PEMA: from the raw .fastq files of 16S rRNA and COI marker genes to the (M)OTU-table, a thorough metabarcoding analysis

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Abstract

Background: Environmental DNA (eDNA) and metabarcoding, allow the identification of a mixture of individuals and launch a new era in bio- and eco-assessment. A number of steps are required to obtain taxonomically assigned (Molecular) Operational Taxonomic Unit ((M)OTU) tables from raw data. For most of these, a plethora of tools is available; each tool's execution parameters need to be tailored to reflect each experiment's idiosyncrasy. Adding to this complexity, for such analyses, the computation capacity of High Performance Computing (HPC) systems is frequently required.

Software containerization technologies ease the sharing and running of software packages across operating systems; thus, they strongly facilitate pipeline development and usage. Likewise are programming languages specialized for big data pipelines, incorporating features like roll-back checkpoints and on-demand partial pipeline execution.

Findings: PEMA is a containerized assembly of key metabarcoding analysis tools with a low effort in setting up, running and customizing to researchers' needs. Based on third party tools, PEMA performs reads' pre-processing, clustering to (M)OTUs and taxonomy assignment for 16S rRNA and COI marker gene data. Due to its simplified parameterisation and checkpoint support, PEMA allows users to explore alternative algorithms for specific steps of the pipeline without the need of a complete re-execution. PEMA was evaluated against previously published datasets and achieved comparable quality results.

Conclusions: Given its time-efficient performance and its quality results, it is suggested that PEMA can be used for accurate eDNA metabarcoding analysis, thus enhancing the applicability of next-generation biodiversity assessment studies.

Keywords:

Pipeline, Container, Docker, Singularity, High Performance Computing, (HPC), eDNA, metabarcoding

Background

Metabarcoding inaugurates a new era in bio- and eco-monitoring. However, from the output of a sequencer to an amplicon study analysis results, it takes a long way.

Well-established pipelines are available to process metabarcoding data (mothur [1], QIIME 2 [2], LotuS [3]) for the case of 16S rRNA marker gene and bacterial communities. However, there is none that can be used in a straightforward way for metabarcoding analysis of eukaryotic organisms. For this to be functional, adaptation to other marker genes (e.g COI) is required. Furthermore, the pipelines mentioned above, although entrenched, they still suffer from a series of hurdles: technical difficulties in installation and use, strict limitations in setting parameters for the algorithms invoked, incompetence in partial re-execution of an analysis, are among the most prominent.

Moreover, given the computational demands of such analyses, access to High Performance Computing (HPC) systems, might be mandatory, for example, to process studies with large number of samples. This is rather timely given the ongoing investment of national and international efforts (for example [4]) to serve the broad biological community via commonly accessible infrastructures.

PEMA is an open-source pipeline that bundles state-of-the-art bioinformatic tools for all necessary steps of amplicon analysis and aims to address the issues mentioned above. It is designed for paired-end sequencing studies and is implemented in the BigDataScript (BDS) [5] programming language. BDS's *ad hoc* task parallelism and task synchronization, supports heavyweight computation which PEMA inherits. In addition, BDS supports *checkpoint* files that can be used for partial re-execution and crash recovery of the pipeline. PEMA builds on this feature to serve tool and parameter exploratory customization for optimal metabarcoding analysis fine tuning. Switching effortlessly between clustering algorithms is a pertinent example. Finally, via the Docker [6] and Singularity [7], the latter HPC-centered, software containerization technologies, PEMA is distributed in an easy to download and install fashion on a range of systems from regular computers, to cloud or HPC environments.

Beyond the technical aspects and from the biology perspective, PEMA supports the metabarcoding analysis of both prokaryotic communities (based on the 16S rRNA marker gene) and eukaryotic ones (based on the COI marker gene).

Two clustering algorithms, Swarm v2 [8] and CROP [9], are employed for the clustering of reads in Molecular Operational Taxonomic Units (MOTUs) in the COI marker gene case. VSEARCH [10] is used for the 16S rRNA gene case. Taxonomy assignment is performed in an alignment-based approach, making use of the CREST LCAClassifier [11] and the Silva database [12]. For the COI marker gene, the RDPClassifier [13] and the MIDORI database [14] are used. In the 16S marker gene case, phylogeny-based assignment is also supported, based on RAxML-ng [15], EPA-ng [16] and Silva, as well as ecological and phylogenetic analysis via the "phyloseq" R package [17].

All the pipeline-controlling and third-party-module parameters are defined in a plain *parameter-value* *pair* text file. Its straightforward format eases the analysis fine tuning complementary to the aforementioned *checkpoint* mechanism. A tutorial about PEMA and installation guidance can be found on PEMA's GitHub repository (https://github.com/hariszaf/pema).

Implementation

PEMA's architecture comprises four main parts taking place in tandem (Figure 1). Detailed description of the tools invoked by PEMA and their licences is included in Additional file 1: Supplementary Methods.

Part 1: Quality control and pre-processing of raw data

Before all else, FastQC [18] is used to obtain an overall read-quality summary. Beyond this visual inspection and to correct the errors are produced by a sequencer, PEMA incorporates a number of tools. Trimmomatic [19] implements a series of trimming steps, namely: either to remove parts of the sequences corresponding to the adapters or the primers, or to trim and crop parts of the reads, or even remove a read completely, when it fails to the quality filtering sequences. BayesHammer [20], an algorithm of the SPAdes assembly toolkit [21], determines specific-position errors, where a particular base has been called incorrectly and revises them. PANDAseq [22] assembles the overlapping paired-end reads and then the 'obiuniq' program of OBITools [23] groups all the identical sequences in every sample, keeping a track of their abundances. The VSEARCH package [10] is invoked for the chimera removal.

Part 2: (M)OTUs clustering

Quality controlled and processed sequences are subsequently clustered into (M)OTUs. For the case of 16S rRNA marker gene, VSEARCH [10] is used. Among the VSEARCH clustering options, PEMA supports --*cluster_size* and --*cluster_unoise*.

For the COI marker gene, two different clustering algorithms are included. First, Swarm v2 [8], a fast and robust algorithm that produces fine-scale MOTUs, free of arbitrary global clustering thresholds and input-order dependency. As the Swarm v2 algorithm is not affected by chimeras (F. Mahé, personal communication), when Swarm v2 is selected, chimera removal occurs after the clustering. This leads to a computational time gain as chimeras are sought among MOTUs instead of unclustered reads.

Second, CROP [9], an unsupervised probabilistic Bayesian clustering algorithm that models the clustering process using Birth-death Markov chain Monte Carlo (MCMC). The CROP clustering algorithm is adjusted by a series of parameters need to be tuned by the user (namely *b*, *e* and *z*). These parameters depend on specific dataset properties like the length and the number of reads). PEMA, automatically adjusts *b*, *e* and *z* by collecting such information and applying the CROP recommended parameter-setting rules [9].

Any singletons occurring among the (M)OTUs after this step are removed.

Part 3: Taxonomy assignment

Alignment-based taxonomy assignment is supported for both the 16S rRNA and the COI marker gene analyses. In the 16S rRNA marker gene alignment-based case, the LCAClassifier algorithm of the CREST set of resources and tools [11], is used together with the Silva database [12] to assign taxonomy to the OTUs. Two versions of Silva are included in PEMA: 128 (Sept 29, 2016) and 132 (Dec 13, 2017).

For the COI marker gene, PEMA uses the RDPClassifier [13] and the MIDORI reference to assign taxonomy of the MOTUs. The MIDORI dataset [14] contains quality controlled metazoan mitochondrial gene sequences from GenBank [24].

Intended primarily for studies from less explored environments, phylogeny-based assignment is available for 16S rRNA marker gene data. PEMA maps OTUs to a custom reference tree of 1000 Silva-derived consensus sequences (created using RAxML-ng [15] and gappa (phat algorithm) [25], Figure 2A). PaPaRa [26] and EPA-ng [16] combine the OTU clustering output and the reference tree to produce a phylogeny-aware alignment and map the 16S rRNA OTUs to the custom reference tree. Beyond the context of PEMA, users may visualize the output with tree viewers like iTOL [27] (Figure 2B).

Part 4: (M)OTU and sample abundance tables, biodiversity downstream analysis

In both the 16S rRNA and COI marker gene analysis, an (M)OTU-table is returned by PEMA. For each sample of the analysis, a subfolder containing statistics about the quality of its reads, as well as the taxonomies and their abundances, is also generated.

For the 16S rRNA marker gene case, a downstream analysis of the OTUs including alpha- and beta-diversity analysis, taxonomic composition, statistical comparisons and calculation of correlations between samples is also supported, thanks to the “phyloseq” R package [17]. When this option is selected, then besides phyloseq’s output, a Multiple Sequence Alignment (MSA) and a phylogenetic tree of the OTUs are returned; for the MSA, the MAFFT [28] aligner is invoked while the latter is being built by RAxML-ng [15].

PEMA container-based installation

An easy way of installing PEMA is via its containers. A dockerized PEMA version is available at https://hub.docker.com/r/hariszaf/pema. Singularity users can *pull* the PEMA image from https://singularity-hub.org/collections/2295. Between the two containers, the Singularity-based one is recommended for HPC environments due to Singularity’s improved security and file accessing properties [29]. For detailed documentation, visit https://github.com/hariszaf/pema.

PEMA output

All PEMA-related files (i.e. intermediate files, final output, *checkpoint* files and per-analysis-parameters) are grouped in distinct (self-explanatory) subfolders per major PEMA pipeline step. In the last subfolder, the results are further split in folders per sample. This eases further analysis both within the PEMA framework (like partial re-execution for parameter exploration) or beyond.

Results and discussion

Evaluation

To evaluate PEMA, two publicly available datasets from published studies were used. For the 16S rRNA marker gene, the dataset reported by study [30] was used while for the COI case, the one of [31] (accession numbers: PRJEB20211 and PRJEB13009 respectively). In both cases, the respective .fastq files were downloaded from ENA-EBI using ‘ENA File Downloader version 1.2’ [32]. For both cases, PEMA was run on the in-house HPC cluster.

Comparison to existing software

By the means of evaluation, PEMA's features were compared with those QIIME 2 [2], mothur [1], and LotuS [3]. Table 1 presents a detailed comparison among the four tool features in terms of marker gene support, diversity and phylogeny analysis capability, parameter setting and mode of execution, operation system availability and HPC suitability. As shown, PEMA is equally feature-rich, if not richer in certain feature categories to the other software packages. In particular, PEMA's support for COI marker gene studies is distinctive; two methods for the taxonomy assignment are supported and PEMA's easy-parameter setting, step-by-step execution and container distribution render it user and analysis friendly.

Table 1: Pipeline comparison.

Feature	LotuS	QIIME 2	mothur	PEMA
16S rRNA	☑	☑	☑	☑
COI	☐	☐	☐	☑
ITS	☑	☑	☐	☐
diversity indices	☐	☑	☑	☑
alignment-based taxonomy assignment	☑	☑	☑	☑
phylogenetic- based taxonomy assignment	☑	☑	☐	☑
parameters assigned in the command line	☑	☑	☑	☐
parameters assigned through a text file	☑	☐	☐	☑
step-by-step execution	☑	☑	☑	☑
all steps in one go possible	☑	☐	☐	☑
available for any OS (Linux,	☐	☑	☑	☑

OSX, Windows)				
traditional application installation	☑	☑	☑	☑
available as a virtual machine	□	☑	□	□
available as a container	□	☑	□	☑
available for HPC as a container	□	□	□	☑

Comparison of the basic features of the different pipelines.

COI marker gene analysis evaluation

The selected study [31] created two COI libraries of different sizes: COIS (235 bp amplicon size) and COIF (658 bp amplicon size). The sequencing reads of COIS were selected for PEMA's evaluation; the COIF sequencing read pairs had no overlap so as to be merged.

Regarding the creation of the MOTU table, [31] used VSEARCH [10] with a clustering at 97% similarity threshold. Afterwards, the BLAST+ (megablast) algorithm [33] was used against a manually created database including all NCBI GenBank COI sequences of length >100 bp (June 2015) while excluding environmental sequences and higher taxonomic level information [31]. As discussed in the publication, this approach resulted in 138 unique MOTUs out of which 73 were assigned to species level. For PEMA's evaluation, the chosen clustering algorithm was Swarm v2, using different options for the cluster radius (*d*) parameter (Table 2); according to [8], this is the most important parameter as it affects the number of MOTUs that are being created. The resulting MOTUs were classified against the MIDORI reference database [14] using RDPClassifier [13]. The results of the processing of the sequences are shown in Additional file 2: Table S1.

As shown in Table 2, PEMA resulted in 83 species level MOTUs with a cluster radius (*d*) of 2, which is very similar to that of the published study (i.e. 73 species). Although both the clustering algorithm and the taxonomy assignment methods were different between [31] and the present study, the results regarding the number of unique species present in the samples are in agreement to a considerable extent.

Table 2: PEMA's output and executional time.

	d **= 1**	***d*** **= 2**	***d*** **= 3**	***d*** **= 10**	***d*** **= 13**
MOTUs after preprocess and clustering steps	83791	59833	33227	7384	4829
MOTUs after chimera removal	80347	57863	32539	7339	4796
Non singletons MOTUs	6381	4947	2658	1914	1634
Assigned species	62	83	86	86	84
Executional time (h)	02:01:35	02:09:49	01:51:44	02:17:26	02:31:15

PEMA's output and executional time (using a 20 core node) for different values of Swarm's *d* parameter.

The taxonomic assignment of the retrieved MOTUs is shown in Figures 3-4. Certain .fastq files contained very few reads, such as those for sample ERR1308241, and therefore resulted in zero MOTUs upon the completion of PEMA; thus, these samples are not included in Figure 3. It is worth mentioning that four of the 138 MOTUs were found in both the positive control samples of the published study as well as the present study (Table 3). Also, in three cases, PEMA resulted in the same genera as the positive control samples of the published study (Table 3).

Table 3: Comparison of the taxonomy of retrieved MOTUs among PEMA and [31] .

	PEMA	**[31]**
Taxonomies identical to species level	*Ablabesmyia monilis* *Crangonyx pseudogracilis* *Radix* sp. Chironomidae sp.	*Ablabesmyia monilis* *Crangonyx pseudogracilis* *Radix* sp. Chironomidae sp.
Taxonomies identical to genus level	*Ancylus* sp. *Athripsodes aterrimus*, *Athripsodes*	*Ancylus fluviatilis* *Athripsodes albifrons*

	cinereus	
	Chironomus sp., *Chironomus anthracinus*, *Chironomus pseudothummi*, *Chironomus riparius*	*Chironomus tentans*

The computational time required by PEMA for the completion of the analysis is also shown in Table 2. Regardless of the value of the *d* parameter, all analyses were completed in about 2 hours, ie. adequately fast to allow parameter testing and customization.

16S rRNA marker gene analysis evaluation

To evaluate PEMA's performance, a comparative analysis of the [30] dataset with QIIME 2 [2], mothur [1], LotuS [3] and PEMA was conducted.

It is known that the choice of parameters affects the output of each analysis; therefore, it is expected that different user choices might distort the derived outputs. For this reason and for a direct comparison of the pipelines, we have included all the commands and parameters chosen in the framework of this study in the Additional file 1: Supplementary Methods. The results of the processing of the sequences by PEMA are shown in Additional file 3: Table S2. All analyses were conducted on identical Dell M630 nodes (128GB RAM, 20 physical Intel Xeon 2.60GHz cores). LotuS, mothur and QIIME 2 operated in a single thread (core) fashion. PEMA, given the BDS intrinsic parallelization [5], operated with up to the maximum number of node cores (in this case 20).

The execution time and the reported OTU number of each tool are presented in Table 4. LotuS and PEMA resulted in a final number of OTUs comparable to that of [30]. Clearly, due to PEMA's parallel-execution support, the analysis time can be significantly reduced (~1.5 hours in this case).

Table 4: OTU predictions and executional time for the different pipelines.

	LotuS	mothur	QIIME 2		PEMA	[30]
			Deblur	DADA2		
Number of OTUs	9849	142669	517	1023	6028	7050
Executional time (h)	~9	~67 (~56 if the reference database is already built)	~2.5	~5	~1.5	~26

The executional time is depending on the parameters chosen for each software (see Additional file 1: Supplementary Methods).

Due to the non-full overlap of the sequence reads, mothur resulted in an inflated number of OTUs; thus, is was excluded from further analyses. The results of all the pipelines were analyzed with the phyloseq script that is provided with PEMA. The taxonomic assignment of the PEMA retrieved OTUs is shown in Figure 5. The phyla that were found in the samples are similar to the ones that were found in [30]. Although the lowest number of OTUs was found in the marine station (Kal) (Table 5), which is not in accordance with [30], the general trend of the decreasing number of OTUs with the increasing salinity was observed as it was in [30]. Notably, this result was not observed with the other tested pipelines (Table 5). Furthermore, each of the pipelines resulted in a different taxonomic profile (Additional files 4-6: Figure S1-3) with an extreme case of missing the Order of Betaproteobacteriales (Additional files 7-9: Figure S4-6).

Table 5: Diversity indices of the samples.

	LotuS		Deblur		DADA2		PEMA	
Samples	**OTUs**	**N**	**OTUs**	**N**	**OTUs**	**N**	**OTUs**	**N**

L_LOout_A	2451	67640	156	5878	177	47954	791	17888
L_LOout_B	3432	95835	180	5978	248	62416	1182	19571
L_LOout_C	2987	97592	180	9216	221	59346	1048	24947
L_LOinA	3656	85882	200	6284	264	62253	1176	18346
L_LOinB	2935	76545	173	6357	194	46191	954	18750
L_LOinC	3149	71222	183	5849	219	49890	1012	15940
S_KalA	2467	59039	82	2991	173	46418	668	10086
S_KalB	2940	57091	89	2107	192	49715	802	7702
S_KalC	2898	61191	101	2934	214	51321	769	8840
R_ARDelta_A	3079	53780	97	1341	259	53237	737	4386
R_ARDelta_B	3671	63857	117	1893	271	62477	836	5716
R_ARDelta_C	3323	50640	100	1389	230	48650	703	4247
R_AR_A	3084	58311	152	3000	269	52689	1058	10208
R_AR_B	4011	84370	199	3931	304	81589	1546	14058
R_AR_C	3674	97462	196	5290	343	83000	1501	16819
R_ARO_A	3079	63781	123	1993	324	60752	1000	7381
R_ARO_B	3178	92008	183	5352	359	64824	1307	17052
R_ARO_C	3248	89992	186	5540	307	61082	1255	16730

OTUs: total number of OTUs. N: total microbial relative abundance values. All sample libraries that start with “L” correspond to samples collected from lagoons, with “S” from the sea while “R” stands for the riverine samples. AR: Arachthos. ARO: Arachthos Neochori. ARDelta: Arachthos Delta. LOin: Logarou station inside the lagoon. LOout: Logarou station in the channel connecting the lagoon to the gulf. Kal: Kalamitsi. A, B, C replicate samples.

Moreover, when the PERMANOVA analysis was run for the results of PEMA, LotuS and DADA2, it was clear that the microbial community composition was significantly different in each of the three sampled habitats (i.e. River, Lagoon, Sea) (PERMANOVA: F.Model = 7.0718, $p < 0.001$; F.Model = 6.5901, $p < 0.001$, F.Model = 2.2484, $p < 0.05$, respectively), which is in accordance with [30]. However, this was not the case with Deblur (PERMANOVA: $p > 0.05$).

Overall, PEMA's output is in accordance with [30] and if all the different outputs are taken into consideration, it can be concluded that it performed better than the other tested pipelines in capturing the microbial community diversity and composition of the samples.

Beyond environmental ecology, on-going and future work

PEMA is mainly intended to support eDNA metabarcoding analysis and be directly applicable to next-generation biodiversity/ecological assessment studies. Given that community composition analysis may also serve additional research fields, eg. microbial pathology, the potential impact of such pipelines is expected to be much higher. On-going PEMA work focuses on serving a wide scientific audience and on making it applicable to more types of studies. The easy set up and execution of PEMA, allows users to work closely with national and European HPC/e-infrastructures (e.g. ELIXIR Greece [34], LifeWatch ERIC[35], EMBRC ERIC[36]). The aim of this effort is to outreach their communities and address both ongoing as well as future analysis needs.

In a mid- to meso-term perspective, pipeline development work will support the analysis of the ITS and 18S rRNA marker gene studies too. When operational, a holistic biodiversity assessment approach would be possible through PEMA and eDNA; the analysis of the most commonly used marker genes (16S rRNA, ITS, COI/18S rRNA) for each of the greatest taxonomic groups (Bacteria, Fungi, Eukaryotes) would be implemented by the same pipeline. Finally, it is our intention to allow *ad hoc* and in-house databases to be used as reference for the (M)OTU taxonomy assignment.

Conclusions

PEMA is an accurate, execution friendly and fast pipeline for the metabarcoding analysis of the 16S rRNA and COI marker genes. It provides a per-sample analysis output, different taxonomy assignment methods and graphics-based biodiversity/ecological analysis. This way, in addition to (M)OTU calling, it provides users with both an informative study overview and detailed result snapshots.

PEMA's user friendliness derives from the easy and with minimal number of installation and execution commands. In addition, PEMA's strategic choice of a single parameter file, implementation programming language, and multiple container-type distribution, grant it with speed (running in parallel), on-demand partial pipeline enactment, and provision for HPC-system-based sharing.

All the aforementioned features, render PEMA attractive for biodiversity/ecological assessment analyses. Applications may mainly concern environmental ecology with possible extensions to fields like microbial pathology and gut microbiome, inline with modern research needs, from low volume to big data.

Availability of supporting source code and requirements

Project name: PEMA

Project home page: https://github.com/hariszaf/pema

Archived version: see project home page (github repository)

Operating system(s): Platform independent

Programming language: BigDataScript

Other requirements: Singularity (in case of HPC usage)

License: GNU GPLv3 (for 3rd party components separate licenses apply)

Any restrictions to use by non-academics: licence needed

Availability of supporting data

The sequence data that support the findings of this study are available in European Nucleotide Archive (ENA) with the study accession numbers PRJEB20211 (http://www.ebi.ac.uk/ena/data/view/PRJEB20211) and PRJEB13009 (https://www.ebi.ac.uk/ena/data/view/PRJEB13009).

Declarations

List of abbreviations

BDS: BigDataScript

COI: Cytochrome Oxidase Subunit 1

eDNA: Environmental DNA

MOTU: Molecular Operational Taxonomic Unit (species equivalent for Eukaryotes)

HPC: High Performance Computing

MCMC: Markov chain Monte Carlo

MSA: Multiple Sequence Alignment

OTU: Operational Taxonomic Unit (species equivalent for prokaryotes)

PEMA: a Pipeline for Environmental DNA Metabarcoding Analysis

SSU: Small Subunit

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

HZ conceived and designed the pipeline, performed its containerization, analyzed and interpreted the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper. HQV offered support in the HPC preparation and setup and in 3rd party component usage. KV and CA conceived the idea and reviewed drafts of the paper. PT conceived the idea, proposed the usage of the programming language and reviewed drafts of the paper. CP conceived the idea, prepared figures and/or table and reviewed drafts of the paper. AP offered support in HPC and in 3rd party components. EP conceived the idea, assisted with programming and setup and reviewed drafts of the paper. All authors read and approved the final manuscript.

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Figure legends

Figure 1: PEMA comprises four parts. The first step (top left) is the quality control and pre-processing of the Illumina sequencing reads. This step is common for both 16S rRNA and COI marker genes. The second step (top right) is the clustering of reads to (M)OTUs. The third step (bottom left) is the taxonomy assignment to the generated (M)OTUs. In the fourth step (bottom right), the results of the metabarcoding analysis are provided to the user and visualized.

Figure 2: Phylogeny-based taxonomy assignment. A: Building a reference tree for the phylogeny-based taxonomy assignment to 16S rRNA marker gene OTUs: from the latest edition of Silva SSU, all entries referring to Bacteria and Archaea were used and using "art" algorithm, 10000 consensus taxa were kept. B: Using PaPaRa and the OTUs that come up from every analysis, an MSA was made and EPA-ng took over the phylogeny based taxonomy assignment.

Figure 3: MOTUs bar plot at the lowest possible taxonomic level. Bar plot depicting the taxonomy of the retrieved MOTUs with confidence estimate equal or higher than 0.97 at the lowest possible

taxonomic level.

Figure 4: MOTUs bar plot at the species level. Bar plot depicting the taxonomy of the retrieved MOTUs with confidence estimate equal or higher than 0.97 at the species level.

Figure 5: OTUs bar plot at the Phylum level. Bar plot depicting the taxonomy of the retrieved OTUs from PEMA at the Phylum level.

Additional files

Additional file 1: Supplementary Methods: Description of tools invoked by PEMA and their licences. Description of the commands, along with their parameters, used to run PEMA, mothur, LotuS and QIIME 2.

Additional file 2: Table S1: Number of sequences after each pre-processing step for the case of COI, dataset from [31].

Additional file 3: Table S2: Number of sequences after each pre-processing step for the case of 16S rRNA gene.

Additional file 4: Figure S1: Bar plot depicting the taxonomy of the retrieved OTUs from LotuS at the Phylum level.

Additional file 5: Figure S2: Bar plot depicting the taxonomy of the retrieved OTUs from QIIME 2 using Deblur at the Phylum level.

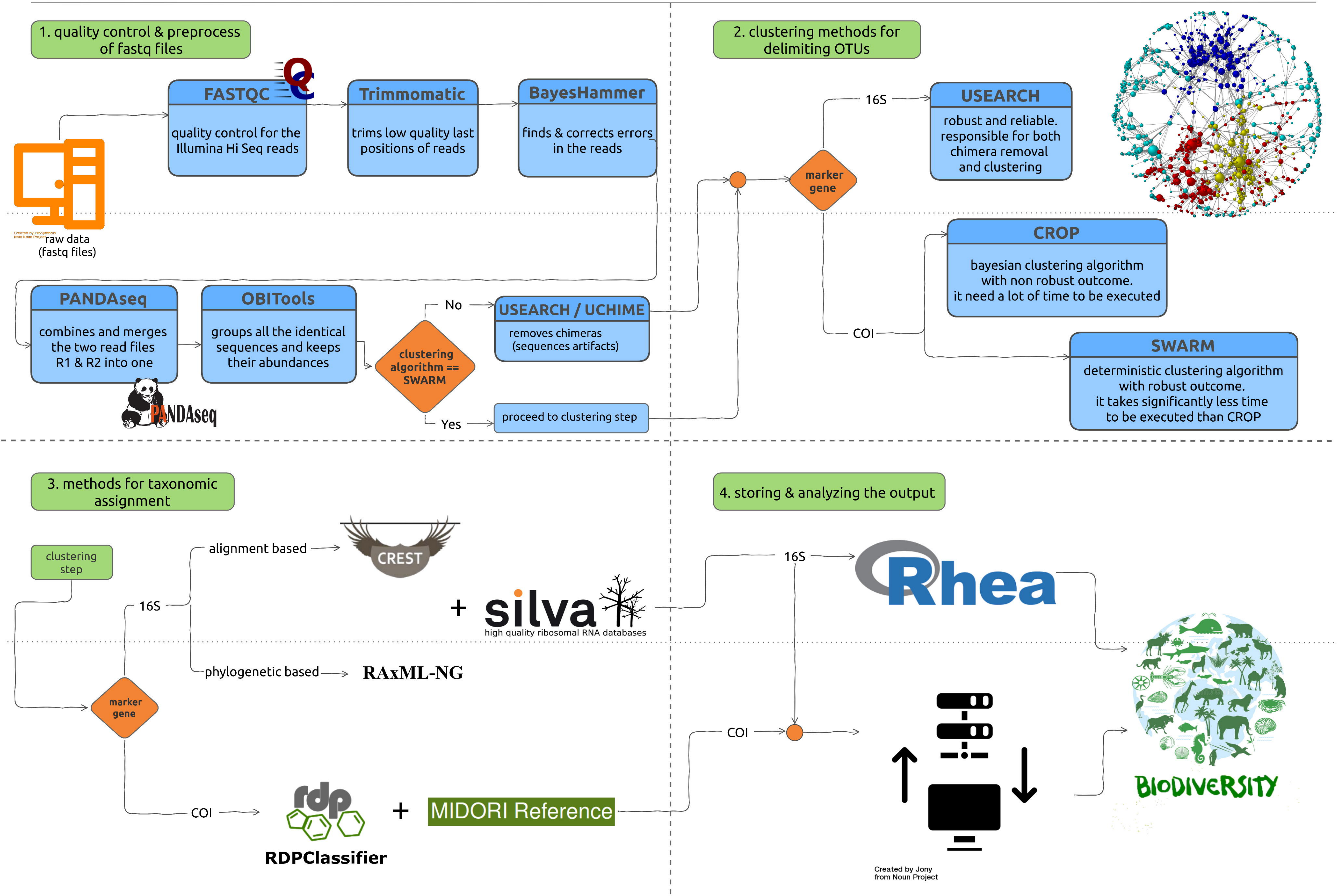
Additional file 6: Figure S3: Bar plot depicting the taxonomy of the retrieved OTUs from QIIME 2 using DADA2 at the Phylum level.

Additional file 7: Figure S4: Bar plot depicting the taxonomy of the retrieved OTUs from LotuS at the class of Betaproteobacteriales.

Additional file 8: Figure S5: Bar plot depicting the taxonomy of the retrieved OTUs from QIIME 2 using Deblur at the class of Betaproteobacteriales.

Additional file 9: Figure S6: Bar plot depicting the taxonomy of the retrieved OTUs from PEMA at the class of Betaproteobacteriales.

P.E.M.A. in a nutshell



1. quality control & preprocess of fastq files

Created by ProSymbols from Noun Project

raw data
(fastq files)

FASTQC
quality control for the Illumina Hi Seq reads

Trimmomatic
trims low quality last positions of reads

BayesHammer
finds & corrects errors in the reads

PANDAseq
combines and merges the two read files R1 & R2 into one

OBITools
groups all the identical sequences and keeps their abundances

clustering algorithm == SWARM

No → **USEARCH / UCHIME**
removes chimeras
(sequences artifacts)

Yes → proceed to clustering step

2. clustering methods for delimiting OTUs

marker gene

16S → **USEARCH**
robust and reliable. responsible for both chimera removal and clustering

COI → **CROP**
bayesian clustering algorithm with non robust outcome.
it need a lot of time to be executed

COI → **SWARM**
deterministic clustering algorithm with robust outcome.
it takes significantly less time to be executed than CROP

3. methods for taxonomic assignment

clustering step

marker gene

16S → alignment based → CREST + silva high quality ribosomal RNA databases

16S → phylogenetic based → RAxML-NG

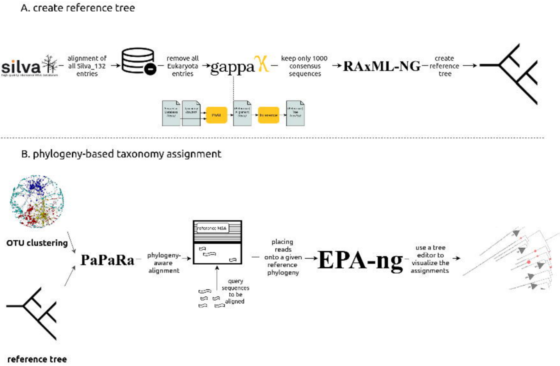
COI → RDPClassifier + MIDORI Reference

4. storing & analyzing the output

16S → Rhea → BIODIVERSITY

COI → BIODIVERSITY

Created by Jony
from Noun Project

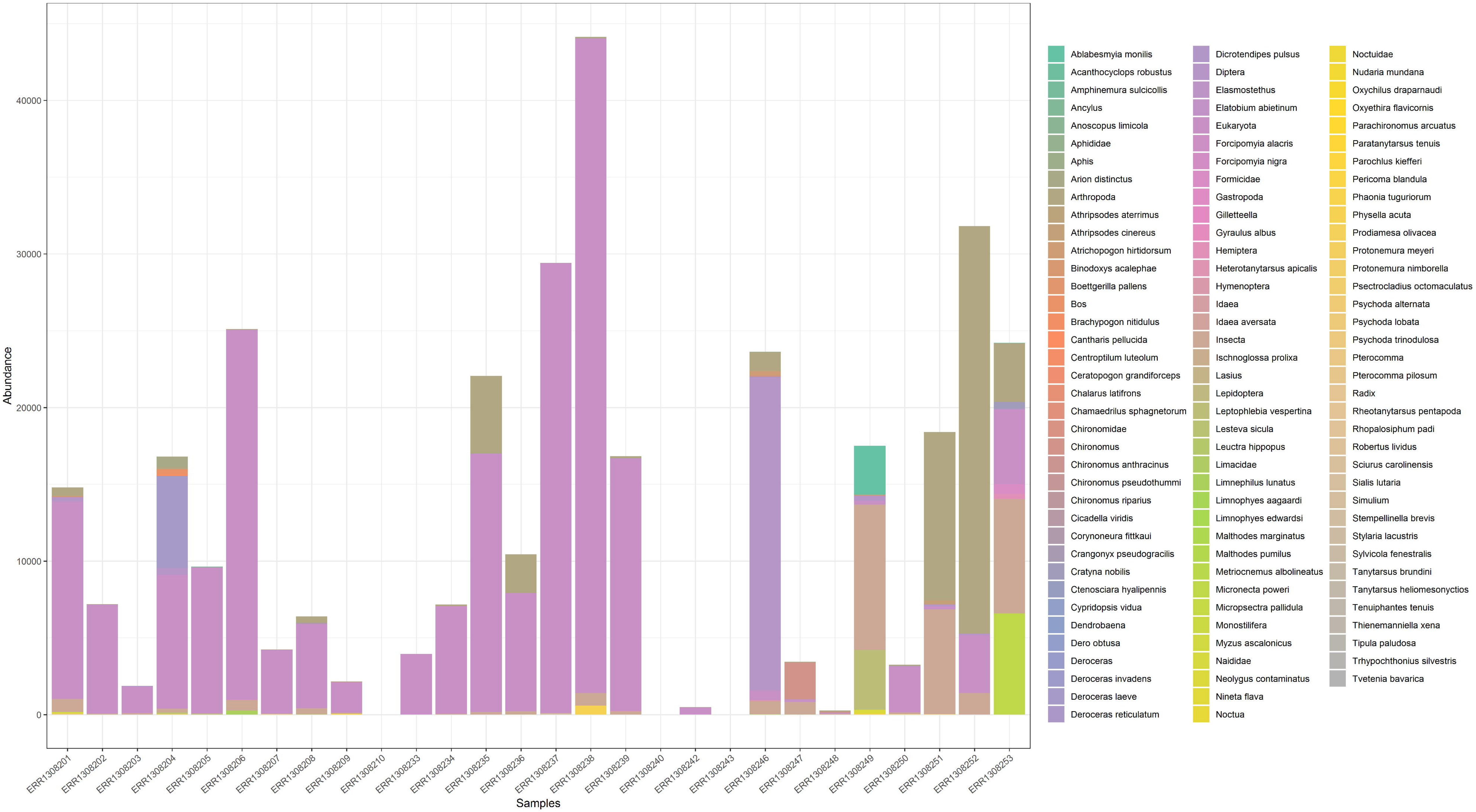


A. create reference tree

silva
high quality ribosomal RNA databases
alignment of all Silva_132 entries
remove all Eukaryota entries
gappa
keep only 1000 consensus sequences
RAxML-NG
create reference tree
PhAT

B. phylogeny-based taxonomy assignment

OTU clustering
reference tree
PaPaRa
phylogeny-aware alignment
reference MSA
query sequences to be aligned
placing reads onto a given reference phylogeny
EPA-ng
use a tree editor to visualize the assignments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20000
10000
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ERR1308247
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ERR1308249
ERR1308250
ERR1308251
ERR1308252
ERR1308253
Ablabesmyia monilis
Acanthocyclops robustus
Amphinemura sulcicollis
Ancylus
Anoscopus limicola
Aphididae
Aphis
Arion distinctus
Arthropoda
Athripsodes aterrimus
Athripsodes cinereus
Atrichopogon hirtidorsum
Binodoxys acalephae
Boettgerilla pallens
Bos
Brachypogon nitidulus
Cantharis pellucida
Centroptilum luteolum
Ceratopogon grandiforceps
Chalarus latifrons
Chamaedrilus sphagnetorum
Chironomidae
Chironomus
Chironomus anthracinus
Chironomus pseudothummi
Chironomus riparius
Cicadella viridis
Corynoneura fittkaui
Crangonyx pseudogracilis
Cratyna nobilis
Ctenosciara hyalipennis
Cypridopsis vidua
Dendrobaena
Dero obtusa
Deroceras
Deroceras invadens
Deroceras laeve
Deroceras reticulatum
Dicrotendipes pulsus
Diptera
Elasmostethus
Elatobium abietinum
Eukaryota
Forcipomyia alacris
Forcipomyia nigra
Formicidae
Gastropoda
Gilletteella
Gyraulus albus
Hemiptera
Heterotanytarsus apicalis
Hymenoptera
Idaea
Idaea aversata
Insecta
Ischnoglossa prolixa
Lasius
Lepidoptera
Leptophlebia vespertina
Lesteva sicula
Leuctra hippopus
Limacidae
Limnephilus lunatus
Limnophyes aagaardi
Limnophyes edwardsi
Malthodes marginatus
Malthodes pumilus
Metriocnemus albolineatus
Micronecta poweri
Micropsectra pallidula
Monostilifera
Myzus ascalonicus
Naididae
Neolygus contaminatus
Nineta flava
Noctua
Noctuidae
Nudaria mundana
Oxychilus draparnaudi
Oxyethira flavicornis
Parachironomus arcuatus
Paratanytarsus tenuis
Parochlus kiefferi
Pericoma blandula
Phaonia tuguriorum
Physella acuta
Prodiamesa olivacea
Protonemura meyeri
Protonemura nimborella
Psectrocladius octomaculatus
Psychoda alternata
Psychoda lobata
Psychoda trinodulosa
Pterocomma
Pterocomma pilosum
Radix
Rheotanytarsus pentapoda
Rhopalosiphum padi
Robertus lividus
Sciurus carolinensis
Sialis lutaria
Simulium
Stempellinella brevis
Stylaria lacustris
Sylvicola fenestralis
Tanytarsus brundini
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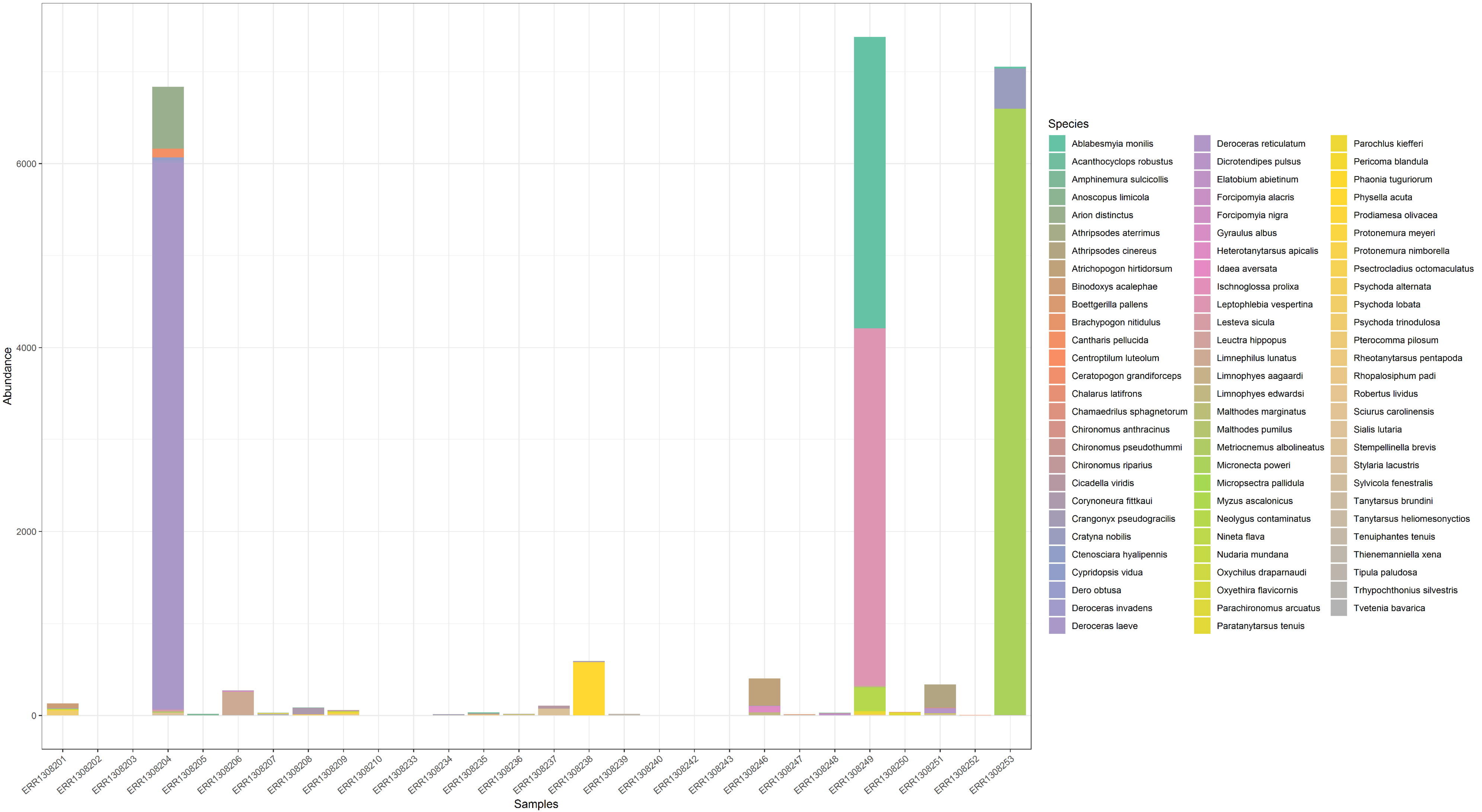
Tipula paludosa
Trhypochthonius silvestris
Tvetenia bavarica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ERR1308253
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Ablabesmyia monilis
Acanthocyclops robustus
Amphinemura sulcicollis
Anoscopus limicola
Arion distinctus
Athripsodes aterrimus
Athripsodes cinereus
Atrichopogon hirtidorsum
Binodoxys acalephae
Boettgerilla pallens
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Cantharis pellucida
Centroptilum luteolum
Ceratopogon grandiforceps
Chalarus latifrons
Chamaedrilus sphagnetorum
Chironomus anthracinus
Chironomus pseudothummi
Chironomus riparius
Cicadella viridis
Corynoneura fittkaui
Crangonyx pseudogracilis
Cratyna nobilis
Ctenosciara hyalipennis
Cypridopsis vidua
Dero obtusa
Deroceras invadens
Deroceras laeve
Deroceras reticulatum
Dicrotendipes pulsus
Elatobium abietinum
Forcipomyia alacris
Forcipomyia nigra
Gyraulus albus
Heterotanytarsus apicalis
Idaea aversata
Ischnoglossa prolixa
Leptophlebia vespertina
Lesteva sicula
Leuctra hippopus
Limnephilus lunatus
Limnophyes aagaardi
Limnophyes edwardsi
Malthodes marginatus
Malthodes pumilus
Metriocnemus albolineatus
Micronecta poweri
Micropsectra pallidula
Myzus ascalonicus
Neolygus contaminatus
Nineta flava
Nudaria mundana
Oxychilus draparnaudi
Oxyethira flavicornis
Parachironomus arcuatus
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Pericoma blandula
Phaonia tuguriorum
Physella acuta
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Protonemura nimborella
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Psychoda trinodulosa
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Robertus lividus
Sciurus carolinensis
Sialis lutaria
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Stylaria lacustris
Sylvicola fenestralis
Tanytarsus brundini
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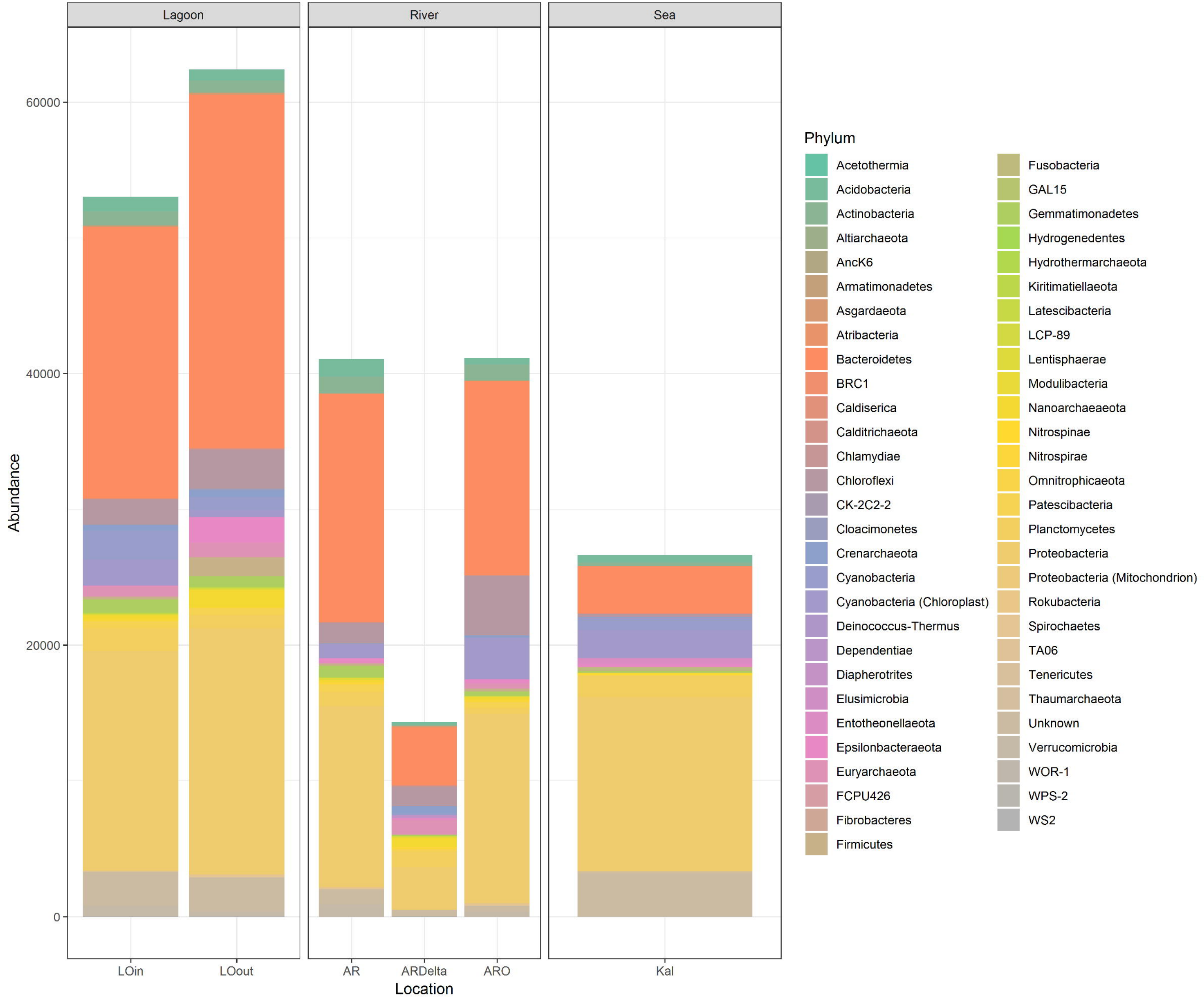
Tvetenia bavarica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LOout
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ARDelta
ARO
Kal
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Acetothermia
Acidobacteria
Actinobacteria
Altiarchaeota
AncK6
Armatimonadetes
Asgardaeota
Atribacteria
Bacteroidetes
BRC1
Caldiserica
Calditrichaeota
Chlamydiae
Chloroflexi
CK-2C2-2
Cloacimonetes
Crenarchaeota
Cyanobacteria
Cyanobacteria (Chloroplast)
Deinococcus-Thermus
Dependentiae
Diapherotrites
Elusimicrobia
Entotheonellaeota
Epsilonbacteraeota
Euryarchaeota
FCPU426
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Nanoarchaeaeota
Nitrospinae
Nitrospirae
Omnitrophicaeota
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Planctomycetes
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