Blobology: exploring raw genome data for contaminants, symbionts, and parasites using taxon-annotated GC-coverage plots

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INTRODUCTION

The raw power of new sequencing methods has permitted the expansion of genome science into a wide range of new biological systems. In particular, the technologies permit genome sampling from wild organisms and communities of organisms. This approach was unthinkable in the era of Sanger-sequenced genomes, as the per-base cost precluded deep sampling of mixed starting materials in order to assemble the genome or transcriptome of a particular target organism. However most species of interest are not easily separable from their environments, either because they cannot yet be cultured, or because they are very intimately involved with a host or other commensal and parasitic organisms. In our research program, focused on the genome biology of the phylum Nematoda and related animals (Blaxter et al., 2012; Godel et al., 2012; Kumar et al., 2012; Wang et al., 2012), we are frequently faced with DNA samples and thus genome sequence datasets from wild isolates of target species where a significant proportion of the sequence data derives from the non-nematode components of the ecosystem. For example, tissue-dwelling nematodes often ingest the cells of their host animals or plants, and immune reactions can involve the adherence and crosslinking of host cells to parasite surfaces. Even free-living nematodes, feeding on bacteria or fungi, can come with attached or ingested food, as difficult-to-remove biofilms, or sequestered in the animals’ intestines. These mixed samples are akin to low-complexity metagenomes, where a metagenome sample all the replications present in an ecological sample. We have frequently observed DNA samples that are “contaminated” with the genomes of other species: components of food, commensal organisms, parasites and pathogens, or laboratory contaminants. It is particularly common to observe bacterial genomic contamination of eukaryotic samples.

Generating the raw data for a de novo genome assembly project for a target eukaryotic species is relatively easy. This democratization of access to large-scale data has allowed many research teams to plan to assemble the genomes of non-model organisms. These new genome targets are very different from the traditional, inbred, laboratory-reared model organisms. They are often small, and cannot be isolated free of their environment — whether ingested food, the surrounding host organism of parasites, or commensal and symbiotic organisms attached to or within the individuals sampled. Preparation of pure DNA originating from a single species can be technically impossible, but assembly of mixed-organism DNA can be difficult, as most genome assemblers perform poorly when faced with multiple genomes in different stoichiometries. This class of problem is common in metagenomic datasets that deliberately try to capture all the genomes present in an environment, but replicon assembly is not often the goal of such programs. Here we present an approach to extracting, from mixed DNA sequence data, subsets that correspond to single species’ genomes and thus improving genome assembly. We use both numerical (proportion of GC bases and read coverage) and biological (best-matching sequence in annotated databases) indicators to aid partitioning of draft assembly contigs, and the reads that contribute to those contigs, into distinct bins that can then be subjected to rigorous, optimized assembly, through the use of taxon-annotated GC-coverage plots (TAGC plots). We also present Blobplorer, a tool that aids exploration and selection of subsets from TAGC-annotated data. Partitioning the data in this way can rescue poorly assembled genomes, and reveal unexpected symbionts and commensals in eukaryotic genome projects. The TAGC plot pipeline script is available from https://github.com/blaxterlab/blobology, and the Blobplorer tool from https://github.com/mojones/Blobplorer.

Keywords: next-generation sequencing, metagenomics, assembly, parasites, symbionts, commensals, contaminants
of the non-target genetic material (and the genes and functions inferred from the sequence) to the reported target genome. There are several issues that preclude simple co-assembly of raw low complexity metagenome data. The first is that most assemblers, and particularly de Brujin assemblers, assume a particular modal read coverage of the genome to be assembled. If the contaminating genomes are at different molar concentrations then the internal logic of the assembler may optimize the output to an erroneous modal coverage. For example, a raw read dataset of a parasite of vertebrates might contain 45% parasite, 49% host, and 10% bacterial reads. If the parasite genome is 100 Mb, the vertebrate 3000 Mb and the bacterium 5 Mb, the genomes will be present at molar ratios of one parasite to 0.03 host to approximately five bacteria. Assemblers will find the bacterial replicons easier to assemble, at the expense of the desired parasite genome. Secondly, different genomes can have very different inherent “assembleability,” and in particular bacterial genomes (with high proportional content of protein-coding sequence, and low repeat content) are more easily assembled than are highly repetitive and gene-poor eukaryotes. Lastly, different genomes can have very different proportions of G and C bases, and mixing low GC genome data with balanced GC genome data may result in assemblies biased toward the mid-GC range.

We here present an effective solution to these problems. By performing a very preliminary assembly, with no attempt to optimize the output, and then classifying the resulting contigs by coverage (a proxy for relative molarity of the genomes in the mix), relative GC content (separating genomes with distinct biases), and best similarity match in public databases (separating data by age (a proxy for relative molarity of the genomes in the mix), content of protein-coding sequence, and low repeat content) metrics. The method is agnostic as to which assembler is used for this step. In this paper we focus on ABySS (Simpson et al., 2009), but we have also present use of MetaSPADE (Aronesty, 2011; Table 1) with a trimming threshold of quality of 20, discarding reads shorter than 50 b. A total of 136.3 M read pairs totaling 26.9 Gb remained after these trimming steps (Table 2). A full analysis of the genome of Drosophila immittis sequencing data have been described previously (Godel et al., 2012).

TOOLS USED IN THE TAGC PLOT PIPELINE

The TAGC plot pipeline uses a number of external tools (Table 1). Some of the external tools are easily substituted with the user’s preferred option. The core processing is carried out using a Perl script, gc_cov_annotate.pl and an R script makeblobplot.R (Table 1). The output includes a tab-separated value (TSV) format file with a single header row followed by one row per contig. The first three columns of each row give the sequence ID, length, and GC content. There follow an arbitrary number of columns, whose field headers begin with the string “cov_” giving the coverage for each library. After these come an arbitrary number of taxonomic annotation columns, whose field headers begin with the string “taxlevel_”.

BLOBSPLORER

Blobplorer takes as input the text file produced by gc_cov_annotate.pl. The tool can process and display text files from any source as long as they conform to the format defined above. Blobplorer is implemented as a single web page, with the processing and visualization code written in JavaScript. jQuery is used to update the plot in response to interface events and Raphael to draw the plot itself. Blobplorer uses the HTML5 file API, allowing it to be distributed as a static web page which does not require a server-side component: all processing is carried out by the browser, so the tool can be run simply by opening a local copy of the page.

RESULTS

OVERVIEW OF THE TAGC PLOT (OR BLOBPLOT) METHOD

The TAGC plot method is simple to perform (Figure 1). The user first collects and filters their raw genome sequencing data as for any standard assembly project. A preliminary assembly is then generated, without any attempt to optimize parameters. This assembly serves to reduce the complexity of the data from tens or hundreds of millions of short reads down to tens or hundreds of thousands of longer, contiguated sequences (contigs). The reduced complexity dataset is easier to screen, partly because of the smaller number of analytic steps needed, but also because the longer sequences are a better substrate for assessment of numerical (GC proportion, coverage) and biological (similarity to known sequences) metrics. The method is agnostic as to which assembler is used for this step. In this paper we present use of ABySS (Simpson et al., 2009), but we have also used Velvet (Zerbino and Birney, 2008) and CLCBio assembly-Cell (http://www.clcbio.com/products/clc-assembly-cell/) in the past. There is no need to extensively scaffold the assembly, and we have used mate-pair data given to the assembler as “single-end” for TAGC plot analyses in the D. immitis example.
<table>
<thead>
<tr>
<th>Tool or resource name</th>
<th>Version</th>
<th>Reference</th>
<th>Source website</th>
<th>Additional parameters used</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data QC/filtering</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preliminary assembly and read mapping</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Abyss                        | 1.3.6   | Simpson et al. (2009) | http://www.bcgsc.ca/platform/bioinfo/software/abyss | k-mer of 61
The user might care to change the k-mer value depending on the quality and length of their read data; it is not necessary to optimize this value. The program can also be run treating any paired (mate or paired-end) data as single-end. |                                                                         |
The settings used are designed to map reads uniquely and quickly |                                                                         |
| **Taxonomic annotation**     |         |                    |                                     |                                                                |                                                                         |
NCBI nt March 1, 2013
| **TAGC plot scripts**        |         |                    |                                     |                                                                |                                                                         |
| gc_cov_annotate.pl           | 1.0     | This work          | https://github.com/blastnlab/blockology | 0.01 taxlevel_order
0.01 is the threshold of displaying annotated contigs, and taxlevel_order sets the taxon level to display |                                                                         |
| makeblobplot R               | 1.0     | This work          | https://github.com/blastnlab/blockology |                                                                  |                                                                         |
Table 1 | Continued

<table>
<thead>
<tr>
<th>Tool or resource name</th>
<th>Version</th>
<th>Reference</th>
<th>Source website</th>
<th>Additional parameters used</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
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<tr>
<td>JQuery</td>
<td>1.8.2</td>
<td><a href="http://jquery.com/">http://jquery.com/</a></td>
<td><a href="http://code.jquery.com/jquery-1.8.2.js">http://code.jquery.com/jquery-1.8.2.js</a></td>
<td>Additional JQuery plugins used: jquery-ui, dropkick, tagsinput, placeholder, chardin.js</td>
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<td><strong>Assembly validation</strong></td>
<td></td>
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<tr>
<td>bristlecone proteome</td>
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<tr>
<td>Caenorhabditis sp. 5</td>
<td>NEMBASE4</td>
<td>Elsworth et al. (2011)</td>
<td><a href="http://www.wormbase.org/genebrowser/database/">http://www.wormbase.org/genebrowser/database/</a></td>
<td>See <a href="http://www.wormbase.org/genome/">http://www.wormbase.org/genome/</a></td>
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<tr>
<td>EST assembly</td>
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<td></td>
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</tr>
<tr>
<td>Caenorhabditis sp. 5</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA-Seq transcriptome</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>assembly</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CEGMA</td>
<td>2.4</td>
<td>Panu et al. (2007)</td>
<td><a href="http://korflab.ucdavis.edu/datasets/cegma/">http://korflab.ucdavis.edu/datasets/cegma/</a></td>
<td>Unpublished data from the Caenorhabditis sp. 5 genome project</td>
<td></td>
</tr>
</tbody>
</table>
The average GC content of each contig in the preliminary assembly is calculated. The raw reads are mapped back to this preliminary assembly and the resulting alignment BAM file used to calculate average read-depth coverage for each contig. We use Bowtie 2 (Langmead and Salzberg, 2012) here. It is also possible to use other read mappers that output BAM format, or to use read or k-mer coverage metrics reported by the assembler directly. The contigs from the preliminary assembly are compared to the NCBI non-redundant nucleotide (nt) database using the megablast option in the BLAST+ suite (Ye et al., 2006; Johnson et al., 2008) to identify a best species hit. It is also possible to construct custom local databases if the taxonomy of the “contaminants” is known, but use of the complete NCBI nt database is recommended as this also results in detection of unexpected contaminants. GC content, read-coverage, and taxonomic information are then combined to generate a standard format file, which is visualized as a TAGC plot. The TAGC plot is then reviewed, and strategies for removal of contaminants, extraction of required reads, and other binning operations defined. The TAGC plot data can also be viewed in Blobshelfer, a JavaScript tool that permits exploration and selection of contig sets interactively in a web browser.

**EXAMPLE OF TAGC PLOT USE IN FILTERING DATA FOR ASSEMBLING**

*Caenorhabditis* sp. 5

Here we demonstrate the use of the method to generate TAGC plots for the sequencing of the free-living nematode *Caenorhabditis* sp. 5 (see http://nematodes.org/genomes/caenorhabditis_sp5/). *Caenorhabditis* sp. 5 is an as-yet unnamed species, found in eastern Asia, a member of the briggsae subgroup of the genus *Caenorhabditis* (Kroonke et al., 2011). All the scripts used are available at https://github.com/blaxterlab/bloshelfer along with an accompanying bash script that can be run to replay the results below, or modified to run the pipeline on a different read set.

A preliminary assembly was performed on the adapter- and quality-trimmed reads using ABySS (Simpson et al., 2009) with default options and a k-mer of 61 with the 300 and 600 bp libraries provided as separate inputs. We used ABySS because it is an accurate result, but BLAST+ (Ye et al., 2006; Johnson et al., 2008). We generated a two-column table with the contig ID in the first column and the taxonomy ID of the species of origin of the best hit (lowest e-value) using the BLAST+ output formatting controls (see Table 1). Other tools such as MEGAN (Huson et al., 2007; Huson and Weber, 2013) or exonerate (Slater and Birney, 2005) might have provided more accurate results, but BLAST+ is convenient because it is very fast, natively parallel, and provides species taxonomy IDs in tabular form in one step. While we queried all 12,264 sequences in the preliminary *Caenorhabditis* sp. 5 assembly against NCBI nt, a randomly selected subset from preliminary assemblies with many hundreds of thousands of assembled sequences can speed up this part of the process with little reduction in final ability to screen for contaminants.

**TAXONOMIC ANNOTATION OF THE PRIMARY ASSEMBLY**

We identified the taxonomic attribution of the best-matching sequence in the NCBI nt database using BLAST+ megablast (Ye et al., 2006; Johnson et al., 2008). We generated a two-column table with the contig ID in the first column and the taxonomy ID of the species of origin of the best hit (lowest e-value) using the BLAST+ output formatting controls (see Table 1). Other tools such as MEGAN (Huson et al., 2007; Huson and Weber, 2013) or exonerate (Slater and Birney, 2005) might have provided more accurate results, but BLAST+ is convenient because it is very fast, natively parallel, and provides species taxonomy IDs in tabular form in one step. While we queried all 12,264 sequences in the preliminary *Caenorhabditis* sp. 5 assembly against NCBI nt, a randomly selected subset from preliminary assemblies with many hundreds of thousands of assembled sequences can speed up this part of the process with little reduction in final ability to screen for contaminants.

**MAKING AND INTERPRETING TAGC PLOTS**

A custom Perl script, gc_cov_annotate.pl, was used to collate the three input types: the assembly FASTA file, the alignment BAM files, and the tabular sequence-to-species mapping file, and produce a single data file that was visualized using the ggplot2 graphics library (Wickham, 2009) in R. The output (Figures 2 and 3) includes separate panels for each library read file and colors contigs plotted in the GC-coverage space by the most abundantly represented taxa matched. Unmatched contigs are shaded gray. In the case of the *Caenorhabditis* sp. 5 TAGC plots (Figure 2A), there were no major differences between the two independent libraries other than in average read depth, as expected. The TAGC plots...
show a major “blob” of contigs with high (~100-fold) coverage and 35–50% GC, with predominant taxonomic identification as Rhabditida (the order containing Caenorhabditis). The apparent skew in this blob, with contigs of lower mean GC having lower coverage, is typical of Illumina datasets, as there are biases due to library preparation and solid-phase PCR that result in under-representation of low GC sequences. Note also that there are some contigs, annotated as Rhabditida, with very high coverages (up to 2000-fold). These represent either repeats, or the mitochondrial genome. To the right, at higher GC, are a set of blobs with distinct coverage means, and distinct consistent taxonomic assignments (to orders of bacteria, including Pseudomonadales, Xanthomonadales, Actinomycetales, and Burkholderiales). These blobs derive from contaminating bacterial species, some at low levels (Pseudomonadales at ~10-fold, or one genome to every 10 Caenorhabditis sp. 5 genomes) and some at higher levels (such as
FIGURE 2 | TAGC plot of Caenorhabditis sp. 5 preliminary assembly. (A) A TAGC plot was constructed as described in the text from the ABySS assembly of the full Illumina dataset for Caenorhabditis sp. 5. The three panels are (left) 300 bp library, (middle) 600 bp library, and (right) both libraries combined, mapped to an assembly that used the combined data. Individual contigs are plotted based on their GC content (x-axis) and their read coverage (y-axis; logarithmic scale). Contigs are colored according to taxonomic order of their best-megablast match to the NCBI nt database (with E-value cutoff = 1e−50). Any taxonomic order annotation associated with 1% or more of annotated contigs is marked with a color; contigs without an annotation from these are in gray. (B) The TAGC plot from an ABySS assembly of the Caenorhabditis sp. 5 data after removal of the bacterial contaminants. Annotation as in part (A).
FIGURE 3 | TAGC plot of Dirofilaria immitis and its Wolbachia endosymbiont. The four panels display TAGC plots for (upper left) a paired end library from the “Pavia” male nematode, (upper right) a mate pair library from the “Pavia” male nematode, (lower left) a paired end library from the “Athens” female nematode and (lower right) all data combined. The specified read sets were aligned to an assembly generated from the paired end data. The TAGC plots were taxonomically annotated, and contigs with best similarity to Spirurida (the order to which D. immitis belong) and Rickettsiales (the alphaproteobacterial order to which Wolbachia belong) were highlighted in color. Other conventions as in Figure 2.

from the “color by” drop-down menu. Clicking the “Download as SVG” button will generate a copy of the plot in scalar vector graphics (SVG) format, which can be opened in a scalar vector drawing package for further processing (for example, to create publication-ready graphics). Once the data have been loaded and displayed, groups of contigs can be defined by drawing ellipses on the plot. To draw an ellipse, the user clicks once on the plot to define the center, and then moves the cursor to define the shape of the ellipse. They then click a second time, and move the cursor to define the rotation of the ellipse. Clicking for the third and final time on the plot completes the definition of the ellipse. Multiple ellipses can be drawn in this way to define a set of contigs. Clicking the “highlight selected” button will confirm the selection visually by shading the selected contigs in red, while clicking the “download contig ids” button will generate a text file containing the identifiers of the selected contigs which can be downloaded for further
The second preliminary (i.e., non-optimized) assembly of the *Caenorhabditis* sp. 5 genome derived from the cleaned read data contained 10,120 contigs, with an N50 of 31.4 kb (Table 3). It scored equivalently to the first assembly in biological measures of completeness (including mapping to *Caenorhabditis* sp. 5 expressed sequence tags (Elsworth et al., 2011), representation of matches to the proteome of the closely related *Caenorhabditis briggsae* (Stein et al., 2003; Yook et al., 2012), and screening with the Core Eukaryotic Genes Mapping Approach, CEGMA; Parra et al., 2007). Each of these metrics of biological completeness were essentially unaffected by the removal of 25 Mb of contaminating bacterial sequence. The reduction in N50 is partly a product of the removal of the more-easily assembled bacterial data (which had an N50 of ~45 kb). We would expect the N50 to be improved on reassembly under optimal parameters.

Multi-genome coassemblies can contain errors. One risk with the TAGC plot method is that sequences erroneously constructed or scaffolded may contain DNA from more than one genome. Removal of all of a contig because one part matches an identified undesired contaminant risks discarding good data. We recommend a conservative approach, for example only discarding contigs that are tagged as having their best megablast match to a contaminant if there is no match better than a relatively permissive cutoff to the target taxon. Similarly it is sometimes difficult to tell where the blobs from the contaminants end and that from the target starts. The *Caenorhabditis* sp. 5 example had relatively clear separation between bacterial and nematode blobs, but this should not be expected in every case. Again, a conservative approach is warranted, retaining the maximal amount of target data.

**IDENTIFYING SYMBIONTS AND LATERAL GENE TRANSFERS WITH TAGC PLOTS**

As indicated above, TAGC plots are also useful for separating several desired target genomes from a mixed dataset. In the case of bacterial symbionts of eukaryotes, this then permits independent, optimized assembly of host and symbiont. We illustrate this

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### Table 3 | Assembly statistics for *Caenorhabditis* sp. 5.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Preliminary assembly</th>
<th>Contigs removed from preliminary assembly</th>
<th>Assembly of data after removal of reads mapping to contaminant contigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Span (bp)</td>
<td>160,970,414</td>
<td>25,566,044</td>
<td>135,501,369</td>
</tr>
<tr>
<td>Number of contigs*</td>
<td>12,264</td>
<td>2,148</td>
<td>10,120</td>
</tr>
<tr>
<td>NGS of contigs (bp)</td>
<td>32,806</td>
<td>44,991</td>
<td>31,396</td>
</tr>
<tr>
<td>CEGMA completeness</td>
<td>97.58%</td>
<td>–</td>
<td>96.37%</td>
</tr>
<tr>
<td>Representation of <em>Caenorhabditis</em> sp. 5 EST transcriptome**</td>
<td>98.1%</td>
<td>–</td>
<td>98.1%</td>
</tr>
<tr>
<td>Representation of <em>Caenorhabditis</em> sp. 5 RNA-Seq transcriptome***</td>
<td>97.41%</td>
<td>–</td>
<td>97.42%</td>
</tr>
<tr>
<td>Matches to <em>Caenorhabditis</em> <em>briggsae</em> proteome****</td>
<td>79.04%</td>
<td>–</td>
<td>79.04%</td>
</tr>
</tbody>
</table>

*Os scaffolds, as the contigs may contain “N” base calls.
**The *Caenorhabditis* sp. 5 expressed sequence tag dataset includes 2,200 unigene sequences.
***The *Caenorhabditis* sp. 5 RNA-Seq transcriptome assembly contains 33,786 unigene sequences.
****Caenorhabditis briggsae is the closest fully sequenced *Caenorhabditis* species to *Caenorhabditis* sp. 5. Its proteome contains 21,981 entries.
We have presented an approach to interpreting and cleaning raw datasets. In a metagenome study, the "target" is usually all the data. Can avoid compromising costly downstream analyses with rogue infection, early removal of contaminant genes from a target assembly can approach each genome and find local optima. In addition for each constituent genome, split-data assembly projects. Rather than achieve a global optimum that in fact is not needed different assembly parameter sets into independent assembly contamination of target genomes with other DNA. They assist in TAGC plots are very useful in pre-screening pilot datasets before in the context of targeted sequencing of "contaminated" samples. What distinguishes the TAGC plot approach is the abundance k-mers from de Bruijn graphs to simplify resolution. Commonly used in genome assembly to remove low- and high-volume sequence datasets to improve both assembly met-rics and biological interpretation. The ideas behind this approach commonly used in genome assembly to remove low- and high-volume sequence datasets to improve both assembly metrics and biological interpretation. The ideas behind this approach here with data from the sequencing of the genome of the dog heartworm, D. immitis (genome size ∼95 Mb), which carries an apparently obligate symbiont, the rickettsial alphaproteobacteria Wolbachia peptidiphilus (W) (genome size ∼1 Mb; Godel et al., 2012). Fragments of the W genome are present in the nematode nuclear genome, horizontally transferred from this germline-transmitted symbiont. In this case therefore, simple separation by taxonomic annotation of the contigs may risk confounding true W contigs with nuclear insertions. For D. immitis, we generated datasets from two different nematodes, including male (“Pavia”, where Wolbachia abundance is low) and female (“Athens”, where abundance is higher). In the TAGC plots of the different libraries (Figure 3) distinct blobs annotated as Rickettsiales in origin were found at different relative coverage in each library. In the "Athens" library the Rickettisia W blob is clearly separable from the nuclear D. immitis blob, as it has approximately 18-fold greater coverage. Also evident in the "Athens" data is a low coverage blob of higher GC content. This blob is derived from the canine host of D. immitis. A simple coverage cutoff along with a selection for megablast matches to Rickettisia resulted in a high-quality W reads set that generated a much better assembly (reducing the number of contigs from 63 to only two, one of 920 kb and one of 1 kb; Comandatore et al., 2013). Similarly, removal of the dog contamination, and filtering the W reads generated a better D. immitis assembly. This procedure also usefully left the W nuclear insertion-derived read data in the nucleosome read set, permitting investigation of laterally transferred fragments (Godel et al., 2012).

DISCUSSION
We have presented an approach to interpreting and cleaning raw high-volume sequence datasets to improve both assembly met-rics and biological interpretation. The ideas behind this approach are not new. Difference in GC proportion is used by several raw data quality-control tools, such as fastqc (Andrews, 2010), to identify potential problems in raw read data. Coverage filters are commonly used in genome assembly to remove low- and high-abundance k-mers from de Bruijn graphs to simplify resolution. Taxonomic annotation is commonly used post assembly to identify contaminants. What distinguishes the TAGC plot approach is the combining of these measures in screening preliminary assemblies in the context of targeted sequencing of "contaminated" samples. TAGC plots are very useful in pre-screening pilot datasets before proceeding to bulk sequencing, as they can identify unexpected contamination of target genomes with other DNA. They assist in generating better assemblies by separating different genomes that need different assembly parameter sets into independent assembly projects. Rather than achieve a global optimum that in fact is not at all optimal for each constituent genome, split-data assembly can approach each genome and find local optima. In addi-tion, early removal of contaminant genes from a target assembly can avoid compromising costly downstream analyses with rogue data.

The problem of multi-genome datasets is at the core of the huge effort that has gone into development of assemblies capable of delivering biologically meaningful results from metagenomic datasets. In a metagenome study, the "target" is usually all the genomes in the environment studied, and an important analytical goal is the identification of which genes in the environment are present on the same replicons, and thus likely to be active within a single membrane-bound organism. To approach the binning of metagenome data, several groups have used approaches similar to TAGC plots, integrating coverage, GC, and taxonomic affin-ity to propose potential linkages between contigs. Importantly, some authors have in addition used higher-dimensional vectors of base composition patterns than simple nt counts. A major locus of activity has been in the use of multidimensional dinucleotide, trinucleotide and, most commonly, tetranucleotide composition vectors (4NCV; Teeling et al., 2004; Slater and Birney, 2005; Chat-terji et al., 2007; Emmersen et al., 2007; Dick et al., 2009; Willner et al., 2009; Ghosh et al., 2011; Lamprea-Burgunder et al., 2011; Brison et al., 2012; Saced et al., 2012; Strous et al., 2012). Hexa-nucleotide counting has also been used to separate simple mixtures of a few species (Hraber and Weller, 2001). Where whole-genome sequence training data are available, 4NCV are extremely pow-erful in binning new data into “known” groups. Applied de novo to metagenomic data, 4NCV can be used to inform hypotheses of association between sequences. The limitation in the 4NCV approach is that the vectors are most informative when derived from long sequences (tens of kilobases) and become less discrimi-natory when derived from short contigs or reads. The best available 4NCV tool, MetaWatt (Strous et al., 2012), uses machine learning to cluster contigs into bins of coherent coverage, GC proportion, 4NCV, and taxonomic annotation. It has a highly featured graph-ical user interface that aids exploration and selection of binned data. In our hands, the tool is effective but hard to use with larger eukaryotic datasets, as it over-splits the datasets, and is partic-ularly slow to respond when a large number of bins and their contigs are selected. It is clear that addition of 4NCV (or simi-lar high-dimensional nt pattern information) to the TAGC plot approach could be very valuable, particularly if efficient methods of unsupervised binning could be developed. Other tools designed to split raw or assembled data into bins that putatively derive from distinct species have been proposed that might serve as useful post TAGC-plot approaches. Support vector machines informed by corpora of training data can be used to separate mixed-origin assemblies based prior expectations of species content (Rudd and Teko, 2005; Emmersen et al., 2007). Another development might be to use a read or k-mer normalization method such as kmmer (Brown et al., 2012) to first equalize the effective molar-ity of the genomes, and then simply use taxonomic matching (and/or 4NCV) to separate the contigs into putative single-genome bins.

The TAGC plot method has been used in several recent genome assembly efforts, largely thus far in nematodes (because of our lab-oratory’s interests and contacts). We and colleagues have used it in assembly of several species’ genomes, and in isolation of their Wolbachia symbionts (Kumar and Blaxter, 2011; Godel et al., 2012; Kumar, 2012; Comandatore et al., 2013; see also http://nematod.es for open access genomes from additional species). Schwarz et al. (2013) used TAGC plots to clean up their Haemonchus contor-tus read sets before assembly. We have also used TAGC plots to examine transcriptome assemblies, though obviously the coverage dimension in these data reflects gene expression levels rather than...
genome coverage, and have found them useful, particularly when screening infected hosts sequenced to reveal both host and parasite/pathogen transcription (Heitlinger et al., 2013). Edinburgh Genomics3 use TAGC plots as a standard part of their data quality-control pipeline, particularly for ecologically or environmentally focused genomics projects where the species of interest is new to genome analysis.

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AUTHOR CONTRIBUTIONS
The TAGC plot software was devised by Mark Blaxter and Sujai Kumar and written by Sujai Kumar in consultation with Mark Blaxter, Martin Jones, Georgios Koutsovoulos, and Michael Clarke. Biobsploader was written by Martin Jones. The software was tested and improved by Sujai Kumar, Georgios Koutsovoulos, Michael Clarke, and Mark Blaxter. All authors contributed to the writing of the manuscript.

REFERENCES


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