Bayesian mixture analysis for metagenomic community profiling

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ABSTRACT

Deep sequencing of clinical samples is now an established tool for the detection of infectious pathogens, with direct medical applications. The large amount of data generated provides an opportunity to detect species even at very low levels, provided that computational tools can effectively interpret potentially complex metagenomic mixtures. Data interpretation is complicated by the fact that short sequencing reads can match multiple organisms and by the lack of completeness of existing databases, in particular for viral pathogens. This interpretation problem can be formulated statistically as a mixture model, where the species of origin of each read is missing, but the complete knowledge of all species present in the mixture helps with the individual reads assignment. Several analytical tools have been proposed to approximately solve this computational problem. Here, we show that the use of parallel Monte Carlo Markov chains (MCMC) for the exploration of the species space enables the identification of the set of species most likely to contribute to the mixture. The added accuracy comes at a cost of increased computation time. Our approach is useful for solving complex mixtures involving several related species. We designed our method specifically for the analysis of deep transcriptome sequencing datasets and with a particular focus on viral pathogen detection, but the principles are applicable more generally to all types of metagenomics mixtures. The code is available on github (http://github.com/smorfopoulou/metaMix) and the process is currently being implemented in a user friendly R package (metaMix, to be submitted to CRAN).

INTRODUCTION

Metagenomics can be defined as the analysis of a collection of DNA or RNA sequences originating from a single sample. In practice, its scope is broad and includes the analysis of a diverse set of samples such as gut microbiome (1), (2), environmental (3) or clinical (4), (5), (6) samples. Among these applications, the discovery of viral pathogens is clearly relevant for clinical practice (7), (8). The traditional process of characterizing a virus through potentially difficult and time consuming culture techniques is being revolutionized by advances in high throughput sequencing. Potential benefits of sequence driven methodologies include a more rapid turnaround time (9), combined with a largely unbiased approach in species detection, including the opportunity for unexpected discoveries.

The analysis of shotgun sequencing data from metagenomic mixtures raises complex computational challenges. Part of the difficulty stems from the read length limitation of existing deep DNA sequencing technologies, an issue compounded by the extensive level of homology across viral and bacterial species. Another complication is the divergence of the microbial DNA sequences from the publicly available references. As a consequence, the assignment of a sequencing read to a database organism is often unclear. Lastly, the number of reads originating from a disease causing pathogen can be low (10). The pathogen contribution to the mixture depends on the biological context, the timing of sample extraction and the type of pathogen considered. Therefore, highly sensitive computational approaches are required.

A first analytical problem is read classification, that is the assignment of a given sequencing read to a species. Several tools have been developed and these belong to two broadly defined classes: composition-based and similarity-based approaches. The read classification based on sequence composition relies on the intrinsic features of the reads, such as CG content or oligonucleotide distributions. Methods include PhyloPythia (11), Phymm and PhymmBL (12), MetaCluster (13). These tend to focus on major classes in a dataset and may not perform well on low-abundance populations (14). Additionally, results are usually reliable for longer reads only (12).

Similarity based methods, using homology search algorithms such as BLAST (15), are considered the most effective methods for read classification (12), at least when other species from the same genus are known. One of the most popular tools using the output of a similarity search algorithm is MEGAN (16). MEGAN addresses ambiguous matches by assigning reads that have multiple possible assignments to several species to the taxonomic group containing all these species, or else their lowest common ancestor (LCA). This approach is accurate on a higher taxonomic level. However, it is lacking a formal solution to resolving ambiguous matches. A weakness of the similarity based methods is the appearance of a long tail of species, supported only by a few reads. This results from the classification being decided one read at a time, in contrast to considering all reads simultaneously. A different approach specifically focusing on the relative abundance estimation task using a non-negative LASSO approach is implemented in GASiC (17). This method

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corrects the observed alignment-based abundances using the reference genomes similarities.

Methods focused on the statistical inference of the set of present species as well as the estimation of their relative proportions, incorporate knowledge from all reads to assign each individual read to a species. From a statistical standpoint, this identification and quantification question can be thought of as an application of mixture models. These ideas have been applied in the metagenomics context in frequentist (GRAMMy (18)) and Bayesian (Pathoscope (19)) settings. GRAMMy formulates the problem as a finite mixture model, using the Expectation-Maximization (EM) algorithm to estimate the relative genome abundances. Pathoscope refines this process by penalizing reads with ambiguous matches in the presence of reads with unique matches and enforcing parsimony within a Bayesian context. Both methods work with unassembled sequence data and they are not currently setup to incorporate an initial short read assembly step, which could be achieved by assigning a higher weight to contigs formed by multiple reads.

A mixture model is concerned with the species relative abundance estimation, as well as the read to species assignment. A related but distinct question concerns the set of species which should be included in the mixture model. This question is closely related to the biological question of asking what species are present in the mixture. Including all species flagged as potential match by the read classification can introduce a large number of species (often $>1{,}000$). Mixture models will, in this situation, identify a large number of species at low levels. This interpretation is appropriate in some applications. In many other cases, the expectation is that the underlying species set should be parsimonious and that some divergence with database species or sequencing errors can explain a large fraction of the non matching reads.

Hence, a better statistical formulation of the community profiling problem is the exploration of the candidate organisms state-space. In this context, non nested models can be compared based on their marginal likelihood. Within this Bayesian framework, readily interpretable probabilities, such as the posterior probabilities of species sets can be used to quantify the support for a species in the mixture. Finally, more complex hypotheses regarding for example the number of viral species or the joint presence of two distinct organisms can be investigated.

The main challenge behind such a formulation is computational. For a large number of potential species, there is a combinatoric explosion of the potential subsets to be considered. Efficient computational strategies are required to make this problem tractable. Here we show that this inference can be achieved for modern scale metagenomics datasets. Our strategy is based on parallel tempering, a Monte Carlo Markov Chain technique, which enables the use of parallel computing to speed up the inference. Our tool are being implemented in a user friendly R package (metaMix) that produces posterior probabilities for various models and the relative abundances under each model. We demonstrate its value using several clinical and artificial datasets.

MATERIALS AND METHODS

Bioinformatics pre-processing

Prior to running the mixture model for metagenomic profiling, several steps are required to process the short read sequence data (Figure 1). The pipeline uses publicly available bioinformatics tools for each preprocessing step. This bioinformatics pipeline has been automated and the scripts are available for public use (`http://github.com/smorfopoulou/metaMix`).

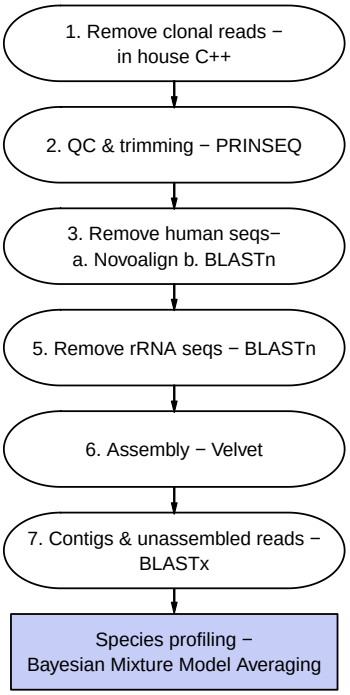

Figure 1. Pipeline steps for species identification. The step of removing the rRNA sequences is applicable only when the aim is viral discovery.

The first step is the removal of clonal reads using an in house C++ script. We then use PRINSEQ (20) to apply read based QC (low quality and complexity) and 3'end trimming. For metagenomic analysis of human samples, reads originating from the human host are not relevant for our research question. We therefore remove human host reads, using a two-step approach to limit computation time: initially a short read aligner (`novoalign`, www.novocraft.com), followed by BLASTn. The next step is only applicable when the focus is on virus discovery using transcriptome reads. We remove ribosomal RNA sequences, using BLASTn against the Silva rRNA database (http://www.arb-silva.de/).

The remaining reads are assembled into contigs using the Velvet short read assembler (21). For each contig we record the number of reads required for its assembly, using this information at the stage of species abundance estimation. A Velvet tuning parameter is the user defined k-mer length that specifies the extent of overlap required to assemble read pairs. Metagenomic assembly is not a straightforward task, as short k-mers work best with the low abundance organisms, while long k-mers with the highly abundance ones. The shorter the k-mer the greater the chance of spurious overlaps, hence we

choose relatively high k-mer length, in order to avoid chimeric contigs.

For each contig and un-assembled read we record the potentially originating species, using the nucleotide to protein homology matching tool BLASTx. We use BLASTx due to the higher level of conservation expected at the protein level compared to nucleotides. This choice is guided by our focus in viral pathogens and viruses have high genetic diversity and divergence (7).

This step generates a sparse similarity matrix between the read sequences and the protein sequences (species as columns, reads and contigs as rows), which is stored using the R package Matrix to handle sparse matrices.

The statistical method described in the remainder of this section considers the competing models that could accommodate our observed data, that is the BLASTx results and compares them. The different models represent different sets of species being present in the sample. The method works on two levels of inference: in the first instance we assume a set of species to be present in the sample and we estimate this model's parameters given the data. The other level of inference is the model comparison so as to assess the more plausible model. The process is iterated in order to explore the model state space.

Model specification assuming a fixed set of species

Assuming a given set of K species from which the reads can originate, the metagenomic problem can be summarized as a mixture problem, for which the assignment of the sequencing reads to species is unknown and must be determined. The data consist of N sequencing reads $\boldsymbol{X}=(x_1,\ldots,x_N)$, and for a given read x_i the likelihood is written as:

$$p(x_i|\boldsymbol{w},K)=p(x_i|\boldsymbol{w})=\sum_{j=1}^{K} w_j f_j(x_i) \qquad \textbf{(1)}$$

where $\boldsymbol{w}=(w_1,\ldots,w_K)$ represent the proportion of each of the K species in the mixture. These mixture weights are constrained such that $0\leq w_j\leq 1$ and $\sum_j w_j=1$. In practice, we also add a category (species $K+1$) which we refer to as the "unknown" category, and captures the fact that some reads cannot be assigned to any species.

Additionally $f_j(x_i) = P(x_i|x_i \text{ from species } j) = p_{ij}$ is the probability of observing the read x_i conditional on the assumption that it originated from species j. We model this probability using the number of mismatches m between the translated read sequence and the reference protein sequence and a Poisson distribution with parameter λ for that number of mismatches. As the read x_i could start from any point in the reference protein sequence, we write:

$$p_{ij}=\frac{\text{Pois}(m;\lambda)}{l_p} \qquad \textbf{(2)}$$

where l_p is the length of the reference protein.

Therefore for a given set of K species, the p_{ij} probabilities are regarded as known and the mixture weights will be estimated.

Combining the above we see that when we know the set of species K, the mixture distribution gives the probability of observing read x_i: $\sum_{j=1}^{K} w_j p_{ij}$, namely equation (**1**).

We therefore write the likelihood of the dataset $\boldsymbol{X}$ as a sum of K^n terms:

$$P(\boldsymbol{X}|\boldsymbol{w})=\prod_{i=1}^{n}[\sum_{j=1}^{K} w_j p_{ij}] \qquad \textbf{(3)}$$

Estimation of mixture weights

Assuming a fixed set of species, the posterior probability distribution of the weights $\boldsymbol{w}$ given the read data X is:

$$P(\boldsymbol{w}|X)=\frac{P(X|\boldsymbol{w})\pi(\boldsymbol{w})}{P(X)} \overset{(3)}{=} \frac{\prod_i[\sum_j w_j p_{ij}]\pi(\boldsymbol{w})}{\int \prod_i[\sum_j w_j p_{ij}]\pi(\boldsymbol{w})d\boldsymbol{w}} \qquad \textbf{(4)}$$

A practical prior for the mixing parameters $\boldsymbol{w}$ is the Dirichlet distribution owing to its conjugate status to the multinomial distribution. Despite the use of conjugate priors, the probabilistic assignment of reads to species involves the expansion of the likelihood into K^n terms which is computationally infeasible through direct computation. An efficient estimation can be performed by the introduction of unobserved latent variables that code for the read assignments. In this framework, either the Gibbs sampler (22), a Monte Carlo Markov Chain technique, or the Expectation-Maximization (EM) (23) algorithm can be used to estimate the mixture weights $\boldsymbol{w}$. EM returns a point estimate for $\boldsymbol{w}$ while the Gibbs sampler the distribution of $\boldsymbol{w}$. Both methods were implemented and provided comparable results (Supplementary Text for details of implementation).

Marginal likelihood estimation

Each combination of species corresponds to a finite mixture model for which the marginal likelihood can be estimated. Marginal likelihood comparison has a central role in comparing different models $\{M_1,\ldots,M_m\}$. To compute the marginal likelihood $P(X|M_k)$ for the mixture model M_k one has to average over the parameters with respect to the prior distribution $\pi(\boldsymbol{\theta_k}|M_k)$, where $\boldsymbol{\theta_k}$ are the model parameters:

$$P(X|M_k)=\int_{\boldsymbol{\theta_k}} P(X|\boldsymbol{\theta_k},M_k)\pi(\boldsymbol{\theta_k}|M_k)d\boldsymbol{\theta_k} \qquad \textbf{(5)}$$

The posterior probability of the model M_k is:

$$P(M_k|X)\propto P(X|M_k)P(M_k) \qquad \textbf{(6)}$$

where $P(M_k)$ is the prior belief we hold for each model. The prior can be specified depending on the context but the basis of our interpretation is that parsimonious models with a limited number of species are more likely. Thus in this Bayesian framework our default prior uses a penalty limiting the number of species in the model.

We approximate this penalty factor based on a user-defined parameter r that represents the species read support required by the user to believe in the presence of this species. We

compute the logarithmic penalty value as the log-likelihood difference between two models: one where all N reads belong to the "unknown" category and one where r reads have a perfect match to some unspecified species and the remaining $N-r$ reads belong to the "unknown" category. The p_{ij} probabilities for the r reads originating from this unspecified species are approximated by 1/(median protein length in the reference database). This parameter essentially reflects how many reads are required to provide credible support that a species is present in the mixture and acts as a probabilistic threshold as opposed to a deterministic one applied on a ranked list.

From now on, when we refer to the marginal likelihood, we mean the marginal likelihood for a specific model and we forego conditioning on the model M_k in the notation. Additionally, in our mixture model p_{ij} are always regarded as known, therefore the model parameters $\boldsymbol{\theta_k}$ are the mixture weights $\boldsymbol{w}$. Hence (**5**) becomes:

$$P(X)=\int_{\boldsymbol{w}} P(X|\boldsymbol{w})\pi(\boldsymbol{w})d\boldsymbol{w} \overset{(3)}{=} \int_{w}\prod_{i}[\sum_{j} w_j p_{ij}]\pi(\boldsymbol{w})d\boldsymbol{w} \tag{7}$$

Approximating the marginal likelihood is a task both difficult and time-consuming. We chose the Defensive Importance Sampling technique (24) for the relative simple implementation compared to other approaches. This is crucial as we perform this approximation numerous times, for every species combination we consider. For more details on IS, see the Supplementary Text.

However the goal of this work is to deliver results in a clinical setting within an actionable time-frame. We wish to speed up the computation without compromising the accuracy and the sensitivity of the results. For that reason, we use a point estimate of the marginal likelihood by means of the Expectation-Maximization (EM) algorithm. The different approaches were used on a benchmark dataset. The resulting taxonomic assignment as well as the species relative abundance estimates were similar between them, with the EM approach resulting in a 13-fold speed increase (Supplementary Text).

Model comparison: exploring the set of present species

We use a Monte Carlo Markov Chain (MCMC) to explore the set of present species of size 2^S-1, where S is the total number of potential species. In practice we observe that S can be greater than 1,000. The MCMC must explore the state-space in a clinically useful timespan. Therefore we reduce the size of the state-space, by decreasing the number of S species to the low hundreds. We achieve this by fitting a mixture model with S categories, considering all potential species simultaneously. Post fitting, we retain only the species categories that are not empty, that is categories that have at least one read assigned to them.

Let us assume that at step t, we deal with a set of species that corresponds to the mixture model M_k. At the next step $(t+1)$, we either add or remove a species and the new set corresponds to the mixture model M_l. The step proposing the model M_l is accepted with probability:

$$A(M_k \rightarrow M_l)=min\{1,\frac{P(X|M_l)^{(t+1)}P(M_l)}{P(X|M_k)^{(t)}P(M_k)}\frac{q(M_l\rightarrow M_k)}{q(M_k\rightarrow M_l)}\} \tag{8}$$

where $q(M_l \rightarrow M_k)$ is the probability of transitioning from model M_l to model M_k. In other words, this is the probability of adding or removing the species to the M_k set of species that took us to the M_l set of species.

If the step is accepted, then the chain moves to the new proposed state M_l. Otherwise if not accepted, the chain's current state becomes the previous state of the chain, i.e the set of species remains unchanged.

metaMix outputs log-likelihood traceplots so that the user can visually inspect the mixing of the chain. The default setting is to discard the first 20% of the iterations as burn-in. We concentrate on the rest to study the distribution over the model choices and perform model averaging. We can then summarize appropriately the posterior distribution and answer the important questions of interest. Examples of such questions include: what species have probability p or greater being included in the set of present species? what is the probability of having the n specific closely related strains in the set of present species? Depending on the biological context, one may ask numerous similar or other case-specific questions.

Optimized implementation: parallel tempering

We observed that simple MCMC does not efficiently explore the complex model state space, as evidenced by the poor mixing of the chain (Figure 2).

In order to overcome this and take advantage of parallel computing, we run multiple chains and allow exchange moves between them. This method is called parallel tempering MCMC (25). Within the parallel setting, each chain simulates from the posterior distribution $g(M)$=$P(M|X)$ raised to a temperature $T\epsilon(0,1]$, where model M represents a set of species being present. The different temperature levels result in tempered versions of the posterior distribution $P(M_k|X)^T$. When $T=1$ the draws are from the posterior distribution. On the other hand, at higher temperatures the posterior spreads out its mass and becomes flatter. In practice that means that distributions at higher temperatures are easily sampled, improving the mixing. We are interesting in studying the original posterior distribution with $T=1$.

We implemented two types of moves. The first is the mutation step, which simply is the within chain move we described in the previous section. This is accepted with probability given by (**8**). The other is the exchange step, a between chains move. This Metropolis-Hastings move proposes to swap the value of two chains k and $k+1$, adjacent in terms of T. Suppose that the values of the two chains are M_k and M_{k+1} respectively, corresponding to two different sets of species. The move is accepted with probability (26):

$$A=min\{1,\frac{g_k(M_{k+1})}{g_k(M_k)}\frac{g_{k+1}(M_k)}{g_{k+1}(M_{k+1})}\} \tag{9}$$

Since $g_k(M_k)$=$P(M_k|X)^T$ and $g_{k+1}(M_{k+1})$ = $P(M_{k+1}|X)^{T+1}$, it follows that when M_{k+1} represents

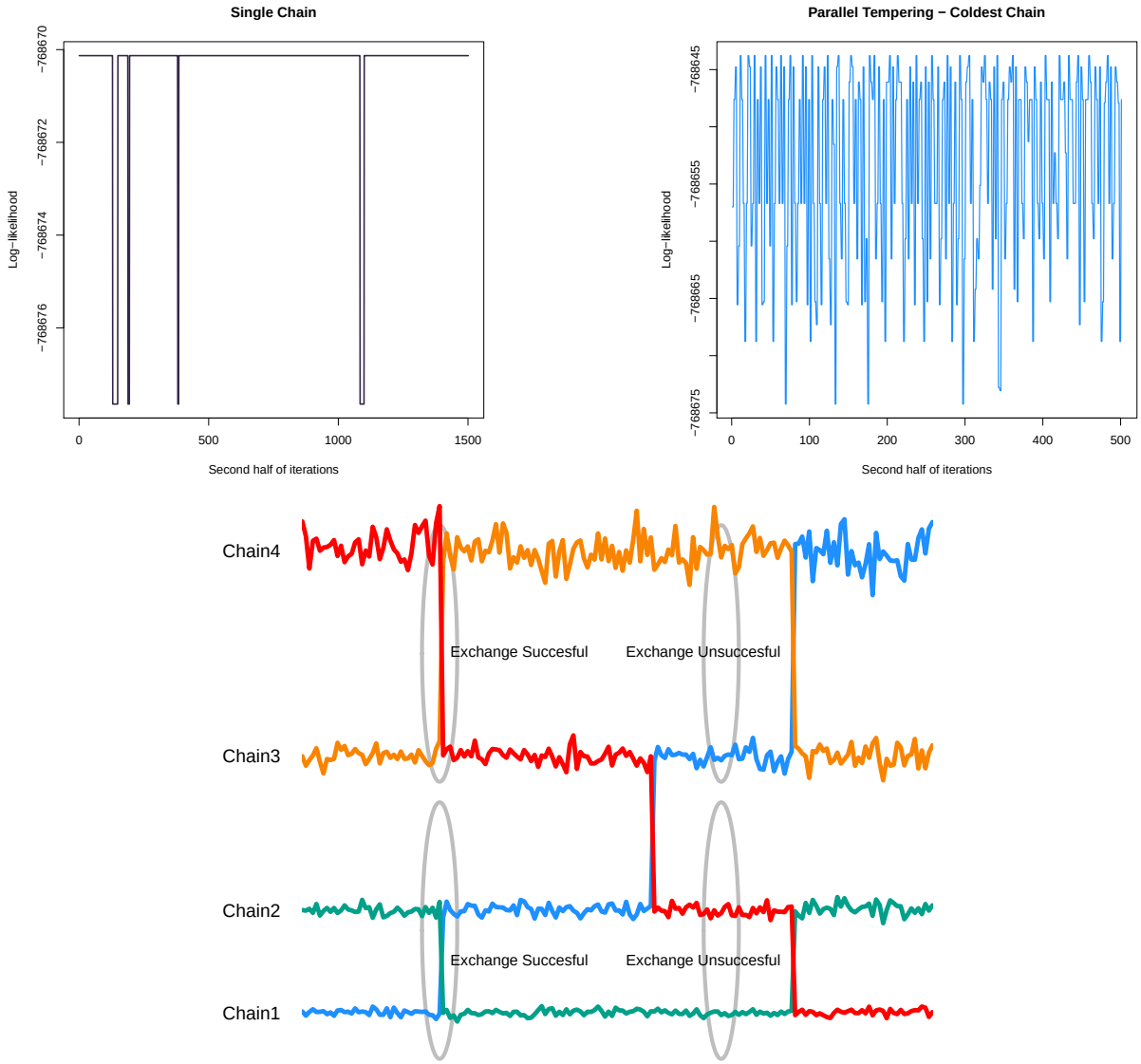

Figure 2. **a.** Log-likelihood trace plot for single chain MCMC and **b.** for PT chain at temperature T=1. **c.** Schematic of parallel tempering. Exchanges are attempted between chains of neighboring temperatures, where Chain1 at $T_1 = 1$, $T_1 < T_2 < T_3 < T_4$.

a set of species of higher probability than the one M_k represents, the exchange move will always be accepted (Figure 2).

This allows moves between separate modes, ensuring a global exploration of the model state space. Eventually "hot" and "cold" chains will progress towards a global mode.

RESULTS

We first applied our method to a reference artificial dataset, for which the community composition and the read assignment is known. We then analyzed two clinical samples generated for diagnosis purpose.

We compare our results with the ones produced by MEGAN version 5.3 and Pathoscope 2.0. Both methods are similarity-based. This property and more specifically their flexibility to work with BLASTx output, makes them better candidates for viral discovery compared to other composition-based methods.

From the mixture model methods, we have chosen Pathoscope. We were also interested in comparing our results to the ones by GRAMMy, which was the first metagenomic method to use the idea of the mixture model method. However it can work only with nucleotide-nucleotide comparisons (BLASTn), which is suboptimal for viral discovery. Additionally, it works only with unassembled reads and it requires that these are of the same fixed length. Lastly, a time consuming pre-structured genome and taxon directory is required. For these reasons, GRAMMy was not included in the comparison.

Default parameters were used for all methods, unless stated otherwise. For metaMix the organisms reported have a posterior probability greater than 0.9. For a detailed discussion

on default settings and practical considerations for metaMix, please see the Supplementary Text.

It is important to state that there is a trade-off between the value of the read support parameter and the number of present species in the output summary table. The user's choice can be informed by the biological context at each instance. Familiarity with the studied community allows for a better understanding on what constitutes real signal and what noise.

As an example, for the typical human clinical sample where the sample collection might have occurred some time after the infection has taken place, a low value in order to adopt a sensitive approach makes sense. This is because it is plausible to expect traces of the infecting pathogen in such a sample. We set the default value for viral identification in human clinical samples to be $\mathtt{r}=10$.

In a highly complex environmental metagenomic community where there is a plethora of species of similar abundances, the choice becomes less straightforward especially in the case of closely related strains. A large `r` value can result in the method merging together strains that are differentiated by fewer reads than `r`. On the other hand a low `r` can have the opposite effect, whereby the methods splits a strain into two or more strains, by moving a few reads from one strain to a very similar one with which they have equally good matches. We set the default value for general community profiling in environmental samples to be $\mathtt{r}=30$, however we report comparative results for different values.

FAMeS simHC dataset - closely related strains

The FAMeS artificial datasets (`http://fames.jgi-psf.org/description.html`), are simulated metagenomic datasets composed of random reads from 113 isolate microbial genomes present in IMG (Integrated Microbial Genomes) and sequenced at the DOE Joint Genome Institute. They are a popular choice to use as benchmark datasets for various metagenomics methods. Their suitability rises from the fact that the number of species that form the metagenomic community is known as well as their relative abundances. The FAMeS datasets have been designed to model real metagenomic communities in terms of complexity and phylogenetic composition.

There are three datasets: simHC, simMC, simLC corresponding to high, medium and low complexity of the metagenomic community respectively. Three methods were applied to simHC, as this is the highest complexity dataset, with many closely related strains with similar abundances and no dominant species. The lowest abundance is 255 reads out of 118,000 reads. The bioinformatics processing in this instance consisted of a BLASTn comparison to all NCBI bacterial genomes. The number of genomes mapped, retrieved from the the BLASTn output was ∼2,500.

metaMix outperforms Pathoscope and MEGAN in the community profiling task and consequently in the relative abundance estimation, as shown in the results below.

metaMix To limit the complexity of the fit, we used a two step procedure described in the Methods section. We first fitted the mixture model with the complete set of 2,500 species and a limited run length of 500 iterations. Based on this analysis, we identified 1,312 species supported by at least one read and explored this state space. To limit the computational time, we also considered a stronger approximation, including only the 374 potential species supported by at least 10 sequencing reads. Both approaches generated similar results, albeit the more complex one with 1,312 potential species required the quadruple of the computation time (12h instead of 3h). metaMix identified 116 species, detecting successfully all the members of the metagenomic community (Table S1). These were detected on the strain level except in four instances where a different strain of the same species, or different species within the same genus was detected. There were four false positives (Table S1).

Pathoscope Pathoscope identified 47 species. Of these 42 are members of the metagenomic community. 42 are the exact same strain, while 3 are either the same species but different strain or same genus but different species. However it fails to detect 68 species that are actually present in the mixture. Tuning the parameter that enforces the parsimonious results (any thetaPrior greater than 10), thereby removing the unique read penalty Pathoscope behaves as a standard mixture model and identifies 165 species. With these settings, it identifies all but one members of the community. The organisms are identified at the strain level, except in three instances where it identified different species within the same genus. The major interpretation issue is the presence of a long tail of species (54 species) that are actually not present in the mixture (Supplementary Table S1). Pathoscope produced the results in one minute.

MEGAN MEGAN identified 232 species. It discovered all original species of the community on the strain level, except for 9 instances where it identified a lowest common ancestor (LCA). Aside from the lack of strain/species specificity for 8% of the community members, the main issue is the long tail of false positives species. In the species summary provided by MEGAN, there are 119 species which are not actually present, but supported by a sufficient number of reads (that is 50 reads) for MEGAN to include these in the output. It produced the results in less than one minute.

Table 1. Number of species identified for the FAMeS simHC dataset.

True	MEGAN	Pathoscope	metaMix
113	232	165	116

Our primary aim is a diagnostic for the presence/absence of species in the mixture. As a secondary aim, we are also interested in estimating accurately the relative abundance of the present organisms. We can assess the abundance estimates produced by the methods by using error measures such as the relative root mean square error, RRMSE and the average relative error, AVGRE. For all methods, when the exact strain was not identified but the correct species or genus was, we used this abundance.

$$\mathbf{RRMSE}=\sqrt{\frac{1}{K}\sum_{j=1}^{K}\left(\frac{|w_j-t_j|}{t_j}\right)^2} \qquad \textbf{(10)}$$

$$\mathbf{AVGRE} = \frac{1}{K}\sum_{j=1}^{K}\left(\frac{|w_j - t_j|}{t_j}\right) \quad (11)$$

where t_j is the true abundance of species j and w_j the estimated abundance.

Table 2. Measures of estimation accuracy of relative abundances for the FAMeS simHC dataset.

Method	RRMSE	AVGRE
metaMix	17	8.5
Pathoscope	36.6	29.7
MEGAN	35.9	18

Importance of read support parameter We then assessed the importance of the read support parameter `r` on the output of metaMix. We ran metaMix on the benchmark simHC FAMeS dataset with `r` = {10, 20, 30, 50} reads (Table 3, Figure 3). We observe that as `r` decreases, a few more related strains from the reference database that are not in the community are retained in the output. As `r` increases two similar strains are merged into one.

We compared these results with the output of Pathoscope and MEGAN. None of these methods have a read support parameter serving the same purpose as metaMix, so we tuned the most relevant parameters in these tools. Pathoscope has a thetaPrior parameter that enforces a unique read penalty. This parameter represents the read pseudocounts for the non-unique matches and the default setting is zero which allows for non informative priors. Using the default setting Pathoscope identifies 47 taxa. When thetaP's value is in (1,7) it identifies 22 taxa, while with thetaP>7 it identifies 165. With this latter setting which is the one we chose for the comparison, Pathoscope behaves as a standard mixture model.

MEGAN has a "Min Support" parameter which sets a threshold for the number of reads that must be assigned to a taxon so that it appears in the result. Any read assigned to a taxon not having the required support is pushed up the taxonomy until a taxon is found that has sufficient support. We used Min support = {10, 20, 30, 50} reads. The respective number of taxa in the summary files were 250, 243, 236, 232.

We then also applied a post-run read count threshold to both methods' output summary. We set the threshold for 10,20,30 reads respectively, disregarding taxa that have less than that number of reads assigned to them. In all instances metaMix produces a community profile closer to the real one. Pathoscope finds $\sim$20 more false positives while MEGAN $\sim$40 more compared to metaMix at the same read support level.

The simHC from the FAMeS datasets is a highly complicated dataset, not very similar to the typical human clinical sample. The differences are the large number of organisms, the presence of closely related strains of similar abundances, as well as the lack of viruses. Nevertheless, it is an essential dataset to use as a benchmark for examining the performance of the methods in a situation of closely related strains in the sample.

Table 3. Number of species in the simHC FAMeS dataset by metaMix, Pathoscope and MEGAN

Read Support	metaMix	Pathoscope	MEGAN
50	115	131	147
30	116	131	156
20	119	141	166
10	137	155	188

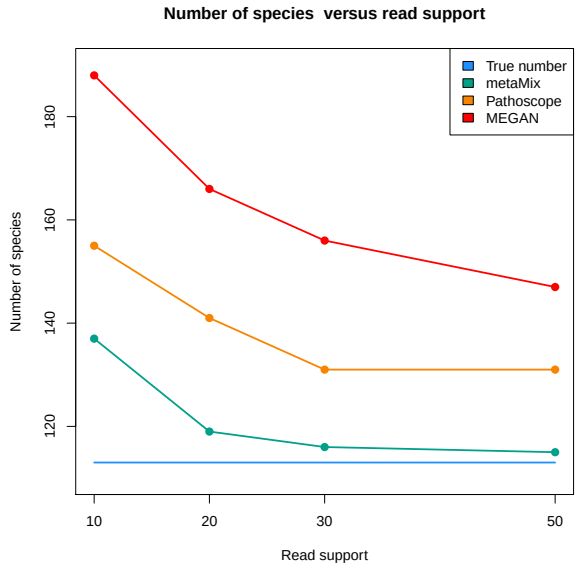

Figure 3. Number of species in the simulated simHC FAMeS dataset detected by metaMix, Pathoscope and MEGAN, as a function of the minimum number of reads required for each species to appear in the output.
For metaMix that is `r`={10, 20, 30, 50} reads, for Pathoscope thetaPrior$>7+$ post-run threshold ={10, 20, 30, 50} reads, for MEGAN "Min Support" + post-run threshold ={10, 20, 30, 50} reads.

Human clinical sample - low viral load

To test metaMix in a clinical setting with a low viral load, we used a brain biopsy RNA-Seq dataset from an undiagnosed encephalitis patient (UCL Hospital, data provided as part of a collaboration with Professor Breuer, UCL). Total RNA was purified from the biopsy and polyA RNA was separated for sequencing library preparation. The Illumina Miseq instrument generated 20 million paired-end reads. We processed the raw data using the bioinformatics pipeline described above. Subsequently we were left with $\sim$75,000 non-host reads and contigs. Based on the BLASTx output there were 1,298 potential species.

metaMix Following the initial processing, we used metaMix for species identification and abundance estimation. We first fitted the all-species mixture model which identified 57

Table 4. Human clinical sample - novel virus.
Comparison of community profile: metaMix - Pathoscope.

		metaMix		Pathoscope
Taxon Identifier	Scientific Name	Assigned Reads	Posterior Probability	Final best hit read numbers
374840	*Enterobacteria phage phiX174 sensu lato*	61758	1	65327
NA	*unknown*	8869	1	NA
9606	*Homo sapiens*	264	1	554
28090	*Acinetobacter lwoffii*	98	1	126
469	*Acinetobacter*	82	1	123
13690	*Sphingobium yanoikuyae*	72	1	135
56946	*Afipia broomeae*	48	1	77
618	*Serratia odorifera*	47	1	-
199310	*Escherichia coli CFT073*	46	1	35
645687	*Astrovirus VA1*	46	1	46
409438	*Escherichia coli SE11*	22	1	49
1747	*Propionibacterium acnes*	17	0.93	35
133448	*Citrobacter youngae*	-	-	169
1282	*Staphylococcus epidermidis*	-	-	10
28211	*Alphaproteobacteria*	-	-	10
28037	*Streptococcus mitis*	-	-	8
562	*Escherichia coli*	-	-	8
509173	*Acinetobacter baumannii AYE*	-	-	7
41297	*Sphingomonadaceae*	-	-	6
40214	*Acinetobacter johnsonii*	-	-	6
29391	*Gemella morbillorum*	-	-	5
76122	*Alloprevotella tannerae*	-	-	4
652103	*Rhodopseudomonas palustris DX-1*	-	-	2
268747	*Prochlorococcus phage P-SSM4*	-	-	2

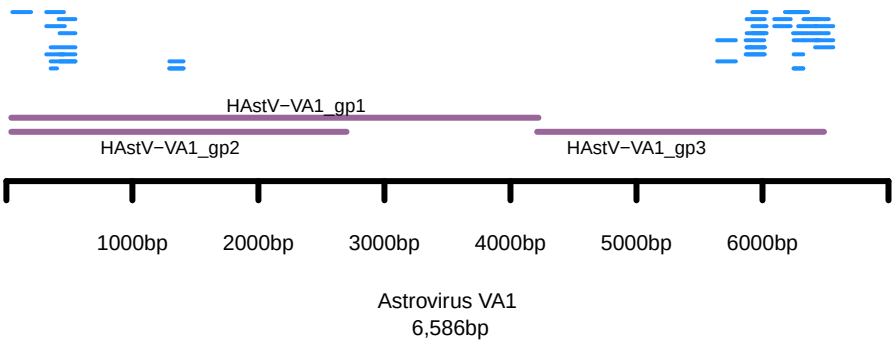

Figure 4. Human clinical sample - novel virus.
The reads (blue lines) assigned by metaMix to Astrovirus VA1, aligned to its genome. The purple lines represent the genes of the virus.

species having one or more reads assigned to them. We then explored this reduced state-space. The resulting species profile is shown in Table 2; the 12 metaMix entries correspond to 9 species. metaMix identified two viruses confidently. The most abundant virus was the phiX bacteriophage: ϕX174 bacteriophage is routinely used for quality control of the Illumina high throughput sequencing. Five short assembled contigs (44 reads) with length ranging between 167bp and 471bp and two non-assembled reads (46 reads overall) were assigned to the Astrovirus VA1 sequence with 97% sequence identity and a probability score of 1 (Figure 4). metaMix also identified a number of bacteria supported by a few reads. These are either known laboratory reagent contaminants or human skin associated contaminants (27). The analysis completed in 20 minutes.

After adding the Astrovirus VA1 and the bacteriophage phiX species in our mixture model, we found no evidence for additional viruses in the data. The presence of the

Astrovirus was confirmed using real-time RT-PCR (Lockwood et al, submitted). Sequencing of the Astrovirus genome with primers designed using the PCR product confirmed the fact that we identified a novel species.

Pathoscope Pathoscope identified 22 taxa, corresponding to 15 species and some genera/families (Table 4). It also assigned all 46 reads to the Astrovirus. Almost all the species identified from metaMix were identified by Pathoscope, with an additional 9 taxa supported by few reads. As the method can only properly work with unassembled sequence data, an extra BLASTx similarity step had to be performed for the 91,516 reads that had contributed to the 679 assembled contigs. After that Pathoscope produced the results in less than one minute.

MEGAN MEGAN identified 19 taxa and did not detect the Astrovirus signal. We modified the minimum read support parameter from 50 reads to 10 to increase sensitivity. MEGAN then identified 25 taxa, including the Astrovirus VA1. The remaining 24 were mostly genera, relevant to the species detected by metaMix and Pathoscope. MEGAN produced the results in less than one minute.

Human clinical sample - species absent from the database

We then compared the performance of the three methods in a scenario where sequences present in the sample are absent from our reference database.
We analyzed a second brain biopsy sample from an undiagnosed patient. 32 million RNA-Seq reads were obtained using the HiSeq instrument. Following initial processing using our bionformatics pipeline, we were left with 1,261,575 non host reads and contigs for subsequent analyses. There were 3,150 potential species based on the BLASTx output.

metaMix The all-species mixture model identified 80 species having one or more reads assigned to them. We then explored this reduced state-space. The metaMix resulting species profile consisted of 7 species (Supplementary Table S2). Two different coronaviruses strains were identified: Human coronavirus OC43 and Bovine coronavirus, with almost half a million reads assigned to each. The high read count assigned to both coronaviruses, as well as the similar read classification probabilities (Supplementary figure S1) suggest a single but novel strain which is not included in our reference database.

We followed up on the sequences assigned to the ”unknown category”, i.e approximately 155K reads, by looking for nucleotide similarity with NR-NT using BLASTn. Approximately half of the reads originated from an untranslated region of the Coronavirus genome, which is not captured by the protein reference database. The remaining reads matched confidently to either *Danio rerio* (zebrafish) sequences or *Gallus gallus* (chicken), two organisms whose proteins are not in the human microbiome reference we are using. The zebrafish and chicken matches were explained as barcode leakage resulting from multiplexing on the same flowcell zebrafish and chicken RNA-Seq libraries. metaMix appropriately assigned these reads to the ”unknown” category, producing a clean probabilistic summary (Supplementary Table S2). The method ran in 7.5 hours.

In this instance, the metaMix results emphasize the importance of being able to deal with missing reference sequences that do not have a closely related strain or species in the same database.

Pathoscope Pathoscope identified 177 species in this sample (Supplementary Table S2). We optimized the value of the unique read penalty parameter and we achieved the best results with the thetaPrior parameter set within the range 10-100. With these settings, the method identified 52 species (Supplementary Table S2). Our assessment is that Pathoscope is confused by the lack of completeness of databases combined with the absence of an ”unknown” category, which prevents it from dealing with these unassigned reads sensibly. Pathoscope completed its analysis in 10 minutes.

MEGAN MEGAN assigned the reads to 30 taxa. These included some species and genera but most were families (Supplementary Table S2). Approximately 250K reads could not be assigned to any taxonomic level. MEGAN run in 8 minutes.

DISCUSSION

In this work we present metaMix, a sensitive method for metagenomic species identification and abundance estimation. The method is implemented in a R package (package in preparation and to be released on CRAN, scripts currently available at `http://github.com/smorfopoulou/metaMix`). Using a Bayesian mixture model framework, we account for model uncertainty by performing model averaging and we resolve ambiguous assignments by considering all reads simultaneously. A key feature of the method is that it provides probabilities that answer pertinent biological questions, in particular the posterior probability for the presence of a species in the mixture. Additionally it accurately quantifies the relative proportions of the organisms.

This general framework is designed to address interpretation issues associated with closely related strains in the sample, low abundance organisms and absence of genomes from the reference database. We show that metaMix outperforms other methods in the community profiling task, particularly when complex structures with closely related strains are studied. It also outperforms the other methods at the task of relative abundance estimation. The method can deal with either unassembled reads or assembled contigs or both, allowing for flexibility of choice for the bioinformatics pre-processing. In practice, the choice of bioinformatics processing prior to the application of our Bayesian mixture analysis must be optimized for each application, and our processing pipeline has been designed with viral sequence identification from transcriptome sequencing as a main goal. Nevertheless, as demonstrated by our analysis of the artificial bacterial community dataset, the method can be applied in other contexts.

The sensitivity and general applicability of metaMix comes at an increased computational cost, requiring access to a multi-core computer to run efficiently. For the datasets presented here, the computation time remained manageable and did not exceed a few hours, using 12 cores to run 12 parallel chains. Nevertheless, a limitation of metaMix is the increased processing time for very large datasets. Speed

related improvements can be implemented in scenarios where the species ambiguity concerns only a small proportion of the read set. Reads with certain assignments can be flagged prior to the MCMC exploration of the state-space. Then only their assignment information can be carried forward, thereby reducing the size of the relevant R object.

metaMix effectively complements faster but more approximate methods designed to perform similar tasks. It is most useful for complex datasets for which the interpretation is challenging.

SUPPLEMENTARY DATA

Supplementary Text, Supplementary tables S1, S2, S3, Supplementary figure S1.

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