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1 # VTAM: A robust pipeline for validating
2 # metabarcoding data using internal controls

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14 Running title: VTAM metabarcoding pipeline
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17 ## Abstract

18 1. Metabarcoding studies should be carefully designed to minimize false
19 positives and false negative occurrences. The use of internal controls,
20 replicates, and several overlapping markers is expected to improve the
21 bioinformatics data analysis.
22 2. VTAM is a tool to perform all steps of data curation from raw fastq data to
23 taxonomically assigned ASV (Amplicon Sequence Variant or simply variant)
24 table. It addresses all known technical error types and includes other features
25 rarely present in existing pipelines for validating metabarcoding data:
26 Filtering parameters are obtained from internal control samples; cross-
27 sample contamination and tag-jump are controlled; technical replicates are
28 used to ensure repeatability; it handles data obtained from several
29 overlapping markers.
30 3. Two datasets were analysed by VTAM and the results were compared to
31 those obtained with a pipeline based on DADA2. The false positive
32 occurrences in samples were considerably higher when curated by DADA2,
33 which is likely due to the lack of control for tag-jump and cross-sample
34 contamination.
35 4. VTAM is a robust tool to validate metabarcoding data and improve
36 traceability, reproducibility, and comparability between runs and datasets.

37

38 Keywords: metabarcoding, mock sample, negative control, replicates, taxonomic
39 assignation, false positives, false negatives

40

41 ## 1 Introduction

42 Metabarcoding has become a powerful approach to study biodiversity from
43 environmental samples (including gut content or faecal samples). Metabarcoding,
44 however, is prone to some pitfalls, and consequently, every metabarcoding study
45 should be designed in a from-benchtop-to-desktop way (from sampling to data
46 analysis) to minimize the bias of each step on the outcome (Alberdi, Aizpurua,
47 Gilbert, & Bohmann, 2018; Cristescu & Hebert, 2018; Zinger et al., 2019). Several
48 papers have called for good practice in study design, data production and analyses
49 to ensure repeatability and comparability between studies. Notably, the importance
50 of mock community samples, negative controls, and replicates is frequently
51 highlighted (Alberdi et al., 2018; Bakker, 2018; Cristescu & Hebert, 2018;

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52 O'Rourke, Bokulich, Jusino, MacManes, & Foster, 2020). However, their use in
53 bioinformatics pipelines is often limited to the verification of expectations.
54 In this study, we present the bioinformatics pipeline, VTAM (Validation and
55 Taxonomic Assignation of Metabarcoding data) that effectively integrates negative
56 controls, mock communities and technical replicates to control experimental
57 fluctuations (e.g. sequencing depth, PCR stochasticity) and validate metabarcoding
58 data.
59 A recent study on the effect of different steps of data curation on spatial
60 partitioning of biodiversity listed the following potential problems: Sequencing and
61 PCR errors, presence of highly spurious sequences, chimeras, internal or external
62 contamination and dysfunctional PCRs (Calderón-Sanou, Münkemüller, Boyer,
63 Zinger, & Thuiller, 2020). They showed that exhaustive curation and ensuring
64 repeatability by technical replicates are necessary, especially for biodiversity
65 measurements. Ideally, a metabarcoding workflow should address all of these
66 technical errors. Existing tools, however, are either highly flexible but too complex
67 or they do not include the curation of all potential biases (Mahé, Rognes, Quince,
68 de Vargas, & Dunthorn, 2014; Boyer et al., 2016; Callahan et al., 2016; Edgar,
69 2016b; Rognes, Flouri, Nichols, Quince, & Mahé, 2016; Bolyen et al., 2019). The
70 filtering steps of VTAM aim to address these points and include several additional
71 features that are unique or rarely found in existing pipelines: (i) the use of internal
72 controls and (ii) replicates to optimize filtering parameter values; (iii) the
73 integration of multiple overlapping markers and (iv) filtration to address cross-
74 sample contamination, including tag-jumps. Finally, VTAM is a variant-based
75 filtering pipeline (such as other denoising methods: Callahan et al., 2016; Edgar,
76 2016b) that deals with amplicon sequence variants (ASVs).

77 # 2 Features

78 ## 2.1 Implementation

79 VTAM is based on the method described in Corse et al. 2017. It is a command-line
80 application that runs on Linux, MacOS or Windows Subsystem for Linux (WSL).
81 VTAM is implemented in Python3, using a Conda environment to ensure
82 repeatability and easy installation of VTAM and these third-party applications:
83 WopMars (https://wopmars.readthedocs.io), NCBI BLAST, Vsearch (Rognes et al.,
84 2016), Cutadapt (Martin, 2011). Data is stored in an SQLite database that ensures
85 traceability.
86

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87 ## 2.2 Workflow

88 Table 1 summarizes the different commands and steps of VTAM, their purpose and
89 the related error types.

90 ### 2.2.1 Pre-processing (optional)

91 An example of the data structure is illustrated in Fig. 1.
92 Paired-end FASTQ files are merged, reads are trimmed and demultiplexed
93 according to forward and reverse tag combinations.

94 ### 2.2.2 Filtering

95 Demultiplexed reads are dereplicated and ASVs are stored in an SQLite database.
96 All occurrences are characterized by their read count.
97 *FilterLFN:* eliminates occurrences likely due to Low Frequency Noise. Occurrences
98 are filtered out if they have low read counts (i) in absolute terms (N_{ijk} is small,
99 where N_{ijk} is the read count of variant i in sample j and replicate k), (ii) compared
100 to the total number of reads of the sample-replicate (N_{ijk}/N_{jk}) or (iii) compared to
101 the total number of reads of the variant (N_{ijk}/N_i).
102 *FilterMinReplicateNumber*: Occurrences are retained only if the ASV is present in
103 at least a user-defined number of replicates.
104 *FilterPCRerror*: ASVs with one difference from another ASV of the same sample
105 are filtered out if the proportion of their read counts is below a user-defined
106 threshold value.
107 *FilterChimera* runs the uchime3_denovo chimera filtering implemented in *vsearch*.
108 *FilterRenkonen* removes whole replicates that are too different compared to other
109 replicates in the same sample.
110 *FilterIndel* and *FilterCodonStop* are intended to detect pseudogenes and should
111 only be used for coding markers. *FilterIndel* eliminates all variants, with aberrant
112 length, where the modulo three of the length is different from the majority.
113 *FilterCodonStop* eliminates all variants that have codon STOP in all reading frames
114 of the direct strand.
115 The output of the filters is an ASV table with validated variants in lines, samples in
116 columns and the sum of read counts over replicates in the cells.

117 ### 2.2.3 Taxonomic assignation

118 Taxonomic assignation is based on the Lowest Taxonomic Group method described
119 in detail in Supporting Information 1. The taxonomic reference database has a
120 BLAST format with taxonomic identifiers so that custom databases or the complete
121 NCBI nucleotide database can be used by VTAM. A custom taxonomic reference

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122 database of COI sequences mined from NCBI nucleotide and BOLD
123 (https://www.boldsystems.org/) databases is available with the program.

124 2.2.4 Parameter optimization

125 Users should first identify expected and unexpected occurrences based on the first
126 filtration with default parameters. The optimization step will guide users to choose
127 parameter values that maximize the number of expected occurrences in the dataset
128 and minimize the number of unexpected occurrences (false positives). Parameters
129 are optimized for the three LFN filters and the FilterPCRerror. Optimized
130 parameters can then be used to repeat the filtering steps.

131 2.2.5 Pool runs/markers

132 A run is FASTQ data from a sequencing run and a marker is a region of a locus
133 amplified by a primer pair. The pool command produces an ASV table with any
134 number of run-marker combinations. When more than one overlapping marker is
135 used, ASVs identical to their overlapping parts are pooled to the same line.

136 3 Benchmarking

137 VTAM was tested with two published metabarcoding datasets: a fish dataset
138 obtained from fish faecal samples (Corse et al., 2017), and a bat dataset obtained
139 from bat guano samples (Galan et al., 2018) . Both datasets included negative
140 controls, mock samples and three PCR replicates. A fragment of the COI gene was
141 amplified using two overlapping markers in the fish dataset, and one in the bat
142 dataset (See details in the original studies).
143 Both datasets were analysed by VTAM. The fish dataset was analysed separately for
144 the two markers and the results of both markers were pooled together.
145 Both datasets were also analysed with the DADA2 denoising algorithm (Callahan et
146 al., 2016), one of the most widely used methods for metabarcoding data curation.
147 The output of DADA2 was filtered by LULU (Frøslev et al., 2017) to further
148 eliminate probable false positive occurrences. Then the three replicates of each
149 sample were pooled (as in VTAM), only accepting the occurrence if it was present
150 in at least two replicates (Supporting information 2).
151 We compared the α-diversity and β-diversity obtained for the environmental
152 samples to address the effect of the curation pipelines on diversity estimations. α-
153 diversity was estimated using both ASV richness and cluster richness (clusters
154 aggregate ASVs with <3% divergence), and β-diversity was summarized using the
155 Bray-Curtis pairwise dissimilarity index. (Supporting information 3).
156 In the fish dataset, all expected variants in the mock samples were validated by
157 both pipelines. However, in the bat dataset, two expected variants had very low

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158 read abundance (2-18 reads/replicate), which were in the range of the number of
159 reads in the negative controls (ten out of the 19 negative controls had at least one
160 read count greater than 18). Therefore, we ignored these two expected variants in
161 the Bulk France mock sample, and we optimized the VTAM parameters to retain all
162 other expected occurrences.
163 After filtering with VTAM, the number of false positives in the mock samples was
164 markedly lower than with DADA2 (Table 2). Similarly, ASV and cluster richness
165 were on average two times lower with VTAM than with DADA2 in environmental
166 samples (Fig. 2A and B). In contrast, dissimilarities between samples were higher
167 with VTAM (Fig. 2D). In both pipelines, most clusters contained a single ASV
168 (Supporting information 3; Fig. 2C).

169 4 Discussion

170 Metabarcoding is known to be prone to two types of errors: false negatives and
171 false positives. Based on controls (negative and mock samples), VTAM aims to find
172 a compromise between these two error types by minimizing false positive
173 occurrences while retaining expected variants in mock samples to avoid false
174 negatives. Therefore, the mock samples should contain both well and weakly
175 amplified taxa, where the abundance, i.e. the number of reads, of weakly amplified
176 taxa is marginally higher than those found in negative samples. This should ensure
177 finding filtering parameter values that simultaneously minimize false positives and
178 false negatives. Additionally, in large-scale studies with more than one sequencing
179 run, the use of identical mock samples in all runs can ensure comparability among
180 runs if they consistently yield the same results.
181 The use of technical replicates is another important tool to limit false positives and
182 false negatives (Alberdi et al. 2018, Corse et al. 2017). False positives can be
183 strongly reduced by only accepting variants in a sample if they are present in at
184 least a certain number of replicates. This strategy is strongly advised to reduce
185 experimental stochasticity and validate ASV occurrences. Furthermore, removing
186 replicates with radically different compositions (Renkonen filter) further reduces
187 the effect of experimental stochasticity (De Barba et al., 2014). Additionally, false
188 negatives can be further reduced by amplifying several markers (Corse et al.,
189 2019). If the different markers overlap, VTAM can pool sequences that are
190 identical in their overlapping regions. This integrates the results of different
191 markers unambiguously.
192 While false positive occurrences due to sequencing and PCR errors are generally
193 well detected by denoising pipelines such as DADA2, tag-jump and cross-sample

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194 contamination are rarely taken into account (but see Boyer et al., 2016; Edgar,
195 2016a). However, failing to filter out these artefacts is likely to inflate false
196 positive occurrences and artificially increase inter-sample similarities. In fact, the
197 DADA2 based pipeline produced ASV and cluster richness per sample that was on
198 average twice as high as with VTAM and even higher for some samples (Fig. 2 A,
199 B). On the other hand, dissimilarities between samples were lower after DADA2
200 filtration. Additionally, the near 1:1 correlation between ASV and cluster richness
201 in both pipelines indicated that most clusters contained just one ASV per sample.
202 This supports the notion that diversity inflation in DADA2 resulted from unfiltered
203 tag-jump contaminations rather than PCR or sequencing errors as this would have
204 produced more ASVs that belong to the same cluster. Our VTAM pipeline,
205 therefore, appears more appropriate for comparing the diversity between samples
206 and for investigating the biological responses to environmental change.

207 5 Conclusions

208 The VTAM metabarcoding pipeline aims to address known technical errors during
209 data analysis (Table 1) to validate metabarcoding data. It is a complete pipeline
210 from raw FASTQ data to curated ASV tables with taxonomic assignments.
211 The implementation of VTAM provides several advantages such as using a Conda
212 environment to facilitate the installation, data storage in SQLite database for
213 traceability and the possibility to run one or several sequencing run-marker
214 combinations using the same command. VTAM includes features rarely considered
215 in most metabarcoding pipelines, and we believe it provides a useful tool for the
216 analysis and validation of metabarcoding data for conducting robust analyses of
217 biodiversity.

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225 Authors' contributions

226 EM, EC, VD conceived the ideas and designed the methodology. EM and AG
227 conceived the software architecture and tested the VTAM. AG, TD and RM
228 developed the VTAM software; AG contributed to the WopMars software

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229 development. EM, AG, VD and EC wrote the manuscript. All authors contributed
230 critically to the draft and approved the final version of the manuscript.
231

232 Data Availability

233 VTAM is available at https://github.com/aitgon/vtam. A detailed user manual is
234 found at https://vtam.readthedocs.io.
235 Empirical data used in this paper are available from the Dryad Digital Repository
236 https://datadryad.org/stash/dataset/doi:10.5061/dryad.f40v5 and
237 https://datadryad.org/stash/dataset/doi:10.5061/dryad.kv02g .

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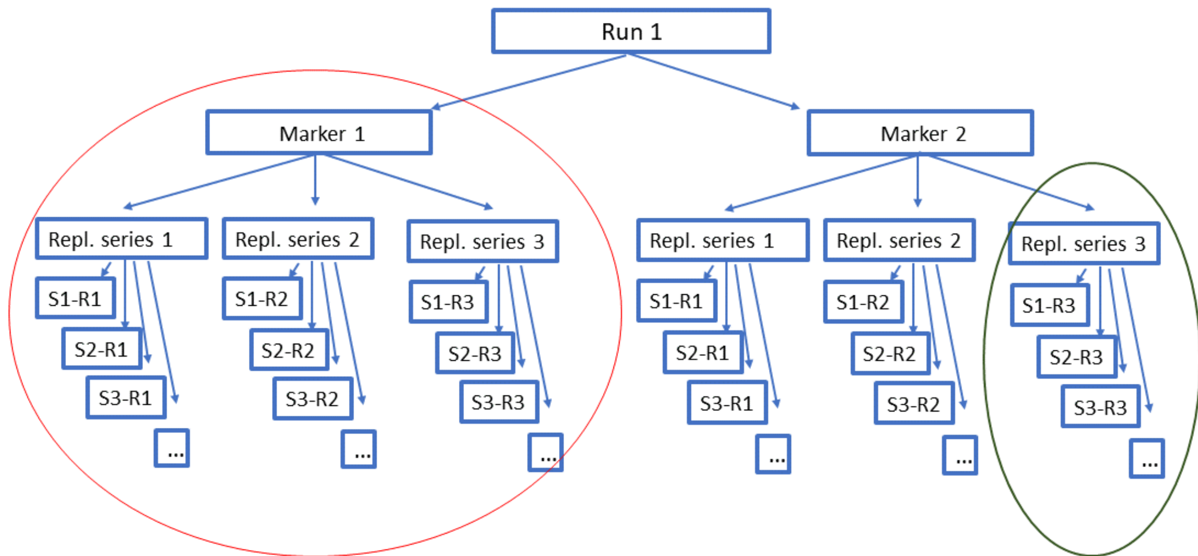
305 # Figures and tables

306



307

308 **Figure 1**. An example of a data structure with one run, two markers and
309 three replicates for each sample. S1-R1: Replicate1 of Sample1. Replicates
310 are not essential but strongly recommended. Samples should include at least
311 one mock sample and one negative control.

312

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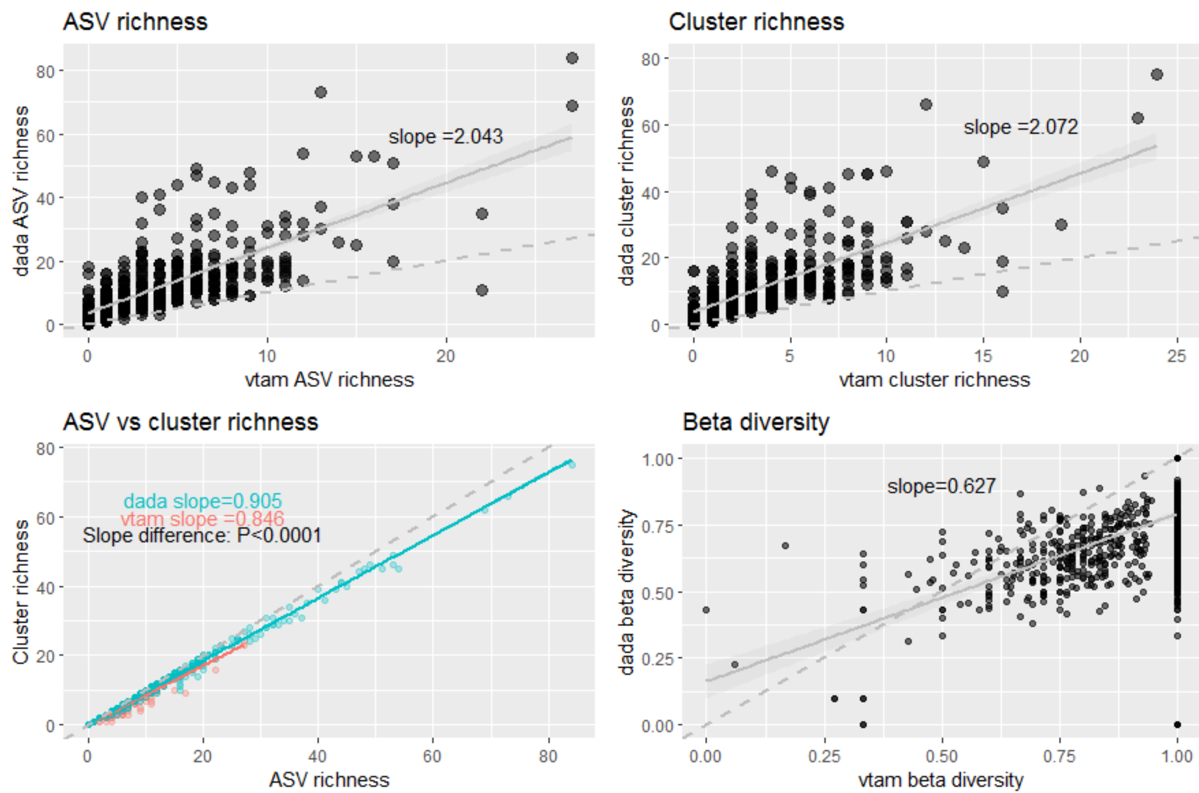
313



314

315 **Figure 2**. Diversity estimates from the fish and bat datasets, based on the
316 VTAM and DADA2-based pipelines. A) ASV richness per sample B) cluster
317 richness per sample C) The correlation between ASV and cluster richness. *P*-
318 *value* indicates a significant slope difference between the two pipelines.
319 D) β-diversity was estimated using the Bray-Curtis dissimilarity index
320 calculated for each pairwise sample comparison. Solid lines indicate linear
321 regression lines, hatched lines are the 1:1 reference lines.

322

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323

324 **Table 1.** List of VTAM commands and their roles.

325

VTAM command	VTAM step (Name in Corse et al. 2017)	Role	Error Type
merge		Merges paired-end reads and quality filtering	Sequencing errors
sortreads		Assigns reads to samples	Sequencing errors
filter	Dereplicate	Dereplicates	
filter	Delete singletons	Deletes singletons	Sequencing errors, highly spurious sequences
filter	LFN_variant filter (LFNtag)	Deletes low frequency errors	Tug jump, inter sample contamination
filter	LFN_read_count filter (LFNneg)	Deletes low frequency errors	Sequencing error, light contamination
filter	LFN_sample_replicate filter (LFNpos)	Deletes low frequency errors	Sequencing error, light contamination
filter	FilterMinReplicateNumber	Ensures consistency between replicates	PCR heterogeneity
filter	FilterPCRerror (Obliclean)	Eliminates PCR errors (even if frequent)	PCR errors
filter	FilterChimera	Eliminates chimeras	Chimeras
filter	FilterRenkonen	Eliminates aberrant replicates	Dysfunctional PCRs
filter	FilterIndel (Pseudogene filter)	Eliminates pseudogenes	Pseudogenes, spurious sequences
filter	FilterCondonStop (Pseudogene filter)	Eliminates pseudogenes	Pseudogenes, spurious sequences
taxassign	(LTG)	Assigns variants to taxa	Highly spurious sequences
optimize	OptimizeLFNsampleReplicate	Finds the optimal parameter for the LFN-sample-replicate filter	
optimize	OptimizePCRerror	Finds the optimal parameter for FilterPCRerror	
optimize	OptimizeLFNreadCountAndLFN-variant	Finds the optimal value for LFN-read-count and LFN-variant filters	
pool		Pools the results from different runs/markers	

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329 **Table 2.** Number of false positive occurrences compared to the total number
330 of occurrences. In negative control and mock samples, the count of false
331 positives is precise, since the sample composition is known.

	VTAM Fish	DADA Fish	VTAM Bat	DADA Bat
Negative controls	0/0 (0%)	32/32 (100%)	2/2 (100%)	19/19 (100%)
Mock samples	5/17 (29%)	37/49 (75%)	22/61 (36%)	73/114 (65%)

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334 ## Supporting Information

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336 **SuppInfo1.pdf**

337 Description of the taxonomic assignation and its custom database.

338 **SuppInfo2.pdf**

339 Commands, user input files, and the final ASV tables produced by VTAM
340 and the DADA based pipeline for the fish and the bat datasets.

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342 **SuppInfo3.pdf**

343 Diversity estimation protocol