
micca Documentation

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micca (MICrobial Community Analysis) is a software pipeline for the processing of amplicon sequencing data, **from raw sequences to OTU tables, taxonomy classification and phylogenetic tree** inference. The pipeline can be applied to a range of highly conserved genes/spacers, such as **16S rRNA gene, Internal Transcribed Spacer (ITS) 18S and 28S rRNA**. micca is an open-source, GPLv3-licensed software.

- [Homepage](#)
- [Documentation \(latest\)](#)
- [Issues](#)
- [Github page](#)

Key features:

- supports **single-end** (Roche 454, Illumina MiSeq/HiSeq ,Ion Torrent) and **overlapping paired-end** reads (Illumina MiSeq/HiSeq);
- **multithread** de novo greedy, closed-reference, open-reference and swarm OTU picking protocols;
- **denoising** of Illumina reads;
- **state-of-the-art taxonomic classification** algorithms (RDP and consensus-based classifier);
- fast and memory efficient **NAST** multiple sequence alignment (MSA);
- filters low quality sequences according to the maximum allowed **expected error (EE) rate %**;
- runs on **Linux, Mac OS X and MS Windows** (through Docker containers)
- **simple, easy to use.**

Docker images are available (compmetagen/micca) starting from version 1.2.2, see the documentation ($\geq 1.3.0$) to learn how to use them. [Docker hub page](#).

How to cite: Davide Albanese, Paolo Fontana, Carlotta De Filippo, Duccio Cavalieri and Claudio Donati. **MICCA: a complete and accurate software for taxonomic profiling of metagenomic data**. Scientific Reports 5, Article number: 9743 (2015), doi:10.1038/srep09743, [Link](#). Dataset download: <ftp://ftp.fmach.it/metagenomics/micca/scirep/>.

micca wraps third party software packages and these **should be cited** if they are used:

- VSEARCH (doi: 10.7717/peerj.2584) used in `classify`, `filter`, `mergepairs`, `otu` and `msa` commands
- MUSCLE (doi: 10.1093/nar/gkh340) used in `msa` and `tree` commands
- FastTree (doi: 10.1371/journal.pone.0009490) used in the `tree` command
- Cutadapt (doi: 10.14806/ej.17.1.200) used in the `trim` command
- RDP classifier (doi: 10.1128/AEM.00062-07) used in the `classify` command
- swarm (doi: 10.7717/peerj.1420) used in the `otu` command

1.1 Using Docker (that is, on MS Windows, Mac OS X and Linux!)

The easiest way to run micca is through [Docker](#). Docker works similarly to a virtual machine image, providing a container in which all the software has already been installed, configured and tested.

1. Install Docker for [Linux](#), [Mac OS X](#) or [Windows](#).
2. Run the Docker Quickstart Terminal (Mac OS X, Windows) or the docker daemon (Linux, `sudo service docker start`).
3. Download the latest version:

```
docker pull compmetagen/micca
```

4. Run an instance of the image, mounting the host working directory (e.g. `/Users/davide/micca`) on to the container working directory `/micca`:

```
docker run --rm -ti --user $(id -u):$(id -g) -v /Users/davide/micca:/micca -w /  
↪micca compmetagen/micca /bin/bash
```

You need to write something like `-v //c/Users/davide/micca:/micca` if you are in Windows or `-v /home/davide/micca:/micca` in Linux. The `--rm` option automatically removes the container when it exits.

5. Now you can use micca:

```
name@68f6784e1101:/micca# micca -h
```

Note: The RDP classifier is preinstalled in the Docker image, so you can check the software version by typing `echo $RDPPATH`

1.2 Using pip

At the moment, only Python 2.7 is supported.

1.2.1 On Ubuntu >= 12.04 and Debian >=7

We suggest to install the following packages through the package manager:

```
sudo apt-get update
sudo apt-get install build-essential python-numpy gcc gfortran python-dev libblas-dev
↳ liblapack-dev cython pkg-config libfreetype6 libfreetype6-dev libpng-dev
```

Then, upgrade pip and install setuptools:

```
pip install --upgrade pip
pip install 'setuptools >=14.0'
```

Finally, install micca:

```
sudo pip install micca
```

1.2.2 On Mac OS X

In Mac OS X, we recommend to install Python from [Homebrew](#):

1. Install [Xcode](#);
2. Install [Homebrew](#);
3. Make sure the environment variable `PATH` is properly setted in your `~/ .bash_profile` or `~/ .bashrc`:

```
.. code-block:: sh
```

```
export PATH=/usr/local/bin:$PATH
```

4. Install Python:

```
brew update
brew install python
```

Install the GNU Fortran and the NumPy package:

```
brew install gcc
pip install numpy
```

Finally, install micca:

```
sudo pip install micca
```

1.2.3 Installation problems

- BIOM fatal error: 'numpy/arrayobject.h'. If the installation process returns a message like this:


```

biom/_filter.c:258:10: fatal error: 'numpy/arrayobject.h' file not found
#include "numpy/arrayobject.h"
      ^
1 error generated.
error: command 'clang' failed with exit status 1

```

then you need to run:

```

pip install --global-option=build_ext --global-option="-I/usr/local/lib/python2.7/
↪site-packages/numpy/core/include/" biom-format

```

After that you can install the micca package.

1.3 Install micca from source

In order to install micca from sources (with the standard procedure `python setup.py install`), in addition to Python (≥ 2.7) and NumPy ($\geq 1.8.0$), the following Python packages must be installed:

- SciPy $\geq 0.13.0$
- Pandas $\geq 0.17.0$
- matplotlib $\geq 1.3.0$
- Biopython ≥ 1.50
- cutadapt ≥ 1.9
- biom-format $\geq 1.3.1$

The easiest way to install these packages is to is using pip:

```

sudo pip install 'scipy >=0.13.0' 'pandas >=0.17.0' 'matplotlib >=1.3.0' 'biopython >
↪= 1.50' 'cutadapt >=1.9' 'biom-format >=1.3.1'

```

Download the latest version from <https://github.com/compmetagen/micca/releases> and complete the installation:

```

tar -zxvf micca-X.Y.Z.tar.gz
sudo python setup.py install

```

1.3.1 If you don't have root access

Install micca in a local directory by specifying the `--prefix` argument. Then you need to set the environment variable `PYTHONPATH`:

```

python setup.py install --prefix=/path/to/modules
export PYTHONPATH=$PYTHONPATH:/path/to/modules/lib/python{version}/site-packages

```

Note: In order to export the variable permanently add the command at the bottom of your `~/.bash_profile` or `~/.bashrc` file.

1.4 Testing the installation

```
micca -h
```

1.5 Install RDP classifier (optional)

The RDP Classifier is a naive bayesian classifier for taxonomic assignments (<http://sourceforge.net/projects/rdp-classifier/>). The RDP classifier can be used in the *classify* command (option *-m/--method rdp*).

Warning: Only RDP Classifier version >2.8 is supported. Install the standard Java or Java compatible runtime (sudo apt-get install default-jre in Ubuntu/Debian or go to the [Oracle Java homepage](#) for OS X)

Download and unzip the file (RDP classifier 2.11 2015-09-14):

```
wget https://sourceforge.net/projects/rdp-classifier/files/rdp-classifier/rdp_
↪classifier_2.11.zip
unzip rdp_classifier_2.11.zip
```

Now you must set the environment variable RDPPATH by typing:

```
$ export RDPPATH=/path-to-rdp-classifier/rdp_classifier_2.11/
```

e.g. export RDPPATH=/Users/David/rdp_classifier_2.11.

Note: In order to export the variable permanently add the latest command at the bottom of your *.bashrc* file.

Supported databases

2.1 16S rRNA

- Greengenes ftp://greengenes.microbio.me/greengenes_release/
- Greengenes *Core Set* (useful for NAST) http://greengenes.lbl.gov/Download/Sequence_Data/Fasta_data_files/core_set_aligned.fasta.imputed
- QIIME-formatted SILVA <https://www.arb-silva.de/download/archive/qiime/>

2.2 ITS

- UNITE <https://unite.ut.ee/repository.php> (QIIME releases)

Note: In the QIIME releases of the UNITE database the `_s` in the filename (e.g. `sh_qiime_release_s_DD.MM.YYY.zip`) specifies that the database includes singletons.

Paired-end sequencing - 97% OTU

This tutorial describes a standard micca pipeline for the analysis of overlapping paired-end sequences. The main products of this pipeline are:

- the Operational Taxonomic Units (OTUs), defined clustering the processed sequences at a similarity threshold of 97%;
- an OTU table, containing the number of times each OTU is observed in each sample;
- a taxonomic classification for each OTU;
- an OTU phylogenetic tree.

This pipeline is intended for different platforms, such as **Illumina MiSeq** and **Illumina HiSeq**. Although this tutorial explains how to apply the pipeline to **16S rRNA** amplicons, it can be adapted to others markers gene/spacers, e.g. **Internal Transcribed Spacer (ITS)**, **18S** or **28S**.

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- *Multiple Sequence Alignment (MSA)*
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3.1 Dataset download

The following paired-end 16S rRNA dataset contains **34 samples** in FASTQ format (V3-V4 region, 341F 5'-CCTACGGGNGGCWGCAG-3', 806Rmod 5'-GACTACNVGGGTWTCTAATCC-3').

Samples comes from the paper “Diversity and Cyclical Seasonal Transitions in the Bacterial Community in a Large and Deep Perialpine Lake” were seasonal variations in the bacterioplankton community composition in the lake Garda were analyzed. Sampling was carried out at monthly intervals in three layers representative of the epilimnetic and euphotic zones of the lake, 1, 10, and 20 m. The dataset contains only a subset of the entire study (2014 samples only) and raw data were randomly subsampled at 3000 sequences per sample.

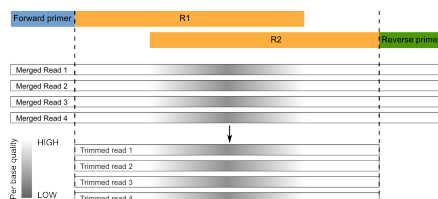
The 2x300-bp paired-end sequencing was carried out on an Illumina MiSeq.

Open a terminal, download the data and prepare the working directory:

```
wget ftp://ftp.fmach.it/metagenomics/micca/examples/garda.tar.gz
tar -zxvf garda.tar.gz
cd garda
```

3.2 Merge paired-end sequences

Overlapping paired-end sequences must be merged to obtain consensus sequences (sometimes called *assembly*). This operation can be performed with the *mergepairs* command.



Moreover, the command merges the samples in a single file where sample names are appended to the sequence identifier, as in *merge* and *split* commands.

Since the sequenced region is about of 465-bp (806-341) and the reads are of 300-bp, the overlap region is quite large ((2x300)-465=135 bp), as rule of thumb we set a minimum overlap length of 100 and maximum number of allowed mismatches of about 1/3, say 30:

```
micca mergepairs -i fastq/*_R1*.fastq -o merged.fastq -l 100 -d 30
```

Note: Starting from micca 1.6.0 staggered read pairs (staggered pairs are pairs where the 3' end of the reverse read has an overhang to the left of the 5' end of the forward read) will be merged by default. To override this feature (and

therefore to discard staggered alignments) set the `-n/--nostagger` option.

Note: *mergepairs* works with FASTQ files only.

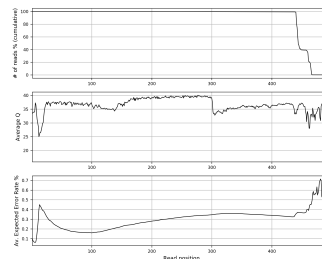
Note: Reverse file names will be constructed by replacing the string `_R1` in the forward file name with `_R2` (typical in Illumina file names, see options `-p/--pattern` and `-e/--repl`).

3.2.1 Compute reads statistics

We can report sequences statistics computed on the file `merged.fastq`. Run the command *stats*:

```
micca stats -i merged.fastq -o stats_merged
```

The command reports in 3 text files and in the relative plots (in PNG format) the length distribution, the Q score distribution and a quality summary. The quality summary plot (*stats_merged/stats_qualsumm_plot.png*) is reported below:



3.3 Primer trimming

Segments which match PCR primers should be now removed. For paired-end (already merged) reads, we recommend to **trim both forward and reverse primers and discard reads that do not contain the forward OR the reverse primer**.

These operations can be performed with the *trim* command:

```
micca trim -i merged.fastq -o trimmed.fastq -w CCTACGGGNGGCWGCAG -r ↵
↵GACTACNVGGGTWCTAATCC -W -R -c
```

The option `-W/--duforward` and `-R/--dureverse` ensures that reads that do not contain the forward or the reverse primer will be discarded. With the option `-c/--searchrc` the command searches reverse complement primers too.

3.4 Quality filtering

Producing high-quality OTUs requires high-quality reads. *filter* filters sequences according to the maximum allowed expected error (EE) rate %. We recommend values $\leq 1\%$.

For paired-end reads, we recommend to merge pairs first, then quality filter using a maximum EE threshold with **no length truncation**.

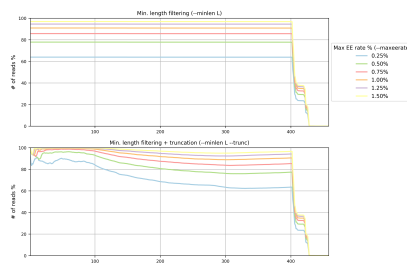
Warning: Parameters for the `filter` command should be chosen using the tool `filterstats`.

3.4.1 Choosing parameters for filtering

The command `filterstats` reports the fraction of reads that would pass for each specified maximum expected error (EE) rate %:

```
micca filterstats -i trimmed.fastq -o filterstats
```

Open the PNG file `filterstats/stats_plot.png`:



In this case (overlapping paired paired-end reads) we are interested in the plot on top (minimum length filtering only). A minimum read length (L) of **400** and a maximum error rate of **0.75%** seems to be a good compromise between the expected error rate and the number of reads remaining. Inspecting the file `filterstats/filterstats_minlen.txt`, you can see that more than **85%** reads will pass the filter:

L	0.25	0.5	0.75	1.0	1.25	1.5
...						
399	63.856	77.766	85.664	90.844	94.484	96.853
400	63.856	77.765	85.661	90.842	94.481	96.850
401	63.842	77.747	85.643	90.822	94.459	96.827
...						

Note: To obtain general sequencing statistics, run the micca command `stats` on the file `trimmed.fastq`.

3.4.2 Filter sequences

Now we can run the `filter` command with the selected parameters:

```
micca filter -i trimmed.fastq -o filtered.fasta -e 0.75 -m 400
```

Note: The maximum number of allowed Ns after truncation can be also specified in `filterstats` and in `filter`.

3.5 OTU picking

To characterize the taxonomic structure of the samples, the sequences are now organized into **Operational Taxonomic Units (OTUs)** at varying levels of identity. An identity of **97%** represent the common working definition of bacterial

species. The *otu* command implements several state-of-the-art approaches for OTU clustering, but in this tutorial we will focus on the **de novo greedy clustering** (see *OTU picking and Denoising*):

```
micca otu -m denovo_greedy -i filtered.fasta -o denovo_greedy_otus -t 4 -c
```

The *otu* command returns several files in the output directory, including the **SV table** (*otutable.txt*) and a FASTA file containing the **representative sequences** (*otus.fasta*).

Note: See *OTU picking and Denoising* to see how to apply the **de novo swarm**, **closed-reference** and the **open-reference** OTU picking strategies to these data.

3.6 Assign taxonomy

Now we can assign taxonomy to each representative sequence using the *classify* command. In this tutorial we use the RDP (<https://doi.org/10.1128/AEM.00062-07>) classifier.

Note: See *Install* on how to install the RDP classifier on your system.

```
micca classify -m rdp -i denovo_greedy_otus/otus.fasta -o denovo_greedy_otus/taxa.txt
```

classify returns a taxonomy file like this:

```
DENOVO1      Bacteria;Cyanobacteria/Chloroplast;Cyanobacteria
DENOVO2      Bacteria;Cyanobacteria/Chloroplast;Cyanobacteria;Family II;Family II;GpIIa
DENOVO3      Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae
DENOVO4      Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;
↳Limnhabitans
...
```

3.7 Infer the phylogenetic tree

These steps are necessary if you want to use phylogenetic-based metrics such as the UniFrac distance (<https://doi.org/10.1128/AEM.01996-06>) in the downstream analysis.

3.7.1 Multiple Sequence Alignment (MSA)

The *msa* command provides two approaches for **MSA**: MUSCLE (<https://doi.org/10.1093/nar/gkh340>) (de novo alignment) and Nearest Alignment Space Termination (NAST) (<https://doi.org/10.1093/nar/gkl244>) (which uses a template alignment). In this tutorial we will use the NAST alignment method. For 16S rRNA sequences, a good template alignment is the Greengenes Core Set:

```
wget ftp://ftp.fmach.it/metagenomics/micca/dbs/core_set.tar.gz
tar -zxvf core_set.tar.gz
```

At this point we can run the *msa* command:

```
micca msa -m nast -i denovo_greedy_otus/otus.fasta -o denovo_greedy_otus/msa.fasta \
  --nast-template core_set_aligned.fasta.imputed --nast-threads 4
```

3.7.2 Build the phylogenetic tree

At this point we can build the phylogenetic tree from the MSA using *tree*:

```
micca tree -i denovo_greedy_otus/msa.fasta -o denovo_greedy_otus/tree.tree
```

Note: The output tree is in *Newick* format.

3.7.3 Midpoint rooting

UniFrac metrics require phylogenetic trees to be rooted. The tree can be rooted (in this case at midpoint between the two most distant tips of the tree) using the *root* command:

```
micca root -i denovo_greedy_otus/tree.tree -o denovo_greedy_otus/tree_rooted.tree
```

Note: Tree can also be rooted with the outgroup clade containing selected targets, see *root*.

3.8 Build the BIOM file

The *Biological Observation Matrix* (BIOM) is a common format for representing OTU tables and metadata and is the core data type for downstream analyses in *QIIME* and in *phyloseq*. *tobiom* converts the OTU table and the taxonomy table produced by the previous steps to the BIOM format. In addition, the *Sample data* can be added:

```
micca tobiom -i denovo_greedy_otus/otutable.txt -o denovo_greedy_otus/tables.biom \
-t denovo_greedy_otus/taxa.txt -s sampledata.txt
```

3.9 Further steps

- *An introduction to the downstream analysis with R and phyloseq*
- *Compute basic statistics, rarefy and summarize OTU/SV tables using micca*

Denoising (Illumina only)

Usually, amplicon sequences are clustered into **Operational Taxonomic Units** (OTUs) using a similarity threshold of 97%, which represents the common working definition of bacterial species.

Another approach consists to identify the **Sequence Variants** (SVs, see *OTU picking and Denoising* for details). This approach avoids clustering sequences at a predefined similarity threshold and usually includes a denoising algorithm in order to identify SVs.

In this tutorial we show how to perform the denoising of Illumina overlapping paired-end sequences in order to detect the SVs. Although this tutorial explains how to apply the pipeline to 16S paired-end Illumina reads, it can be adapted to Illumina single-end sequencing or to others markers gene/spacers, e.g. **Internal Transcribed Spacer (ITS)**, **18S** or **28S**.

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- *Data download and preprocessing*
- *Denoising - Sequence Variants identification*
- *Further steps*

4.1 Data download and preprocessing

In this tutorial we analyze the same dataset used in *Paired-end sequencing - 97% OTU*. Reads merging, primer trimming and quality filtering are the same as in *Paired-end sequencing - 97% OTU*:

```
wget ftp://ftp.fmach.it/metagenomics/micca/examples/garda.tar.gz
tar -zxvf garda.tar.gz
cd garda

micca mergepairs -i fastq/*_R1*.fastq -o merged.fastq -l 100 -d 30
```

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```
micca trim -i merged.fastq -o trimmed.fastq -w CCTACGGGNGGCWGCAG -r ↪
↪GACTACNVGGGTWCTAATCC -W -R -c
micca filter -i trimmed.fastq -o filtered.fasta -e 0.75 -m 400
```

4.2 Denoising - Sequence Variants identification

The *otu* command implements the UNOISE3 protocol (*denovo_unoise*) which includes dereplication, denoising and chimera filtering:

```
micca otu -m denovo_unoise -i filtered.fasta -o denovo_unoise_otus -t 4 -c
```

The *otu* command returns several files in the output directory, including the **SV table** (*otutable.txt*) and a FASTA file containing the **representative sequences** (*otus.fasta*).

Note: See *OTU picking and Denoising* to see how to apply the **de novo swarm**, **closed-reference** and the **open-reference** OTU picking strategies to these data.

4.3 Further steps

- *Assign taxonomy*
- *Infer the phylogenetic tree*
- *Build the BIOM file*
- *An introduction to the downstream analysis with R and phyloseq*
- *Compute basic statistics, rarefy and summarize OTU/SV tables using micca*

Single-end sequencing

This tutorial describes a standard micca pipeline for the analysis of **single-end** amplicon data. This pipeline is intended for different platforms, such as **Roche 454**, **Illumina MiSeq/HiSeq** and **Ion Torrent**. Although this tutorial explains how to apply the pipeline to **16S rRNA** amplicons, it can be adapted to others markers gene/spacers, e.g. **Internal Transcribed Spacer (ITS)**, **18S** or **28S**.

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- *Merge files*
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5.1 Dataset download

The dataset used in this tutorial is taken from the Barelli et al. paper *Habitat fragmentation is associated to gut microbiota diversity of an endangered primate: implications for conservation* (<https://doi.org/doi:10.1038/srep14862>). The dataset contains only a subset of the entire study (Mwanihana samples only) for a total of **15 samples** (in FASTQ format) and **235179 16S rRNA amplicon reads** (V1-V3 hypervariable regions, 27F 5'-AGAGTTTGATCMTGGCTCAG, 533R 5'-TTACCGCGGCTGCTGGCAC). The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+ chemistry.

Open a terminal, download the data and prepare the working directory:

```
wget ftp://ftp.fmach.it/metagenomics/micca/examples/mwanihana.tar.gz
tar -zxvf mwanihana.tar.gz
cd mwanihana
```

5.2 Merge files

Now the FASTQ files must be merged in a single file. This operation can be performed with the `merge` command. Sample names will be included into the sequence identifiers.

```
micca merge -i fastq/*.fastq -o merged.fastq
```

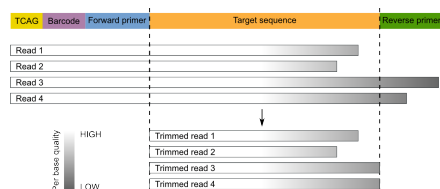
Note: The `merge` command works with FASTQ or FASTA files. If your sequences are in a different format (e.g. SFF or FASTA+QUAL) use `convert` to convert them.

Warning: In the case of multiplexed reads (with 5' barcode sequences) use `split` instead of `merge`. This command will perform demultiplexing and merging at the same time.

Note: In the case of overlapping paired-end reads go to [Paired-end sequencing - 97% OTU](#) or [Denoising \(Illumina only\)](#).

5.3 Primer trimming

Segments which match PCR primers should be now removed. Typical Roche 454 reads start with a sequence key (e.g. TCAG) followed by the barcode (if it was not previously removed) and the forward primer. For these types of data (and in general, for single-end sequencing) we recommend to **trim both forward reverse primers and discard reads that do not contain the forward primer**. Moreover, sequence preceding (for the forward) or succeeding (for the reverse, if found) primers should be removed:



These operations can be performed with the `trim` command:

```
micca trim -i merged.fastq -o trimmed.fastq -w AGAGTTTGATCMTGGCTCAG -r _
↪ GTGCCAGCAGCCGCGGTAA -W
```

The option `-W/--duforward` ensures that reads that do not contain the forward primer will be discarded.

Warning: Do not use the `-R/--dureverse` with single-end reads.

Note: The *trim* command supports IUPAC nucleotide codes and multiple primers. With the option `-c/--searchrc` the command searches reverse complement primers too. *trim* works with FASTQ or FASTA files.

5.4 Quality filtering

Producing high-quality OTUs requires high-quality reads. The *filter* command filters sequences according to the maximum allowed expected error (EE) rate %. We recommend values $\leq 1\%$. Moreover, to obtain good results in clustering (see *otu*), reads should be **truncated at the same length** when they cover partial amplicons or if quality deteriorates towards the end (common when you have long amplicons in 454 or Illumina single-end sequencing).

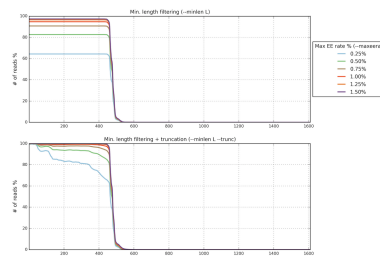
Warning: Parameters for the *filter* command should be chosen using the command *filterstats*.

5.4.1 Choosing parameters for filtering

The command *filterstats* reports the fraction of reads that would pass for each specified maximum expected error (EE) rate %:

```
micca filterstats -i trimmed.fastq -o filterstats
```

Open the PNG file `filterstats/stats_plot.png`:



In this case we are interested in the plot below (minimum length filtering + truncation). A truncation length of **350** and a maximum error rate of **0.5%** seems to be a good compromise between read read length, expected error rate and number of reads remaining. Inspecting the file `filterstats/truncalen_stats.txt`, you can see that more than **92%** reads will pass the filter:

L	0.25	0.5	0.75	1.0	1.25	1.5
...						
349	78.905	92.472	97.425	99.135	99.705	99.897
350	78.639	92.385	97.389	99.126	99.704	99.896
351	78.369	92.300	97.357	99.116	99.700	99.892
...						

Note: To obtain general sequencing statistics, run *stats*.

5.4.2 Filter sequences

Now we can run the *filter* command with the selected parameters:

```
micca filter -i trimmed.fastq -o filtered.fasta -e 0.5 -m 350 -t
```

Note: The maximum number of allowed Ns after truncation can be also specified in *filterstats* and in *filter*.

5.5 OTU picking

To characterize the taxonomic structure of the samples, the sequences are now organized into **Operational Taxonomic Units (OTUs)** at varying levels of identity. An identity of **97%** represent the common working definition of bacterial species. The *otu* command implements several state-of-the-art approaches for OTU clustering, but in this tutorial we will focus on the **de novo greedy clustering** (see *OTU picking and Denoising*):

```
micca otu -i filtered.fasta -o denovo_greedy_otus -d 0.97 -c -t 4
```

The *otu* command returns several files in the output directory, including the **OTU table** (*otutable.txt*) and a FASTA file containing the **representative sequences** (*otus.fasta*).

5.6 Further steps

- *Assign taxonomy*
- *Infer the phylogenetic tree*
- *Build the BIOM file*
- *An introduction to the downstream analysis with R and phyloseq*
- *Compute basic statistics, rarefy and summarize OTU/SV tables using micca*

An introduction to the downstream analysis with R and phyloseq

In this tutorial we describe a [R](#) pipeline for the downstream analysis starting from the output of micca. In particular, we will discuss the following topics:

- rarefaction;
- taxonomy and relative abundances;
- alpha diversity and non-parametric tests;
- beta diversity and PERMANOVA;
- differential abundance testing with DESeq2.

You can download the script [here](#).

Warning:

- This tutorial requires *Paired-end sequencing - 97% OTU* to be done.
- The tutorial is tested on R 3.5.3, phyloseq 1.26.1, ggplot2 3.1.0, vegan 2.5-4 and DESeq2 1.22.2.

6.1 (Optional) Using the Dockerized RStudio environment

The tutorial can be run using a [Docker](#) image with the required packages installed. Run the following command line mounting the host working directory (i.e. the directory containing the micca output files, in this case `/Users/davide/micca`) into the `/home/rstudio/micca` folder:

```
docker run --rm -d -p 8787:8787 -e DISABLE_AUTH=true --name rstudio-micca \
-v /Users/davide/micca:/home/rstudio/micca \
compmetagen/rstudio-micca
```

Open a browser and go to `127.0.0.1:8787`.

Warning: Files stored outside the micca directory will be lost when you stop the container.

You can stop (and destroy) the container using the following line:

```
docker stop rstudio-micca
```

6.2 Import data and preparation

Import the micca processed data (the BIOM file, the phylogenetic tree and the representative sequences) into the R environment using the `import_biom()` function available in [phyloseq](#) library.

```
> library("phyloseq")
> library("ggplot2")
> library("vegan")
> library("DESeq2")
> setwd("/home/rstudio/micca/garda/denovo_greedy_otus") # set the working directory
> ps = import_biom("tables.biom", treefilename="tree_rooted.tree", refseqfilename=
  ↪ "otus.fasta")
> sample_data(ps)$Month <- as.numeric(sample_data(ps)$Month)
> ps
phyloseq-class experiment-level object
otu_table()      OTU Table:             [ 529 taxa and 34 samples ]
sample_data()    Sample Data:          [ 34 samples by 4 sample variables ]
tax_table()      Taxonomy Table:        [ 529 taxa by 6 taxonomic ranks ]
phy_tree()       Phylogenetic Tree:     [ 529 tips and 528 internal nodes ]
refseq()         DNASTringSet:          [ 529 reference sequences ]
```

The `import_biom()` function returns a `phyloseq` object which includes the OTU table (which contains the OTU counts for each sample), the sample data matrix (containing the metadata for each sample), the taxonomy table (the predicted taxonomy for each OTU), the phylogenetic tree, and the OTU representative sequences.

Print the metadata using the `phyloseq` function `sample_data()`:

```
> sample_data(ps)
Sample Data:             [34 samples by 4 sample variables]:
                        Season Depth Month Year
B0214D1-PL1-D1    Winter      1      2    14
B0214D2-PL1-E1    Winter     10      2    14
B0214D3-PL1-F1    Winter     20      2    14
B0314D1-PL1-G1    Spring      1      3    14
B0314D2-PL1-H1    Spring     10      3    14
B0314D3-PL1-A2    Spring     20      3    14
B0414D1-PL1-B2    Spring      1      4    14
B0414D2-PL1-C2    Spring     10      4    14
B0414D3-PL1-D2    Spring     20      4    14
B0514D1-PL1-E2    Spring      1      5    14
B0514D2-PL1-F2    Spring     10      5    14
B0514D3-PL1-G2    Spring     20      5    14
B0614D1-PL1-H2    Summer      1      6    14
B0614D2-PL1-A3    Summer     10      6    14
B0714D2-PL1-B3    Summer     10      7    14
B0714D3-PL1-C3    Summer     20      7    14
B0814D1-PL1-D3    Summer      1      8    14
B0814D2-PL1-E3    Summer     10      8    14
```

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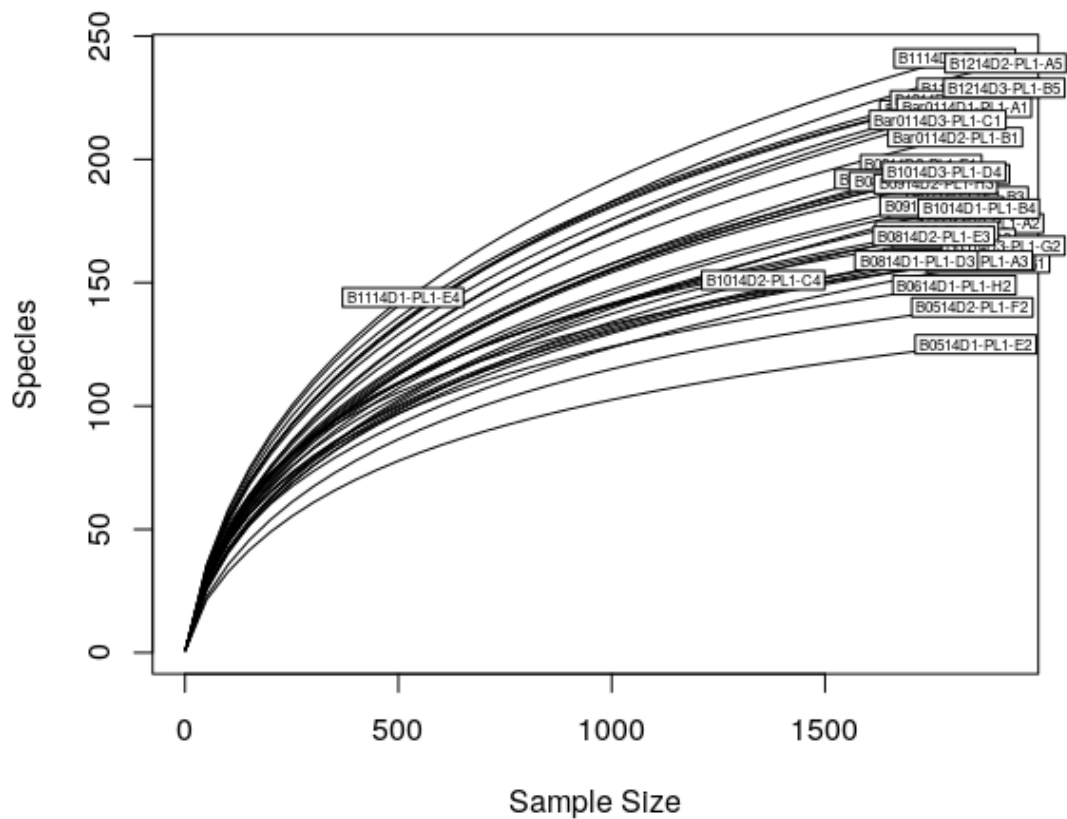
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B0814D3-PL1-F3	Summer	20	8	14
B0914D1-PL1-G3	Fall	1	9	14
B0914D2-PL1-H3	Fall	10	9	14
B0914D3-PL1-A4	Fall	20	9	14
B1014D1-PL1-B4	Fall	1	10	14
B1014D2-PL1-C4	Fall	10	10	14
B1014D3-PL1-D4	Fall	20	10	14
B1114D1-PL1-E4	Fall	1	11	14
B1114D2-PL1-F4	Fall	10	11	14
B1114D3-PL1-G4	Fall	20	11	14
B1214D1-PL1-H4	Winter	1	12	14
B1214D2-PL1-A5	Winter	10	12	14
B1214D3-PL1-B5	Winter	20	12	14
Bar0114D1-PL1-A1	Winter	1	1	14
Bar0114D2-PL1-B1	Winter	10	1	14
Bar0114D3-PL1-C1	Winter	20	1	14

The sample data contains 4 features for each sample: the season of sampling, the sampling depth (in m), the month and the year of sampling .

Plot the rarefaction curves using vegan function `rarecurve()` :

```
> rarecurve(t(otu_table(ps)), step=50, cex=0.5)
```



`otu_table()` is a phyloseq function which extract the OTU table from the phyloseq object.

Rarefy the samples without replacement. Rarefaction is used to simulate even number of reads per sample. In this example, the rarefaction depth chosen is the 90% of the minimum sample depth in the dataset (in this case 459 reads per sample).

```
> # rarefy without replacement
> ps.rarefied = rarefy_even_depth(ps, rngseed=1, sample.size=0.9*min(sample_sums(ps)),
  ↪ replace=F)
```

Warning:

- Rarefaction can waste a lot of data and would not be necessary. See <https://doi.org/10.1371/journal.pcbi.1003531>.
- Remember to set the random seed (`rngseed`) for repeatable experiments.

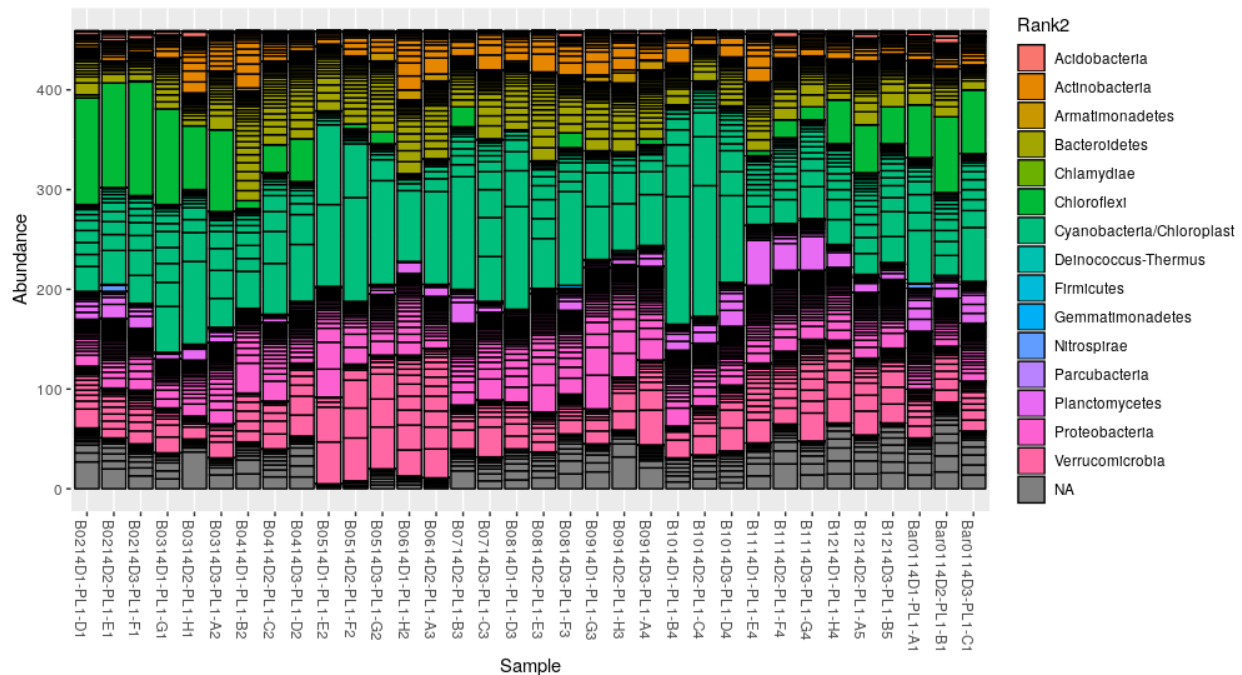
Exercise

Plot the samples depths before and after the rarefaction using the phyloseq function `sample_sums()`.

6.3 Plot abundances

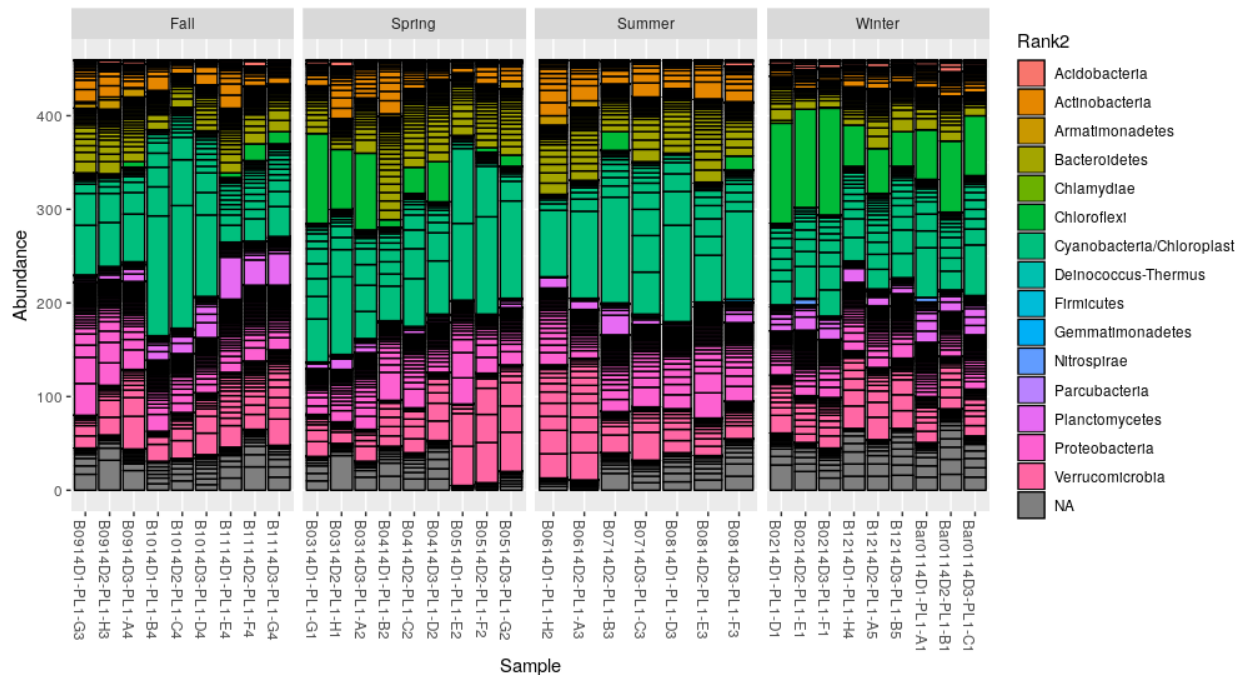
Using the rarefied dataset, make a stacked barplot of the abundances (read counts) and color each OTU (i.e. each bar) according its classified phylum (in this case Rank2):

```
> plot_bar(ps.rarefied, fill="Rank2")
```



The `plot_bar()` function returns a `ggplot2` object that can be customized with additional options, in this case we separate the samples in 4 panels according to the season:

```
> plot_bar(ps.rarefied, fill="Rank2") + facet_wrap(~Season, scales="free_x", nrow=1)
```

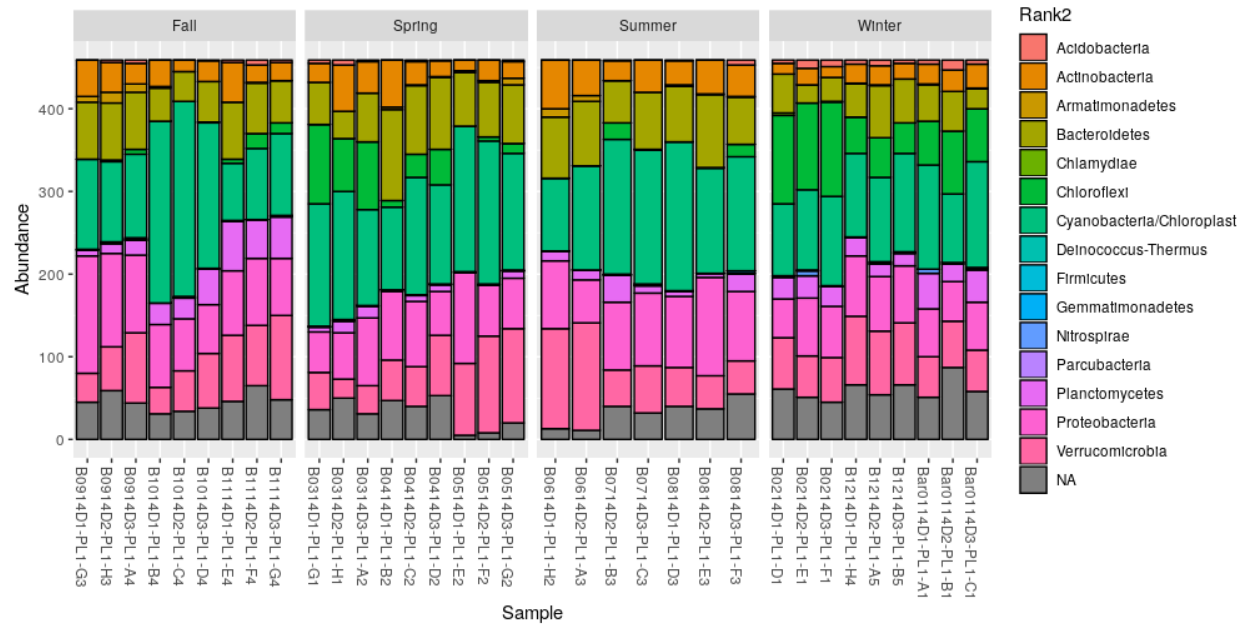


Alternatively, we can merge the OTUs at the phylum level and build a new `phyloseq` object. Given a taxonomic rank (in this case the phylum), the `phyloseq` function `tax_glom` merges the OTUs with the same taxonomy, summing the abundances:

```
> ps.phylum = tax_glom(ps.rarefied, taxrank="Rank2", NArm=FALSE)
> ps.phylum
phyloseq-class experiment-level object
otu_table() OTU Table: [ 35 taxa and 34 samples ]
sample_data() Sample Data: [ 34 samples by 4 sample variables ]
tax_table() Taxonomy Table: [ 35 taxa by 6 taxonomic ranks ]
phy_tree() Phylogenetic Tree: [ 35 tips and 34 internal nodes ]
refseq() DNASTringSet: [ 35 reference sequences ]
```

The option `NArm` set to `FALSE` forces the function to keep the unclassified OTUs at the phylum level. Now we can make a cleaner bar plot:

```
> plot_bar(ps.phylum, fill="Rank2") + facet_wrap(~Season, scales="free_x", nrow=1)
```



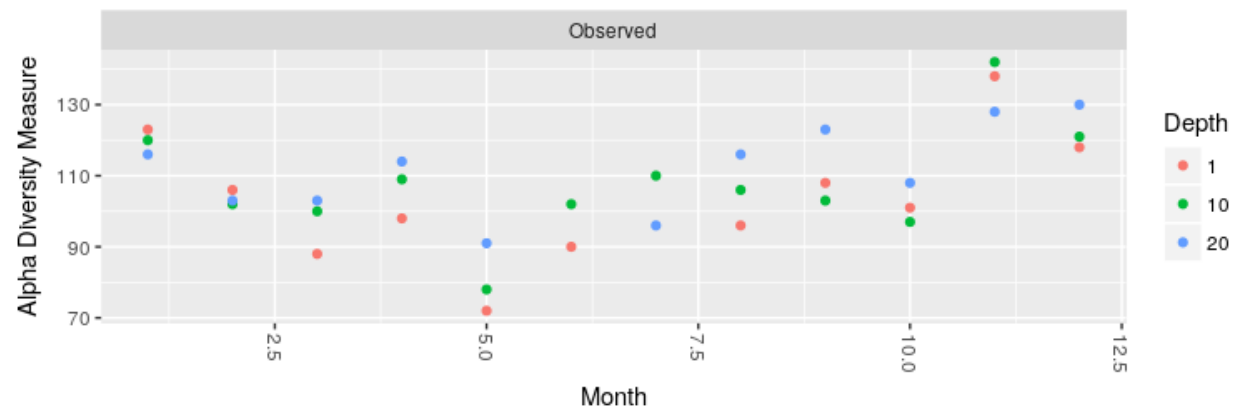
Exercise

Make a stacked barplot at class level (Rank3).

6.4 Alpha diversity

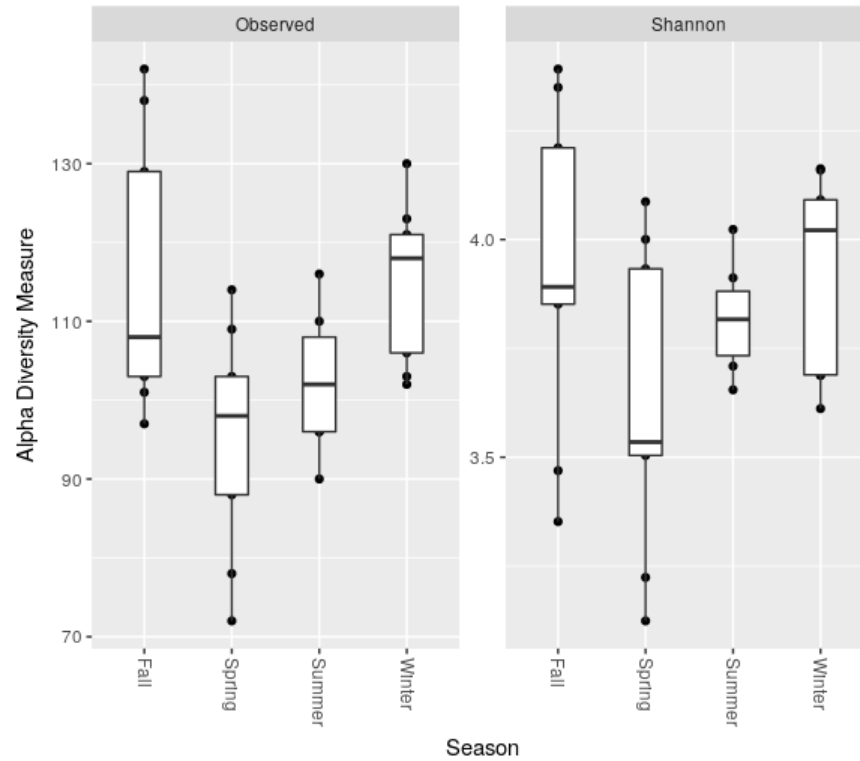
Plot the number of OTUs at each month coloring the points according to the sampling depth:

```
> plot_richness(ps.rarefied, x="Month", color="Depth", measures=c("Observed"))
```



Make a boxplot of the number of OTUs and the Shannon entropy grouping the different months by season:

```
> plot_richness(ps.rarefied, x="Season", measures=c("Observed", "Shannon")) + geom_
  ↪ boxplot()
```



We can export a `data.frame` containing a number of standard alpha diversity estimates using the `phyloseq` function `estimate_richness()`

```
> rich = estimate_richness(ps.rarefied)
> rich
```

	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson
↪InvSimpson Fisher							
B0214D1.PL1.D1	106	197.8667	35.57985	188.3066	8.170040	3.687611	0.9299652
↪14.27862 43.21532							
B0214D2.PL1.E1	102	143.1304	16.39579	161.0871	6.968287	3.689071	0.9314271
↪14.58303 40.65808							
B0214D3.PL1.F1	103	184.0588	30.82336	190.4337	7.690088	3.611560	0.9227125
↪12.93871 41.28956							
B0314D1.PL1.G1	88	137.4000	21.40127	142.2737	6.479689	3.534831	0.9325188
↪14.81895 32.34465							
B0314D2.PL1.H1	100	222.7692	47.63464	203.5988	7.938369	3.504056	0.9304873
↪14.38587 39.41058							
B0314D3.PL1.A2	103	178.2000	30.13564	160.8535	6.547177	3.787005	0.9486475
↪19.47324 41.28956							
B0414D1.PL1.B2	98	143.0000	20.26436	136.2743	5.823351	4.086749	0.9750571
↪40.09153 38.18345							
B0414D2.PL1.C2	109	224.9091	47.96245	172.8367	7.082246	4.000190	0.9664754
↪29.82883 45.18882							
B0414D3.PL1.D2	114	186.5455	26.01395	211.5217	8.993286	3.932662	0.9602954
↪25.18601 48.58680							
B0514D1.PL1.E2	72	99.1875	13.13050	109.1346	6.234068	3.124113	0.9126215
↪11.44446 23.97705							
B0514D2.PL1.F2	78	109.1667	14.13628	122.0444	6.234465	3.223947	0.9125835
↪11.43949 26.97943							
B0514D3.PL1.G2	91	128.0588	16.43157	126.6355	5.731954	3.524923	0.9258547
↪13.48704 34.04531							

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B0614D1.PL1.H2	90	123.0000	15.00832	128.4771	5.792422	3.816668	0.9577323	└
↪23.65873 33.47364								
B0614D2.PL1.A3	102	151.2857	19.37303	167.6238	7.074761	3.757622	0.9423821	└
↪17.35571 40.65808								
B0714D2.PL1.B3	110	172.6364	23.12117	187.8670	8.028159	3.709128	0.9258547	└
↪13.48704 45.85743								
B0714D3.PL1.C3	96	141.5556	19.02400	151.4630	6.818280	3.850288	0.9634946	└
↪27.39319 36.97645								
B0814D1.PL1.D3	96	178.5000	34.94025	155.8289	6.447118	3.654719	0.9326233	└
↪14.84192 36.97645								
B0814D2.PL1.E3	106	155.5000	19.71247	162.2091	6.744816	4.022988	0.9689815	└
↪32.23887 43.21532								
B0814D3.PL1.F3	116	216.6471	36.87625	215.7956	8.770340	3.911931	0.9456952	└
↪18.41456 49.98502								
B0914D1.PL1.G3	108	168.2727	22.42221	201.5552	9.294336	3.891102	0.9617763	└
↪26.16180 44.52562								
B0914D2.PL1.H3	103	162.3684	23.12990	180.7485	8.445071	3.886107	0.9643964	└
↪28.08706 41.28956								
B0914D3.PL1.A4	123	178.0000	19.47167	199.4132	8.292517	4.090999	0.9670545	└
↪30.35312 55.06042								
B1014D1.PL1.B4	101	173.5263	27.19010	193.4237	8.337151	3.469170	0.9060428	└
↪10.64314 40.03176								
B1014D2.PL1.C4	97	251.0000	63.34083	207.5726	8.807031	3.352156	0.8968440	└
↪9.69406 37.57745								
B1014D3.PL1.D4	108	180.0588	27.98694	171.2683	6.839082	3.851583	0.9479830	└
↪19.22447 44.52562								
B1114D1.PL1.E4	138	244.6364	35.62005	235.2076	8.598060	4.349086	0.9764620	└
↪42.48457 66.94886								
B1114D2.PL1.F4	142	217.6774	24.31684	250.3584	9.765194	4.391405	0.9794808	└
↪48.73491 70.36907								
B1114D3.PL1.G4	129	206.5385	26.10650	225.4320	8.773816	4.210509	0.9742881	└
↪38.89256 59.64440								
B1214D1.PL1.H4	118	240.0625	44.22653	241.1003	9.310808	4.091076	0.9714972	└
↪35.08426 51.40601								
B1214D2.PL1.A5	121	185.5652	23.38079	199.4590	8.499590	4.159264	0.9720763	└
↪35.81183 53.58096								
B1214D3.PL1.B5	130	256.1364	40.94272	298.4156	10.584524	4.162425	0.9733673	└
↪37.54785 60.43014								
Bar0114D1.PL1.A1	123	190.7778	23.19105	215.1598	8.974270	4.021200	0.9614251	└
↪25.92359 55.06042								
Bar0114D2.PL1.B1	120	216.3158	34.30966	222.7492	9.064837	4.028745	0.9586721	└
↪24.19674 52.85012								
Bar0114D3.PL1.C1	116	187.8696	25.47702	221.1842	8.864324	3.932334	0.9560141	└
↪22.73454 49.98502								

Test whether the observed number of OTUs differs significantly between seasons. We make a non-parametric test, the Wilcoxon rank-sum test (Mann-Whitney):

```
> pairwise.wilcox.test(rich$Observed, sample_data(ps.rarefied)$Season)

Pairwise comparisons using Wilcoxon rank sum test

data:  rich$Observed and metasample_data(ps.rarefied)$Season

      Fall  Spring Summer
Spring 0.112 -      -
```

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```

Summer 0.270 0.681 -
Winter 1.000 0.025 0.112

P value adjustment method: holm

```

By default, the function `pairwise.wilcox.test()` reports the pairwise adjusted (Holm) p-values.

Exercise

Repeat the test on the Shannon indexes.

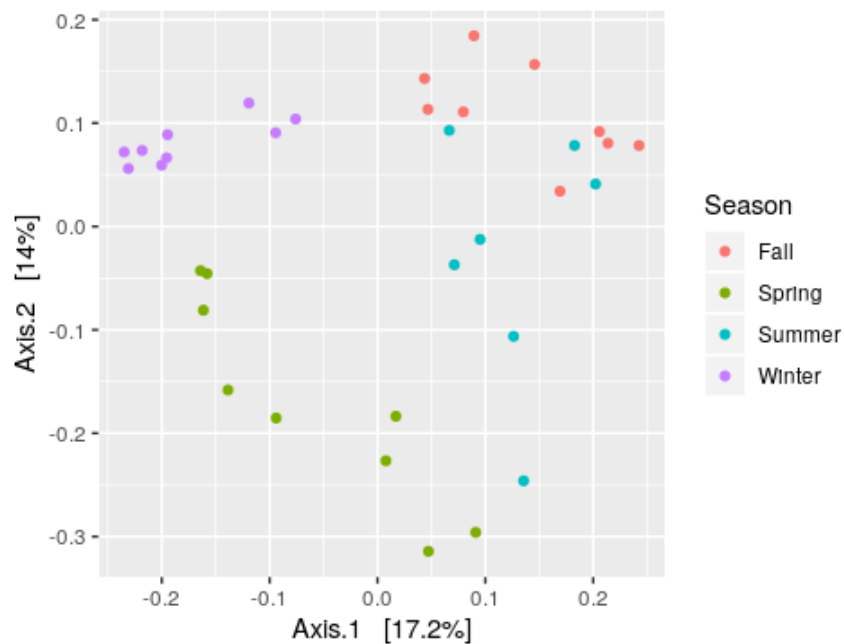
6.5 Beta diversity

Plot the PCoA using the unweighted UniFrac as distance:

```

> # PCoA plot using the unweighted UniFrac as distance
> wunifrac_dist = phyloseq::distance(ps.rarefied, method="unifrac", weighted=F)
> ordination = ordinate(ps.rarefied, method="PCoA", distance=wunifrac_dist)
> plot_ordination(ps.rarefied, ordination, color="Season") + theme(aspect.ratio=1)

```



Test whether the seasons differ significantly from each other using the permutational ANOVA (PERMANOVA) analysis:

```

> adonis(wunifrac_dist ~ sample_data(ps.rarefied)$Season)

```

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```

Call:
adonis(formula = wunifrac_dist ~ sample_data(ps.rarefied)$Season)

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

              Df SumsOfSqs MeanSqs F.Model    R2 Pr(>F)
sample_data(ps.rarefied)$Season  3    1.3011 0.43372  4.1604 0.29381 0.001 ***
Residuals                      30    3.1274 0.10425             0.70619
Total                          33    4.4286             1.00000
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

Exercise

Make the PCoA and the PERMANOVA using the Bray-Curtis dissimilarity instead.

6.6 OTU differential abundance testing with DESeq2

To test the differences at OTU level between seasons using DESeq2, we need to convert the `Season` column into factor. Note that we use the data without rarefaction (i.e. `ps` object):

```
> sample_data(ps)$Season <- as.factor(sample_data(ps)$Season)
```

Convert the phyloseq object to a DESeqDataSet and run DESeq2:

```
> ds = phyloseq_to_deseq2(ps, ~ Season)
> ds = DESeq(ds)
```

Extract the result table from the `ds` object using the DESeq2 function `results` and filter the OTUs using a False Discovery Rate (FDR) cutoff of 0.01. In this example we return the significantly differentially abundant OTU between the seasons “Spring” and “Fall”:

```

> alpha = 0.01
> res = results(ds, contrast=c("Season", "Spring", "Fall"), alpha=alpha)
> res = res[order(res$padj, na.last=NA), ]
> res_sig = res[(res$padj < alpha), ]
> res_sig
log2 fold change (MLE): Season Spring vs Fall
Wald test p-value: Season Spring vs Fall
DataFrame with 62 rows and 6 columns
      baseMean  log2FoldChange  lfcSE      stat
  <numeric>    <numeric>      <numeric> <numeric>
1 DENOV017  22.7436598625802 -4.1529844728879 0.552035702386233 -7.52303601911288 5.
2 35186717121325e-14 1.24163318372147e-11
3 DENOV035  10.6015033917283 -7.36751901929925 1.01933372324247 -7.22777913779147 4.
4 90956301343594e-13 5.6950930955857e-11
5 DENOV091  5.31287448011852 -6.51255526618412 0.947998700432628 -6.86979345352695 6.
6 42949270405053e-12 4.97214102446574e-10

```

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```

DENOVO2  82.4704545010533 -4.14259840011034 0.673404296938788 -6.15172552201119 7.
↳66444402875036e-10 4.44537753667521e-08
DENOVO7  15.6311735008548 5.91263059667889 0.979789881740526 6.0345903819455 1.
↳59366414316775e-09 7.39460162429838e-08
...
DENOVO83 3.63662006180492 1.92505847356698 0.617438877584007 3.11781221341228 0.
↳00182198852945677 0.00728795411782707
DENOVO89 2.68296393708501 2.84137889985046 0.912892035548744 3.11250267195342 0.
↳00185508334637251 0.00729456502302411
DENOVO72 4.86241695816352 2.71763740147229 0.895564240058129 3.03455327927775 0.
↳00240892202480818 0.00931449849592497
DENOVO21 17.208142677795 -1.1266184329166 0.373108760004578 -3.01954430901804 0.
↳002531552600065 0.00962820005270621
DENOVO55 6.24723247307275 2.09415598552554 0.695335908667259 3.01171845063975 0.
↳00259773414843998 0.00972055358771089

```

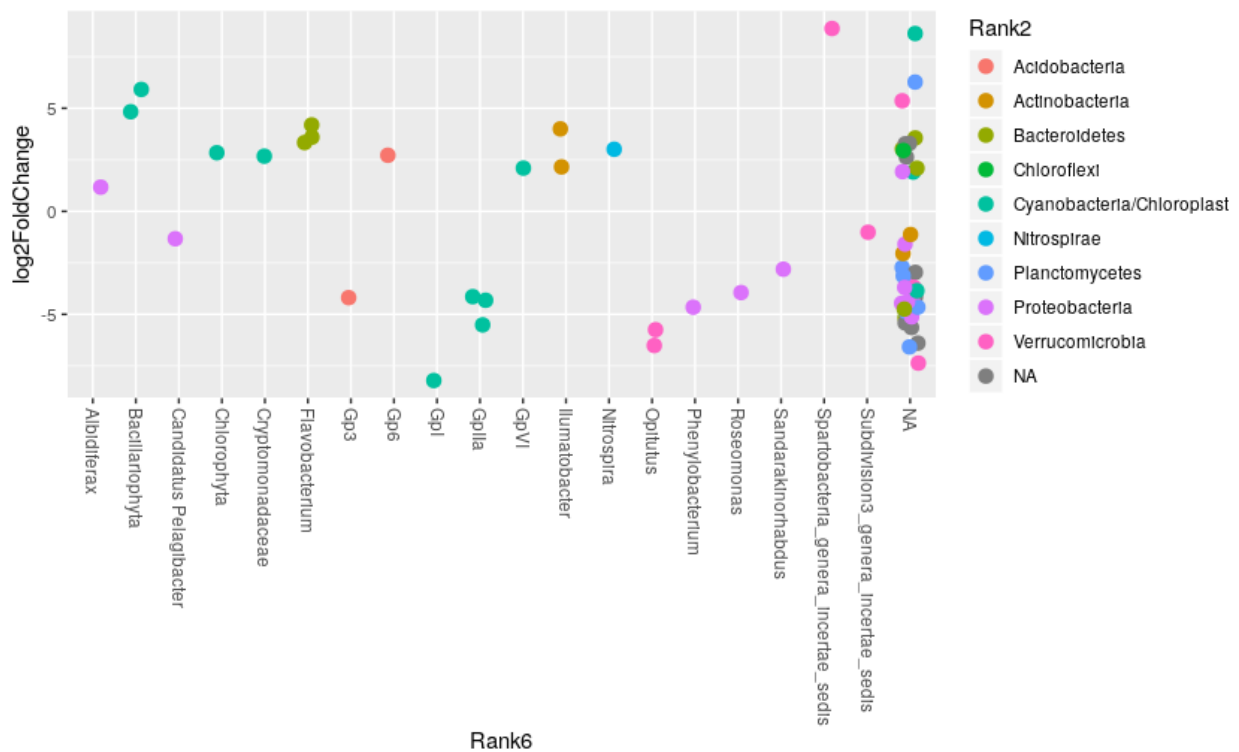
The result table reports base means across samples, log2 fold changes, standard errors, test statistics, p-values and adjusted p-values.

Make a genus vs log2FC plot of the significant OTUs:

```

> res_sig = cbind(as(res_sig, "data.frame"), as(tax_table(ps)[rownames(res_sig), ],
↳"matrix"))
> ggplot(res_sig, aes(x=Rank6, y=log2FoldChange, color=Rank2)) +
  geom_jitter(size=3, width = 0.2) +
  theme(axis.text.x = element_text(angle = -90, hjust = 0, vjust=0.5))

```



Exercise

Test the differences between summer and fall and compare the results with those above.

CHAPTER 7

Compute basic statistics, rarefy and summarize OTU/SV tables using micca

Note: This tutorial requires *Paired-end sequencing - 97% OTU* to be done.

The command `tablestats` reports a sample summary, an OTU summary and the rarefaction curves for the input OTU/SV table:

```
micca tablestats -i denovo_greedy_otus/otutable.txt -o tablestats
```

Inspecting the file `tablestats/tablestats_samplesumm.txt` you can see that the less abundant sample contains 512 reads:

Sample	Depth	NOTU	NSingle
B1114D1-PL1-E4	512	145	68
B1014D2-PL1-C4	1356	152	57
B0214D3-PL1-F1	1665	192	74
...

Note: Rarefaction curves can be inspected through `tablestats/tablestats_rarecurve.txt` and `tablestats/tablestats_rarecurve_plot.png`.

To compare different samples, the OTU/SV table must be subsampled (rarefied) using the command `tablerare`. In this case we are interested in rarefy the table with the depth of the less abundant sample (B1114D1-PL1-E4):

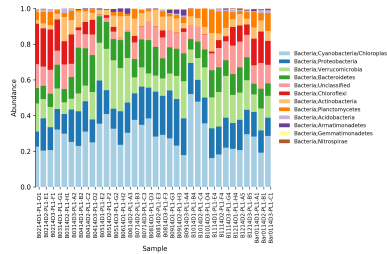
```
micca tablerare -i denovo_greedy_otus/otutable.txt -o denovo_greedy_otus/otutable_
↪rare.txt -d 500
```

Now we can summarize communities by their taxonomic composition. The `tabletotax` creates in the output directory a table for each taxonomic level (`taxtable1.txt`, ..., `taxtableN.txt`). OTU counts are summed together if they have the same taxonomy at the considered level.

```
micca tabletotax -i denovo_greedy_otus/otutable_rare.txt -t denovo_greedy_otus/taxa.
↪txt -o taxtables
```

Finally, we can generate a relative abundance bar plot from generated taxa tables, using the command `tablebar`. In this case only the bar plot relative to the taxonomy level 2 (phylum) will be generated:

```
micca tablebar -i taxtables/taxtable2.txt -o taxtables/taxtable2.png
```



Picking OTUs for use in PICRUSt

PICRUSt (doi: 10.1038/nbt.2676) is a software designed to predict metagenome functional content from marker gene (e.g., 16S rRNA) surveys and full genomes. This tutorial covers how to pick OTUs from 16S rRNA sequences data to use with PICRUSt.

Note: Requires *Quality filtering* in *Single-end sequencing* to be done and the PICRUSt software to be installed in your system. Warning: PICRUSt 1.0.0 requires the biom-format package v1.3.1 to be installed in your system (from the command line run: `pip install biom-format==1.3.1`, for more information see <http://biom-format.org/>).

PICRUSt requires an *Closed-reference* OTU table computed against the Greengenes reference (clustered at 97% identity). Download the reference database (Greengenes, version 2013/05), clustered at 97% identity:

```
wget ftp://ftp.fmach.it/metagenomics/micca/dbs/gg_2013_05.tar.gz
tar -zxvf gg_2013_05.tar.gz
```

Run the micca closed-reference protocol:

```
micca otu -m closed_ref -i filtered.fasta -o closed_ref_otus -r 97_otus.fasta -d 0.97
↪ -t 4
cd closed_ref_otus
```

Report the sample summary:

```
micca tablestats -i otutable.txt -o tablestats
head tablestats/tablestats_samplesumm.txt
```

Sample	Depth	NOTU	NSingle
Mw_03	1084	132	39
Mw_06	1387	122	27
Mw_11	1485	155	44
Mw_07	1528	150	36
Mw_01	1537	143	35
Mw_15	1565	144	35

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Mw_14	1610	149	42
Mw_02	1670	143	43
Mw_12	1710	153	54

Rarefy the OTU table for the PICRUSt analysis is always a good idea (see <https://groups.google.com/forum/#!topic/picrust-users/ev5uZGUIPrQ>), so we will rarefy the table at 1084 sequences per sample using *tablerare*:

```
micca tablerare -i otutable.txt -o otutable_rare.txt -d 1084
```

Convert the rarefied OTU table into the BIOM format replacing the OTU IDs with the original sequence IDs using the *tobiom* command:

```
micca tobiom -i otutable_rare.txt -o tables.biom -u otuids.txt
```

Normalize the OTU table by dividing each OTU by the known/predicted 16S copy number abundance using the PICRUSt script `normalize_by_copy_number.py`:

```
normalize_by_copy_number.py -i tables.biom -o normalized_otus.biom
```

Create the final metagenome functional predictions using the PICRUSt script `predict_metagenomes.py`:

```
predict_metagenomes.py -i normalized_otus.biom -o metagenome_predictions.biom
```

Now you can analyze the PICRUSt predicted metagenome as described in http://picrust.github.io/picrust/tutorials/downstream_analysis.html#downstream-analysis-guide.

OTU picking and Denoising

Usually, amplicon sequences are clustered into **Operational Taxonomic Units (OTUs)** using a similarity threshold of **97%**, which represents the common working definition of bacterial species.

Another approach consists to define the so-called **Sequence Variants (SVs)**, a.k.a Amplicon Sequence Variants - ASVs, Exact Sequence Variants ESVs, zero-radius OTUs - ZOTU, unique sequence variants or sub-OTUs). This approach avoids clustering sequences at a predefined similarity threshold and usually includes a denoising algorithm in order to identify SVs (see **UNOISE**, **DADA2**, **Deblur**, **oligotyping** and **swarm**).

The *otu* command assigns similar sequences (marker genes such as 16S rRNA and the fungal ITS region) to operational taxonomic units or sequence variants (OTUs or SVs).

- *Methods*
 - *De novo greedy*
 - *De novo UNOISE*
 - *Closed-reference*
 - *Open-reference*
 - *De novo swarm*
- *Definition of identity*
- *Output files*

9.1 Methods

9.1.1 De novo greedy

In denovo greedy clustering (parameter `--method denovo_greedy`), sequences are clustered without relying on an external reference database, using an approach similar to the UPARSE pipeline (<https://doi.org/10.1038/nmeth.>

2604) and tested in <https://doi.org/10.7287/peerj.preprints.1466v1>. This protocol includes in a single command dereplication, clustering and chimera filtering:

1. Dereplication. Predict sequence abundances of each sequence by dereplication, order by abundance and discard sequences with abundance value smaller than MINSIZE (option `-s/--minsize`, default value 2);
2. Greedy clustering. Distance (DGC) and abundance-based (AGC) strategies are supported (option `--greedy`, see <https://doi.org/10.1186/s40168-015-0081-x> and <https://doi.org/10.7287/peerj.preprints.1466v1>). Therefore, the candidate representative sequences are obtained;
3. Chimera filtering (optional). Remove chimeric sequences from the representatives performing a de novo chimera detection (option `--rmchim`);
4. Map sequences. Map sequences to the representatives.

Example:

```
micca otu -m denovo_greedy -i filtered.fasta -o denovo_greedy_otus -d 0.97 -c -t 4
```

9.1.2 De novo UNOISE

Denoise amplicon sequences using the [UNOISE3](#) protocol. The method is designed for Illumina (paired or unpaired) reads. This protocol includes in a single command dereplication, denoising and chimera filtering:

1. Dereplication; Predict sequence abundances of each sequence by dereplication, order by abundance and discard sequences with abundance value smaller than MINSIZE (option `-s/--minsize`, default value 8);
2. Denoising;
3. Chimera filtering (optional);
4. Map sequences. Map sequences to the representatives.

Example:

```
micca otu -m denovo_unoise -i filtered.fasta -o denovo_unoise_otus -c -t 4
```

9.1.3 Closed-reference

Sequences are clustered against an external reference database and reads that could not be matched are discarded (method `closed_ref`). Example:

Download the reference database (Greengenes), clustered at 97% identity:

```
wget ftp://ftp.fmach.it/metagenomics/micca/dbs/gg_2013_05.tar.gz
tar -zxvf gg_2013_05.tar.gz
```

Run the closed-reference protocol:

```
micca otu -m closed_ref -i filtered.fasta -o closed_ref_otus -r 97_otus.fasta -d 0.97 -t 4
```

Simply perform a sequence ID matching with the reference taxonomy file (see [classify](#)):

```
cd closed_ref_otus
micca classify -m otuid -i otuids.txt -o taxa.txt -x ../97_otu_taxonomy.txt
```

9.1.4 Open-reference

Open-reference clustering (`open_ref`): sequences are clustered against an external reference database (as in *Closed-reference*) and reads that could not be matched are clustered with the *De novo greedy* protocol. Example:

Download the reference database (Greengenes), clustered at 97% identity:

```
wget ftp://ftp.fmach.it/metagenomics/micca/dbs/gg_2013_05.tar.gz
tar -zxvf gg_2013_05.tar.gz
```

Run the open-reference protocol:

```
micca otu -m open_ref -i filtered.fasta -o open_ref_otus -r 97_otus.fasta -d 0.97 -t 4 -c
```

Run the VSEARCH-based consensus classifier or the RDP classifier (see *classify*):

```
cd open_ref_otus
micca classify -m cons -i otus.fasta -o taxa.txt -r ../97_otus.fasta -x ../97_otu_taxonomy.txt -t 4
```

9.1.5 De novo swarm

In denovo swarm clustering (doi: 10.7717/peerj.593, doi: 10.7717/peerj.1420, <https://github.com/torognes/swarm>, parameter `--method denovo_swarm`), sequences are clustered without relying on an external reference database. From <https://github.com/torognes/swarm>:

The purpose of swarm is to provide a novel clustering algorithm that handles massive sets of amplicons. Results of traditional clustering algorithms are strongly input-order dependent, and rely on an arbitrary global clustering threshold. swarm results are resilient to input-order changes and rely on a small local linking threshold `d`, representing the maximum number of differences between two amplicons. swarm forms stable, high-resolution clusters, with a high yield of biological information.

`otu` includes in a single command dereplication, clustering and de novo chimera filtering:

1. Dereplication. Predict sequence abundances of each sequence by dereplication, order by abundance and discard sequences with abundance value smaller than `MINSIZE` (option `--minsize` default value is 1, i.e. no filtering);
2. Swarm clustering. Fastidious option is recommended (`--swarm-fastidious`);
3. Chimera filtering (optional).

Warning: Removing ambiguous nucleotides (N) (with the option `--maxns 0` in *filter*) is mandatory if you use the de novo swarm clustering method.

Example:

```
micca filter -i trimmed.fastq -o filtered.fasta -e 0.5 -m 350 -t --maxns 0
micca otu -m denovo_swarm -i filtered.fasta -o otus_denovo_swarm -c --minsize 1 --
  swarm-fastidious -t 4
```

9.2 Definition of identity

In micca, the pairwise identity (except for ‘de novo swarm’ and ‘denovo unoise’) is defined as the edit distance excluding terminal gaps (same as in USEARCH and BLAST):

$$\frac{\text{\# matching columns}}{\text{alignment length} - \text{terminal gaps}}$$

9.3 Output files

The *otu* command returns in a single directory 5 files:

otutable.txt TAB-delimited file, containing the number of times an OTU is found in each sample (OTU x sample, see *Supported file formats*):

OTU	Mw_01	Mw_02	Mw_03	...
DENOVO1	151	178	177	...
DENOVO2	339	181	142	...
DENOVO3	533	305	63	...
...

otus.fasta FASTA containing the representative sequences (OTUs):

```
>DENOVO1
GACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGGGG...
>DENOVO2
GATGAACGCTAGCTACAGGCTTAACACATGCAAGTCGAGGGGCA...
>DENOVO3
AGTGAACGCTGGCGACGTGGTTAAGACATGCAAGTCGAGCGGTA...
...
```

otuids.txt TAB-delimited file which maps the OTU ids to original sequence ids:

DENOVO1	IS0AYJS04JQKIS;sample=Mw_01
DENOVO2	IS0AYJS04JL6RS;sample=Mw_01
DENOVO3	IS0AYJS04H4XNN;sample=Mw_01
...	

hits.txt TAB-separated file, three-columns, where each column contains: the matching sequence, the representative (seed) and the identity (if available, see *Definition of identity*):

IS0AYJS04JE658;sample=Mw_01; IS0AYJS04I4XYN;sample=Mw_01	99.4
IS0AYJS04JPH34;sample=Mw_01; IS0AYJS04JVUBC;sample=Mw_01	98.0
IS0AYJS04I67XN;sample=Mw_01; IS0AYJS04JVUBC;sample=Mw_01	99.7
...	

otuschim.fasta (only for ‘denovo_greedy’, ‘denovo_swarm’ and ‘open_ref’ methods, when *-c/--rmchim* is specified) FASTA file containing the chimeric otus.

Warning: Trimming the sequences to a fixed position before clustering is strongly recommended when they cover partial amplicons or if quality deteriorates towards the end (common when you have long amplicons and single-end sequencing), see *Quality filtering*.

Note: De novo OTUs are renamed to `DENOVO [N]` and reference OTUs to `REF [N]`.

Supported file formats

10.1 Sequence files

FASTA and **FASTQ** Sanger/Illumina 1.8+ format (phred+33) formats are supported. *micca* provides the *convert* command to convert between sequence file formats.

10.2 Taxonomy files

Taxonomy files map sequence IDs to taxonomy. Input taxonomy files must be TAB-delimited files where rows are either in the form:

1. SEQID[TAB]k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;
g__;
2. SEQID[TAB]Bacteria;Firmicutes;Clostridia;Clostridiales;;;;
3. SEQID[TAB]Bacteria;Firmicutes;Clostridia;Clostridiales
4. SEQID[TAB]D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;
D_3__Clostridiales;D_4__;D_5__;

Compatible taxonomy files are:

- Greengenes (<http://greengenes.secondgenome.com/downloads>);
- QIIME-formatted SILVA (<https://www.arb-silva.de/download/archive/qiime/>);
- UNITE (<https://unite.ut.ee/repository.php>);
- Human Oral Microbiome Database (HOMD) (<http://www.homd.org/>).

The output taxonomy file returned by *classify* is a TAB-delimited file where each row is in the format:

```
SEQID[TAB]Bacteria;Firmicutes;Clostridia;Clostridiales
```

10.3 OTU/SV tables and taxonomy tables

The OTU table returned by *otu* is an OTU x sample, TAB-delimited text file, containing the number of times an OTU is found in each sample:

OTU	Mw_01	Mw_02	Mw_03	...
DENOVO1	151	178	177	...
DENOVO2	339	181	142	...
DENOVO3	533	305	63	...
DENOVO4	166	299	115	...
...

The *tabletotax* command returns the “taxonomy tables” for each taxonomic level, e.g.:

OTU	Mw_01	Mw_02	Mw_03	...
Bacteria;Bacteroidetes	1363	1543	1168	...
Bacteria;Cyanobacteria/Chloroplast	0	0	0	...
Bacteria;Firmicutes	6257	5780	6761	...
Bacteria;Lentisphaerae	0	1	0	...
...

10.4 Sample data

The sample data file contains all of the information about the samples. In QIIME this file is called *Mapping File*. In micca, the sample data file must be a TAB-delimited text file (a row for each sample). The first column must be the sample identifier (assigned in *merge*, *split* or *mergepairs*):

ID	Group	Altitude
Mw_01	Mw1	492
Mw_02	Mw1	492
Mw_09	Mw1	492
Mw_12	Mw1	492
...

10.5 Phylogenetic tree

Only the *Newick format* is supported.

10.6 BIOM file

The *tobiom* command generates OTU/SV tables in the biom version 1.0 JSON file format (http://biom-format.org/documentation/format_versions/biom-1.0.html).

11.1 Version 1.7.0

- VSEARCH updated to version 2.7.1;
- Denoising protocol added to the otu command for Illumina reads (UNOISE);
- -S/-chim-abskew option added to micca otu command;
- Documentation updated;
- multithread option added to the mergepairs command.

11.2 Version 1.6.2 (bug fix release)

- Definitely fix the “new-line error” in classify;
- Fix bar plots in “stats”.

11.3 Version 1.6.1 (bug fix release)

- Fix the “new-line error” in classify.

11.4 Version 1.6.0

- swarm updated to version 2.1.12;
- Now the mergepairs command allows merging staggered reads by default. With the new option -n/--nostagger the command produces the same results of the previous versions (≤ 1.5);
- classify and tabletotax commands now strip the D_X__ prefix from the Silva taxonomy files;

- Documentation updated;
- Fix: remove duplicate file closing in `micca.api.merge()`.

11.5 Version 1.5.0

- Now the NAST algorithm trims candidate sequences to that which is bound by the beginning and end points of the alignment span; with the new option `--nast-notrim` in `micca msa` produces the same results of the previous version ($\leq 1.4.0$);
- Y-scale in `micca tablebar` when `--raw` fixed;
- “text file busy error” in `micca msa (nast)` fixed;
- pandas deprecation warnings fixed;
- documentation updated.

11.6 Version 1.4.0

- No-filter option added to `micca.api.msa.nast()` (do not remove positions which are gaps in every sequence) and to the `msa` command (`--nast-nofilter` option);
- documentation improved.

11.7 Version 1.3.0

- Swarm clustering algorithm added to `micca otu`;
- `micca.api.otu.denovo_swarm()` function added;
- `micca v1.3.0` includes: VSEARCH v1.9.5, MUSCLE v3.8.31, FastTree v2.1.8, swarm v2.1.8;
- documentation updated.

11.8 Version 1.2.2

- Now `micca` can generate plots with matplotlib when the `DISPLAY` environment variable is undefined;
- `MANIFEST.in`, Dockerfile updated.

11.9 Version 1.2.1

- Dockerfile added;
- Documentation improved.

11.10 Version 1.2.0

- Hits output file option added to `micca.api.msa.nast()` (`hits_fn` option) and to the `msa` command (`--nast-hits` option);
- `setup.py` improved.

11.11 Version 1.1.0

- Strand option added in `classify` (consensus-based classifier), `msa` (NAST) and `otu` (closed-reference and open reference OTU picking protocols) commands. Now these commands search both strand (default) instead the plus strand only.

11.12 Version 1.0.0

- micca 1.0.0 includes: VSEARCH v1.9.5, MUSCLE v3.8.31, FastTree v2.1.8.

CHAPTER 12

classify

```
usage: micca classify [-h] -i FILE -o FILE [-m {cons,rdp,otuid}] [-r FILE]
                        [-x FILE] [--cons-id CONS_ID]
                        [--cons-maxhits CONS_MAXHITS]
                        [--cons-minfrac CONS_MINFRAC]
                        [--cons-mincov CONS_MINCOV] [--cons-strand {both,plus}]
                        [--cons-threads THREADS]
                        [--rdp-gene {16srrna,fungallsu,fungalits_warcup,fungalits_unite}]
                        [--rdp-maxmem GB] [--rdp-minconf RDP_MINCONF]
```

micca classify assigns taxonomy for each sequence in the input file and provides three methods for classification:

* VSEARCH-based consensus classifier (cons): input sequences are searched in the reference database with VSEARCH (<https://github.com/torognes/vsearch>). For each query sequence the method retrieves up to 'cons-maxhits' hits (i.e. identity >= 'cons-id'). Then, the most specific taxonomic label that is associated with at least 'cons-minfrac' of the hits is assigned. The method is similar to the UCLUST-based consensus taxonomy assigner presented in doi: 10.7287/peerj.preprints.934v2 and available in QIIME.

* RDP classifier (rdp): only RDP classifier version >= 2.8 is supported (doi:10.1128/AEM.00062-07). In order to use this classifier RDP must be installed (download at <http://sourceforge.net/projects/rdp-classifier/files/rdp-classifier/>) and the RDPPATH environment variable setted. The available databases (--rdp-gene) are:

- 16S (16srrna)
- Fungal LSU (28S) (fungallsu)
- Warcup ITS (fungalits_warcup, doi: 10.3852/14-293)
- UNITE ITS (fungalits_unite)

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For more information about the RDP classifier go to
<http://rdp.cme.msu.edu/classifier/classifier.jsp>

* OTU ID classifier (otuid): simply perform a sequence ID matching with the reference taxonomy file. Recommended strategy when the closed reference clustering (`--method closedref` in micca-otu) was performed. OTU ID classifier requires a tab-delimited file where the first column contains the current OTU ids and the second column the reference taxonomy ids (see `otuids.txt` in micca-otu), e.g.:

```
REF1[TAB]1110191
REF2[TAB]1104777
REF3[TAB]1078527
...
```

The input reference taxonomy file (`--ref-tax`) should be a tab-delimited file where rows are either in the form:

```
1. SEQID[TAB]k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__;
2. SEQID[TAB]Bacteria;Firmicutes;Clostridia;Clostridiales;;;
3. SEQID[TAB]Bacteria;Firmicutes;Clostridia;Clostridiales
4. SEQID[TAB]D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__;D_
  ↪5__;
```

Compatible reference database are Greengenes
(<http://greengenes.secondgenome.com/downloads>), QIIME-formatted SILVA
(<https://www.arb-silva.de/download/archive/qiime/>) and UNITE
(<https://unite.ut.ee/repository.php>).

The output file is a tab-delimited file where each row is in the format:

```
SEQID[TAB]Bacteria;Firmicutes;Clostridia;Clostridiales
```

optional arguments:

`-h, --help` show this help message and exit

arguments:

```
-i FILE, --input FILE          input FASTA file (for 'cons' and 'rdp') or a tab-
                                delimited OTU ids file (for 'otuid') (required).

-o FILE, --output FILE         output taxonomy file (required).

-m {cons,rdp,otuid}, --method {cons,rdp,otuid}
                                classification method (default cons)

-r FILE, --ref FILE            reference sequences in FASTA format, required for
                                'cons' classifier.

-x FILE, --ref-tax FILE        tab-separated reference taxonomy file, required for
                                'cons' and 'otuid' classifiers.
```

VSEARCH-based consensus classifierspecific options:

```
--cons-id CONS_ID            sequence identity threshold (0.0 to 1.0, default 0.9).
--cons-maxhits CONS_MAXHITS
                                number of hits to consider (>=1, default 3).
--cons-minfrac CONS_MINFRAC
                                for each taxonomic rank, a specific taxa will be
```

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```

        assigned if it is present in at least MINFRAC of the
        hits (0.0 to 1.0, default 0.5).
--cons-mincov CONS_MINCOV
        reject sequence if the fraction of alignment to the
        reference sequence is lower than MINCOV. This
        parameter prevents low-coverage alignments at the end
        of the sequences (default 0.75).
--cons-strand {both,plus}
        search both strands or the plus strand only (default
        both).
--cons-threads THREADS
        number of threads to use (1 to 256, default 1).

RDP Classifier/Database specific options:
--rdp-gene {16srrna,fungallsu,fungalits_warcup,fungalits_unite}
        marker gene/region
--rdp-maxmem GB
        maximum memory size for the java virtual machine in GB
        (default 2)
--rdp-minconf RDP_MINCONF
        minimum confidence value to assign taxonomy to a
        sequence (default 0.8)

```

Examples

Classification of 16S sequences using the consensus classifier and Greengenes:

```

micca classify -m cons -i input.fasta -o tax.txt \
--ref greengenes_2013_05/rep_set/97_otus.fasta \
--ref-tax greengenes_2013_05/taxonomy/97_otu_taxonomy.txt

```

Classification of ITS sequences using the RDP classifier and the UNITE database:

```

micca classify -m rdp --rdp-gene fungalits_unite -i input.fasta \
-o tax.txt

```

OTU ID matching after the closed reference OTU picking protocol:

```

micca classify -m otuid -i otuids.txt -o tax.txt \
--ref-tax greengenes_2013_05/taxonomy/97_otu_taxonomy.txt

```


CHAPTER 13

convert

```
usage: micca convert [-h] -i FILE -o FILE [-q FILE] [-d DEFAULTQ]
                  [-f INPUT_FORMAT] [-F OUTPUT_FORMAT]

micca convert converts between sequence file formats. See
http://biopython.org/wiki/SeqIO#File\_Formats for a comprehensive list
of the supported file formats.

Supported input formats:
abi, abi-trim, ace, embl, embl-cds, fasta, fasta-qual, fastq, fastq-illumina,
fastq-sanger, fastq-solexa, gb, genbank, genbank-cds, ig, imgt, pdb-atom,
pdb-seqres, phd, pir, qual, seqxml, sff, sff-trim, swiss, tab, uniprot-xml

Supported output formats:
embl, fasta, fastq, fastq-illumina, fastq-sanger, fastq-solexa, gb, genbank,
imgt, phd, qual, seqxml, sff, tab

optional arguments:
-h, --help            show this help message and exit

arguments:
-i FILE, --input FILE
                        input sequence file (required).
-o FILE, --output FILE
                        output sequence file (required).
-q FILE, --qual FILE  input quality file (required for 'fasta-qual' input
                        format).
-d DEFAULTQ, --defaultq DEFAULTQ
                        default phred quality score for format-without-quality
                        to format-with-quality conversion (default 40).
-f INPUT_FORMAT, --input-format INPUT_FORMAT
                        input file format (default fastq).
-F OUTPUT_FORMAT, --output-format OUTPUT_FORMAT
                        input file format (default fasta).
```

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Examples

Convert FASTA+QUAL files into a FASTQ (Sanger/Illumina 1.8+) file:

```
micca convert -i input.fasta -q input.qual -o output.fastq \
-f fasta-qual -F fastq
```

Convert a SFF file into a FASTQ (Sanger/Illumina 1.8+) file:

```
micca convert -i input.sff -o output.fastq -f sff -F fastq
```

CHAPTER 14

filter

```
usage: micca filter [-h] -i FILE -o FILE [-e MAXEERATE] [-m MINLEN] [-t]
                  [-n MAXNS] [-f {fastq,fasta}]
```

micca filter filters sequences according to the maximum allowed expected error (EE) rate %. Optionally, you can:

- * discard sequences that are shorter than the specified length (suggested for Illumina overlapping paired-end (already merged) reads) (option --minlen MINLEN);

- * discard sequences that are shorter than the specified length AND truncate sequences that are longer (suggested for Illumina and 454 unpaired reads) (options --minlen MINLEN --trunc);

- * discard sequences that contain more than a specified number of Ns (--maxns).

Sequences are first shortened and then filtered. Overlapping paired reads with should be merged first (using micca-mergepairs) and then filtered.

The expected error (EE) rate % in a sequence of length L is defined as (doi: 10.1093/bioinformatics/btv401):

$$\text{EE rate \%} = \frac{\text{sum(error probabilities)}}{L} * 100$$

Before filtering, run 'micca filterstats' to see how many reads will pass the filter at different minimum lengths with or without truncation, given a maximum allowed expected error rate % and maximum allowed number of Ns.

micca-filter is based on VSEARCH (<https://github.com/torognes/vsearch>).

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```
optional arguments:
-h, --help            show this help message and exit

arguments:
-i FILE, --input FILE      input FASTQ file, Sanger/Illumina 1.8+ format
                           (phred+33) (required).
-o FILE, --output FILE    output FASTA/FASTQ file (required).
-e MAXEERATE, --maxeerate MAXEERATE
                           discard sequences with more than the specified expected
                           error rate % (values <=1%, i.e. less or equal than one
                           error per 100 bases, are highly recommended).
                           Sequences are discarded after truncation (if enabled)
                           (default 1).
-m MINLEN, --minlen MINLEN
                           discard sequences that are shorter than MINLEN
                           (default 1).
-t, --trunc            truncate sequences that are longer than MINLEN
                           (disabled by default).
-n MAXNS, --maxns MAXNS
                           discard sequences with more than the specified number
                           of Ns. Sequences are discarded after truncation
                           (disabled by default).
-f {fastq,fasta}, --output-format {fastq,fasta}
                           file format (default fasta).
```

Examples

Truncate reads at 300 bp, discard low quality sequences
(with EE rate > 0.5%) and write a FASTA file:

```
micca filter -i reads.fastq -o filtered.fasta -m 300 -t -e 0.5
```

CHAPTER 15

filterstats

```
usage: micca filterstats [-h] -i FILE [-o DIR] [-t TOPN]
                        [-e MAXEERATES [MAXEERATES ...]] [-n MAXNS]

micca filterstats reports the fraction of reads that would pass for each
specified maximum expected error (EE) rate %% and the maximum number of
allowed Ns after:

* discarding sequences that are shorter than the specified length
(suggested for Illumina overlapping paired-end (already merged)
reads);

* discarding sequences that are shorter than the specified length AND
truncating sequences that are longer (suggested for Illumina and 454
unpaired reads);

Parameters for the 'micca filter' command should be chosen for each
sequencing run using this tool.

micca filterstats returns in the output directory 3 files:

* filterstats_minlen.txt: fraction of reads that would pass the filter after
the minimum length filtering;
* filterstats_truncclen.txt: fraction of reads that would pass the filter after
the minimum length filtering + truncation;
* filterstats_plot.png: plot in PNG format.

optional arguments:
-h, --help            show this help message and exit

arguments:
-i FILE, --input FILE      input FASTQ file, Sanger/Illumina 1.8+ format
                           (phred+33) (required).
-o DIR, --output DIR      output directory (default .).
```

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```
-t TOPN, --topn TOPN  perform statistics on the first TOPN sequences
                        (disabled by default)
-e MAXEERATES [MAXEERATES ...], --maxeeres MAXEERATES [MAXEERATES ...]
                        max expected error rates (%). (default [0.25, 0.5,
                        0.75, 1, 1.25, 1.5])
-n MAXNS, --maxns MAXNS
                        max number of Ns. (disabled by default).
```

Examples

Compute filter statistics on the top 10000 sequences, predicting the fraction of reads that would pass for each maximum EE error rate (default values):

```
micca filterstats -i input.fastq -o stats -t 10000
```

CHAPTER 16

merge

```
usage: micca merge [-h] -i FILE [FILE ...] -o FILE [-s SEP] [-f {fastq,fasta}]
```

micca merge merges several FASTQ or FASTA files in a single file. Different samples will be merged in a single file and sample names will be appended to the sequence identifier (e.g. >SEQID;sample=SAMPLENAME). Sample names are defined as the leftmost part of the file name splitted by the first occurrence of '.' (-s/--sep option). Whitespace characters in names will be replaced with a single character underscore ('_').

optional arguments:

-h, --help show this help message and exit

arguments:

-i FILE [FILE ...], --input FILE [FILE ...]
input FASTQ/FASTA file(s) (required).

-o FILE, --output FILE
output FASTQ/FASTA file (required).

-s SEP, --sep SEP Sample names are defined as the leftmost part of the file name splitted by the first occurrence of 'SEP' (default .)

-f {fastq,fasta}, --format {fastq,fasta}
file format (default fastq).

Examples

Merge files in FASTA format:

```
micca merge -i in1.fasta in2.fasta in3.fasta -o merged.fasta \
-f fasta
```


CHAPTER 17

mergepairs

```
usage: micca mergepairs [-h] -i FILE [FILE ...] -o FILE [-r FILE]
                        [-l MINOVLEN] [-d MAXDIFFS] [-p PATTERN] [-e REPL]
                        [-s SEP] [-n] [--notmerged-fwd FILE]
                        [--notmerged-rev FILE] [-t THREADS]
```

micca mergepairs merges paired-end sequence reads into one sequence.

A single merging of a pair of FASTQ files can be simply performed using both `-i/--input` and `-r/--reverse` options.

When the option `-r/--reverse` is not specified:

1. you can indicate several forward files (with the option `-i/--input`);

2. the reverse file name will be constructed by replacing the string `'_R1'` in the forward file name with `'_R2'` (typical in Illumina file names, see options `-p/--pattern` and `-e/--repl`);

3. after the merging of the paired reads, different samples will be merged in a single file and sample names will be appended to the sequence identifier (e.g. `>SEQID;sample=SAMPLENAME`), as in 'micca merge' and 'micca split'. Sample names are defined as the leftmost part of the file name splitted by the first occurrence of `'_'` (`-s/--sep` option). Whitespace characters in names will be replaced with a single character underscore `'_'`).

micca mergepairs wraps VSEARCH (<https://github.com/torognes/vsearch>). Statistical testing of significance is performed in a way similar to PEAR (doi: 10.1093/bioinformatics/btt593). The quality of merged bases is computed as in USEARCH (doi: 10.1093/bioinformatics/btv401).

By default staggered read pairs (staggered pairs are pairs where the 3' end of the reverse read has an overhang to the left of the 5' end of the forward read) will be merged. To override this feature (and

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therefore to discard staggered alignments) set the `-n/--nostagger` option.

optional arguments:

`-h, --help` show this help message and exit

arguments:

`-i FILE [FILE ...], --input FILE [FILE ...]`
forward FASTQ file(s), Sanger/Illumina 1.8+ format (phred+33) (required).

`-o FILE, --output FILE`
output FASTQ file (required).

`-r FILE, --reverse FILE`
reverse FASTQ file, Sanger/Illumina 1.8+ format (phred+33).

`-l MINOVLEN, --minovlen MINOVLEN`
minimum overlap length (default 32).

`-d MAXDIFFS, --maxdiffs MAXDIFFS`
maximum number of allowed mismatches in the overlap region (default 8).

`-p PATTERN, --pattern PATTERN`
when the reverse filename is not specified, it will be constructed by replacing 'PATTERN' in the forward file name with 'REPL' (default `_R1`).

`-e REPL, --repl REPL` when the reverse filename is not specified, it will be constructed by replacing 'PATTERN' in the forward file name with 'REPL' (default `_R2`).

`-s SEP, --sep SEP` when the reverse file name is not specified, sample names are appended to the sequence identifier (e.g. `>SEQID;sample=SAMPLENAME`). Sample names are defined as the leftmost part of the file name splitted by the first occurrence of 'SEP' (default `_`).

`-n, --nostagger` forbid the merging of staggered read pairs. Without this option the command will merge staggered read pairs and the 3' overhang of the reverse read will be not included in the merged sequence.

`--notmerged-fwd FILE` write not merged forward reads.

`--notmerged-rev FILE` write not merged reverse reads.

`-t THREADS, --threads THREADS`
number of threads to use (1 to 256, default 1).

Examples

Merge reads with a minimum overlap length of 50 and maximum number of allowed mismatches of 3:

```
micca mergepairs -i reads1.fastq -r reads2.fastq -o merged.fastq \
-l 50 -d 3
```

Merge several illumina paired reads (typically named `*_R1*.fastq` and `*_R2*.fastq`):

```
micca mergepairs -i *_R1*.fastq -o merged.fastq --notmerged-fwd \
notmerged_fwd.fastq --notmerged-rev notmerged_rev.fastq
```

CHAPTER 18

msa

```
usage: micca msa [-h] -i FILE -o FILE [-m {muscle,nast}]
               [--muscle-maxiters MUSCLE_MAXITERS] [--nast-template FILE]
               [--nast-id NAST_ID] [--nast-threads NAST_THREADS]
               [--nast-mincov NAST_MINCOV] [--nast-strand {both,plus}]
               [--nast-notaligned FILE] [--nast-hits FILE] [--nast-nofilter]
               [--nast-notrim]
```

micca msa performs a multiple sequence alignment (MSA) on the input file in FASTA format. micca msa provides two approaches for MSA:

* MUSCLE (doi: 10.1093/nar/gkh340). It is one of the most widely-used multiple sequence alignment software;

* Nearest Alignment Space Termination (NAST) (doi: 10.1093/nar/gkl244). MICCA provides a very fast and memory efficient implementation of the NAST algorithm. The algorithm is based on VSEARCH (<https://github.com/torognes/vsearch>). It requires a pre-aligned database of sequences (--nast-template). For 16S data, a good template file is the Greengenes Core Set (http://greengenes.lbl.gov/Download/Sequence_Data/Fasta_data_files/core_set_aligned.fasta.imputed).

optional arguments:

-h, --help show this help message and exit

arguments:

-i FILE, --input FILE input FASTA file (required).
-o FILE, --output FILE output MSA file in FASTA format (required).
-m {muscle,nast}, --method {muscle,nast} multiple sequence alignment method (default muscle).

MUSCLE specific options:

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```
--muscle-maxiters MUSCLE_MAXITERS
                        maximum number of MUSCLE iterations. Set to 2 for a
                        good compromise between speed and accuracy (>=1
                        default 16).

NAST specific options:
--nast-template FILE    multiple sequence alignment template file in FASTA
                        format.
--nast-id NAST_ID        sequence identity threshold to consider a sequence a
                        match (0.0 to 1.0, default 0.75).
--nast-threads NAST_THREADS
                        number of threads to use (1 to 256, default 1).
--nast-mincov NAST_MINCOV
                        reject sequence if the fraction of alignment to the
                        template sequence is lower than MINCOV. This parameter
                        prevents low-coverage alignments at the end of the
                        sequences (default 0.75).
--nast-strand {both,plus}
                        search both strands or the plus strand only (default
                        both).
--nast-notaligned FILE
                        write not aligned sequences in FASTA format.
--nast-hits FILE         write hits on a TAB delimited file with the query
                        sequence id, the template sequence id and the
                        identity.
--nast-nofilter          do not remove positions which are gaps in every
                        sequence (useful if you want to apply a Lane mask
                        filter before the tree inference).
--nast-notrim           force to align the entire candidate sequence (i.e. do
                        not trim the candidate sequence to that which is bound
                        by the beginning and end points of the alignment
                        span
```

Examples

De novo MSA using MUSCLE:

```
micca msa -i input.fasta -o msa.fasta
```

Template-based MSA using NAST, the Greengenes alignment as template (clustered at 97% similarity) 4 threads and a sequence identity threshold of 75%:

```
micca msa -i input.fasta -o msa.fasta -m nast --nast-threads 4 \
--nast-template greengenes_2013_05/rep_set_aligned/97_otus.fasta
```

See *OTU picking and Denoising* for details.

```
usage: micca otu [-h] -i FILE [-o DIR] [-r FILE]
                [-m {denovo_greedy,denovo_unoise,denovo_swarm,closed_ref,open_ref}]
                [-d ID] [-n MINCOV] [-t THREADS] [-g {dgc,agc}] [-s MINSIZE]
                [-a {both,plus}] [-c] [-S CHIM_ABSKEW]
                [--swarm-differences SWARM_DIFFERENCES] [--swarm-fastidious]
                [--unoise-alpha UNOISE_ALPHA]
```

micca otu assigns similar sequences (marker genes such as 16S rRNA and the fungal ITS region) to operational taxonomic units (OTUs) or sequence variants (SVs).

Trimming the sequences to a fixed position before clustering is **strongly recommended** when they cover partial amplicons or if quality deteriorates towards the end (common when you have long amplicons and single-end sequencing).

Removing ambiguous nucleotides 'N' (with the option `--maxns 0` in micca filter) is mandatory if you use the de novo swarm clustering method.

micca otu provides the following protocols:

* de novo greedy clustering (denovo_greedy): useful for the identification of 97% OTUs;

* de novo unoise (denovo_unoise): denoise Illumina sequences using the UNOISE3 protocol;

* de novo swarm (denovo_swarm): a robust and fast clustering method (deprecated, it will be removed in version 1.8.0);

* closed-reference clustering (closed_ref): sequences are clustered against an external reference database and reads that could not be

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matched are discarded.

* open-reference clustering (open_ref): sequences are clustered against an external reference database and reads that could not be matched are clustered with the 'de novo greedy' protocol.

Outputs:

* otutable.txt: OTU x sample, TAB-separated OTU table file, containing the number of times an OTU is found in each sample.

* otus.fasta: FASTA file containing the representative sequences (OTUs);

* otuids.txt: OTU ids to original sequence ids (tab-delimited text file);

* hits.txt: three-columns, TAB-separated file with matching sequence, representative (seed) and identity (if available, else '*');

* otuschim.fasta (only for 'denovo_greedy', 'denovo_swarm' and 'open_ref' when --rmchim is specified): FASTA file containing the chimeric otus.

optional arguments:

-h, --help show this help message and exit

arguments:

-i FILE, --input FILE
input fasta file (required).

-o DIR, --output DIR output directory (default .).

-r FILE, --ref FILE reference sequences in fasta format, required for 'closed_ref' and 'open_ref' clustering methods.

-m {denovo_greedy,denovo_unoise,denovo_swarm,closed_ref,open_ref}, --method {denovo_greedy,denovo_unoise,denovo_swarm,closed_ref,open_ref}
clustering method (default denovo_greedy)

-d ID, --id ID sequence identity threshold (for 'denovo_greedy', 'closed_ref' and 'open_ref', 0.0 to 1.0, default 0.97).

-n MINCOV, --mincov MINCOV
reject sequence if the fraction of alignment to the reference sequence is lower than MINCOV (for 'closed_ref' and 'open_ref' clustering methods, default 0.75).

-t THREADS, --threads THREADS
number of threads to use (1 to 256, default 1).

-g {dgc,agc}, --greedy {dgc,agc}
greedy clustering strategy, distance (DGC) or abundance-based (AGC) (for 'denovo_greedy' and 'open_ref' clustering methods) (default dgc).

-s MINSIZE, --minsize MINSIZE
discard sequences with an abundance value smaller than MINSIZE after dereplication (≥ 1 , default values are 2 for 'denovo_greedy' and 'open_ref', 1 for 'denovo_swarm' and 8 for 'denovo_unoise').

-a {both,plus}, --strand {both,plus}
search both strands or the plus strand only (for 'closed_ref' and 'open_ref' clustering methods,

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default both).

Chimera removal specific options:

```
-c, --rmchim          remove chimeric sequences (ignored in method
                        'closed_ref'
-S CHIM_ABSKEW, --chim-abskew CHIM_ABSKEW
                        abundance skew. It is used to distinguish in a three-
                        way alignment which sequence is the chimera and which
                        are the parents. If CHIM_ABSKEW=2.0, the parents
                        should be at least 2 times more abundant than their
                        chimera (defaults values are 16.0 for 'denovo_unoise',
                        2.0 otherwise).
```

Swarm specific options:

```
--swarm-differences SWARM_DIFFERENCES
                        maximum number of differences allowed between two
                        amplicons. Commonly used d values are 1 (linear
                        complexity algorithm), 2 or 3, rarely higher. (>=0,
                        default 1).
--swarm-fastidious    when working with SWARM_DIFFERENCES=1, perform a
                        second clustering pass to reduce the number of small
                        OTUs (recommended option).
```

UNOISE specific options:

```
--unoise-alpha UNOISE_ALPHA
                        specify the alpha parameter (default 2.0).
```

Examples

De novo clustering with a 97% similarity threshold and remove chimeric OTUs:

```
micca otu -i input.fasta --method denovo_greedy --id 0.97 -c
```

Open-reference OTU picking protocol with a 97% similarity threshold, without removing chimeras in the de novo protocol step and using 8 threads:

```
micca otu -i input.fasta --method open_ref --threads 8 --id 0.97 \
--ref greengenes_2013_05/rep_set/97_otus.fasta
```

De novo swarm clustering with the protocol using 4 threads:

```
micca otu -i input.fasta --method denovo_swarm --threads 4 \
--swarm-fastidious --rmchim --minsize 1
```



```
usage: micca root [-h] -i FILE -o FILE [-m {midpoint,outgroup}]
                [-t TARGETS [TARGETS ...]]

micca root reroot the input tree:

* at the calculated midpoint between the two most distant tips of the
tree (--method midpoint);

* with the outgroup clade containing the given taxa (leaf nodes),
i.e. the common ancestor of the outgroup (--method outgroup).

optional arguments:
-h, --help            show this help message and exit

arguments:
-i FILE, --input FILE    input FASTA file (required).
-o FILE, --output FILE   output MSA file in FASTA format (required).
-m {midpoint,outgroup}, --method {midpoint,outgroup}
                        rooting method (default midpoint).
-t TARGETS [TARGETS ...], --targets TARGETS [TARGETS ...]
                        list of targets defining the outgroup (required for
                        the outgroup method).
```

Examples

Midpoint rooting:

```
micca root -i input.tree -o input_rooted.tree
```

Rooting with outgroup:

```
micca root -i input.tree -o input_rooted.tree -m outgroup DENOVO1 DENOVO2
```


CHAPTER 21

split

```
usage: micca split [-h] -i FILE -o FILE -b FILE [-n FILE] [-c FILE] [-s N]
                  [-e MAXE] [-t] [-f {fastq,fasta}]
```

micca split assign the multiplexed reads to samples based on their 5' nucleotide barcode (demultiplexing) provided by the FASTA file (`--barcode`). micca split creates a single FASTQ or FASTA file with sample information (e.g. `>SEQID;sample=SAMPLENAME`) appended to the sequence identifier. Barcode and the sequence preceding it is removed by default, e.g.:

Barcode file:	Input file:
<code>>SAMPLE1</code>	<code>>SEQ1</code>
TCAGTCAG	TCAGTCAGGCCACGGCTAACTAC...
...	...

the output will be:

```
>SEQ1;sample=SAMPLE1
GCCACGGCTAACTAC...
...
```

optional arguments:

`-h, --help` show this help message and exit

arguments:

`-i FILE, --input FILE` input FASTQ/FASTA file (required).
`-o FILE, --output FILE` output FASTQ/FASTA file (required).
`-b FILE, --barcode FILE` barcode file in FASTA format (required).
`-n FILE, --notmatched FILE` write reads in which no barcode was found.

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```
-c FILE, --counts FILE      write barcode counts in a tab-delimited file.
-s N, --skip N              skip N bases before barcode matching (e.g. if your
                             sequences start with the control sequence 'TCAG'
                             followed by the barcode, set to 4) (>=0, default 0).
-e MAXE, --maxe MAXE       maximum number of allowed errors (>=0, default 1).
-t, --notrim               do not trim barcodes and the sequence preceding it
                             from sequences.
-f {fastq,fasta}, --format {fastq,fasta}
                             file format (default fastq).
```

Examples

Split 'reads.fastq' and write the notmatched sequences in the file 'notmatched.fastq':

```
micca split -i input.fastq -o splitted.fastq -b barcode.fasta \
-n notmatched.fastq
```

```
usage: micca stats [-h] -i FILE [-o DIR] [-n TOPN]
```

micca stats reports statistics on reads in a FASTQ file. micca stats returns in the output directory 3 tab-delimited text files:

- * stats_lendist.txt: length distribution;
- * stats_qualdist.txt: Q score distribution;
- * stats_qualsumm.txt: quality summary. For each read position, the following statistics are reported:
 - L: read position;
 - NPctCum: percent of reads with at least L bases;
 - QAv: average Q score at position L;
 - EERatePctAv: average expected error (EE) rate %.

Moreover, micca stats returns the respective plots in PNG format, stats_lendist_plot.png, stats_qualdist_plot.png, and stats_qualsumm_plot.png.

optional arguments:

-h, --help show this help message and exit

arguments:

-i FILE, --input FILE input FASTQ file, Sanger/Illumina 1.8+ format (phred+33) (required).

-o DIR, --output DIR output directory (default .).

-n TOPN, --topn TOPN perform statistics only on the first TOPN sequences (disabled by default).

Examples

Compute statistics on the top 10000 sequences of input.fastq:

```
micca stats -i input.fastq -o stats -n 10000
```



```
usage: micca tablebar [-h] -i FILE -o FILE [-r] [-t N] [--xticklabelsize SIZE]
                        [-f {pgf,ps,svg,rgba,raw,svgz,pdf,eps,png}]

micca tablebar generates a relative abundance bar plot from
OTU or taxa tables. The table must be an OTU/taxon x sample,
TAB-separated file (see 'micca otu').

optional arguments:
-h, --help                show this help message and exit

arguments:
-i FILE, --input FILE      input OTU table file (required).
-o FILE, --output FILE     output image file (required).
-r, --raw                  plot raw values (i.e. counts) instead of relative
                           abundances.
-t N, --topn N             plot the top N abundant taxa (default 12).
--xticklabelsize SIZE      x tick label size (default 8).
-f {pgf,ps,svg,rgba,raw,svgz,pdf,eps,png}, --format {pgf,ps,svg,rgba,raw,svgz,pdf,eps,
↪png}                      output file format (default png).

Example

    micca tablebar -i otutable.txt -o otutable_plot.png
```

tablerare

```
usage: micca tablerare [-h] -i FILE -o FILE -d DEPTH [-r] [-s SEED]
```

Rarefy an OTU table by subsampling, with or without replacement. Samples that have fewer counts than the depth are omitted from the output table. OTUs that are not present in at least one sample are omitted from the output table.

optional arguments:

-h, --help show this help message and exit

arguments:

-i FILE, --input FILE input OTU table file (required).
-o FILE, --output FILE output rarefied OTU table file (required).
-d DEPTH, --depth DEPTH sample depth (>0, required).
-r, --replace subsample with replacement.
-s SEED, --seed SEED random seed (default 0).

Examples

Rarefy an OTU table at a depth of 1000 sequences/sample:

```
micca tablerare -i otutable.txt -o otutable_rare.txt -d 1000
```


tablestats

```
usage: micca tablestats [-h] -i FILE [-o DIR] [-t STEP] [-r] [-s SEED]
```

micca tablestats reports a sample summary, an OTU summary and the rarefaction curves for the input OTU table. The rarefaction curves are evaluated using the interval of 'step' (-t/--step) sample depths, always including 1 and the total sample size.

micca filterstats returns in the output directory 4 files:

- * tablestats_samplesumm.txt: samples summary;
- * tablestats_otusumm.txt: OTUs summary;
- * tablestats_rarecurve.txt: rarefaction curves in text format.
- * tablestats_rarecurve_plot.txt: rarefaction curves in png format.

optional arguments:

-h, --help show this help message and exit

arguments:

-i FILE, --input FILE input FASTQ file, Sanger/Illumina 1.8+ format (phred+33) (required).

-o DIR, --output DIR output directory (default .).

-t STEP, --step STEP sample depth interval (for rarefaction curves, default 500).

-r, --replace subsample with replacement (for rarefaction curves).

-s SEED, --seed SEED random seed (for rarefaction curves, default 0).

Examples

Compute OTU table statistics on otutable.txt:

```
micca tablestats -i otutable.txt -o tablestats
```


CHAPTER 26

tabletotax

```
usage: micca tabletotax [-h] -i FILE -t FILE [-o DIR]
```

Given an OTU table and a taxonomy file, micca tabletotax creates in the output directory a table for each taxonomic level (taxtable1.txt, ..., taxtableN.txt). OTU counts are summed together if they have the same taxonomy at the considered level.

The OTU table must be an OTU x sample, TAB-separated OTU table file (see 'micca otu'). The taxonomy file must be a tab-delimited file where rows are either in the form (see 'micca classify'):

```
1. SEQID[TAB]k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__;
2. SEQID[TAB]Bacteria;Firmicutes;Clostridia;Clostridiales;;;
3. SEQID[TAB]Bacteria;Firmicutes;Clostridia;Clostridiales
4. SEQID[TAB]D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__;D_
  ↪5__;
```

optional arguments:

```
-h, --help          show this help message and exit
```

arguments:

```
-i FILE, --input FILE          input OTU table file (required).
-t FILE, --tax FILE            input taxonomy file (required).
-o DIR, --output DIR          output directory (default .).
```

Examples

```
micca tabletotax -i otutable.txt -t tax.txt -o taxtables
```



```
usage: micca tobiom [-h] -i FILE -o FILE [-t FILE] [-s FILE] [-u FILE]

micca tobiom converts the micca OTU table into BIOM Version
1.0 (JSON) format. Optionally, taxonomy and/or sample
information can be added. When you convert on
(closed-reference) OTU table for PICRUSt, replace OTU IDs with
the original sequence IDs use the option -u/--otuids.

optional arguments:
-h, --help            show this help message and exit

arguments:
-i FILE, --input FILE      input OTU table file (required).
-o FILE, --output FILE     output BIOM file Version 1.0 (JSON) (required).
-t FILE, --tax FILE       add taxonomy information from a taxonomy file.
-s FILE, --sampledata FILE add sample information from a sample data file.
-u FILE, --otuids FILE    replace OTU IDs with the original sequence IDs. Useful
                           when the closed-reference OTU picking protocol was
                           performed for PICRUSt

Example

micca tobiom -i otutable.txt -o output.biom -t tax.txt -s sampledata.txt
```



```
usage: micca tree [-h] -i FILE -o FILE [-m {fasttree,muscle}] [--fasttree-gtr]
                  [--fasttree-fastest]
                  [--muscle-cluster {upgmb,upgma,neighborjoining}]
```

micca tree infers phylogenetic trees from alignments. It provides two methods:

- * FastTree (doi: 10.1371/journal.pone.0009490);
- * MUSCLE (doi: 10.1093/nar/gkl244).

optional arguments:

-h, --help show this help message and exit

arguments:

-i FILE, --input FILE input FASTA file (required).
 -o FILE, --output FILE output tree in Newick format (required).
 -m {fasttree,muscle}, --method {fasttree,muscle} tree inference method (default fasttree).

FastTree specific options:

--fasttree-gtr use the generalized time-reversible (GTR)+CAT model instead of Jukes-Cantor+CAT (default False).
 --fasttree-fastest speed up the neighbor joining phase and reduce memory usage recommended for >50,000 sequences) (default False).

MUSCLE specific options:

--muscle-cluster {upgmb,upgma,neighborjoining} clustering algorithm (default upgmb).

Examples

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Tree inference using FastTree and the generalized time-reversible (GTR)+CAT model:

```
micca tree -i input.fasta -o tree.tree --fasttree-gtr
```

```
usage: micca trim [-h] -i FILE -o FILE [-w FORWARD [FORWARD ...]]
                [-r REVERSE [REVERSE ...]] [-e MAXERATE] [-c] [-W] [-R]
                [-f {fastq,fasta}]

micca trim trims forward and reverse primers from a FASTQ/FASTA file
using Cutadapt (doi: 10.14806/ej.17.1.200) internally. Primer and the
sequence preceding (for forward) or succeeding (for reverse) it are
removed. Optionally, reads that do not contain the primers (untrimmed
reads) can be discarded with the options -W/--duforward and
-R/--dureverse. Recommended options are:

* always discard reads that do not contain the forward primer
(-W/--duforward option);

* for overlapping paired-end (already merged) reads, also discard
reads that do not contain the reverse primer (using both
-W/--duforward and -R/--dureverse options).

IUPAC codes and multiple primers are supported.

optional arguments:
-h, --help            show this help message and exit

arguments:
-i FILE, --input FILE
                        input FASTQ or FASTA file (required).
-o FILE, --output FILE
                        output FASTQ or FASTA file (required).
-w FORWARD [FORWARD ...], --forward FORWARD [FORWARD ...]
                        trim forward primer(s). Only the best matching primer
                        is removed.
-r REVERSE [REVERSE ...], --reverse REVERSE [REVERSE ...]
                        trim reverse primer(s). Only the best matching primer
                        is removed.
```

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```
-e MAXERATE, --maxerate MAXERATE
                        maximum allowed error rate (default 0.1).
-c, --searchrc         search reverse complement primers too (default False).
-W, --duforward        discard untrimmed reads (reads that do not contain the
                        forward primer) (always recommended) (default False).
-R, --dureverse        discard untrimmed reads (reads that do not contain the
                        reverse primer) (suggested option for overlapping
                        paired-end already merged reads) (default False).
-f {fastq,fasta}, --format {fastq,fasta}
                        file format (default fastq).
```

Examples

454 or Illumina single-end reads: trim forward primer and discard reads that do not contain it. Moreover, trim reverse primer:

```
micca trim -i input.fastq -o trimmed.fastq -w AGGATTAGATACCCTGGTA \
-r CRRACGAGCTGACGAC -W
```

Illumina overlapping paired-end (already merged) reads: trim forward and reverse primers. Reads that do not contain the forward or the reverse primer will be discarded:

```
micca trim -i reads.fastq -o trimmed.fastq -w AGGATTAGATACCCTGGTA \
-r CRRACGAGCTGACGAC -W -R
```