BLAST-based validation of metagenomic sequence assignments

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Abstract

When performing bioforensic casework, it is important to be able to reliably detect the presence of a particular organism in a metagenomic sample, even if the organism is only present in a trace amount. For this task, it is common to use a sequence classification program that determines the taxonomic affiliation of individual sequence reads by comparing them to reference database sequences. As metagenomic data sets often consist of millions or billions of reads that need to be compared to reference databases containing millions of sequences, such sequence classification programs typically use search heuristics and databases with reduced sequence diversity to speed up the analysis, which can lead to incorrect assignments. Thus, in a bioforensic setting where correct assignments are paramount, assignments of interest made by "first-pass" classifiers should be confirmed using the most precise methods and comprehensive databases available. In this study we present a BLAST-based method for validating the assignments made by faster sequence classification programs, with optimal parameters for filtering of BLAST results determined via simulation of sequence reads from genomes of interest, and we apply the method to the detection of four pathogenic organisms. The software implementing the method is open source and freely available.

Keywords: BLAST; metagenomics; sequence classification; taxonomic assignment; bioforensics; validation

Introduction

In metagenomic analysis, comparing the genomic sequence content of a sample to a reference database is fundamental to understanding which organisms present in the sample were sequenced. There exist many bioinformatics software programs that perform this classification task; some programs only estimate overall taxonomic composition and abundance in the sample [16, 23], while other programs assign a taxonomic label to each metagenomic sequence [3, 11, 14, 15, 22, 26]. In a bioforensic setting, one is often concerned with reliably detecting the presence of a particular organism in a metagenomic sample, which may only be present in a trace amount. For this task, one typically uses the latter class of programs just mentioned, which determine the taxonomic affiliation

of each sequence using a reference database [6, 17, 18] and a taxonomy [4]. A canonical metagenomic sequence classification workflow is shown in Figure 1.

When classifying sequences, there is a general trade-off between sensitivity (the proportion of the total number of sequences assigned correctly) and precision (the proportion of assigned sequences assigned correctly), as well as between classification performance (combined sensitivity and precision) and computational resource requirements. Modern metagenomic sequence classification programs often use relatively fast heuristics and databases with limited sequence diversity to increase analysis speed, as metagenomic data sets often consist of millions or billions of sequences that need to be compared to millions of database sequences. Thus, while they are useful in performing a "first-pass" analysis, in a bioforensic setting it is important to validate the assignments of interest made by such programs using the most precise methods available. One could choose to validate only the assignments made to the taxonomic clade of interest (e.g., *Bacillus anthracis*), but depending on the compute capacity one has access to, one might choose to validate all assignments subsumed by a higher ranking taxon (e.g., the *B. cereus* group or the *Bacillus* genus), which would enable the detection of possible false negative assignments as well as false positive assignments made by the first-pass classifier.

In this study, we present a method that uses BLAST [8] (the "gold standard" for sequence comparison), the NCBI non-redundant nucleotide database [9] (`nt`), and the NCBI taxonomy [9] to validate the assignments made by faster sequence classification programs. For simplicity, we refer to the taxonomic clade of interest in each example as the "target taxon", and we assume all metagenomic sequences are paired-end reads generated by the Illumina HiSeq 2500 sequencer (no assembled sequences). The basic validation procedure involves comparing each read against the `nt` database using BLASTN, and then filtering and interpreting the BLAST results based on data collected from metagenomic simulations aimed at optimising detection of the target taxon. The validation workflow is shown in Figure 2.

We developed two basic ways of filtering BLAST results. The first is an "absolute" filter, which specifies that BLAST hits not meeting a particular criterion are removed. Various possible criteria include minimum alignment length, minimum alignment percent identity, or maximum E-value. Of these three filters, this study only uses the E-value filter, as E-value is fundamentally a composite of alignment length and alignment similarity. (Our software supports the use of all of these filters, however, either individually or in combination.) If the best BLAST hit matches the target taxon after application of the absolute filters, it is then possible to apply a "relative" filter by computing the difference in E-value or bit score between the best hit and the best hit to a *non-target* taxon (should the latter exist). As very small E-values are typically rounded to zero, our software uses relative bit scores in this context for maximum applicability; we call this quantity the "bit score difference", abbreviated b. If b is greater than or equal to an experimentally determined threshold, then we have validated the assignment of the read to the target taxon. Examples of the application of the bit score difference filter are given in Figures 3 and 4.

When deciding which filters to use and how they should be parameterised, an optimality criterion is needed. In the execution of bioforensic casework, it is important that any assignments made are correct, so ideally one would like to eliminate any incorrect assignments of reads to the target taxon while correctly assigning as many reads as possible. Due to inherent variation in the degree of interrelatedness among genomes, optimal filter parameter values need to be set differently for each target taxon. Here we present examples aimed at detecting a variety of pathogenic target taxa including *Bacillus anthracis*, *Clostridium botulinum*, pathogenic *Escherichia coli*, and *Yersinia pestis*. In order to determine optimal filter settings we must know the true origin of our test sequences; thus, we simulate reads from each target taxon genome, BLAST

them against `nt`, and evaluate classification performance under different combinations of filter parameter settings. The parameter optimization workflow is shown in Figure 5. In the experiments that follow, a true positive (TP) is a simulated read from the target taxon assigned correctly (either assigned to the target taxon directly or to a more specific taxon beneath the target); a false positive (FP) is a simulated read from a non-target taxon that is incorrectly assigned to the target taxon; and a false negative (FN) is a simulated read from the target taxon assigned incorrectly (i.e., assigned to a taxon that is not part of the target taxon lineage).

The count of true positives and false negatives can be easily determined by parsing the BLAST output associated with the target taxon. Determining the count of false positives is more difficult; naively, it would require simulating reads and evaluating BLAST results for every non-target taxon in the database, but we currently regard this as computationally prohibitive. Instead, we offer two alternatives. The first, which we call "near neighbour", uses the genome in the database that is most globally similar to the target taxon as a proxy for all non-target database taxa. The intuition behind this approach is that a misclassified read (presumably due to sequencing error) is most likely to originate from a database genome that is very similar to the target taxon. Thus, with the near neighbour approach, we choose filters and parameter values that maximize sensitivity (TP/read count) while maintaining perfect precision ($TP/(TP + FP)$). The potential weakness of this approach is that there could be a region of local similarity to the target taxon in a database genome that is not the near neighbour. Thus, we offer a second approach that does not rely on selecting other genomes from the database, which we call the "false negatives" approach. This approach relies on the observation that if the sequencing error process is symmetric — i.e., the probability of an erroneous A to C substitution is the same as that of C to A, insertions are as probable as deletions, and so on — then the process that gives rise to false negatives can be treated as equivalent to the process that gives rise to false positives. While it is known that in practice this assumption of symmetry is violated [24], it may nonetheless suffice to use FN as a proxy for FP in this context. Thus, with the false negatives approach, one only needs to simulate reads from the target taxon, and maximize sensitivity while maintaining perfect precision, which is redefined as ($TP/(TP + FN)$). Deciding which of the two heuristics is more effective would unfortunately require comparison to a provably optimal procedure; thus, we present simulation results from both the near neighbour and false negatives approaches and describe the patterns we observe.

Related work

Platypus Conquistador

An existing software tool, "Platypus Conquistador" [10], also uses BLAST to validate the classification of particular sequences of interest. Platypus requires the user to split their reference sequences into two databases: a database containing only sequences of possible interest, and a database composed of potential "background" sequences. BLAST queries are performed against both databases, and hits may be filtered by various combinations of percent identity or alignment length values, which need to be provided by the user. After filtering, query sequences with hits to the "interest" database are checked to see if they also have hits to the "background" database; if so, the bit scores of the respective best hits are compared and are roughly categorised as "equal", "interest > background", etc. While this could be a helpful diagnostic tool, there is no guidance provided to the user as to what parameter values to use or what difference in bit scores between interest and background should be regarded as significant. Furthermore, this tool no longer appears to be actively developed.

Genomic purity assessment

Whereas in this study we are concerned with the precision with which individual reads are classified so as to be confident in the detection of a target taxon in a metagenomic sample, a recently published study [19] addresses a different, but related problem, that of detecting contaminant organisms in ostensibly axenic (non-metagenomic) samples. Specifically, Olson et al. develop methods to determine the proportion of a contaminant required to be present in an otherwise pure material such that the contaminant can be detected with standard metagenomic sequence classification tools. As with our study, they simulate reads with ART [13] software (in their case from both "material" and potential contaminant genomes) to set up conditions under which sequence classification performance can be assessed. PathoScope [12] is used instead of BLAST for read classification. In general, they find that their method is able to identify contaminants present in a proportion of at least 10^{-3} for most contaminant-material combinations tested.

Outlier detection in BLAST hits

Shah et al. [25] have developed a method that detects outliers among BLAST hits in order to separate the hits most closely related to the query from hits that are phylogenetically more distant using a modified form of Bayesian Integral Log Odds (BILD) scores [2] and a multiple alignment scoring function. In this way, they separate sequences with confident taxonomic assignments from sequences that should be analyzed further. The method was developed for and tested on 16S rRNA data, and thus is currently not applicable to whole genome sequencing (WGS) data sets. As a general-purpose filter, however, it can be used with any organism containing 16S rRNA data, whereas our methods are optimized for detection of specific taxa. It is also interesting to note that in the Shah et al. study, BLAST is used as a first-pass classifier and subsequent analysis is performed with TIPP [20], whereas in the paradigm we present here, a much faster classifier than BLAST is used for a first-pass (e.g., Kraken [26]), and then BLAST is used for validation.

Results and discussion

To demonstrate the BLAST-based validation procedure, we selected four target taxa, all of which are biological agents that could conceivably be of interest in a bioforensic setting. The first is *Bacillus anthracis*, the bacterium that causes anthrax. The second is *Clostridium botulinum*, a bacterium capable of producing the lethal botulinum neurotoxin. The third is a pathogenic strain of *Escherichia coli*, *E. coli* O157:H7 str. Sakai, a bacterium that has been associated with major outbreaks of foodborne illness. The fourth and final target taxon is *Yersinia pestis*, the bacterium that causes bubonic plague. Thus, three out of the four target taxa represent particular species to be identified (*B. anthracis*, *C. botulinum*, and *Y. pestis*), whereas one target taxon represents a particular strain to be identified (*E. coli* O157:H7 str. Sakai). Additional information about the target taxa and selected near neighbours is provided in Table 1.

For each target taxon and near neighbour, two experiments were performed. The first experiment simulated 10× coverage of 250-bp reads from the target and near neighbour genomes, and tested 81 different combinations of filter parameter values — i.e., all combinations of maximum E-value (E) = $\{10^0, 10^{-1}, 10^{-2}, 10^{-4}, 10^{-8}, 10^{-16}, 10^{-32}, 10^{-64}, 10^{-128}\}$ and bit score difference (b) = $\{0, 1, 2, 4, 8, 16, 32, 64, 128\}$. Optimal filter parameters were chosen to maximize sensitivity (detection of the target taxon) while maintaining perfect precision (zero incorrect read assignments); the results are shown in Table 2.

We observe that when requiring perfect precision, sensitivity was highest for identification of *C. botulinum* (≈99%), followed by much lower sensitivity for *B. anthracis* and

Y. pestis. These results are understandable, as it is well established that the species that comprise *B. cereus sensu lato* have very similar genomic content [5], and that *Y. pestis* and *Y. pseudotuberculosis* are also very closely related [1]. Sensitivity was lowest for identification of *E. coli* O157:H7 str. Sakai (≈0.4% for near neighbour and ≈0.08% for false negatives). Again, this result is congruent with the expectation that strain-level identification would be substantially more challenging than species-level identification. Regarding the two methods of computing FP, we observe that the "false negatives" approach uniformly recommends stricter filter parameter values than the "near neighbour" approach. This pattern is consistent with the idea that the false negatives approach attempts to prevent spurious assignments to the target taxon genome by using the genomic content of all other taxa in the reference database, whereas the near neighbour approach uses only a single taxon that is closely related to the target as a proxy for all other database taxa, thus being more permissive. Because the reads in this experiment were simulated from genomes that were present in the reference database, almost all read alignments had equally good scores, so the absolute E-value filter had little or no effect until it was set so stringently that it eliminated all TP ($E = 10^{-128}$). Thus, here we report the most stringent parameter values that yielded exactly the same optimal values of sensitivity and precision. In the case of *B. anthracis*, we observe that sensitivity increased from ≈0.8% to ≈8.9% when allowing exactly one FN assignment (precision ≈99.9995%; Table 2). This suggests that if one is willing to relax the perfect precision requirement very slightly, it may be possible to make significant gains in sensitivity. Finally, it is interesting to note that in multiple cases, $b = 8$ maximized sensitivity while achieving perfect precision. This likely represented a "sweet spot" (at least as compared to $b = 4$ or $b = 16$) for the level of taxonomic specificity represented by the selected target taxa.

The second experiment was identical to the first, except that a simulated read length of 150 bp was used, thus making the classification task more challenging. The results are shown in Table 3. Under optimal filter parameter values, we observe that sensitivity in detecting each target taxon decreased relative to the 250-bp experiment (e.g., in the case of *C. botulinum*, sensitivity decreased from ≈99% to ≈97%). Also, optimal values for the E-value and bit score difference filters varied somewhat relative to the 250-bp experiment, although it was always the case that $E \leq 10^{-64}$ and $b \leq 8$.

In a final experiment, clade-level exclusion [7] of either species (*B. anthracis*) or strains (*C. botulinum* A str. ATCC 3502 and *Y. pestis* CO92) was performed to assess classification performance in the hypothetical scenario where the taxon one has sequence data for is not represented in the reference database. In these tests, we simulated 250-bp reads from the *excluded* taxon, omitted the excluded taxon from the reference database during BLAST searches, and then obtained optimal filter parameter values for classification of the target taxon, which in this case was the taxon immediately above the excluded taxon in taxonomic rank. Only the false negatives method of computing FP was used; the results are shown in Table 4. We observe that in this experiment, it was not always possible to achieve perfect precision — maximum precision for identification of the *B. cereus* group when excluding *B. anthracis* was ≈93.9%, and maximum precision for identification of *C. botulinum* when excluding *C. botulinum* A str. ATCC 3502 was ≈99.9%. We note that sensitivity for identification of *C. botulinum* decreased from ≈98.5% in the first experiment (Table 2) to ≈90.5% in the clade-level exclusion experiment (Table 4). By contrast, sensitivity for identification of *Y. pestis* hardly decreased at all (≈10.6% vs. ≈10.5%).

Conclusion

We have presented a BLAST-based method to validate specific assignments of interest made by faster classification programs, which involves using simulations to determine optimal parameter values to use for filtering BLAST results. We demonstrated the parameter optimization process for four different pathogenic organisms, and showed that optimal parameter values and resulting values of sensitivity and precision varied significantly depending on the selected taxon, taxonomic rank, read length, and if the taxon sequenced was represented in the reference database. Once optimal BLAST filter parameter values for a particular taxon have been determined by a procedure similar to the one presented here, they can be subsequently used to perform validation of sequence assignments to that taxon, provided that the attributes of the sequences being classified and the attributes of the reference database are sufficiently similar to those used in the optimization process. Potential users of the software will find scripts for parsing BLAST results, performing parameter sweeps, and assigning final taxon labels to sequences at https://github.com/bioforensics/blast-validate.

Methods

Read simulation

For read simulation we used ART [13] (version 2016-06-05). For 150-bp reads, we used the built-in HS25 quality profile with an insert size of 200 ± 10 bp (mean $\pm$ standard deviation). For 250-bp reads, we used a custom Illumina HiSeq quality profile that we generated from recent runs of our HiSeq 2500, and we also specified an insert size of 868 ± 408 bp determined from recent library preparations. We provided this information so that the simulated reads would have characteristics that closely matched what we would expect to obtain from a real HiSeq run in our laboratory, thus ensuring that the simulation results would be maximally useful to us. We recommend that others who may emulate our procedures customise the attributes of their simulated reads to correspond to the real data they anticipate analysing.

Near neighbour determination

We used the "Genome neighbor report" feature of the NCBI Genome database [9] to determine, for each target taxon, the most closely related complete genome of a different species or strain, as appropriate (Table 1).

Similarity searches

We used BLASTN from the BLAST+ package [8] (version 2.2.25+) together with the NCBI non-redundant nucleotide database [9] (downloaded February 2017) for all classification experiments. Default parameters were used, except when excluding taxa from the reference database, in which case the `-negative_gilist` option was added. The BLAST computation was distributed over many cluster nodes to complete the analyses in a timely manner.

BLAST result parsing

BLAST result parsing and final taxonomic assignment of each read was performed with a custom Perl script that is capable of querying the NCBI taxonomy database [9]. If a target taxon was supplied, assignments to the target taxon lineage that were more specific than the target taxon were simply reassigned to the target taxon. BLAST hits that did not

meet the criteria specified by the absolute filters (minimum alignment length, minimum alignment percent identity, or maximum E-value) were removed, as were hits to the "other sequences" clade (NCBI taxon ID 28384), which were presumed to be erroneous. To finally classify each read, the lowest common ancestor (LCA) algorithm [14] was applied to the remaining hits that had a difference in bit score from the best hit less than a specified amount. If multiple parameter values were supplied for one or more filters, the script parsed the BLAST results once for each possible combination of parameter values and wrote the results to separate files, thus enabling the user to efficiently perform parameter sweeps. The final output from the script was one or more files, each containing the final LCA classification of each read for a particular combination of filter parameter values.

Evaluating results

Counts of true positives, false positives, and false negatives (from which sensitivity and precision were derived) were obtained using a custom Perl script that parsed the LCA files produced by the BLAST result parser.

Software and data availability

All genome data used in this study is available from the NCBI RefSeq [21] database (assembly accessions provided in Table 1). The software implementing the methods described in this study is available online at the following URL: https://github.com/bioforensics/blast-validate

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Author contributions statement

ALB conceived the experiments and all authors participated in the intellectual development of the project. BDO, DDS, and ALB wrote the software. All authors took part in conducting the experiments and analysing the results. ALB drafted the manuscript and all authors reviewed and revised the text.

Additional information

Competing financial interests

The authors declare no competing financial interests.

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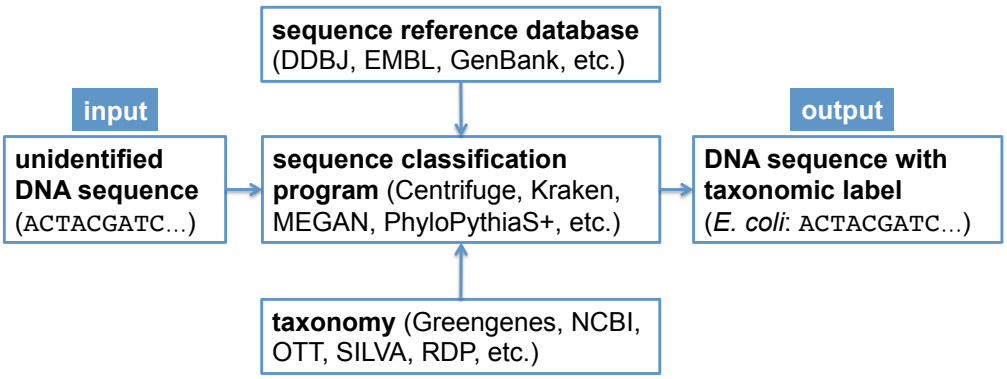

Figure 1. Canonical workflow for the classification of metagenomic sequences.

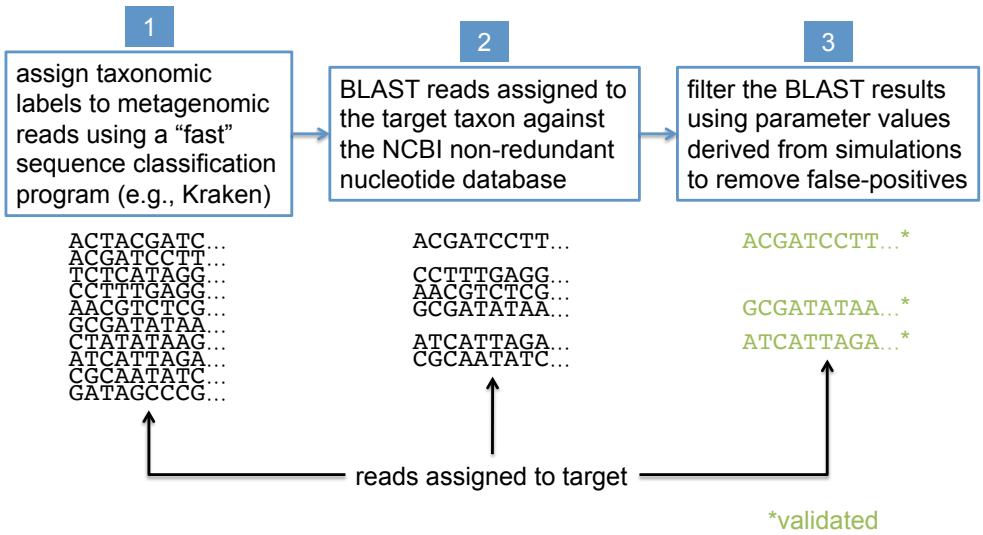

Figure 2. Workflow for BLAST-based validation of taxonomic assignments.

```
# BLASTN 2.2.25+
# Query: NC_003997.3-1994/1
# Database: /data/ncbi/blast/db/nt
# Fields: query id, subject id, % identity, alignment length, mismatches, gap opens, q. start, q. end, s. start, s. end, evalue, bit score, taxon
# 372 hits found
NC_003997.3-1994/1	gi|1043168508|gb|CP012728.1|	100.00	250	0	0	1	250	1704042	1703793	2e-123	452	Bacillus anthracis
NC_003997.3-1994/1	gi|1043163212|gb|CP012727.1|	100.00	250	0	0	1	250	1704042	1703793	2e-123	452	Bacillus anthracis
NC_003997.3-1994/1	gi|1043157917|gb|CP012726.1|	100.00	250	0	0	1	250	1704041	1703792	2e-123	452	Bacillus anthracis
NC_003997.3-1994/1	gi|1043152620|gb|CP012725.1|	100.00	250	0	0	1	250	1704043	1703794	2e-123	452	Bacillus anthracis
NC_003997.3-1994/1	gi|1043147323|gb|CP012724.1|	100.00	250	0	0	1	250	1704043	1703794	2e-123	452	Bacillus anthracis
NC_003997.3-1994/1	gi|589079678|gb|CP006742.1|	100.00	250	0	0	1	250	1690737	1690488	2e-123	452	Bacillus anthracis str. SVA11
NC_003997.3-1994/1	gi|570716017|gb|CP001970.1|	100.00	250	0	0	1	250	1703973	1703724	2e-123	452	Bacillus anthracis str. A16
NC_003997.3-1994/1	gi|570710095|gb|CP001974.1|	100.00	250	0	0	1	250	1703925	1703676	2e-123	452	Bacillus anthracis str. A16R
NC_003997.3-1994/1	gi|384383725|gb|CP002091.1|	100.00	250	0	0	1	250	1694749	1694500	2e-123	452	Bacillus anthracis str. H9401
NC_003997.3-1994/1	gi|229264291|gb|CP001598.1|	100.00	250	0	0	1	250	1703999	1703750	2e-123	452	Bacillus anthracis str. A0248
NC_003997.3-1994/1	gi|227002338|gb|CP001215.1|	100.00	250	0	0	1	250	2535117	2535366	2e-123	452	Bacillus anthracis str. CDC 684
NC_003997.3-1994/1	gi|218534755|gb|CP001283.1|	100.00	250	0	0	1	250	1762619	1762370	2e-123	452	Bacillus cereus AH820
...
```

- **bit score of the best hit to the target taxon (*B. anthracis*) = 452**
- **bit score of the best hit to a non-target taxon (*B. cereus*) = 452**
- **452 – 452 = 0; minimum required bit score difference determined by simulation = 8**
- **therefore, this read is <u>not</u> assigned to the target taxon (*B. anthracis*)**

Figure 3. Demonstration of the bit score difference filter (first example).

```
# BLASTN 2.2.25+
# Query: NC_003997.3-1992/1
# Database: /data/ncbi/blast/db/nt
# Fields: query id, subject id, % identity, alignment length, mismatches, gap opens, q. start, q. end, s. start, s. end, evalue, bit score, taxon
# 307 hits found
NC_003997.3-1992/1	gi|753450531|gb|CP009541.1|	100.00	250	0	0	1	250	322480	322231	2e-123	452	Bacillus anthracis str. Sterne
NC_003997.3-1992/1	gi|753270362|gb|CP009315.1|	100.00	250	0	0	1	250	4108350	4108599	2e-123	452	Bacillus anthracis str. Turkey32
NC_003997.3-1992/1	gi|673992625|gb|CP007666.1|	100.00	250	0	0	1	250	1176391	1176640	2e-123	452	Bacillus anthracis str. Vollum
NC_003997.3-1992/1	gi|589079678|gb|CP006742.1|	100.00	250	0	0	1	250	2289646	2289895	2e-123	452	Bacillus anthracis str. SVA11
NC_003997.3-1992/1	gi|570716017|gb|CP001970.1|	100.00	250	0	0	1	250	2303524	2303773	2e-123	452	Bacillus anthracis str. A16
NC_003997.3-1992/1	gi|570710095|gb|CP001974.1|	100.00	250	0	0	1	250	2303013	2303262	2e-123	452	Bacillus anthracis str. A16R
NC_003997.3-1992/1	gi|384383725|gb|CP002091.1|	100.00	250	0	0	1	250	2293805	2294054	2e-123	452	Bacillus anthracis str. H9401
NC_003997.3-1992/1	gi|229264291|gb|CP001598.1|	100.00	250	0	0	1	250	2303542	2303791	2e-123	452	Bacillus anthracis str. A0248
NC_003997.3-1992/1	gi|227002338|gb|CP001215.1|	100.00	250	0	0	1	250	1935455	1935206	2e-123	452	Bacillus anthracis str. CDC 684
NC_003997.3-1992/1	gi|49176966|gb|AE017225.1|	100.00	250	0	0	1	250	2303569	2303818	2e-123	452	Bacillus anthracis str. Sterne
NC_003997.3-1992/1	gi|30260185|gb|AE016879.1|	100.00	250	0	0	1	250	2303518	2303767	2e-123	452	Bacillus anthracis str. Ames
NC_003997.3-1992/1	gi|675832311|gb|CP008853.1|	99.60	250	1	0	1	250	2303095	2303344	9e-122	446	Bacillus anthracis
NC_003997.3-1992/1	gi|755995789|gb|CP009605.1|	99.20	250	2	0	1	250	1841890	1841641	1e-120	443	Bacillus cereus
...
```

- **bit score of the best hit to the target taxon (*B. anthracis*) = 452**
- **bit score of the best hit to a non-target taxon (*B. cereus*) = 443**
- **452 – 443 = 9; minimum required bit score difference determined by simulation = 8**
- **therefore, this read <u>is</u> assigned to the target taxon (*B. anthracis*)**

Figure 4. Demonstration of the bit score difference filter (second example).

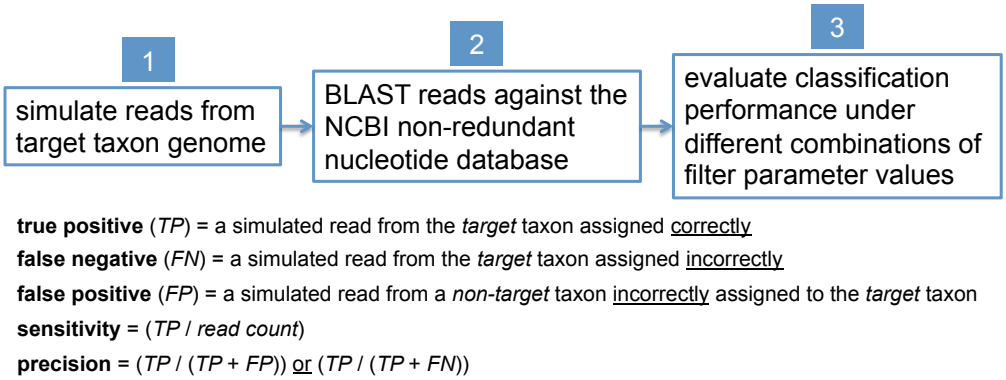

Figure 5. Workflow for determining optimal BLAST filter parameter values.

Taxon	Taxonomic rank	Type	NCBI Taxonomy ID	RefSeq assembly accession
Bacillus cereus group	species group		86661	N/A
B. anthracis	species	target	1392	N/A
B. cereus	species	near neighbour	1396	N/A
B. anthracis Ames Ancestor	strain		261594	GCF_000008445
B. cereus JEM-2	strain		N/A	GCF_001941925
Clostridium	genus		1485	N/A
C. botulinum	species	target	1491	N/A
C. sporogenes	species	near neighbour	1509	N/A
C. botulinum A str. ATCC 3502	strain		413999	GCF_000063585
C. sporogenes NCIMB 10696	strain		N/A	GCF_000973705
Escherichia	genus		561	N/A
E. coli	species		562	N/A
E. coli O157:H7 str. Sakai	strain	target	386585	GCF_000008865
E. coli SRCC 1675	strain	near neighbour	N/A	GCF_001612495
Yersinia pseudotuberculosis complex	species group		1649845	N/A
Y. pestis	species	target	632	N/A
Y. pseudotuberculosis	species	near neighbour	633	N/A
Y. pestis CO92	strain		214092	GCF_000009065
Y. pseudotuberculosis PB1/+	strain		502801	GCF_000834475

Table 1. Taxonomic data and metadata for target taxa and near neighbour species or strains.

Target taxon	Taxonomic rank	Number of reads	Method of computing FP	Maximum E-value	Bit score difference	Sensitivity	Precision
B. anthracis	species	220,140	near neighbour	10^{-64}	8	0.088539	1.0
			false negatives	10^{-64}	8	0.088539	0.999995
			false negatives	10^{-64}	128	0.007954	1.0
C. botulinum	species	156,120	near neighbour	10^{-1}	0	0.990802	1.0
			false negatives	10^{-64}	8	0.985050	1.0
E. coli O157:H7 str. Sakai	strain	223,760	near neighbour	10^{-64}	1	0.003745	1.0
			false negatives	10^{-64}	8	0.000822	1.0
Y. pestis	species	193,170	near neighbour	10^{-1}	2	0.186753	1.0
			false negatives	10^{-64}	8	0.105596	1.0

Table 2. Experimental results using simulated 250-bp reads.

Target taxon	Taxonomic rank	Number of reads	Method of computing FP	Maximum E-value	Bit score difference	Sensitivity	Precision
B. anthracis	species	366,920	near neighbour	10^{-32}	8	0.046070	1.0
			false negatives	10^{-32}	8	0.046070	1.0
C. botulinum	species	260,200	near neighbour	10^{-1}	0	0.981295	1.0
			false negatives	10^{-32}	8	0.964316	1.0
E. coli O157:H7 str. Sakai	strain	372,960	near neighbour	10^{-1}	2	0.002298	1.0
			false negatives	10^{-64}	8	0.000483	1.0
Y. pestis	species	321,970	near neighbour	10^{-32}	8	0.062009	1.0
			false negatives	10^{-32}	8	0.062009	1.0

Table 3. Experimental results using simulated 150-bp reads.

Target taxon	Taxonomic rank	Excluded taxon	Number of reads	Method of computing FP	Maximum E-value	Bit score difference	Sensitivity	Precision
B. cereus group	species group	*B. anthracis*	220,140	false negatives	10^{-64}	64	0.163546	0.939339
C. botulinum	species	*C. botulinum* A str. ATCC 3502	156,120	false negatives	10^{-64}	32	0.904926	0.999385
Y. pestis	species	*Y. pestis* CO92	193,170	false negatives	10^{-64}	8	0.104944	1.0

Table 4. Clade-level exclusion experimental results using simulated 250-bp reads.