then metagenomics would require tools similar to those used for genomic analysis. Since this is currently infeasible, however, new tools must be developed.

The role of contigs and data fidelity

While consensus sequences are useful for *de novo* sequencing or resequencing in clonal genomics, they are of limited use for metagenomics. Using a consensus sequence built from many reads shields us from individual read errors but also obscures community structure throughout the taxonomic tree by collapsing strain-level, species-level, genus-level and family-level variation that could help with pangenome comparisons.

Clonal genomics uses consensus sequences to increase quality by integrating over multiple observations (reads); however, since metagenomic sequence data represents a random subsample of genetic information from many potential genomes, each single read potentially provides new data. This situation not only presents a challenge to sequencing providers; it also complicates making any statements about variation. For instance, what allows us to distinguish variation from a read error in a complex microbial community at low sequence coverage? We need ways of determining the quality of a sequencing run and the fidelity of any derived statements. Artifacts from the sequencing process need to be determined for the analysis of each dataset as a fundamental metric.

Researchers interested in microbial community composition and microbial community function look at contigs as a means to an end rather than an end product. We assume here that a contig from a metagenomic assembly is 'virtual' unless proven real by *in vitro* methods such as PCR amplification or restriction analysis. A contig that is potentially a hybrid of many strains and even species, while informative, has only limited information content once one is interested in the composition of the strains or species within that contig. However, using recruiting of short reads to a contig is a computationally inexpensive method for displaying species or strain differences.

In the days of Sanger-based clonal genomics, the read coverage of the target genome proved useful as a means of understanding the expected quality of the assembled consensus sequence. The number of gaps in the (un)finished genome sequence was used as another indicator of quality. Metagenomics lacks a corresponding instrument.

We believe that the scientific question motivating the sequencing needs to govern the depth (and type?) of sequencing applied, rather than the simple model 'more is better' and monetary considerations.

One of the fundamental tenets that bioinformaticians should hold true is that the tools they develop must be useful to and usable by the intended audience. In microbial metagenomics, one of the key groups is microbial ecologists. It is therefore essential that the dialog between these groups be open and capable of enabling continuous feedback; new tool developments quickly change the playing field and create new requirements. One good example is the migration from providing a catalog of functions and taxonomic composition to creating an extrapolation of these catalogs to additional metrics of community capability. Predictive elative Metabolic Turnover, PRMT [12••], converts metagenomic sequence data into relative metrics for the consumption or production of specific metabolites. Bioinformaticists should focus on exploring new ways to interpret metagenomic sequence data, with continuous reference to the needs of the community.

The information required by researchers can be generalized to who is out there and what are they doing.

Who is out there? or 'Binning' to taxonomic units

Binning tools were initially developed to allow placing of metagenomic contigs into taxonomic units [13•]. These tools typically require contigs of at least 1000 bp (the longer the better) to allow training the models used for classification [14]. It is not clear to us that current assembly algorithms produce contigs that are well suited to binning, since the algorithms optimize a mathematical function, constructing the shortest common superstring [15] without preserving species or strain identities in the process. Binning of long Sanger reads with the assumption that the data were from a clonal source made sense. Binning a contig created from short reads without considering all the implications of studying a complex mix of species and/or strains is fundamentally flawed and likely to reflect artifacts of the assembly process. In many ways it is accurate to say that genomicists pretend a metagenomes consists of a series of clonal contigs, rather than accepting the new challenge of analyzing a complex mixture of genomes.

[1] Even with perfect 16s amplicon based characterization of microbial communities, strain variants cannot be distinguished based on single-gene amplicons. One OTU as detected by a primer targeting a ribosomal gene is likely to consist of many different strains.

While this assumption might hold true for very simple systems such as acid mine drainage, initial unpublished data convince us that most currently used assembly algorithms [16••,17••,18,19] do not account for the presence of strain-level or species-level variation in a metagenomic mixture. One of the main assumptions deeply embedded into modern assembly tools is that in order to avoid being confused by repetitive elements, the assembly tools need to detect coverage and eliminate regions of higher coverage (read repeats) from the initial rounds of the assembly and add them back in later. However, doing so in a metagenome where defining the 'average abundance' is an impossible task, accepting only a small coverage band for assembly is introducing a serious bias into the data. It is arguable that assembly therefore should not be used to create contigs that can subsequently be binned to taxonomic units. We feel, however, that using contigs as artificial constructs for the purpose of binning reads is acceptable as long as it is understood that the contigs probably do not exist in nature.

What are they doing? or Functional assignment and finding genes

Using BLASTX [20] for defining functions of short DNA reads is tempting but computationally not tractable [21]. Alternative codes such as BLAT [22] or RAPSearch [23] are computationally friendlier but involve some loss of sensitivity. BLAT and RAPSearch also require an additional gene prediction stage such as FragGeneScan, MetaGeneMark, Metagene, or MetaGeneAnnotator [24••,25–27]. (BLASTX performs the prediction of potential coding genes implicitly but is limited to genes with representatives in the known protein databases.) Methods based on other classification techniques abound [28,29] but have not gained a lot of traction because the community has, so far, not sufficiently validated the results.

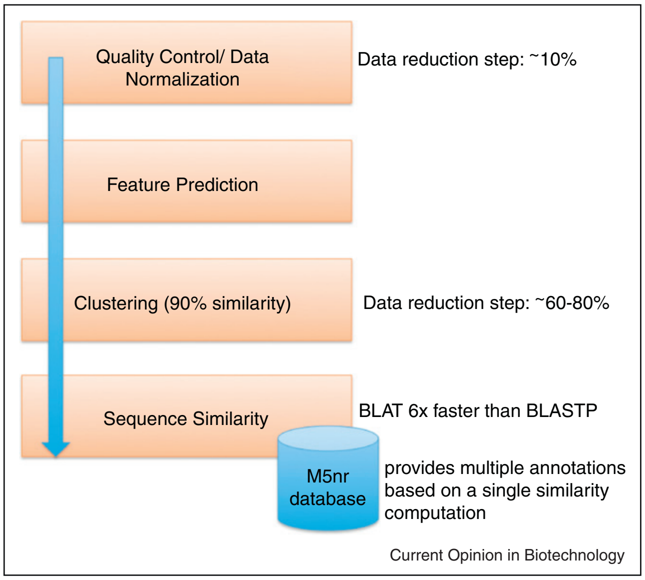
The computational cost of metagenome sequence analysis is so high that only a subset of the traditional tool set from clonal genomics is typically applied. While finding short, noncoding RNAs is clearly interesting to many and might lead to numerous new insights into the biology of the biome studied [30], the computational cost is high. The cost for running BLASTX analysis for large datasets on Amazon's EC2 cloud [21] is several times the cost of running the sequencing instrument, with sequencing cost dropping much faster than computing cost. Running analyses that are significantly more expensive than BLASTX, such as CRISPR [31•] or RFAM [32], is not current practice, with the trend going toward reducing the cost of computation.

Sharing data and analysis results

The cost-imbalance associated with bioinformatics analysis vs. data generation means that ideally the community should analyze a dataset only once and rely on a community archive to distribute the raw data and the analysis results. This culture of trusting third-party analysis results is currently not well established in metagenomics. Traditional data products (e.g. GenBank files) are not well suited to the requirements of metagenomics and fail, for example, to represent community properties. For metagenomics to meet the new challenges of the 'data bonanza' and to flourish, the sharing of data and analysis results needs to be established so the community of analysis providers (MG-RAST, IMG/M, or CAMERA [33•,34•,35]) do not spend their time and resources reanalyzing the same data. In the past this has been made difficult by the fact that the community sequence archives (SRA) [36] were not built to primarily support the current metagenomics use-cases. Important progress in this area has been seen with MG-RAST [33•], which has taken over the role of the de facto community archive and makes both raw data and computational results available for download and further analysis.

Sharing of data and results is clearly part of the solution as our ability to produce data grows faster than our computers [13•,37]. We can do this only if we create reliable data products and include more than mere sequence data. Derived data and provenance information needs to be exchangeable between groups and analysis providers in standard formats. In this context the Genome Standards Consortium [38••] has been a pioneer in standards development and in the context of their M5 project [39] started to work on exchangeable data formats. This will enable researchers to download data and analysis results.

Figure 1


Quality Control/ Data Normalization
Data reduction step: ~10%
Feature Prediction
Clustering (90% similarity)
Data reduction step: ~60-80%
Sequence Similarity
BLAT 6x faster than BLASTP
M5nr database
provides multiple annotations based on a single similarity computation
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The MG-RAST system has made performance improvements to deal with the onslaught of metagenomics sequence data. This figure outlines, at a high level, the data reduction steps as well as changes in tools being used to speed computation. Overall, MG-RAST has seen a 40–60× improvement in speed throughout.

In addition it will enable the development of a series of downstream analysis and visualization tools.

Future directions

Metagenomic analytics is a rapidly changing field, with major revisions to both tools and analysis pipelines occurring frequently. We expect the major trends of data growth and algorithmic complexity to hold for at least a few years. For the foreseeable future, analysis strategy will be guided by tradeoffs among scalability, sensitivity and performance. These decisions must be made carefully, with specific goals, in order to determine the proper approach. MG-RAST has attempted to balance performance improvements with sensitivity of analysis (Figure 1). Studies such as the acid mine drainage study were in the past possible only on very simple microbial communities. With easier access to sequencing, we believe that by improving the tools available to the community we will see many such studies in the coming years, continuing to improve our understanding of the microbial world surrounding us.

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