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SimpleMetaPipeline: Breaking the bioinformatics bottleneck in metabarcoding

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Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online.

SimpleMetaPipeline-2.0.1.zip
SimpleMetaPackage-1.2.2.zip

Abstract

1. The democratisation of next-generation sequencing has vastly increased the availability of sequencing data from metabarcoding. However, to effectively prepare these metabarcoding data for subsequent analysis, researchers must consistently apply several different bioinformatic tools – from denoising and clustering to assignment. This often creates a bioinformatics bottleneck in workflows due to three challenges: A) integrating different tools; B) the inability to easily modify and rerun bioinformatic pipelines involving non-scripted ("point-and-click") elements; and C) the multiple outputs that may be required of a single dataset (e.g. Amplicon Sequence Variants (ASVs) and Operational Taxonomic Units (OTUs)), which often results in users running pipelines multiple times.
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Data/Code for peer review statement

No data is used in this manuscript. Code for both SimpleMetaPipeline and SimpleMetaPackage are archived on Zenodo and development versions are available at GitHub repositories, these links have been removed for peer review, but will be reinserted for publication. For the purposes of peer review zip files of each repository have been provided. All code is anonymised.

Key words

Bioinformatics pipeline; metabarcoding; next-generation sequencing; Autonomous Reef Monitoring Structures; eDNA; R

1 Introduction

There is a growing interest in applying next-generation sequencing to a wide range of ecological questions. Metabarcoding or marker gene amplicon sequencing can now rapidly deliver an in-depth and complementary perspective on ecological communities to that provided by traditional biomonitoring (Porath-Krause et al., 2022). The declining cost of these approaches has resulted in increasing adoption across ecological specialisms, thus generating vast amounts of raw sequencing data (Kodama et al., 2012). This includes published data, which can often be utilised to answer questions quite different from those the original authors intended if the data is published accessibly (Shea et al., 2023), and if it can be readily reanalysed.

However, there is a bottleneck in using these approaches for high-throughput environmental monitoring at the bioinformatics step, which is required to convert raw sequencing data into annotated community matrices that can be used in analysis (Porath-Krause et al., 2022). The importance of this bottleneck is amplified by the need to apply consistent bioinformatic processing in order to compare datasets, and thus the common need for bioinformatics to be rerun on published raw sequencing data for meta-analyses. This bioinformatics bottleneck is due to challenges in three areas. (i) Ease-of-use (Bolyen et al., 2019), that is, the extent to which the integration of different tools with a variety of native formats is facilitated. (ii) Reproducibility (Powers and Hampton, 2019; Wratten et al., 2021), in general the ability to re-generate identical results from raw data, and in this case specifically the ability to easily modify and rerun bioinformatic pipelines using non-scripted ("point-and-click") elements. And (iii) parallel outputs (e.g. Antich et al., 2021), which relates to users needing to run pipelines multiple times to generate different sequence annotations. Herein we define annotations as any information generated about a sequence, including with which other sequences from the dataset they form clusters, and any taxa to which they can be assigned.

Existing tools currently tend to trade-off ease-of-use against reproducibility. They either provide GUIs and other point-and-click solutions to increase users' accessibility (see e.g. mifish (Sato et al., 2018); q2galaxy (Bolyen et al,. 2019); and APSCALE (Buchner et al., 2022)), thereby limiting reproducibility. Alternatively they are entirely scripted, thereby enhancing reproducibility but requiring computing skills beyond those of the general user (e.g. mothur (Schloss and Westcott, 2011) and other QIIME2 interfaces (Bolyen et al,. 2019)). It should be noted that in the case of QIIME2 extensive documentation and an active user community and forum provide an excellent learning opportunity for new users.

To our knowledge, none of the existing tools enable the easy and efficient generation of parallel outputs. Examples of parallel outputs include the concurrent generation of both Amplicon Sequence Variants (ASVs, also known as Exact Sequence Variants (ESVs)), and Operational Taxonomic Units (OTUs), or taxonomic assignments from multiple assignment algorithms. Parallel outputs are important as it is now common practice for metabarcoding studies to present results for both ASVs and OTUs as a way to explore the influence of taxonomic resolution on their results (Antich et al., 2021). Furthermore, the taxonomic assignment of sequences is a source of uncertainty in metabarcoding studies as all methods have their strengths and weaknesses (Hleap et al., 2021); and comparing the assignments from multiple assignment algorithms is one way to address this. This need for parallel outputs can be problematic if running a pipeline multiple times to generate parallel outputs introduces slight differences, making results incomparable. This problem is avoidable if identical commands are run within identical computing environments, but achieving this manually requires computational knowledge, can be time consuming, and is subject to user error that can be impossible to trace (Gruning et al., 2018; Mangul et al., 2019).

Here we present SimpleMetaPipeline, an easy-to-use, entirely scripted bioinformatics pipeline producing parallel outputs. It is open-source, implemented in R, and combines well-established bioinformatics tools. Implementing the pipeline in R helps make the source code more accessible to users, given the widespread use of R in ecology, and is appropriate given that multiple bioinformatic tools are native to R (DADA2, LULU and IDTAXA). It should be noted that scripted (i.e. non-interactive) pipelines in R are highly shareable, maintainable and reusable – as is the case for any programming language – unlike interactive command line pipelines (Djaffardjy et al., 2023). SimpleMetaPipeline requires a single short R script, defining all parameters, to be run alongside a correctly formatted directory of raw fastq files, including as many Illumina sequencing runs as desired. From this, the pipeline reproducibly generates a sequence data table containing denoised ASVs as rows, and columns containing all parallel clustering and assignment annotations.

SimpleMetaPipeline is novel in three important ways. First, it is both easy to use, requiring only a single R script to be run, and has guaranteed reproducibility from this single R script, where other pipelines focus on either ease-of-use or reproducibility. Second, it combines existing bioinformatic tools unavailable in other pipelines, e.g. combining IDTAXA assignment (Murali et al., 2018) with LULU sequence curation (Frøslev et al., 2017). Third, it utilises an underlying sequence data table structure to efficiently handle parallel outputs. Specifically, SimpleMetaPipeline retains all bioinformatic annotations produced in an accessible form in the output. This has the added benefit of enabling testing for agreement between the parallel outputs of multiple algorithms, providing new opportunities to improve inferences from next-generation sequencing data.

2 Overview and workflow

SimpleMetaPipeline integrates bioinformatics tools to trim, denoise, cluster and taxonomically assign raw, demultiplexed, input amplicon datasets from multiple Illumina sequencing runs. These tools include: Cutadapt v3.5 (trimming; Martin, 2011); DADA2 v1.24.0 (denoising; Callahan et al., 2016); VSEARCH v.2.4.1 (clustering; Rognes et al., 2016); Swarm v3.1 (clustering; Mahé, 2015); LULU v0.1.0 (clustering; Frøslev et al., 2017); DECIPHER v2.24.0 (taxonomy assignment with the IDTAXA function; Murali et al., 2018); and BLAST v.2.9.0-2 (taxonomy assignment; Atschul et al., 1990).

The pipeline starts by using DADA2's robust error estimation to generate a reliable list of all ASVs present and their frequencies across samples (Callahan et al., 2016). All subsequent tools in the pipeline are then applied to these ASVs, and their standard outputs are captured. Firstly, LULU is used to annotate each ASV with the "curated ASV" to which it belongs (Frøslev et al., 2017). LULU curation uses sequence similarity and distribution to cluster sequences together, these clusters are thus sometimes referred to as "distribution-based OTUs" (Frøslev et al., 2017). Secondly, either VSEARCH or Swarm (according to user-specified preference) is used to annotate each ASV with the OTU to which it belongs (note that these are similarity-based OTUs specifically; Mahé, 2015; Rognes et al., 2016); then (in the only step not applying directly to ASVs) LULU is applied to "curate" these OTUs, and each ASV is then annotated with the "curated OTU" to which it belongs (Frøslev et al., 2017). Even in this case information is recorded for each ASV independently. Thus, there are always three types of clusters produced by SimpleMetaPipeline, depending on the option chosen these will either be LULU, VSEARCH, and VSEARCH+LULU; or LULU, Swarm, and Swarm+LULU. (SimpleMetaPipeline is not designed to compare VSEARCH and Swarm clusters within a single pipeline run).

Finally, if desired, the pipeline will assign taxonomy to ASVs. IDTAXA can be used to annotate each ASV with a k-mer-based taxonomic assignment (Murali et al., 2018). BLAST can be used to annotate each ASV with a similarity-based taxonomic assignment (Atschul et al., 1990). This creates a range of information about each ASV, including both the assignments themselves and various metrics quantifying the degree of uncertainty associated with these assignments. We provide a workflow diagram to illustrate the input data required; steps in the pipeline; and outputs (Figure 1).

Bioinformatic tools were chosen based on their frequency of use and their complementarity. Crucially no preference was given to tools based on their native format. Combining DADA2, VSEARCH, Swarm and LULU in a single pipeline provides all of the most commonly used sequence and clustering outputs in parallel (e.g. Antich et al., 2021; Brandt et al., 2021). IDTAXA and BLAST were combined as they determine taxonomic assignment of sequences in radically different, but widely accepted and well-justified ways, with BLAST tending to minimise under-classifications and IDTAXA minimising over-classifications (Altschul et al., 1990; Murali et al., 2018). Comparing the two assignments can thus increase the confidence in an assignment (if a conservative approach is taken where agreement between algorithms is required), or help understand the degree of uncertainty (e.g. by calculating the proportion of ASVs in a cluster which received the same assignment from both algorithms at a given rank).

The pipeline requires each of the tools previously listed to be installed, along with R version 4.2 (R Core Team, 2022) and the following R packages: SeqinR v4.2-16 (Charif and Lobry, 2007), ShortRead v1.54.0 (Wilkonson et al., 2008), gridExtra v.2.3 (Auguie et al., 2017), ggplot2 v.3.4.0 (Wickham, 2011), and dplyr v1.1.1 (Wickham et al., 2023). SimpleMetaPipeline source code is

available for UNIX/Linux and macOS environments and is archived here: [*anonymised*]. The development version can be accessed on GitHub at [*anonymised*], where installation instructions are available.

A supporting R package is also provided, which can quickly and reproducibly generate a variety of standardised annotated community matrices from the parallel outputs stored in a sequence data table (e.g. matrices reflecting OTUs or ASVs, or including taxonomic assignments produced by IDTAXA or those produced by BLAST). This division of functionality between pipeline and package is thus crucial to enabling efficient handling of parallel outputs. Specifically, the package generates "phyloseq objects", derived from the Phyloseq R package commonly used in the analysis of metabarcoding data (McMurdie and Holmes, 2013). The package source code is archived here: [*anonymised*] and the development version can be accessed on GitHub at [*anonymised*] where installation instructions are available.

3 Input data preparation and parameter choices

3.1 Control scripts

SimpleMetaPipeline requires running a single R script, known as a control script. An example control script is provided in the codebase with sensible defaults (or guidance where a sensible default value is impossible) for all adjustable parameters. The example also includes detailed descriptions of what each adjustable parameter controls and links to underlying tools where applicable. Where parameters for underlying tools do not appear in the control script they are not adjustable and the default values are used.

3.2 Demultiplexed fastq directory

SimpleMetaPipeline accepts demultiplexed paired-end or single read fastq or fastq.gz files, with each R1/R2 pair or single read file named by sample. These files can be generated from any marker gene amplicon, and SimpleMetaPipeline has been tested with COI gene, 18S rRNA gene, 16S rRNA gene, ITS rRNA gene, 23s rRNA gene and 12s rRNA gene marker datasets. The fastq files from each Illumina sequencing run should be stored in separate directories. This is important as it allows DADA2 denoising to learn error rates for each sequencing run independently (Callahan et al., 2016). In some cases, samples may appear multiple times across a batch of sequencing runs (as commonly occurs in multi-run experiments to address low quality or failed sequencing of certain samples). SimpleMetaPipeline can handle this scenario as a unique sequencing run identifier is automatically appended to each sample name, allowing decisions about how to handle these duplicates to be made downstream, without needing to rerun bioinformatics.

3.3 Taxonomic assignment

An appropriate IDTAXA classifier and/or BLAST database, generated from any reference library one wishes to use, will need to be provided alongside the fastq files if sequence classification is required. Details of how to generate IDTAXA classifiers and BLAST databases are provided by each of these tools respectively (Altschul et al., 1990; Murali et al., 2018).

4 Outputs

4.1 Sequence data table

SimpleMetaPipeline outputs a sequence data table with ASVs as rows and information on each ASV generated by the pipeline as columns. Columns contain ASV annotations themselves - e.g.

OTU2, or *Taxa3* – and useful information about these annotations (Table 1). This information includes a variety of assignment certainty measures provided by the underlying algorithms: sequence similarity and e-value from the BLAST algorithm and assignment confidences from the IDTAXA algorithm; as well as TRUE/FALSE values showing whether ASVs were identified as representative sequences of their clusters.

4.2 Diagnostic outputs

SimpleMetaPipeline generates additional outputs that enable the inspection of performance of different steps in the pipeline. These diagnostic outputs include a set of tables displaying: 1) a count of all primer sequences removed by cutadapt; 2) the number of dereplicated sequences in each sample at each DADA2 step (input, filtering, denoising, merging and chimera removal); 3) the distribution of ASV lengths (number of bases); and 4) the number of clusters produced under each parallel clustering approach. Further, standard diagnostic figures are provided from DADA2 (quality profiles and error plots) and IDTAXA (taxonomic assignment plot).

5 Examples and benchmarking

5.1 Comparison and multi-algorithm agreement

Sequence data tables, as output by SimpleMetaPipeline, enable easy comparison between clustering and assignment methods. This allows testing for multi-algorithm agreement to better understand uncertainties in annotations. Such tests can be conducted for agreement between 1) clustering algorithms, 2) assignment algorithms, and 3) clustering and assignment algorithms (Figure 2). The concept of multi-algorithm agreement tests is that the different annotations given to ASVs by the robust and widely-used, yet methodologically distinct, algorithms deployed in SimpleMetaPipeline each contain information about the biology of the ASV.

In the case of two clustering algorithms there is no straightforward rule which can be applied to require agreement. However, the variation between clustering algorithms can be used to interrogate clusters of interest to understand their potential relationship to other clusters and internal sequence diversity. In the case of two assignment algorithms SimpleMetaPackage enables the application of the conservative rule of, for each sequence at each taxonomic rank, only accepting a taxonomic assignment agreed upon by both algorithms. In the case of agreement between clustering and assignment algorithms (e.g. testing whether all sequences in a cluster receive the same assignment) SimpleMetaPackage enables phyloseq objects to be generated with clusters receiving taxonomic assignments only if the proportion of their reads receiving that annotation is above a user specified threshold. For example, if this threshold is set to 85% for a given rank then, for each cluster at that taxonomic rank, an assignment is only accepted if at least 85% of reads from that cluster have received the assignment at that rank.

5.2 Benchmarking speed and memory

Run times and resource requirements for multi-step bioinformatic processing of metabarcoding data vary depending on marker genes, sequencing depth, and the number of sequencing runs processed together. If algorithms, bioinformatic parameters and reference databases are also adjustable, as in the case of SimpleMetaPipeline, then this variation is further increased. We do not attempt to exhaustively benchmark how all combinations of these variables influence run times and resource requirements. However, by benchmarking pipeline performance in processing published datasets we provide real world examples of what users can expect.

We conducted all benchmark runs on a laptop with a 4-core CPU and 32GB of RAM. All benchmark runs included all SimpleMetaPipeline steps, including taxonomic assignment, and made

use of different reference databases appropriate to the marker gene. See Table 2 and Supplementary Information for full details. In the case of single Illumina MiSeq runs a relatively shallowly sequenced COI dataset (total raw reads = ca. 11 million; samples = 20) completed in 3.5 hours, whereas a more deeply sequenced 23S rRNA dataset (total raw reads = ca. 22 million; samples = 20) completed in 11.5 hours. Multiple MiSeq runs take substantially longer, for a given depth of sequencing, due to the previously noted requirement that DADA2 learns the error rate for each MiSeq run separately (Callahan et al., 2016). A dataset of four shallowly sequenced 18S rRNA gene MiSeq runs (total raw reads = ca. 15 million; samples = 238), where the sequences were merged before publication substantially speeding up the DADA2 step while reducing its reliability, completed in 13.5 hours. Finally, a dataset of three shallowly sequenced 16S rRNA gene MiSeq runs (total raw reads = ca. 27 million; samples = 110) completed in 32 hours. These figures are intended to provide an indication of orders of magnitude, while making clear that exact results will vary depending on the variables mentioned previously.

The performance of the pipeline is largely dependent on the underlying algorithms that compose it and different algorithms within the pipeline scale differently as the number of input sequences increases. The time required for denoising with DADA2 and assignment with BLAST and IDTAXA scales roughly linearly, but the time required for clustering with LULU, VSEARCH and Swarm scales exponentially. Further, the memory requirements can become large when large numbers of MiSeq runs (>10 runs) are processed together (LULU), or a large taxonomic classifier (>1 GB) is used (IDTAXA) thus requiring the use of a high performance computing cluster. All algorithms used are parallelised, thus enabling substantial speed improvements from the use of additional cores if running the pipeline on a high performance cluster.

6 Concluding remarks

SimpleMetaPipeline provides a novel and accessible tool that generates robust bioinformatic outputs and usable annotated community matrices from raw metabarcoding data. It will be particularly useful for workers with a knowledge of R but a limited background in bioinformatics (a common combination in ecology) and where: (a) multiple sequencing runs need to be compared, as in large projects and meta-analyses; (b) there is uncertainty about what outputs are required; or (c) there is an established need for multiple parallel outputs, such as ASVs and OTUs. It thus represents a valuable open-source addition to the existing library of pipelines, helping democratize bioinformatics in ecology.

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Table 1: An example of sequence data table format if both IDTAXA and BLAST assignment options are selected. Note that this table is transposed to aid presentation. Column names, as output from the pipeline, are abbreviated and do not include spaces.

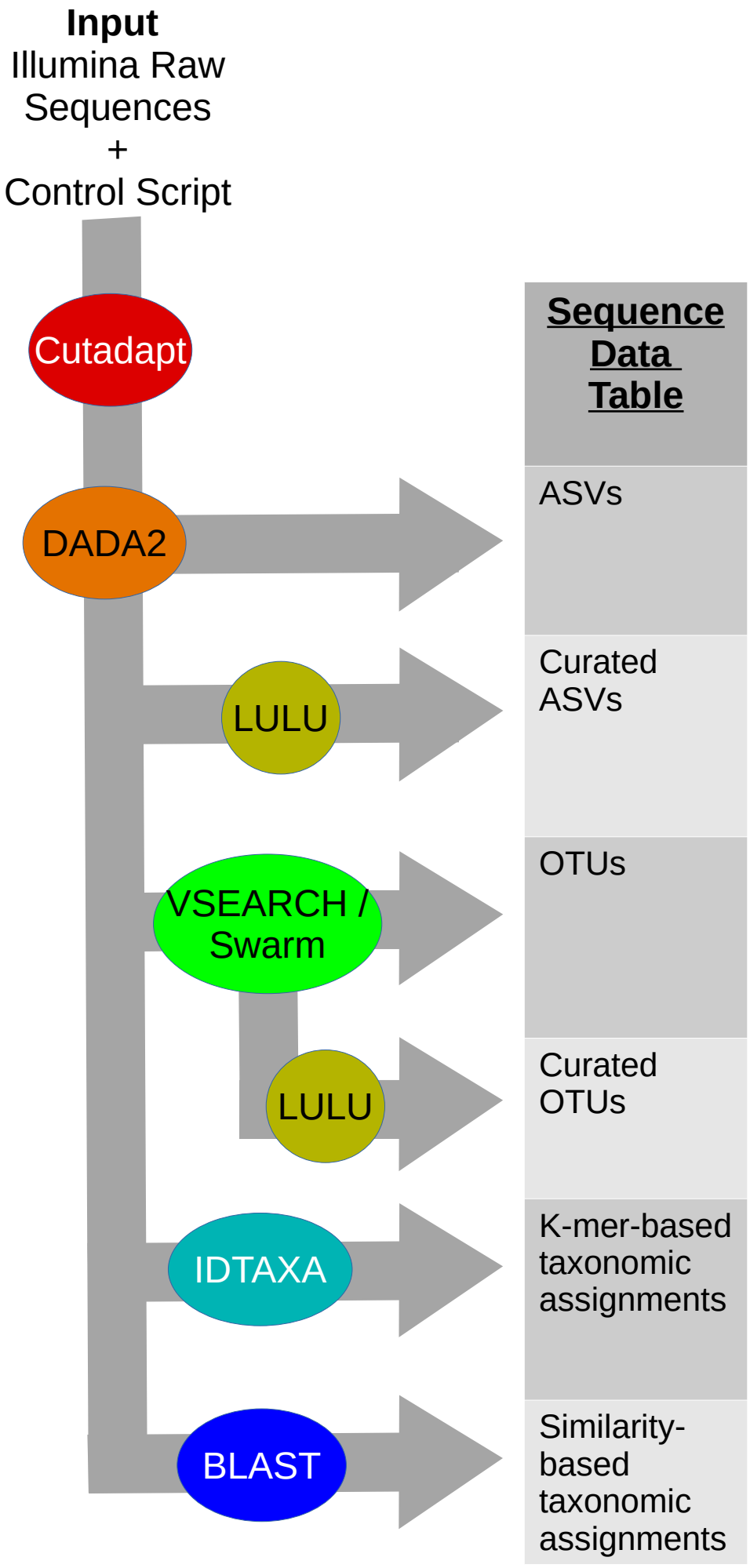
Source of Output	Column Description	Example row content				
		Example row 1	Example row 2	Example row 3	Example row 4	Example row 5
DADA2	ASV Sequence	ASV1 TACG...	ASV2 ATTT...	ASV3 GTAC...	ASV4 CCTT...	ASV5 AAAT…
	Sample 1	11	0	4	589	98
	…	…	…	…	…	…
	Sample n	34	55	0	0	7
LULU	Curated ASV	ASV1	ASV1	ASV2	ASV2	ASV2
	Curated ASV Representative Sequence	1	0	0	0	1
VSEARCH/ Swarm	OTU	OTU1	OTU1	OTU1	OTU2	OTU2
	OTU Representative Sequence	1	0	0	0	1
VSEARCH/ Swarm + LULU	Curated OTU	OTU1	OTU1	OTU1	OTU1	OTU1
	Curated OTU Representative Sequence	1	0	0	0	0
IDTAXA	Rank 1	Taxa1	Taxa2	Taxa1	Taxa3	Taxa3
	…	…	…	…	…	…
	Rank n	Taxa4	NA	Taxa5	Taxa6	Taxa7
	Rank 1 Confidence	100	43	78	81	83
	…	…	…	…	…	…
	Rank n Confidence	46	0	46	55	63
BLAST	Blast Percent Identical	98	77	89	88	92
	Blast evalue	0	0	0	0	0
	Blast Query Coverage	99	100	100	97	58
	Rank 1	Taxa1	Taxa2	Taxa1	Taxa3	Taxa3
	…	…	…	…	…	…
	Rank n	Taxa4	NA	Taxa5	Taxa6	Taxa7

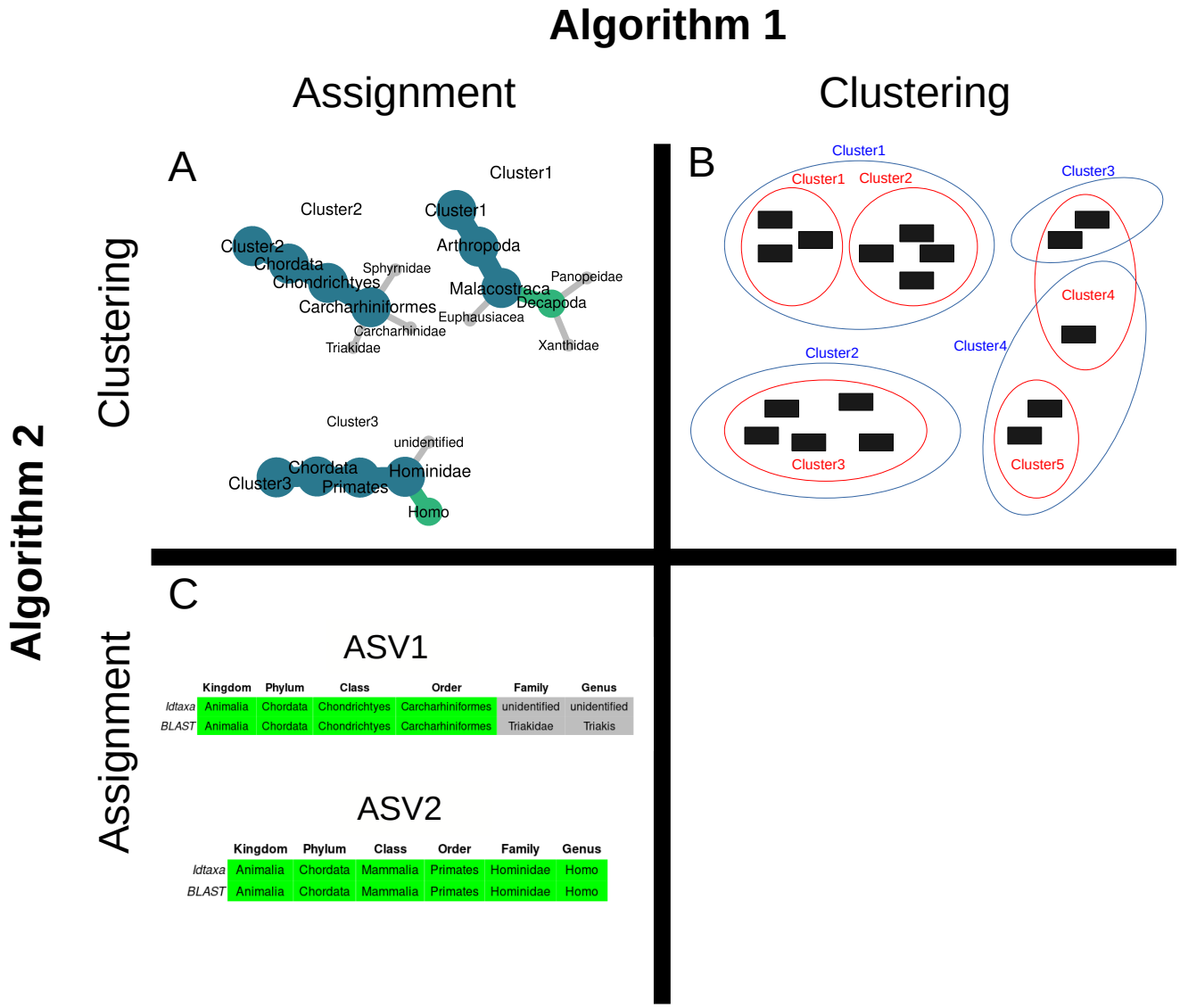
Table 2: SimpleMetaPipeline benchmarking results for published datasets, including taxonomic classification. All benchmarks performed on a laptop with a 4 core CPU and 32GB of RAM. All files were input in fastq.gz format. See Appendix for ControlScripts used in each benchmark run.

Maker Gene	Publication	Reference Database	# MiSeq Runs	FASTQ type	# FASTQ files	Total reads (nearest million)	Amplicon length range	Total size of input (GB)	Time required (hours)
23S rRNA	Williams et al., In press	µgreen-db (Djemel et al., 2020)	1	Paired-end	40	22 million	350-370	9.9	11.5
COI	Steyaert et al., 2020	MIDORI2 (Leray et al., 2022)	1	Paired-end	40	11 million	280-360	5.4	3.5
18S rRNA	DiBattista et al., 2020	SILVA (Quast et al., 2012)	4	Pre-merged	238	15 million	320-430	1.6	13.5
16S rRNA	Williams et al., In press	GTDB (Parks et al., 2022)	3	Paired-end	220	27 million	240-270	4.1	32

Figure 1: Diagram of the SimpleMetaPipeline workflow. Ovals represent the different steps in the pipeline and the order in which they occur – either in series or in parallel. The table on the right represents the format of the output "Sequence Data Table" (as shown in Table 1) in simplified graphical form. Arrows indicate the step in the pipeline where each set of information in the Sequence Data Table is generated.

Figure 2: Varieties of multi-algorithm agreement. Only two-way algorithm agreements are visualised, three-way and four-way algorithm agreement tests are also possible by combining the two-way varieties visualised here. A) Agreement between assignment and clustering algorithms. Three clusters are shown, with the proportion of component ASVs assigned to each taxa at each rank visualised, with taxonomic assignments in large blue circles representing those received by all component ASVs. For example, Cluster1 contains 3 ASVs all assigned to the phylum Arthropoda and class Malacostraca, but they are assigned to different orders (Decapoda and Euphausiaceae). A conservative approach would therefore be to assign the cluster to the class Malacostraca but leave it unidentified at lower ranks. B) Agreement between clustering algorithms. Two parallel clustering outputs are shown (red and blue ovals containing ASVs represented by black bars). For example, the blue Cluster1 contains two red clusters containing 3 and 4 ASVs each. In this case agreement and disagreement between clustering algorithms provides additional information to interrogate the internal structure of, or potential relationships between, specific clusters of interest. C) Agreement between assignment methods. Two ASVs are shown, each receiving an assignment from both IDTAXA and BLAST. ASV1 receives diverging assignments at lower ranks (family and genus), while ASV2 receives the same assignment from both algorithms at all ranks. A conservative approach would therefore assign ASV1 to the Order Charchariniformes but leave it unidentified at lower ranks.


Input
Illumina Raw Sequences
+
Control Script
Cutadapt
DADA2
LULU
VSEARCH / Swarm
LULU
IDTAXA
BLAST
Sequence Data Table
ASVs
Curated ASVs
OTUs
Curated OTUs
K-mer-based taxonomic assignments
Similarity-based taxonomic assignments


Algorithm 1
Assignment
Clustering
Algorithm 2
Clustering
Assignment
A
Cluster1
Cluster2
Cluster1
Cluster2
Chordata
Chondrichtyes
Sphyrnidae
Carcharhiniformes
Carcharhinidae
Triakidae
Arthropoda
Malacostraca
Decapoda
Panopeidae
Euphausiacea
Xanthidae
Cluster3
unidentified
Chordata
Cluster3
Primates
Hominidae
Homo
B
Cluster1
Cluster1
Cluster2
Cluster3
Cluster4
Cluster2
Cluster3
Cluster4
Cluster5
C
ASV1
Kingdom
Phylum
Class
Order
Family
Genus
Idtaxa
Animalia
Chordata
Chondrichtyes
Carcharhiniformes
unidentified
unidentified
BLAST
Animalia
Chordata
Chondrichtyes
Carcharhiniformes
Triakidae
Triakis
ASV2
Kingdom
Phylum
Class
Order
Family
Genus
Idtaxa
Animalia
Chordata
Mammalia
Primates
Hominidae
Homo
BLAST
Animalia
Chordata
Mammalia
Primates
Hominidae
Homo