User's guide: Manual for Metaxa2.2

This is a guide on how to install and use the software utility Metaxa2, version 2.2. The software is written for Unix-like platforms, and should work on nearly all Linux-based systems, as well as macOS.

Contents of this manual

1. Detailed installation instructions

The README.txt file bundled with the script provides a quick installation guide.

In order to install certain packages, you might need to have superuser privileges. For installation on Mac, you have to install the Apple Xcode Command Line Tools package, if that is not already installed, in order to compile the required programs. Please talk to your system administrator if you are unsure about these steps. Note that the package is mandatory and that you should not proceed unless these criteria are fulfilled.

[If you don’t have superuser privileges on your machine: Create a directory within your user directory, e.g. /home/user/bin/, and store all required binaries there. By adding this directory to your PATH, any software placed in the directory will behave as if installed for all users using superuser privileges. If you use the bash shell, you can add a bin directory to your PATH, by adding the line “export PATH=$PATH:$HOME/bin/:.” to the file .profile in your home directory. Note, though, that the process of adding items to one’s PATH varies among systems and shells. Close the terminal and open a new one for this change to take effect.]

Perl needs to be installed on the computer. Most Unix-based systems including Linux and macOS have Perl pre-installed. You can check this by opening a command line terminal and type “perl -v”. In case Perl is not installed, you have to download (http://www.perl.org) and compile the program.

Download and install HMMER version 3 (http://hmmer.janelia.org/software). Version 2 of Metaxa relies on HMMER version 3.1b. Metaxa2 will ***not*** work with earlier versions of HMMER, and as of version 2.2 it by default expects HMMER version 3.1b or later to be installed. Download the HMMER package source code to your preferred directory such as

/home/user/. Open a command line terminal, move into the directory with "cd /home/user/" and unpack the tarball with "tar -xvfz hmmer-3.1b.tar.gz". Now, you need to compile HMMER from source files. To compile it from source, enter the new directory and follow the installation instructions in the file INSTALL.

If you have trouble compiling HMMER, you can try to use the pre-compiled binaries available at the HMMER home page. After downloading and unpacking of the tarball, the binaries are located in the binaries directory contained within the newly created HMMER directory. Move into the binaries directory and move all of its contained files into your preferred bin directory (usually either /usr/local/bin/ or your own bin directory, /home/user/bin/). The HMMER package should now be installed on your computer; you can check this by typing "hmmscan -h" in the terminal and press enter; you should now see HMMER output.

Download and install the BLAST package (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/LATEST/) for sequence similarity searches. Metaxa2.2 works with both the legacy version of BLAST *as well as* BLAST+. It should work with any version of BLAST starting with version 2.2 and later. Download the BLAST package for your operating system to your preferred directory. Open a command line terminal, move into the directory with "cd /home/user/" and unpack the tarball with "tar -xvfz blast-2.2.24-platform.tar.gz". Move into the bin directory inside the newly created BLAST directory, and move all of its contained files into your preferred bin directory. Alternatively, you can add the BLAST bin directory to your PATH. The BLAST package should now be installed on your computer; you can check this by typing "blastall -" in the terminal and press enter; you should now see the listing of BLAST options.

Download and install MAFFT (http://mafft.cbrc.jp/alignment/software/) for multiple alignment. Metaxa2 relies on MAFFT version 7 (although it likely would work also with version 6). MAFFT is not critical for Metaxa2's core functions, but is used for automatically creating alignments for sequences of uncertain taxonomic placement. Instructions for installing MAFFT are available on the MAFFT download page.

Finally, to enable all the features of the Metaxa2 Database Builder introduced in version 2.2, the USEARCH software, version 7 or later, must also be installed. USEARCH can be downloaded from http://drive5.com/usearch/. Use the software download link and download the software to a preferred location. Change the permission of the binary with a name starting with "usearch" using the following command: "chmod +x /usr/bin/usearch........". Move the USEARCH binary into your preferred bin directory and rename it "usearch" (otherwise Metaxa2 will not be able to identify it). Some additional installation instructions can be found here: http://drive5.com/usearch/manual/install.html. If USEARCH is not installed, the Metaxa2 Database Builder will try to find VSEARCH instead as an alternative.

Go to http://microbiology.se/software/metaxa2 in order to download the Metaxa2 package. Download it to your preferred directory. Unpack the downloaded tarball with "tar -xvfz metaxa2_2.2.tar.gz". A directory called Metaxa2 will be created. You will see a number of files and directories inside it, including metaxa2, metaxa2_x, metaxa2_c, install_metaxa2, and the metaxa2_db directory (containing the Hidden Markov Models and BLAST databases), the user's guide, and test input files. Enter the directory, and type "./install_metaxa2". Press enter and follow the on-screen instructions. You will be prompted for whether you have superuser privileges and for the location where you want Metaxa2 to be installed. If Metaxa2 is successfully installed you should see its help message when typing the command "metaxa2 --help".

2. Changes from previous versions of Metaxa

Version 2.2 of Metaxa2 differs from the previous version in a few ways, outlined in the first paragraph below. In addition, Metaxa2 introduced a couple of new features over Metaxa 1.1.2, as well as changing the default behaviour of some options. The main changes are outlined in the following paragraphs.

Changes in Metaxa2 version 2.2

The major enhancement in Metaxa2 version 2.2 is the ability to use custom databases for classification. This means that the user can now make their own database for their own barcoding region of choice, or download additional databases from the Metaxa2 Database Repository (described in a later section of the manual). The selection of other databases is made through the "-g" option already existing in Metaxa2. As part of these changes, we have also updated the classification scoring model for better stringency and sensitivity across multiple databases and different genes. The old scoring system can still be used by specifying the --scoring_model option to "old".

Other minor features introduced in this version include support for compressed input files in the gzip, zip, bzip, and dsrc formats. Detection of compressed input files should be made automatically by Metaxa2 version 2.2 if files with appropriate suffixes are provided as input. We have also improved the capability to auto-detect the location of the classification databases, which should improve the ease-of-use slightly. Finally, the new scoring system allows output reliability scores for each taxonomic rank, which can be done using the "--reltax" option.

Changes in Metaxa2 version 2.1

Metaxa2 version 2.1 incorporates a number of minor changes and improvements. These include a new detection mode for detection of multiple rRNA sequences in e.g. full genomes, an option to specify reference sequences to exclude from the analysis to be able to sort out specific non-target sequences from the dataset (e.g. from a host organism), the possibility to get separate files for paired-end reads matching rRNA for further downstream analysis, and the important addition of the Metaxa2 Diversity Tools (see section 6, further below).

The new genome mode allows Metaxa2 to be used to find multiple rRNA sequences in longer stretches of DNA, such as complete genomes or contigs. It also comes with an automatic option, which processes sequences longer than 2500 bases in the genome mode and shorter sequences in the metagenomic mode.

The new reference option can be used to supply Metaxa2 with a FASTA file with reference sequences that should *not* be included in the output. These sequences can, for example, be the rRNA sequences from a host organism, or from some particular taxon that the user is not interested in studying.

Finally, the default option for the "--align" flag has been changed to "none", since with increasing data set sizes the automatic alignment became a very time-consuming step. To use this capability as present by default in previous versions of Metaxa, add the "--align auto" option to the command line. Metaxa2 2.1 and later is also able to automatically detect whether legacy BLAST or BLAST+ is installed and will (normally) be able to choose which one to use. Still, the "--plus" option can be used to exercise control over what BLAST version to use.

Changes in Metaxa2 over Metaxa versions 1.X

Extraction and classification of LSU rRNA sequences

One of the major new features in Metaxa2 is the addition of a second, frequently used gene in addition to the SSU gene: the large subunit rRNA (LSU), also known as 23S rRNA in prokaryotes and 25S or 28S rRNA in eukaryotes. Toggling the switch from the SSU gene to the LSU gene is done using the "-g lsu" or "--gene lsu" options (both set to "ssu" by default). The operation of Metaxa2 is then the same as when searching for and extracting SSU genes.

The databases of Metaxa2 representing the SSU and LSU genes are kept separately within the metaxa2_db directory. Although the support of Metaxa2 for LSU extractions is reliable and robust, it has not been as extensively tested internally as the SSU extractions. Therefore, we encourage users to report suspicious – or obviously misclassified – entries, so that the LSU support of Metaxa2 can be improved further in future releases.

Completely redesigned system for taxonomic classifications

One of the design goals of Metaxa2 was to be able to make more sensible predictions of the origin of each input sequence identified as an SSU or LSU gene, even at very short read lengths. To achieve this, we completely rewrote the classification engine, which enabled Metaxa2 to produce reliable predictions of the taxonomic affiliation of the extracted SSU/LSU genes. Metaxa2 reports those affiliations to a file with the suffix ".taxonomy.txt". By using the taxonomic data of the best five BLAST matches (by default), Metaxa2 calculates the most accurate taxonomic placement possible for the input sequence, given the affiliations of the matching hits and their degree of identity to the input sequence. Each entry is associated with a taxonomic affiliation/level and three numbers: the percent identity to the best matching BLAST hit, the length of that alignment, and a reliability score. The reliability score is calculated based on the percent identity to the best BLAST hit and how divergent the rest of the BLAST hits are from the first one. The maximum value of this score is 100. The score of 100 is only given if *all five* BLAST matches are 100% identical to the input sequence, and all those matches represent the same taxonomic lineage. This means that the situation outlined above rarely – if ever – occurs on real data. Instead, scores above 80 should be considered trustworthy. The reliability score can then be used to filter out uncertain entries when summarizing the taxonomic predictions with the Metaxa2 Taxonomic Traversal Tool (metaxa2_ttt), described below.

The Metaxa2 Taxonomic Traversal Tool – metaxa2_ttt

Because of the extended abilities of Metaxa2 to classify SSU and LSU sequences in greater detail, a new tool to investigate the organism diversity of the sample at different taxonomic levels has been included in the Metaxa2 package. This tool is called the Metaxa2 Taxonomic Traversal Tool, since it does exactly that – it traverses the ".taxonomy.txt" output file and reports Metaxa's taxonomic predictions according to specified cutoffs. The complete usage of the metaxa2_ttt tool is described in part five of this manual (*Metaxa2 Taxonomic Traversal Tool*). In short, the traversal tool outputs the number of identified SSU/LSU sequences associated with each node in the taxonomic tree, at different levels (roughly corresponding to kingdoms, phyla, classes, orders, families, genera, species, subspecies, etc.)

The "--guess_species" option is now obsolete

Since Metaxa2 now handles taxonomy in a much more elaborate way, the "--guess_species" option has been deemed obsolete. It is, however, still possible to use this option, but a warning message will appear. The function may be deprecated in future versions of Metaxa, and is not actively maintained anymore (and has thus not been tested in the Metaxa2 evaluation phase).

Updated database for classification

The improved classification engine has naturally required us to update and improve upon the underlying databases. The basis of the Metaxa2 databases is the SILVA reference release 111 and Mitozoa release 10. From this data, we have created a curated reference database, in large part by automated means, but also using extensive manual curation and cross-checking in

GreenGenes, CRW, and GenBank. Suspicious or erroneous entries were removed, as were sequences from uncultured or unverified organisms.

Direct input of sequences in FASTQ-format

Metaxa2 now supports input of sequence data sets directly in the FASTQ format. Although Metaxa2 will try to auto-detect the format of the input file, specifying "-f fastq" will force Metaxa2 to read input in FASTQ format. This option might be particularly useful when piping input to Metaxa2 using standard input. Note that FASTQ files are only supported when Metaxa2 is run in pipeline mode, i.e. not when the two tools the Metaxa2 Extractor (metaxa2_x) and the Metaxa2 Classifier (metaxa2_c) are run separately (see section 9). These two tools still expect input in the FASTA format.

Support for paired-end libraries

In addition to FASTQ support, Metaxa2 also handles paired-end libraries. Paired-end data needs to be supplied in two separate files, with sequence 1 in both files corresponding to the two ends of the same read, and so on. If your data is held in a single file, a utility such as the pefcon and pesort tools of the PETKit (http://microbiology.se/software/petkit/) can help you. To enable Metaxa2's paired-end capabilities, use the options "-1 firstfile -2 secondfile" instead of the "-i" option for input. Metaxa2 will automatically orient the reads in the same direction, and utilize a combination of the two ends for the extraction and classification process. Note that the two ends will be output ***together*** as one single read with a spacer, unless the "--split_pairs" option is used.

Improved support for libraries with short read lengths (~100 bp)

Since metagenomics is moving towards shorter read lengths and larger data sets (e.g. Illumina and IonTorrent sequencing), special care has been taken to try to address the short-read issues in previous Metaxa versions. We have tried to optimize the default options for reads down to ~100 bp. This will, however, increase the likelihood of false positive extractions somewhat. Moreover, the optimization towards shorter reads should be kept in mind when using Metaxa2 on longer reads, which might require tweaking of the default options (one may, for example, compare to the default options of Metaxa 1.1.2).

Quality pre-filtering of reads in FASTQ-format

Metaxa2 can use the quality information in the FASTQ files to filter out low-quality reads or read pairs. This is controlled by a range of options described in part three of the manual (*Usage and Commands*).

Support for the modern BLAST+ package

Metaxa2 now incorporates the long sought-after support for the NCBI BLAST+ package, enabling users to move on to what is deemed to be the future of BLAST. In addition, Metaxa 2.2 can select BLAST version automatically depending on which one is installed on the computer, a feature that was introduced in Metaxa2 version 2.1.

Compatibility with HMMER 3.1

Not only does Metaxa2 bring BLAST+ compatibility, it also secures support for HMMER version 3.1b and later.

Changed default priority for scoring best HMMER match

As part of the optimization for larger data sets and shorter reads, Metaxa's priority system for determining the best HMMER match has now been changed from "sum" to "score". To switch back to Metaxa 1.X behaviour, one can use the "--selection_priority sum" option. Be aware of that this might influence other parameters as well!

HMMER's heuristics are now used by default

Another optimization for larger data sets is that the heuristics of HMMER have now been turned on by default. To switch the heuristics off, use the "--heuristics F" option. Note, however, that this will make HMMER *much* slower on large data sets.

3. Usage and commands

For the *very* impatient only: follow the brief installation instructions in the file README.txt. To check for SSU rRNA sequences in the file test.fasta, you would then type "metaxa2 -i test.fasta -o test" on the command line (note that the "-g ssu" option is not mandatory for SSU sequences). To check for LSU rRNA sequences, type "metaxa2 -i test.fasta -o test -g lsu" instead.

For all other users: Metaxa2 accepts input in the FASTA and FASTQ formats. As it pre-processes the input sequences it is possible to input both aligned and unaligned FASTA files, containing both DNA and RNA sequences. It does not matter if the sequence data is given in lowercase or UPPERCASE letters. By default, Metaxa2 outputs ten files; one summary file of the entire run, one "graphical" representation of the hits, one FASTA file of all identified SSU sequences, one FASTA file for each of the six possible origins, and one file containing the predicted taxonomic origin of the extracted sequences. To list all the available options for Metaxa2, type "metaxa2 --help". You can use the test.fasta file that comes bundled with the software for a test run. This file contains 50 randomly selected SSU entries – ten of each origin – as well as 50 randomly selected LSU entries and 10 non-SSU, non-LSU sequences. In the simplest case, Metaxa2 is run by the command "metaxa2 -i input_file -o output". Below is a listing of all options Metaxa2 accepts. Boolean options can be turned on with "T", "true" or "1" and off using "F", "false" or "0".

Main options:

-i {file}	Nucleotide FASTA/FASTQ input file to investigate. Metaxa2 accepts both aligned and unaligned FASTA. If no input is specified, Metaxa2 will read sequences from standard input, which means that FASTA sequences can be piped into Metaxa2. Use this option for single-end read files.
-o {file}	Base for the file names of the output files. Suffixes will be added automatically. Defaults to metaxa_out.
-1 {file}	DNA FASTA/FASTQ input file containing the first reads in the read pairs to investigate. Used instead of the -i option.
-2 {file}	DNA FASTA/FASTQ input file containing the *second* reads in the read pairs to investigate. Use instead of the -i option, and only together with the -1 option. Note that the reads in the files must be in the same order.
--pairfile {file}	As an alternative to using the -1 and -2 options, the user might use -i and --pairfile instead, which has the same meaning.

-f {a, auto, f, fasta, q, fastq, p, paired-end, pa, paired-fasta}	Specifies the format of the input file(s). By default, Metaxa2 will try to auto-detect the format (auto). Synonymous to "--format". For non-paired FASTA input, this option should be "f", while for non-paired FASTQ input it should be "q". For paired FASTQ files specify "p" and for paired-read FASTA input, set the option to "pa".
-g {ssu, lsu}	Specifies if Metaxa2 should identify and extract SSU or LSU rRNA genes. By default, Metaxa2 will look for SSU genes (ssu).
--mode {m, metagenome, g, genome, a, auto}	Controls the Metaxa2 operating mode. Genome (and auto) mode allows the detection of multiple matches on the same DNA sequence. Default is the 'metagenome' mode.
-p {directory}	A path to a directory containing HMM-profile collections representing SSU rRNA conserved regions. By default, Metaxa assumes to find the databases in the metaxa_db directory, located in the same directory as Metaxa itself.
-d {database}	A path to the BLAST database (normally ending with "blast") used for classification. By default, Metaxa assumes to find the databases in the metaxa_db directory, located in the same directory as Metaxa itself.
--date {T or F}	Adds a date and time stamp to the output file. This can be useful e.g. if Metaxa is part of a pipeline where input files with the same name could cause overwriting of important data. Off (F) is set by default.
--plus {T or F}	If enabled, the BLAST search will be performed using BLAST+ (if installed) instead of the legacy blastall engine. By default, blastall is used (F).

FASTQ and Paired-end specific options:

Note that FASTQ format is only supported when Metaxa2 is run in pipeline mode.

-q {value}	Minimum quality value for a nucleotide to be considered "good". Default = 20.
--quality_percent {value}	Percentage of low-quality (below -q value) accepted before filtering/trimming is started. Default=10.
--quality_filter {T or F}	If enabled, Metaxa2 will filter out low-quality reads (below specified -q value), removing them entirely from the input data before running the HMMER searches. Off (F) by default.
--quality_trim {T or F}	If enabled, Metaxa2 will trim bad bases (below -q value) from the 3' end of the read. This is generally to prefer over filtering out the read entirely. Off (F) by default.
--ignore_paired_read {T or F}	When enabled, Metaxa2 will not discard the entire read pair if only one of the reads is of bad quality and would be filtered out. On (T) by default.
--distance {value}	Specifies the distance between the sequence pairs (often referred to as the insert size). Changing this value has little impact on Metaxa2's actual performance. Default = 150.

Sequence selection options:

-t {b, bacteria, a, archaea, e, eukaryota, m, mitochondrial, c, chloroplast, A, all}	A set of profiles to use for the search (comma-separated). Accepts any list of sets, e.g. "bacteria,chloroplast", "m,c" or "eukaryota". Can be used to restrict the search to only a few SSU/LSU types to save time, if one or more of the origins (archaea, bacteria, eukaryota, chloroplast, and

	mitochondria) are not relevant to the dataset under study. Default is to use all (the “all” option).
-E {value}	The domain E-value cutoff that a sequence must obtain in the HMMER-based step to be included in the output. Default = 1.
-S {value}	The domain score cutoff that a sequence that must be obtain in the HMMER-based step to be included in the output. Default = 12.
-N {value}	The minimum number of domains that must match a sequence for it to be included in the output. Setting the value lower than two will increase the number of false positives, while increasing it above two will decrease Metaxa’s detection abilities on fragmentary data. Default = 2.
-M {value}	The number of top BLAST matches that should be considered in the classification to different origins. This setting also affects the number of matches used for taxonomic classification. Default = 5.
-R {value}	Reliability score cutoff for the taxonomic classification. Entries not satisfying the cutoff will be classified at the level above in the taxonomic hierarchy, until the reliability score is above the threshold. Default = 80.
-T {comma-separated values}	Sets the percent identity cutoff to be classified at a certain taxonomic level. If the percent identity to a sequence in the Metaxa2 database is below this cutoff, the sequence will not be classified at that taxonomic level. The order of the values is: Kingdom/Domain,Phylum,Class,Order,Family,Genus,Species Default: 0,60,70,75,85,90,97
-H {value}	The number of points that the predicted origin of the Metaxa Extractor is given. Default is the same as the number of sequences used for classification (-M option above), which is set to 5 by default.
--selection_priority {score, sum, domains, eval}	Determines what will be of highest priority when assessing the origin of the sequence. Options are: - score, which uses the average score of the found hits - sum, which sums the scores for each profile match and divides the sum by the number of profiles of the given type - domains, which uses the number of domains retrieved of a given type - eval, which uses the average E-value of the found hits Default is to use score.
--scoring_model {new, old}	Selects the scoring model to be used for classification. Select 'old' to use the scoring model present in Metaxa2 versions prior to 2.2. Default is “new”, which will use the more stringent and sensitive model.
--search_eval {value}	The actual E-value cutoff used in the HMMER search. High numbers may slow down the process. Should never be set to a lower value than the -E option. Cannot be used in combination with the --search_score option. The default setting is to use score cutoff (see --search_score below), and not E-value cutoff.
--search_score {value}	The score cutoff used in the HMMER search. Low numbers may slow down the process. Should never be set to a higher number than the -S option. Cannot be used in combination with the --search_eval option. Default = 0.
--blast_eval {value}	The E-value cutoff used in the BLAST search. High numbers may slow down the process. Cannot be used in combination with the --blast_score option. Default is 1e-15.

--blast_score {value}	The score cutoff used in the BLAST search. Low numbers may slow down the process. Cannot be used in combination with the --blast_eval option. The default setting is to use E-value cutoff (see --blast_eval above), and not score cutoff.
--blast_wordsize {value}	The word-size used for the BLAST-based classification. Lower numbers will slow down the process significantly, while higher numbers may potentially decrease classification accuracy. Default is 14.
--allow_single_domain {e-value,score or F}	Allow inclusion of sequences that only find a single domain, given that they meet the more stringent E-value and score thresholds specified. By default, single domains are allowed, with an E-value cutoff of 1e-10 and a score cutoff 0 (corresponding to the setting "1e-10,0").
--allow_reorder {T or F}	Allows profiles not to be in the expected order (1-9) on the extracted sequences. If turned off, a file of potential chimeric sequences (with profile matches in the wrong order) is written, allowing for rudimentary chimera detection. This can (and should) be used on full-length sequences. On fragmented sequences, however, the risk of missing true positives increases if this option is turned off. On (T) by default.
--complement {T or F}	If on, Metaxa2 checks both DNA strands for matches to HMM-profiles. On (T) by default.
--cpu {value}	The number of CPU threads to use. Metaxa performs significantly faster using more CPUs. Default is 1.
--multi_thread {T or F}	Enables multi-thread of the HMMER-search. On (T) by default if the number of CPUs is larger than one (--cpu option > 1), if the number of CPUs is one it is off (F).
--heuristics {T or F}	Selects whether to use HMMER's heuristic filtering. On (T) by default. Turning this setting off will decrease speed, but increase precision.
--megablast {T or F}	Uses megablast for classification for better speed but less accuracy. Off (F) by default.
--reference {file}	Enables sorting out sequences from a file of reference entries in the FASTA format. Input sequences matching the reference sequences will be directed to a separate file in the analysis. Default is that this option is not used.
--ref_identity {value}	The sequence percent identity cutoff for a sequence to be considered as being derived from a reference entry (when using the --reference option). Default is 99.

Output options:

--summary {T or F}	If on, Metaxa outputs a summary of results. File suffix is ".summary.txt". On (T) by default.
--graphical {T or F}	If on, Metaxa outputs "graphical" text representations of where in each sequence the conserved domains were found. File suffix is ".graph". On (T) by default.
--fasta {T or F}	If on, FASTA-formatted files containing the extracted SSU sequences are written. One file for each origin is written, plus an extraction file containing all SSUs identified in the first analysis step. On (T) by default.
--split_pairs {T or F}	Outputs the two read pairs in two separate files with suffixes "_1.fasta" and "_2.fasta" instead of as a joint rRNA sequence. Off (F) by default.
--table {T or F}	If on, Metaxa saves table format output of results, separately for the HMMER and BLAST output. File suffixes are ".hmmer.table" and

	".blast.table", respectively. Note that neither of these outputs is the *actual* output of the respective program. To get these files, use the "--save_raw T" (see below). Off (F) by default.
--taxonomy {T or F}	Table format output of probable taxonomic origins for the identified SSU/LSU sequences. File suffix is ".taxonomy.txt". On (T) by default.
--reltax {T or F}	Outputs a table of probable taxonomic origins for sequences, with reliability scores for each rank (RDP Classifier style). Off (F) by default.
--taxlevel {integer}	Forces Metaxa to classify sequences at a certain taxonomy level, regardless of their reliability score. Off (0) by default.
--not_found {T or F}	If on, Metaxa outputs a list of entries that do *not* seem to be SSU sequences. File suffix is "_not_found.txt". Off (F) by default.
--align {a, all, u, uncertain, n, none}	Outputs alignments of BLAST matches to each query for all sequences (a), entries with uncertain classification to origin (u) or never for any query sequence (n). Requires MAFFT to be installed. Default is to output alignments in uncertain cases (u).
--truncate {T or F}	Removes ends of SSU sequences if they are outside of the SSU region. If off, the whole input sequence is saved. On (T) by default.
--guess_species {T or F}	Deprecated option, use the --taxonomy option instead. Kept for compatibility with previous Metaxa versions. Off (F) by default.
--silent {T or F}	Suppresses printing of progress information to screen. Off (F) by default.
--graph_scale {value}	Sets the scale of the graphical output. If the provided value is zero, a percentage view is shown. Default is 0.
--save_raw {T or F}	Saves all raw data for searches etc. instead of removing it when finished. Saves data to a directory with the suffix "_metaxa_raw_output". Off (F) by default.

Information options:

-h	Displays basic usage help.
--help	Displays the help message, complete with all options.
--bugs	Displays the bug fixes and any known bugs in this version of Metaxa.
--license	Displays licensing information.

4. Output files

Metaxa2 outputs a number of files, depending on the selections of the user (see Usage and Commands above). By default, seven FASTA-files, a table of taxonomic classifications, a file containing graphical representation of putative SSU/LSU sequences, and a summary file is written. In addition, tables of BLAST and HMMER results, lists of non-targeted entries, and sequence alignments can be written on request by the user. There is also an option to preserve all the intermediate data generated by the HMMER and BLAST searches.

FASTA-output

Metaxa2 generates one FASTA file for each origin (archaea, bacteria, eukaryota, chloroplast, and mitochondria), one file containing sequences of uncertain origin, and one extraction file with all rRNA sequences identified and extracted in the first step. Sequences in these files are marked according to their origin. A sequence of certain origin may look like this:

```
>gi|117927211 Bacterial 16S SSU rRNA
GTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGA…
```

Note that Metaxa2 has added the type of the SSU sequence ("Bacterial 16S SSU rRNA") to the definition line in the example above.

Sequences whose origin Metaxa2 could not establish with certainty, but for which enough data were available to allow a qualified guess as to the origin of the sequences, are marked with a "#" character at the end of the definition line. An uncertain sequence could look like this:

```
>AABL01000014.4508.5931 Putative Chloroplast 16S SSU rRNA #
GAACGCTAGAAATATACATTACACATGCAAATTTATGATAATATCATAGTGAATAGGTGA…
```

The extraction file contains all sequences identified as rRNA by metaxa2_x (the first step of the analysis). The sequence entries in that file contain information on the rRNA domains that were found in each sequence and what origin that is most likely based on the profile search. An example is shown below:

```
>A16379.1.1496|B Predicted Bacterial 16S SSU rRNA (1447 bp) From domain
V1l to V9r on main strand Found domains: V1l V2l V2r V3l V3r V4l V4r
V5l V5r V6l V7l V8l V8r V9l V9r
CAGGCTTAACACATGCAAGTCGAACGGTAGCACGAAGGACTTGCTCCTTGGGTGACGAGT…
```

Summary

A summary of the Metaxa2 run is written to a file with the suffix ".summary.txt". In this file, the statistics of the run is collected, as are the starting and ending times for the run. Also, lists of the identifiers of extracted SSU sequences are written to this file, one list for each origin. The first section of the file shows the data from the extraction step. The second section is associated with the second classification step. Note that the number of assignment to each origin may differ slightly to what is produced by the metaxa2_ttt tool (see Section 5 below), and may also differ slightly to the numbers produced by earlier versions of Metaxa2, because of the updated classification algorithm (see Section 9). After the second section, the lists of entries of different origins are found. An example of parts of a summary file is shown below:

```
Metaxa run started at Tue Jul 23 10:07:52 2013.
-----------------------------------------------------------------
Number of sequences in input file:        100
Sequences detected as SSU rRNA by Metaxa: 100
  On main strand:            91
  On complementary strand:    9
SSU sequences by preliminary origin:
  Archaea:        0
  Bacteria:       0
  Eukaryota:      0
  Chloroplast:    100
  Mitochondria:   0
  Other:          0
-----------------------------------------------------------------
Number of SSU rRNA sequences to be classified by Metaxa:    100
Number of SSU rRNA having at least one database match:      100
Number of SSU rRNA successfully classified by Metaxa:       100
Number of uncertain classifications of SSU rRNA sequences:  0
Total number of classifications made by Metaxa:             100
Number of SSU rRNA sequences assigned to each origin:
  Archaea:        0
  Bacteria:       0
  Eukaryota:      0
  Chloroplast:    100
  Mitochondria:   0
  Uncertain:      0
-----------------------------------------------------------------
Sequences of archaeal origin (16S):
-----------------------------------------------------------------
Sequences of bacterial origin (16S):
-----------------------------------------------------------------
Sequences of eukaryote origin (18S):
-----------------------------------------------------------------
Sequences of chloroplast origin (16S):
Acorus_americanus_AcamCr001
Aethionema_cordifolium_AecoCr001
...
Welwitschia_mirabilis_WemiC_r001
Zea_mays_ZemaCr113
-----------------------------------------------------------------
Sequences of mitochondrial origin (12S and 16S):
-----------------------------------------------------------------
Sequences of uncertain origin:
-----------------------------------------------------------------
Metaxa run finished at Tue Jul 14 10:08:42 2013.
```

Taxonomy table

One of the new features of Metaxa2 is the substantially improved ability to make taxonomic predictions of identified rRNA sequences. The results of these predictions are written to a file with the suffix ".taxonomy.txt". Each input sequence is represented by one line in this tab-separated, five-column file.

Column	**Description**
ID	The identifier of the query sequence.
Classification	The taxonomic tree for which Metaxa2 has been able to produce a reliable prediction.
Identity	The percent identity to the best BLAST match in the database.
Length	The length of the alignment of the input sequence and the best BLAST match.
Reliability score	The reliability score is calculated based on the percent identity to the best BLAST hit, and how divergent the rest of the BLAST hits are from the first one. The maximal score is 100, and the minimum score is determined by the -R cutoff used (75 by default). Scores above 75 can generally be considered good. For more details see section 9 of the manual.

The taxonomy output file can be used to assess the reliability of individual assignments. For each assignment Metaxa2 makes, the certainty of that assignment is represented by the reliability score. Although this score should not be read as a probability of a correct assignment (the model underlying the computation is not appropriate for that interpretation), it can still be useable to ascertain the quality if assignments. The reliability scores are given in the fifth (and last) column in the taxonomy table (taxonomy has been shortened in the example):

```
AB246684    Eukaryota;(…);Styptosphaera sp. 2013;      100   1801   99.65
AB509380    Bacteria;(…);Bradyrhizobium;               100   1350   98.90
AE000666    Archaea;(…);Methanothermobacter;           100   1408   98.78
```

For the above sequences, the reliability scores are 99.65, 98.90 and 98.78, respectively, that is much above the default cutoff of 75. The other way of assessing individual assignments is by using the "--reltax T" option, which will generate reliability scores at each taxonomic level. These end up in a file with the suffix "taxonomy-reliability.txt". For the above sequences, this would look like this:

```
AB246684    Eukaryota(95.15);Rhizaria(95.15);Radiolaria(95.15);
            Polycystinea(95.15);Spumellaria(95.15);Ssp-05(19.93);
            Styptosphaera(99.65);Styptosphaera sp. 2013(99.65);(0);
AB509380    Bacteria(98.90);Proteobacteria(98.90);
            Alphaproteobacteria(98.90);Rhizobiales(98.90);
            Bradyrhizobiaceae(98.90);Bradyrhizobium(98.90);Rhizobium sp.
            CCBAU 33215(19.78);(0);
AE000666    Archaea(98.78);Euryarchaeota(98.78);Methanobacteria(98.78);
            Methanobacteriales(98.78);Methanobacteriaceae(98.78);
            Methanothermobacter(98.78);Methanothermobacter thermautotrophicus
            str. Delta H(39.61);(0);
```

Graphical representations

Metaxa2 writes graphical (ASCII) representations of where in each sequence the various conserved regions were found to a text file with the suffix ".graph". Separate graphs are written for each origin and strand, which means that each sequence entry may be present more than once in this file, if it has matches to HMM-profiles from more than one origin. This makes it possible to manually inspect how Metaxa2 has evaluated each sequence. The graphical representations look like this:

```
B matches on main strand:
>> id|454_30|gi|50402825|gb|AY687385.1|   403 bp
------------------V5l=====-----V5r=======---------------------------
***********************************************************
```

The first row shows the type of the entries, as well as the strand they are found on. Each entry begins with the characters ">> ", followed by the sequence identifier and the sequence length. Below the identifier row, the sequence graph is shown. By default, all sequences are scaled so that they are of equal length, and the domains are placed according to their *relative* position in the sequence. The characters that are used in the graphical representations are explained in the table below.

Feature	Description
-	Part of the sequence without any conserved domain (i.e., a variable region).
V5l	Start of a conserved domain (here V5, l = leftmost part.; r would be rightmost).
=	Continuation of a conserved domain.
>	Indicates that one conserved domain goes into (overlaps) the next. Domains are normally not overlapping, so this could be an indication of a bad input sequence. (Not exemplified in the graphical representation above.)

The line of asterisks indicates the end of one set of matches. Note that the graph should be viewed with a non-proportional font, such as Courier, if loaded into, e.g., Word.

Extraction results table

The full results of the Metaxa2 extraction are saved to a file with the suffix ".extraction.results". This file consists of tab-separated columns containing various data on each SSU sequence found. The file is easy to import into spreadsheet programs such as Excel. The contents of the columns (from left to right) are explained in this table:

Column	**Description**
ID	The identifier of the query sequence.
Length	The length of the query sequence.
Origin	A one-letter abbreviation of the sequence origin. A = archaeal, B = bacterial, C = chloroplast, E = eukaryote, M = mitochondrial 16S, N = mitochondrial 12S.
Strand	A zero (0) if the SSU/LSU was found on the main strand, a one (1) if it was found on the complementary strand.
Domains	The number of conserved domains for the most likely origin that was found in the sequence.
Average E-value	The average E-value for these domains.
Average score	The average score for these domains.
Start	The first position of the first domain.
End	The last position of the last domain.
First domain	The domain that is located first on the sequence.
Last domain	The domain that is located last on the sequence.
Chimera	The word "Chimeric" is given if the sequence was marked as a potential chimera. This field is left empty if not. Sequences will only be marked as chimeric if the --allow_reorder option is turned off. Note that this is *not* a robust measure against chimeras of all kinds.
Specific origin information	A collection of data of *all* possible origins for the given query. Each entry is a space-separated list, containing the origin type, the number of domains of that type, the average E-value, and the average score, e.g. "N: 4 8.2e-11 43.475"

HMMER summary table

If table output is turned on, Metaxa2 will save statistics of every profile set that the sequence in question matches to in a file with the suffix ".hmmer.table". This file consists of tab-separated columns containing information on the rRNA sequence found. The contents of the columns (from left to right) are explained in this table:

Column	**Description**
ID	The identifier of the query sequence.
Length	The length of the query sequence.
List of hits	Each new column contains information of a profile match. Each column is organized as follows: "<starting position> - <ending position>: <name of matching profile> (<score>, <E-value>)".

As in the graphical output file, the table file is divided into sections. Each section represents one group of sequences and begins with the line "X matches on main strand:", and ends with a line of asterisks.

Classification results table

If table output is turned on, Metaxa2 will save statistics of every BLAST match that the sequence in question produces against the database to a file with the suffix ".blast.table". This file consists of tab-separated columns containing information on the matches found, one BLAST match per line. The contents of the columns (from left to right) are explained in this table:

Column	Description
Query ID	The identifier of the query sequence.
Subject ID	The identifier of the matching database sequence.
Score	The score this match obtained in the classification system
Species	The species name as provided by the reference database
BLAST Score	The BLAST score of the match
E-value	The E-value of the match, as reported by BLAST

Each new query is indicated by a comment line, e.g.:

```
## Query AATT01000235.146421.147977|E
```

List of non-target sequences

If not-found output is turned on, Metaxa2 will write a list of sequences for which no conserved SSU/LSU regions could be found to a file with the suffix "_not_found.txt". The file contains only the identifiers of the non-rRNA sequences.

Sequence alignments

By default, Metaxa2 saves alignments of sequences of uncertain origin to a directory with the suffix "_alignments". The user may specify to instead align all SSU sequences by using the "--align all" option (note that this would increase the runtime (and the required disk space) significantly). The five best BLAST matches are aligned to the query sequence, and saved to an aligned FASTA file with the name "<query identifier>.aligned.fasta".

Chimeric sequences

If the option --allow_reorder is turned off, Metaxa2 will save an additional FASTA file containing sequences that are suspected to be chimeric. These are sequences with domains located in the wrong order. This is useful on full-length or near full-length data sets, but should not be used on short reads as it could increase the number of false negatives when run on short sequences.

Raw data

If the option to save all raw data is turned on, Metaxa2 will save all data from the pre-processing, HMMER-search, BLAST-search, as well as a file of raw statistics into a directory with the suffix "_metaxa_raw_output".

5. Metaxa2 Taxonomic Traversal Tool

In addition to the improved classifier, Metaxa2 also introduces a new bundled tool to further analyze the taxonomy output. This tool, called the Metaxa2 Taxonomic Traversal Tool – metaxa2_ttt, summarizes the taxonomic output of Metaxa2 at different taxonomic levels. The It is designed to be run after Metaxa2 has completed, and uses the ".taxonomy.txt" output file as its input. Simply put, the traversal tool goes through the taxonomic predictions in the ".taxonomy.txt" output file and counts the number of entries associated with each taxonomic level. The levels are, roughly, corresponding to kingdoms, phyla, classes, orders, families, genera, species, and subspecies, in some cases followed by more specific annotations. The traversal tool can also filter the output according to reliability score, alignment length, percent identity to the best BLAST match and/or taxonomic group (for details, see table of options below). The output of metaxa2_ttt consists of a number of tab-separated text files containing group counts at different taxonomic levels (by numbers), and a summary file with the suffix ".taxonomy.summary.txt". Each of the count files has the following format:

```
Bacteria;Actinobacteria;Actinobacteria    5
Bacteria;Firmicutes;Bacilli   14
Bacteria;Firmicutes;Clostridia      1
Bacteria;Proteobacteria;Betaproteobacteria      2
Bacteria;Unclassified Bacteria;     3
```

Each line here represents one node in the taxonomic tree, and the second column contains the number of entries associated with that node. The options for metaxa2_ttt are given in this table:

Options:

-i {file}	Metaxa2 taxonomy output file to process (with suffix ".taxonomy.txt") to use as input for metaxa2_ttt.
-o {file}	Base for the file names of the output files. Suffixes will be added automatically. Default is "metaxa_ttt_out".
-t {b, bacteria, a, archaea, e, eukaryota, m, mitochondrial, c, chloroplast, A, all, o, other}	Include only classifications of this taxonomic origin(s). Several origins can be specified, separated by comma. Default is to use all origins (all).
-r {value}	Reliability cutoff. Entries with reliability scores below this cutoff will be classified as "unknown". Default = 0.
-l {value}	Length cutoff (in bp) for the best BLAST hit. Entries below this cutoff will be classified as "unknown". Default = 50
-d {value}	Percent identity cutoff for the best BLAST hit. Entries below this cutoff will be classified as "unknown". Default = 0.
-m {integer}	Maximum resolution level for taxonomic traversal. Providing a zero (0) to this option will remove this limit. Usually, these levels correspond to Kingdom/Domain (1), Phylum (2), Class (3), Order (4), Family (5), Genus (6) and Species (7). Default is to have no upper limit for traversal (0).

-n {integer}	Minimum resolution level for taxonomic traversal. The kingdom level is defined as 1, and this is the lowest possible setting. Default = 1.
-s {T or F}	If true, metaxa2_ttt will investigate only the last taxonomic level (which in the best case will represent the species level). Note that this setting will classify sequences at very different taxonomic levels! When true, metaxa2_ttt will only output the ".level_1.txt" output file. Default is off (F).
--remove_na {T or F}	If true, sequence entries with no BLAST hits will be set to 'Unknown'. Default is on (T).

Output options:

--summary {T or F}	Outputs a summary of the results. On (T) by default.
--lists {T or F}	Outputs a list of counts for different taxa, one file for each traversal level. On (T) by default.
--separate {T or F}	Outputs the taxonomy file used as input, but separated for the different origins. Off (F) by default.
--unknown {T or F}	Outputs a list of entries designated as unknowns, with their statistics. Off (F) by default.

Information options:

-h	Displays the help message.
--help	Displays the help message.
--bugs	Displays the bug fixes and any known bugs in this version of Metaxa.
--license	Displays licensing information.

It is important to note that the output at the lowest taxonomic level (domain/kingdom) may differ slightly from the numbers showed in the ".summary.txt" file. This is due to that the default length cutoff for metaxa2_ttt is set to 50, while no such cutoff is set for the output to the summary file. In our internal testing, this difference (manifesting itself as a number of unknown sequences in the metaxa2_ttt output) is about 1% or less. The difference can be removed by setting the "-l" option to zero.

6. Metaxa2 Diversity Tools

Included with Metaxa2 since version 2.1 is also a set of other handy tools for taxonomic and diversity studies, collectively known as Metaxa2 Diversity Tools. These currently include four tools for automating and improving the analysis of the taxonomy output from Metaxa2. All these tools works with the *.taxonomy.txt output files of Metaxa2 or the output from the Metaxa2 Taxonomic Traversal Tool (metaxa2_ttt), and are intended to be run after the normal Metaxa2 analysis has finished. The four tools are the Data Collector (metaxa2_dc), the Species Inference tool (metaxa2_si), the Rarefaction analysis tool (metaxa2_rf), and the Uniqueness of Community analyzer (metaxa2_uc). Each tool is briefly described with command-line options explained below.

Metaxa2 Data Collector (metaxa2_dc)

The Metaxa2 Data Collector is designed to merge the output of several *.level_X.txt files from the Metaxa2 Taxonomic Traversal Tool into one large abundance matrix, suitable for further analysis in, for example, R. Note that all arguments not preceded by an option "flag" will be interpreted as input files for metaxa2_dc.

Options:

	All arguments not preceded with an option flag will be interpreted as input files for metaxa2_dc. Note that you can end the command-line by *level_6.txt to include all files ending with "level_6.txt" present in a particular directory.
-o {file}	The output file containing the full abundance matrix. Defaults to "collected_data.txt".
-t {integer}	Column containing the taxon name data. Default is zero (first column).
-c {integer}	Column containing the count data. Default is one 1 (second column).
-r {string}	String to be removed from each file name for use as the sample name. Regular expressions can be used. Default is ".level_[0-9].txt", which (as an example) will extract "Sample1" as the sample name from the file "Sample1.level_6.txt".
-p {string}	Regular expression pattern for selecting the sample name from the file name. Default is ".*", which will cover full file name.

Information options:

-h	Displays the help message.
--help	Displays the help message.
--bugs	Displays the bug fixes and any known bugs in this version of Metaxa.
--license	Displays licensing information.

Metaxa2 Species Inference Tool (metaxa2_si)

The Metaxa2 Species Inference tool can be used to further infer taxon information on, for example, the species level despite a lower reliability score than accepted in the Metaxa2 classifier, using a complementary algorithm. The idea is that if the only species present in, e.g., the Flavobacteriaceae family is *Ornithobacterium rhinotracheale* and a read is assigned to the Flavobacteriaceae family, but not to the species level, that sequence will be inferred to the *Ornithobacterium rhinotracheale* species given that it has more than 97% sequence identity to its best reference match. This can be useful if the user really needs species or genus classifications but many organisms in the studied species group have similar rRNA sequences, making it hard for the Metaxa2 classifier to classify sequences to the species level. The metaxa2_si tool works on the *.taxonomy.txt output from the Metaxa2 software.

Options:

-i {file}	Input taxonomy file from Metaxa2 (*.taxonomy.txt).
-o {file}	The output file containing the full abundance matrix. Defaults to the input name with the suffix ".inferred.txt" appended.

-l {integer}	Taxonomic level for performing inference (1 = domain, 2 = phylum, 3 = class, 4 = order, 5 = family, 6 = genus, 7 = species). Default is 7 (species level).
-c {value}	Percent identity cutoff to closest reference sequence for allowing inference. Default is 97.
--list_all {T or F}	Outputs a list of all possible taxon associations for sequence entries with multiple possible inferences. Off (F) by default.
--multiple {keep,merge,remove,assign}	Decides how to handle entries with multiple possible inferences. The 'keep' option will retain them in the taxonomy file without changes to their taxonomic classification, 'merge' will keep the entries but remove all taxonomic information and replace it with "Multi-origin sequence", 'remove' will exclude entries with multiple possibilities entirely, and 'assign' will randomly assign the entry to a possible taxon within the same taxonomic group. Default is to 'keep' entries (without modifications).
--low_identity {keep,merge,remove}	Decides how to handle entries with sequence identity below the specified cutoff (see the "-c" option). The 'keep' option will retain these entries in the taxonomy file without changes to their taxonomic classification, 'merge' will keep the entries but remove all taxonomic information and replace it with "Low-identity sequence", and 'remove' will exclude entries with low sequence identity entirely. Default is to 'keep' these entries.

Information options:

-h	Displays the help message.
--help	Displays the help message.
--bugs	Displays the bug fixes and any known bugs in this version of Metaxa.
--license	Displays licensing information.

Metaxa2 Rarefaction analysis tool (metaxa2_rf)

The Metaxa2 Rarefaction analysis tool performs a rarefaction analysis based on the output from the Metaxa2 classifier (or the Metaxa2 Species Inference tool). The tool produces three different curves, one for only the observed taxa (the *Observed number of taxa* column), one for the maximum number of taxa possible, given that all unknown entries are individual taxa (the *Theoretical maximum number of taxa* column), and one modelled column that takes the proportion of unknown entries into account and then models the number of different taxa sampled at a particular number of rRNA sequences drawn (the *Modelled number of taxa* column). Depending on the options selected (see below) the Metaxa2 Rarefaction analysis tool can perform rarefaction at different taxonomic levels.

Options:

-i {file}	Input taxonomy file from Metaxa2 (*.taxonomy.txt).
-o {file}	Base for the name of output file(s). Defaults to "metaxa2_rf_out".

Entry selection options:

-t {b, bacteria, a, archaea, e, eukaryota, m, mitochondrial, c, chloroplast, A, all, o, other}	Include only classifications of this origin(s). Several origins can be provided, separated by commas. Default is ‘all’ origins.
-r {value}	Specifies the reliability cutoff. Entries below the cutoff will be regarded as 'unknown' in the rarefaction analysis. Default is 0 (will include all entries).
-l {value}	Specifies the length cutoff (in bp) of the overlap to the best hit. Entries below the cutoff will be regarded as 'unknown' in the rarefaction analysis. Default is 50.
-d {value}	Sets the percent identity cutoff to the best hit. Entries below the cutoff will be regarded as 'unknown' in the rarefaction analysis. Default is 0 (will include all sequences, regardless of identity).
-m {integer}	Maximum resolution level for taxonomic traversal. Zero is unlimited. Default is not to limit this (0).
-n {integer}	Minimum resolution level for taxonomic traversal, starting at level 1. Default is 1 (domain level).
-s {T or F}	Investigate only the last taxonomic level (in good cases corresponding to species resolution). Default is not to do this (F).
-u {T or F}	If turned on, unclassified entries will be treated as unknowns. Default is off (F), which will use as much taxonomic information as possible.
--remove_na {T or F}	Set sequence entries with no BLAST hits to 'Unknown'. Default is to do this (T).

Rarefaction options:

--resamples {integer}	Number of resamplings of the abundance data. Default is 1000.
--write {integer}	Write interval for the sampled list output. Default is 1 (which will write out every line).
--size {integer}	Fix the total number of sequences to this number. Allows for the addition of “artificial” entries to the analysis. By default this is set to the sum of all counts in the dataset.
--scale {integer}	Scale all samples to have this number of sequences. Default is 0, which turns scaling off.
--exclude_rows {list of integers}	A comma-separated list of rows *not* to include in the analysis. Default is not to exclude any entries.

Output options:

--summary {T or F}	Enables output of results summary. On (T) by default.
--lists {T or F}	Enables output of lists of counts for different taxa, one file for each traversal level. On (T) by default.
--separate {T or F}	Will output rarefaction analysis results separately for the different origins. On (T) by default.
--unknown {T or F}	Outputs a list of all entries designated as unknowns, along with their statistics. Off (F) by default.

--sampled {T or F}	Outputs a table containing the number of observations for different taxa at different number of sequences sampled. One file will contain the output for each traversal level. Note that this option can be extremely memory demanding, and that the resulting table can be very large. Off (F) by default.

Information options:

-h	Displays the help message.
--help	Displays the help message.
--bugs	Displays the bug fixes and any known bugs in this version of Metaxa.
--license	Displays licensing information.

Metaxa2 Uniqueness of Community analyzer (metaxa2_uc)

The Metaxa2 Uniqueness of Community analyzer is a tool that allows analysis of whether the community composition of two or more samples or groups is significantly different. Using resampling of the community data, the tool tests the null hypothesis that the taxonomic contents of two communities are drawn from the same set of taxa (given certain abundances). The metaxa2_uc tools works on the output from metaxa2_dc or any abundance matrix with the same structure. The most important parameter to metaxa2_uc is the mode used for resampling. By default, the comparison will be made to a distribution estimated from the average abundances in each group, with a certain degree of variation allowed. This model generates each resampled abundance number as follows:

[Resampled abundance] = [True group relative abundance] ± [Random number between 0 and 1] * 2 * [Standard deviation of group relative abundance]

Options:

-i {file}	Input abundance matrix, e.g. derived from metaxa2_dc.
-o {file}	Base for the name of output file(s). Defaults to "metaxa2_uc_out".
-g {file,string,auto,none,all}	A file or string describing the sample group division, or 'auto' if the groups should be guessed from sample names, or 'none' if all samples should be treated individually, or 'all' if all samples should be treated as they come from the same group. Default is to use all samples as one single group ('all').
-r {value}	Number of resampling rounds for each sample. Default is 10000 (which will give a lowest p-value of 0.0001). Caution: memory usage *and* processing time largely scales with this parameter and it is recommended to lower it if you have hardware constraints.
-s {value}	Number of entries sampled in each resampling round for each sample. Higher number will improve sensitivity to differences. If this option is set to "min" the entries sample will be the number of entries in the smallest sample. Default is 1000.
-c {string}	Sample to compare to. Leave blank to compare to all samples. Use 'groups' to compare groups instead of samples. Default is blank, which will compare all samples to each other.

-w {value}	Within-sample variation cutoff to compare to (corresponding to the proportion of all resampling values included). Can be in the range of 0 to 1. Default is 0.99.
-m {empirical,average,model}	The model to use for the resampling procedure. The 'empirical' mode uses the empirical distribution of abundances from each sample within each group. This mode will give rise to a large variance that may be somewhat more realistic, but not statistical good-practice since it will estimate the parameters from the same data as it analyses. The 'average' mode assumes that the abundances of taxa in every sample of each group are drawn directly from the group average, which gives very small variance, and is thus unrealistic. The 'model' mode, finally, assumes a model that take within-group sample variation into account when drawing from the group average, which gives larger variance but in a more realistic way, and without (extensive) use of the data itself to estimate the parameters. Default is to use the 'model' mode (based on empirical testing of the software performance).
-d {bray,jaccard,euclidean}	Distance/dissimilarity measure to use when comparing samples and groups. The 'bray' option corresponds to the Bray-Curtis dissimilarity, 'jaccard' corresponds to the Jaccard distance, and "euclidean" corresponds to the Euclidean distance between samples/groups. Default is to use Bray-Curtis dissimilarity ('bray').
--binary {T or F}	Use presence/absence for distance/dissimilarity rather than taking abundances into account. Off (F) by default (will use abundance data).
--filter {value}	Filter out abundance values below this cutoff value. Default is 0, which will use all data.

Output options:

--summary {T or F}	Outputs a readable summary file of the results. On (T) by default.
--table {T or F}	Outputs a tab-separated table of the results. Off (F) by default.
--matrix {T or F}	Outputs the results in a matrix format. Off (F) by default.
--resampling_table {T or F}	Outputs the resampling table (which can be huge). Off (F) by default.

Information options:

-h	Displays the help message.
--help	Displays the help message.
--bugs	Displays the bug fixes and any known bugs in this version of Metaxa.
--license	Displays licensing information.

An example workflow based on Metaxa2 and the diversity tools

The following section will outline a simple example workflow for how Metaxa2 can be used in taxonomic and diversity analyses. We will use the test file that comes with Metaxa2 to illustrate how data can be analyzed.

1) Running Metaxa2 on the input data:

```
metaxa2 -i test.fasta -o SSU_EXAMPLE --cpu 2
```

This should generate the following (excerpt from the SSU_EXAMPLE.summary.txt file):

```
Number of SSU rRNA sequences to be classified by Metaxa:        51
Number of SSU rRNA having at least one database match:          50
Number of SSU rRNA successfully classified by Metaxa:           50
Number of uncertain classifications of SSU rRNA sequences:      0
Total number of classifications made by Metaxa:                 50
Number of SSU rRNA sequences assigned to each origin:
  Archaea:      10
  Bacteria:     10
  Eukaryota:    10
  Chloroplast:  10
  Mitochondria: 10
  Uncertain:    0
```

2) In the next step, we will generate some data to compare to. To keep it simple, we will compare to the LSU data from the same file:

```
metaxa2 -i test.fasta -o LSU_EXAMPLE --cpu 2 -g lsu
```

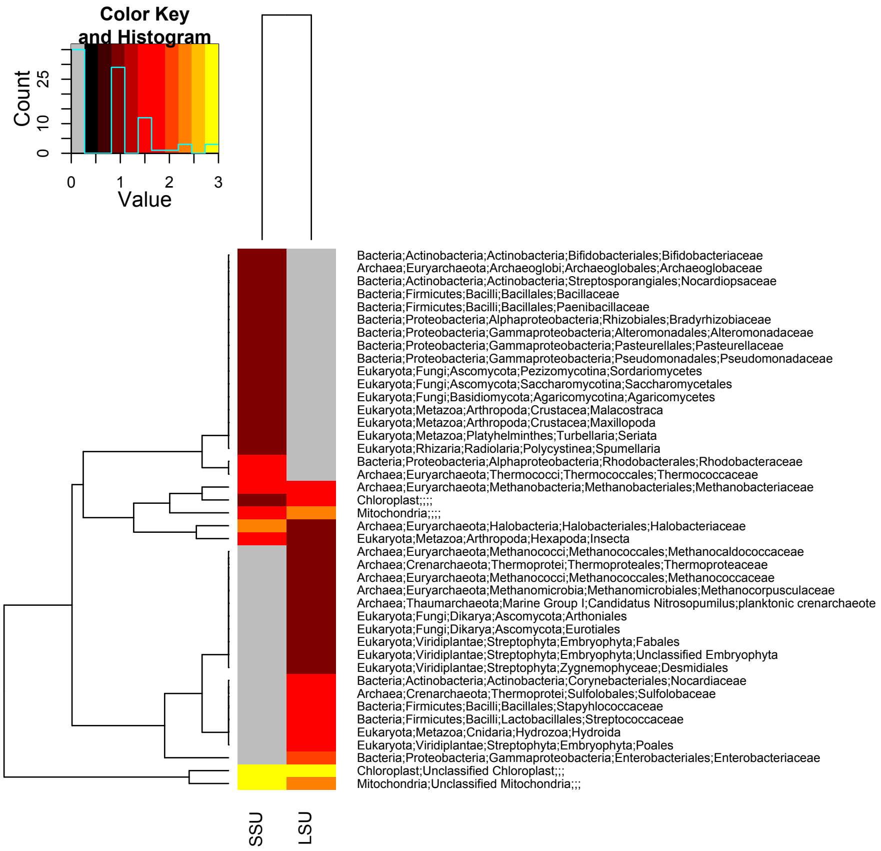
3) Now, we want to count the number of species, genera etc. present in these two datasets. For this we will use the Metaxa2 Taxonomic Traversal Tool (metaxa2_ttt). Note that we use the same base for the output names as for the input:

```
metaxa2_ttt -i SSU_EXAMPLE.taxonomy.txt -o SSU_EXAMPLE
metaxa2_ttt -i LSU_EXAMPLE.taxonomy.txt -o LSU_EXAMPLE
```

4) To be able to compare the LSU and SSU data we have generated, we want to combine the counts to a single abundance matrix. For this, we will use the Metaxa2 Data Collector (metaxa2_dc). Since the data is scarce in this example, we will combine the counts on the family level (level 5). To only get the first three characters of the file name as the header for each column, we use a regular expression for the pattern option (-p "^…"). Notice the neat trick we use to get both files using the wildcard (*) on the command line:

```
metaxa2_dc -o EXAMPLE_COMBINED.txt -p "^..." *level_5.txt
```

From this combined abundance matrix file, we can for example generate a heatmap of family abundances (this particular example was generated in R using the gplots library):



5) We can also use the abundance matrix to test if the SSU and LSU "communities" seem to be drawn from the same species pool, in order to test if they significantly differ. For this we will use the Metaxa2 Uniqueness of Community Analyzer (metaxa2_uc). We will use the "auto" mode to detect the groups, which will separate the LSU and SSU datasets into separate groups. Note that we also turn on the matrix and table output, to be able to analyze and visualize the results more efficiently later. Note also that this tool takes a rather long while to run, even on this small dataset:

```
metaxa2_uc -i EXAMPLE_COMBINED.txt -o EXAMPLE_COMBINED --table T --
matrix T -g auto
```

This analysis will generate the following output, indicating that if we assume that the LSU sequences are drawn from the SSU sequence pool and vice versa, the datasets significantly differ. However, if we assumed that both datasets are drawn from a single, common, family pool, the datasets do not differ significantly to that total pool (as seen when compared to the "_all_" group):

```
Results:
=================================================
Sample Group Bray-Curtis dissimilarity (min-max) p-value Significance
-------------------------------------------------
LSU    LSU    0.347   (0.347-0.348)   < 9.996e-01
LSU    SSU    0.698   (0.685-0.722)   < 9.999e-05     ***
LSU    _all_  0.301   (0.248-0.363)   < 9.984e-01
-------------------------------------------------
Internal distance (LSU, 0.99):  0.347
=================================================
Sample Group Bray-Curtis dissimilarity (min-max) p-value Significance
-----------------------------------------------------
SSU     LSU     0.657   (0.636-0.676)   < 9.999e-05     ***
SSU     SSU     0.348   (0.348-0.350)   < 9.988e-01
SSU     _all_   0.296   (0.239-0.353)   < 9.997e-01
-----------------------------------------------------
Internal distance (SSU, 0.99):  0.348
=====================================================
```

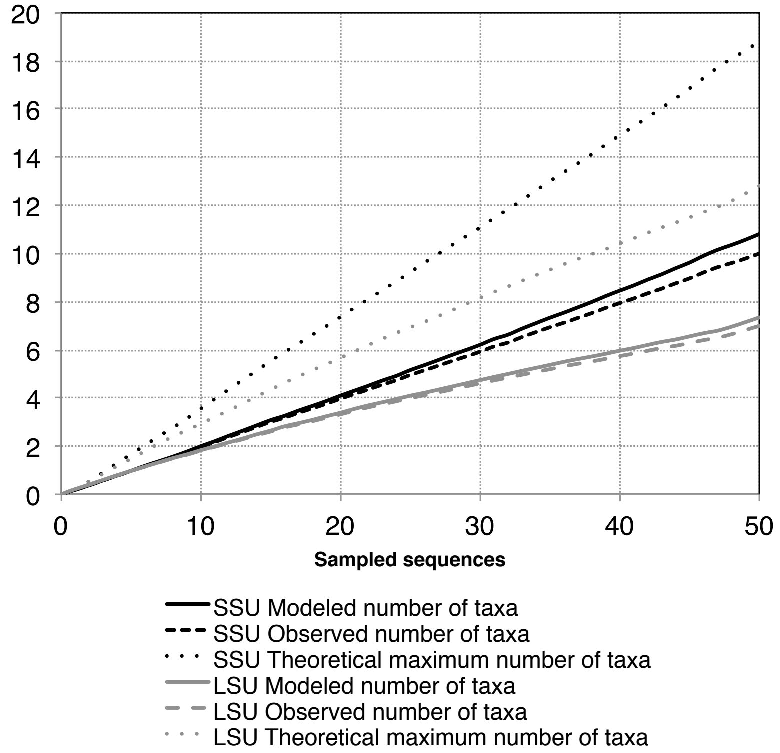
6) As a next step, we might want to try to further infer species classifications using the Metaxa2 Species Inference tool (metaxa2_si). This tool takes the original taxonomy output from Metaxa2 as input, so we will need to return to these files:

```
metaxa2_si -i SSU_EXAMPLE.taxonomy.txt -o SSU_EXAMPLE_INFERRED.txt
metaxa2_si -i LSU_EXAMPLE.taxonomy.txt -o LSU_EXAMPLE_INFERRED.txt
```

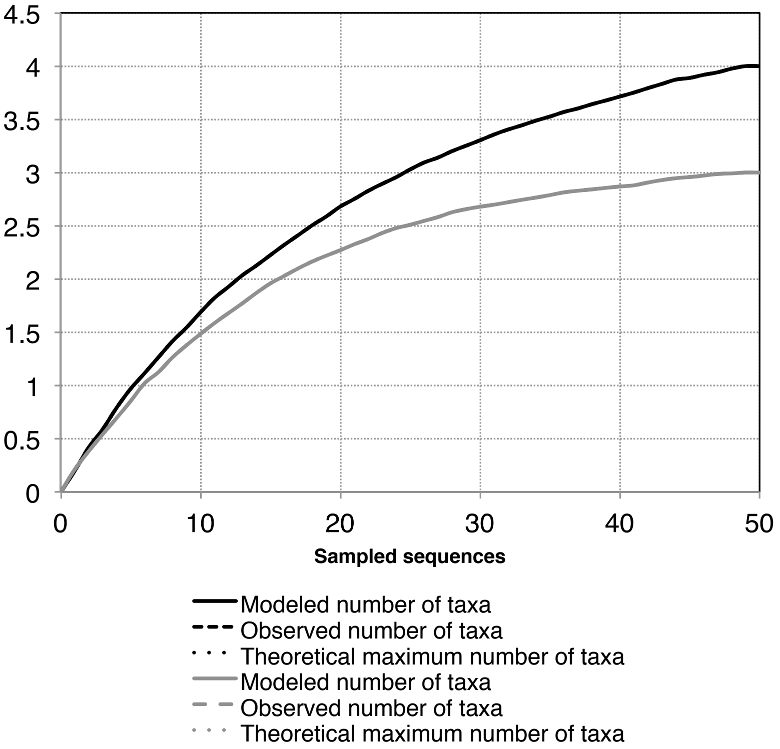
7) Using the inferred species data, we may now attempt to do a rarefaction analysis for each taxonomic level, using the Metaxa2 Rarefaction tool (metaxa2_rf). This tool uses the taxonomy output from the Metaxa2 classifier *or* inferred taxonomy data from the Metaxa2 Species Inference tool as input:

```
metaxa2_rf -i SSU_EXAMPLE_INFERRED.txt -o SSU_EXAMPLE_RF
metaxa2_rf -i LSU_EXAMPLE_INFERRED.txt -o LSU_EXAMPLE_RF
```

This will result in a number of files (one for each organism group and each taxonomy level). By loading the tab-separated SSU_EXAMPLE_RF.bacteria.rf.7.txt file in, e.g., Excel, it is possible to plot rarefaction curves. In this example we have combined it with the rarefaction curves for the LSU data (in grey), both on the species level (level 7):



As you can clearly see, this data is not well saturated, so we can also repeat the analysis on class level (level 3) for the purpose of illustration:



At this level, the modelled and observed curves completely overlap (because all classes in the data has been classified, so there are no unknown entries), and the curve for the LSU seem to start to saturate at around 45, while the SSU curve (black) does not look completely saturated.

It is of course possible to do much more with these tools, but this is an example of how the tools included with Metaxa2 can be used to make simple analyses of community composition and diversity.

7. Metaxa2 Database Builder

Metaxa2 version 2.2 introduced the possibility to build custom databases for any barcoding gene or region. This is achieved through a new tool called the Metaxa2 Database Builder – metaxa2_dbb. The database builder takes a number of FASTA files as input, each corresponding to a taxonomic origin, and a taxonomy file with corresponding sequence identifiers and builds a classification database based on this input.

Options:

-i {file}	DNA FASTA file containing the reference sequences of a single genetic marker (typically a gene) to be used for classification (overrides input options for specific origins below).
-o {directory}	Directory name for the output files. Default name is "new_database".
-g {string}	Gene name for the database (e.g. SSU).
-p {directory}	Use HMMs from the specified directory instead of computing new ones. This will cause the program to build a new BLAST database for classification, but use a pre-defined set of HMM-profiles. Not used by default.
-t {file}	Taxonomy file containing taxonomic information to be parsed. The taxonomy file can be in any of the following formats: Metaxa2, FASTA, ASN1, NCBI XML, INSD XML (see also specific paragraph on the taxonomy file below)
-r {sequence_ID}	Sequence ID of the sequence that should be used as representative sequence for the barcoding gene or region. This sequence is used to define the start and end of the barcoding sequence (see specific paragraph below). If left blank, the first sequence in the input file will be used.
--auto_rep {T or F}	Choose a reference sequence automatically by clustering. This requires USEARCH to be installed. Off (F) by default.
--cpu {integer}	Number of CPUs to be used (will be passed on to other programs). Default = 1.
--save_raw {T or F}	Keep intermediate files after the program finishes. Off (F) by default.
--plus {T or F}	Use BLAST+ instead of legacy BLAST. Off (F) by default.

Origin-specific input options:

-a {file}	DNA FASTA file containing archaeal reference sequences to be used for classification (cannot be combined with the -i option).
-b {file}	DNA FASTA file containing bacterial reference sequences to be used for classification (cannot be combined with the -i option).

-c {file}	DNA FASTA file containing chloroplast reference sequences to be used for classification (cannot be combined with the -i option).
-e {file}	DNA FASTA file containing eukaryote reference sequences to be used for classification (cannot be combined with the -i option).
-m {file}	DNA FASTA file containing mitochondrial reference sequences to be used for classification (cannot be combined with the -i option).
-n {file}	DNA FASTA file containing metazoan mitochondrial (e.g. 12S rRNA) reference sequences to be used for classification (cannot be combined with the -i option).
--other {file}	DNA FASTA file containing reference sequences of other origins to be used for classification (cannot be combined with the -i option).

Database construction parameters:

--full_length {integer}	Number of basepairs to use for full-length definition. Set this option to zero to disable full-length extraction and use all input sequences as they are. Default = 10.
-C {integer}	Conservation score cutoff. The cutoff is set to 4 by default, but is not used unless the "-A" option is set to false (F).
-N {ratio}	Noise cutoff (the minimal proportion of sequences required for a nucleotide to be considered as being present at each position of the alignment). This must be a number between 0 and 1. Default = 0.1.
-A {T or F}	Auto-detect conservation score cutoff. On (T) by default.
-P {ratio}	Minimal conserved proportion of the sequences in the alignment. If this proportion is not met, the program will consider a lower conservation cutoff, until this criterion is satisfied. Default = 0.6.
-L {integer}	Look-ahead length. This is the number of nucleotides that will be considered when determining the start and the end of conserved regions. Default = 5.
-M {integer}	Minimal conserved region length, in basepairs. If this length is not met, a region will not be counted as conserved. Default = 20.
--single_profile {T or F}	Build only one single HMM for the entire alignment from the input sequences, instead of trying to model conserved and variable regions. Off (F) by default.
--mode {divergent, conserved, hybrid}	Selects the mode to build the profile database in. The conserved mode is useful for gene families with highly similar sequences. See the section on the conserved versus divergent and hybrid modes below. Default is divergent.
--dereplicate {ratio or F}	De-replicate the input data using USEARCH before building the database, using the specified identity threshold. Off (F) by default.

Filtration and correction options:

--filter_uncultured {T or F}	Will attempt to filter out sequences that are derived from uncultured organisms. Off (F) by default.
--filter_level {integer}	Will filter out sequences without taxonomic information at the specified level. Off (0) by default (will not discard any sequences).

--correct_taxonomy {T or F}	Will attempt to correct the taxonomic information at order, family, genus, and species level. See the section on taxonomy correction below. Off (F) by default.
--cutoffs {string}	A string of numbers defining the identity cutoffs at different taxonomic levels. Using this option will turn off automatic calculation of these cutoffs. If left blank, the identity cutoffs are determined automatically. Default is blank (off), which will cause the software to automatically calculate cutoffs.
--sample {integer}	The maximum number of sequences to investigate when determining taxonomic cutoffs. Default = 1000.

Evaluation options:

--evaluate {T or F}	Statistically evaluate the performance of the database built. This increases the time requirement for the process dramatically, but produces a measure on the precision and sensitivity of the database. See the Evaluation section below. Off (F) by default.
--iterations {integer}	Number of iterations for the statistical evaluation. Default = 10.
--test_sets {ratio}	Proportion of sequences to leave out for testing. Several values can be specified, separated by commas, which will run the evaluation for each of those proportions. Default = 0.1.
--db {directory}	Skips building the database, and only runs the evaluation on the specified database directory. Not used by default.

Information options:

-h	Displays the help message.
--help	Displays the help message.
--bugs	Displays the bug fixes and any known bugs in this version of Metaxa.
--license	Displays licensing information.

Taxonomy files

The Metaxa2 Database Builder requires the user to supply a file containing taxonomic affiliations of the provided input sequences. This file must associate taxonomic information with *exactly* the same sequence identifiers as in the input files. The database builder accepts a number of input formats for the taxonomy files. The preferred format is to use the same format as Metaxa2 uses for its database files, that is a tab-separated text file with entries organized like this:

```
s3455.B     Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirill
ales;Acetobacteraceae;Acetobacter;Acetobacter aceti IFO3283
s5724.B     Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonad
ales;Moraxellaceae;Acinetobacter;Acinetobacter sp. strain ADP1
```

Note that the identifier and the taxonomic information are separated by a tab, while each level of the taxonomy is separated by a semi-colon. If there are more entries in the taxonomy file than in the sequence file(s) provided, the program will just ignore those extra entries.

The Metaxa2 Database Builder also accepts a FASTA file as taxonomy input, given that its headers are organized as the headers used in the SILVA database "taxonomy" FASTA files:

```
>GAXI01000525.151.1950         Eukaryota;Opisthokonta;Holozoa;Metazoa
(Animalia);Eumetazoa;Bilateria;Arthropoda;Hexapoda;Ellipura;Collembol
a;Tetrodontophora bielanensis (giant springtail)
CCUGGUUGAUCCUGCCAGUAGUCAUAUGCUUGUCUCAAAGAUUAAGCCAUG...
```

Note that this format is highly similar to the Metaxa2 format, but that the sequence identifier and the taxonomy is separated by a space instead of a tab. The actual sequences in this file will be ignored by the database builder.

The database builder also has (limited) support for parsing NCBI GenBank files in the ASN and XML formats, as well as the XML format used by the INSDC. For the ASN format, the database builder will look for the tags "id {", "accession", "taxname" and "lineage", which must be in the file to be able to infer the taxonomy. For the NCBI XML format, the program needs the tags "<Seq-entry>", "<Textseq-id_accession>", "<Org-ref_taxname>", "<BinomialOrgName_genus>", "<BinomialOrgName_species>" and "<OrgName_lineage>" to infer taxonomy, while for the INSD XML format the "<INSDSeq>", "<INSDSeq_locus>", "<INSDSeq_organism>", and "<INSDSeq_taxonomy>" tags are required.

Choosing the reference sequence

An important component of the database builder is the ability to only extract the actual barcoding region from a set of longer sequences (which could in principle be full genomes, although that would make the process *very* slow). To be able to do this, the software needs a reference sequence that is representative of the full barcoding region and runs from where the barcoding region starts (but no earlier) to where it ends (but no further). The program will then use the start and end of that sequence to build a model of how the barcoding region (hopefully conserved) ends look, and extract only that part from all other sequences. It is important to select a reference that is actually representative, otherwise the entire database might be compromised or biased. If you are not sure what would be a representative sequence, there are two possible options – and it is advisable to explore both and investigate which gives the best sensitivity and accuracy. The first is to make the software automatically select a representative through clustering. This requires USEARCH to be installed. From the clusters generated, the database builder will select the centroid sequence of the largest cluster as reference. This functionality is turned on by specifying the "--auto_rep T" option. The other possibility is to turn off the extraction of the barcoding region entirely, which can be done by specifying "--full_length 0". This will, however, include also very long sequences in the database, and thus requires somewhat better quality of the input data. Finally, note that when the database builder is run in divergent mode (see below), the reference sequence is not used.

The different operating modes

There are two different main operating modes of the Metaxa2 Database Builder, as well as a hybrid mode combining the features of the two other modes. The divergent and conserved modes work in almost completely different ways and deal with two different types of barcoding regions. The divergent mode is designed to deal with barcoding regions that exhibit fairly large variation between taxa within the same taxonomic domain. Such regions include, e.g., the eukaryotic ITS region, or the *trnL* gene used for plant barcoding. In the other mode – the conserved mode – a highly conserved barcoding region is expected (at least within the different taxonomic domains). Genes that fall into this category would be, e.g., the 16S SSU rRNA, and the bacterial *rpoB* gene. This option would most likely also be suitable for barcoding within certain groups of e.g. plants, where similarity of the barcoding regions can be expected to be high. There is also a third mode – the hybrid mode – that incorporates features of both the other. The hybrid mode is more experimental in nature, but could be useful in situations where both the other modes perform poorer than desired.

In the divergent (and default) mode, the database builder will start by clustering the input sequences at 20% identity using USEARCH. All clusters generated from this process are then individually aligned using MAFFT. Those alignments are split into two regions, which are used to build two hidden Markov models for each cluster of sequences. These models will be less precise, but more sensitive than those generated in the conserved mode. In the divergent mode, the database builder will attempt to extract full-length sequences from the input data, but this may be less successful than in the conserved mode.

In the conserved mode, on the other hand, the database builder will first extract the barcoding region from the input sequences using models built from a reference sequence provided (see above) and the Metaxa2 extractor. It will then align all the extracted sequences using MAFFT and determine the conservation of each position in the alignment. When the criteria for degree of conservation are met, all conserved regions are extracted individually and are then re-aligned separately using MAFFT. The re-aligned sequences are used to build hidden Markov models representing the conserved regions with HMMER. In this mode, the classification database will only consist of the extracted full-length sequences.

In the hybrid mode, finally, the database builder will cluster the input sequences at 20% identity using USEARCH, and then proceed with the conserved mode approach on each cluster separately.

Taxonomy filtration and correction

The actual taxonomic classification in Metaxa2 is done using a sequence database. It was shown in the original Metaxa2 paper (http://dx.doi.org/10.1111/1755-0998.12399) that replacing the built-in database with a generic non-processed one was detrimental to performance in terms of accuracy. In the database builder, we have tried to incorporate some of the aspects of the manual database curation we did for the built-in database that can be automated. By default, all these filtration steps are turned off, but enabling them might drastically increase the accuracy of classifications based on the database.

The first such aspect is the removal of non-informative sequences derived from unknown specimens and mixed environmental samples. This is done by filtering out all sequences with taxonomic information containing a list of keywords, including "uncultured", "unclassified", "environmental", "contaminant", "unknown", and "metagenome". This may discard some valid entries as well, but in our internal testing it still improves performance relative to leaving them in. This filtration can be turned on with the "--filter_uncultured T" option.

The second automatic method to improve taxonomy that can be applied is taxonomy correction. This process tries to standardize the input taxonomy into seven taxonomic levels (corresponding to domain/kingdom, phylum, class, order, family, genus, and species in bacteria and archaea; in eukaryotes, mitochondria and chloroplasts the division is more complex). The automated correction process also works much better in bacteria and archaea than in the other domains. First, the phylum level is identified by matching the levels against a list of pre-defined phyla. Second, the order level is identified by locating the level with the "-ales" suffix (if found). The class level is defined as being one level above the order level. Thereafter, the family level is identified by locating the level with the "-ceae" suffix (if present). This is followed by finding the level with a Latin binomial, which corresponds to the species level. The genus level is then identified as the level containing only the first part of that binomial. This procedure has a high success rate in parsing out a standard taxonomy from the input taxonomy. However, it is not perfect and will not catch all special cases. Particularly in eukaryotes, it may fail entirely because the taxonomic naming conventions are not that standardized. Therefore, this automatic correction should, in all cases where correct taxonomic information is crucial, be followed by manual checks. The automatic taxonomy correction can be turned on using the option "--correct_taxonomy T".

The final strategy to improve taxonomic accuracy is to filter out all entries with poor taxonomic information, i.e. all sequences that only have, e.g., phylum and class information. This can be done by using the option "--filter_level" followed by an integer, corresponding to the minimum levels of taxonomy an entry must have to be included in the classification database. For example, specifying "--filter_level 7" would require all entries to have taxonomic information all the way to the species level to be included, while "--filter_level 4" would only require taxonomic information to the order level. By default, no such filtering is performed and all sequences are included (corresponding to specifying "--filter_level 0"). It is worth noting that this level filtering occurs after the removal of non-informative sequences and the taxonomy correction.

Evaluating database accuracy

To assess the accuracy of the constructed database, the Metaxa2 Database Builder allows for testing the detection ability and classification accuracy of the constructed database. This is done by sub-dividing the database sequences into subsets and rebuilding the database using a smaller (by default 90%), randomly selected, set of the sequence data. The remaining sequences (10% by default) are then classified using Metaxa2 with the subset database. The number of detections, and the numbers of correctly or incorrectly classified entries are recorded and averaged over a number of iterations (10 by default). This allows for obtaining a picture of the lower end of the accuracy of the database. However, since the evaluation only uses a subset of all sequences included in the full database, the performance of the *full* database actually constructed is likely to be slightly better. The automatic evaluation is time-consuming but gives a good picture of the quality of the database, and if the barcoding region or the input sequences employed were suitable or not (or if the taxonomic information was insufficient). The evaluation can be turned on using the "--evaluate T" option. Note also that the evaluation can be run on an existing Metaxa2 database, without rebuilding it, by specifying the path to the database directory with the "--db" option together with the "--evaluate T" option.

8. The Metaxa2 Database Repository

Metaxa2 2.2 also introduces the database repository, from which the user can download additional databases for Metaxa2. To download new databases from the repository, the metaxa2_install_database command is used. This is a simple piece of software but requires internet access to function.

Options:

-g {string}	Specify the name of the database to install (usually a gene name). If this is not specified, the program will instead show a list of available database options.
-d {directory}	The directory where to install the database. Default is in the metaxa2_db directory in the same directory as Metaxa2 itself. You will need to have write access to this directory for the installation to work.
-r {http-address}	The repository to download database files from. Default is http://microbiology.se/sw/metaxa2_dbs

To install a database, first run the command "metaxa2_install_database" without any options. This will produce a list similar to this one:

```
Metaxa2 -- Database installer
by Johan Bengtsson-Palme, University of Gothenburg
Version: 2.2
This program is distributed under the GNU GPL 3 license, use the --
license option for more information on this license.
-----------------------------------------------------------------
Gene name   Description
SSU   The default SSU rRNA database bundled with Metaxa2 (manually
curated)
LSU   The default LSU rRNA database bundled with Metaxa2 (manually
curated)
SSU_SILVA123.1    A database representing the complete SILVA 123.1
release. Please note that this database is not manually quality-
checked by the Metaxa team.
```

Then, to install the database type, for example:

```
metaxa2_install_database -g SSU_SILVA123.1
```

If the process ends with the message “Finished.” the installation was successful, and you can now use the database for classification like this:

```
metaxa2 -g SSU_SILVA123.1 -i test.fasta -o TEST --cpu 4
```

9. Internal changes in Metaxa2

The original algorithms and design behind Metaxa are described in the manual for Metaxa version 1.1.2 (see part 4: *Algorithm and implementation* in that manual). In the following, we mainly focus on the changes made with respect to previous Metaxa versions, instead of reiterating the information from the previous manual.

If the main design goal for the original version of Metaxa was to achieve fast and accurate extraction of SSU sequences in large data sets, without introducing a large number of false positives, the design goal of Metaxa2 is to do the same task faster, better and for larger data sets. In addition, Metaxa2 adds the ability to extract and classify LSU sequences, as well as to produce more precise taxonomic classifications. Metaxa2 still relies on the HMMER3 software, which allows for extremely fast comparisons, for filtration of the input dataset, and further analysis of just a subset of the input data. Similarly to version 1, Metaxa2 uses multiple HMM profiles representing conserved domains in the SSU sequence. In addition, separate sets of HMM profiles for SSU sequences of archaeal, bacterial, eukaryal, mitochondrial and chloroplast origin are utilized. We have also added HMM profiles representing the LSU gene in these lineages, in very much the same way. To avoid false positive matches, Metaxa2 by default requires at least two such conserved domains to be found in a query sequence. This criterion brings down the false positive rate to close to zero.

The subsequent BLAST-based classification step now has an even more elaborately crafted and curated database, allowing Metaxa2 to make accurate taxonomic predictions that often go down to genus or species levels. The scoring system for assigning the sequences to archaeal, bacterial, eukaryal, mitochondrial, or chloroplast origin still remains in Metaxa2. If the origin of the final classification does not agree with the predicted origin from the HMMER-based step, the sequence classification is marked as uncertain (by applying a “#” to the end of the definition line). The sequence is also marked as uncertain if the difference between the classification scores of the two most likely origins is smaller than the number of analyzed BLAST-matches (by default 5).

Most changes that have been introduced in Metaxa2 are related to either the improved taxonomy engine, or adaptions to modern metagenomics, which is moving towards even larger datasets, but with short read lengths. In general, the internal changes are invisible to the end user, but some of them might have impact on particular usage scenarios.

First of all, the main Metaxa2 program is now able to perform quality filtering of sequences in the FASTQ format (http://en.wikipedia.org/wiki/Fastq). Please note that the filtering is performed before metaxa2_x or metaxa2_c has been started. Thus, none of the individual programs can read FASTQ-format. Instead, they still expect input in FASTA format.

Metaxa2 accommodates for paired-end reads in a special fashion. Similarly to FASTQ input, paired-end libraries must be pre-processed through the first metaxa2 program and cannot be directly inputted into metaxa2_x or metaxa2_c. This is because Metaxa2 re-organizes the input sequences into a special concatenated format, which enables HMMER to work on both ends *at once*. This conversion, however, must happen before the extraction step can begin. This design decision was taken in order to streamline the downstream processing of sequence entries. Although it would be possible to re-write the code so that the individual tools handle FASTQ and paired-end input, it would severely complicate the way Metaxa2 internally manages its files. When Metaxa2 reads a paired-end library, it first concatenates the two reads into one sequence, retaining information on where the original reads begin and end. The paired-end information is saved to a file called "pairinfo.1.txt", which can be retained and accessed by running Metaxa2 with the "--save_raw T" option. The metaxa2_x program reads the concatenated FASTA file and the pair data to create an output file containing the identified rRNA sequences. In this file, Metaxa2 also inserts an insert sequence consisting of repeated "N" characters between the reads in the pair. This file is then used as a regular FASTA file as input to metaxa2_c. Thus, metaxa2_c does not consider the paired reads; rather it looks at each concatenated entry as one single, long FASTA sequence with a stretch of unknown nucleotides in it.

In version 2.0 of Metaxa2, the Metaxa2 Extractor (metaxa2_x) was made "gene-agnostic", meaning that it was no longer hardcoded for SSU genes. In version 2.2 we have taken this ability all the way, and with the new database builder, any marker gene or region could now be used with Metaxa2. Secondly, the extractor has a different way of handling the input sequences; partially inspired by the work we did on ITSx (http://microbiology.se/software/itsx/). In principle, this means that Metaxa2 does not process sequences sequentially. Instead, batches of sequences (the number dependent on the available system memory) are kept in RAM, and processed separately. If you choose to use the "--save_raw T" option, you may notice that there are files containing ".1." and higher numbers among the raw data files. Those are the batches processed by Metaxa2. This way of handling sequences greatly speeds up the Metaxa2 post-processing of HMMER output (which becomes a time-limiting step in typical Illumina datasets). Minor such changes were introduced already in Metaxa 1.1, but have now been fully implemented to accommodate for increasingly larger datasets. The sequence handling has also gone through some minor changes to be able to deal with paired-end sequences. However, this should not affect the sequence handling of single-end sequences.

For version 2.0 of Metaxa, the classifier (metaxa2_c) underwent a complete overhaul in terms of making taxonomic predictions. For version 2.2 of Metaxa2, this classification system has been further improved and now has a more proper mathematical framework. As before, the classification system is built upon the taxonomic information of the (by default) five best BLAST matches to each sequence in the input data (this can be changed using the "-M" option). For each sequence entry, Metaxa2 compares the taxonomic affiliation of the top BLAST match with the four other matches. In each comparison, the percent identity to the query is taken into account. If the BLAST matches point to the same taxonomic origin, the query sequence gets a taxonomic affiliation with a high reliability score (close to 100). If not (that is if the score, by default, is below 75 (the default is changed from 80 to 75 in version 2.2 due to slight differences in how the reliability score is calculated; other cutoff scores can be specified by the "-R" option)), the comparison is repeated at the taxonomic level above (e.g. genus if the last comparison was made on species level), until the score is above 75. In this way, all sequences get a taxonomic affiliation at a trustworthy taxonomic level. This data is written to a file with the suffix ".taxonomy.txt".

As of version 2.2, this calculation is based on the following formula (which produces almost, but not exactly the same scores as the 2.0 and 2.1 versions of Metaxa2 would generate):

$$R = 1 - 10^{-\frac{m^2}{1.0005^{l^{m^{1.1}}}}}$$

where R is the reliability score for the individual entry at a specific taxonomic level, m the sequence identity between the entry and the database match, and l the length of the alignment overlap between the entry and the database match. This number is calculated for every comparison of extracted gene fragments from the queries and database reference sequences. The individual reliability scores for entries that point to the same taxonomic order a given level are thereafter summed, and divided by the total number of valid matches (i.e. matches satisfying the identity cutoff to the database sequences at that level), and finally multiplied by 100 to generate the final overall reliability score of the classification. If this score is larger than the reliability score cutoff (75 by default), the classification is accepted and the algorithm proceeds to the next entry. Otherwise, it will instead recalculate all the reliability scores at the taxonomic level above (e.g. genus if the first classification was done on the species level). The new classification algorithm also influences how the numbers produced in the ".summary.txt" file are derived. This means that the numbers in the summary output can now be exactly reproduced by running the metaxa2_ttt tool with the option "-l 0". In previous versions of Metaxa2, this would not always have yielded the same numbers as in the summary file, but as of Metaxa 2.2 this discrepancy is now removed.

The default settings of Metaxa2 should be useful in most situations. However, since the software has been tweaked to accommodate for analysis of really large datasets, you should consider if they are suitable for your purposes and for your data set. If the data set is small, the user should consider running the software multiple times on the data, with different settings, and analyze the outcome. On larger data sets, it might be more feasible to only run Metaxa2 on a subset of the sequences for testing. The graphical output is very useful for determining whether Metaxa2 performs as desired on long-read (>400 bp) data, as the positions of the recovered conserved domains can be examined easily. If domains are missing, the criteria might be set to be too stringent. If they are not in sequential order (from V1l to V9r for SSU sequences, for example), that might be an indication that there is something wrong with the input sequences.

The HMMER program hmmsearch, used by Metaxa2, uses heuristic filters to increase the search speed. In the interest of speed, Metaxa2 runs hmmsearch as it is, with the "--max" option disabled. To turn off all heuristic filters of HMMER, Metaxa2 can be run with the "--heuristics F" option. This increases detection power at the cost of speed.

10. Running the analysis steps of Metaxa2 separately

The analysis procedure of Metaxa2 is divided into three steps: pre-processing, extraction and classification. These steps are normally run in sequence through the metaxa2 command. However, they can also be run separately if the user wishes. To run the extraction step independently, use the metaxa2_x command. This command takes a subset of the Metaxa2 options (other options will be ignored). To see the available options for the metaxa2_x command, type "metaxa2_x --help" on the command line. To run the classification step on a set of known rRNA sequences, use the command metaxa2_c. The options for metaxa2_c can be seen by typing "metaxa2_c --help" on the command line. Note that the output files obtained when running each step separately will be slightly different from those obtained through running the entire Metaxa2 pipeline. Additionally, FASTQ and paired-end file processing is ***not*** supported when running the analysis steps separately.

11. 'Undocumented' features

Metaxa2 has three undocumented options that can be activated, but they are considered *experimental* and should be used with caution. The first of these allows you to use USEARCH instead of the normal BLAST implementation for the classification step of Metaxa2. The second of these allows using a set of additional HMM profiles for the SSU extraction, and the final option allows seeing the underlying taxonomic data justifying the taxonomic predictions, by adding them to the taxonomy output. Note that particularly the USEARCH support is considered experimental, and it is not recommended that this option is used in sharp analysis.

'Undocumented' options:

-t {o, other}	It is possible to supply an additional set of HMM profiles in an O.hmm file within the HMMs directory. This custom set can be any type of profiles, but the profiles must be named according to the convention in the other HMM files.
--taxonomy {complete}	It is possible to see the underlying taxonomic data that the taxonomic predictions are based upon by using the "--taxonomy complete" option instead of setting it to "--taxonomy T". This data is then added to each entry in the ".taxonomy.txt" output file.
--usearch {version}	Runs USEARCH instead of BLAST for the classification step. Note that you need to specify the USEARCH version used, as the algorithms and options differ. Off (0) by default.
--usearch_bin {path}	Specifies where Metaxa2 should expect to find the USEARCH binary (either the name of the binary or the complete path to it) to be used. Default is 'usearch'.
--ublast {T or F}	Runs the UBLAST algorithm instead of the normal USEARCH algorithm. Default is to do this, since sensitivity is empirically better (T).

12. License information