Metabarcoding, metagenomics

Microbial DNA, environmental DNA (eDNA)

Properties of the DNA fragment used for the barcode:

- variable between species
- conserved (conserved) flanking sites for annealing universal primers across species
- a sufficiently short region that can be sequenced using newer high-throughput technologies (short read sequencing technologies vs long read sequencing technologies)

The term metabarcoding was introduced by Taberlet et al. (2012), and defined as an "automated identification of multiple species from a single bulk sample containing entire organisms or from a single environmental sample containing degraded DNA" (Taberlet et al., 2012). Although the term microbiota has been used interchangeably with the term microbiome, distinctions in the use of the term do exist. ***Microbiome*** *refers to the study of the entirety of the microbial genetic material recovered directly from the environment, also known as shotgun metagenomics, while* ***microbiota*** *refers to the taxonomic composition of the microbial community as determined by metabarcoding analysis (Ursell et al., 2012). While the former term (microbiome) provides information about composition and function of the microbial community, the latter more simply allows one to answer the question: "who is there?".*

Abdelfattah, A., Malacrinò, A., Wisniewski, M., Cacciola, S. O., & Schena, L. (2017). Metabarcoding: A powerful tool to investigate microbial communities and shape future plant protection strategies. *Biological Control*.

Most commonly used genes for metabarcoding:

- prokaryotes: 16S rRNA
- eukaryotes: 18S rRNA, ITS1, ITS2

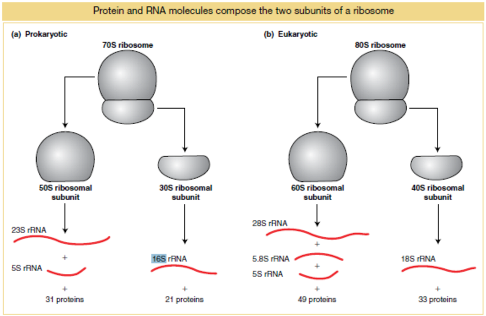


FIGURE 9-10 A ribosome contains a large and a small subunit. Each subunit contains both rRNA of varying lengths and a set of proteins. There are two principal rRNA molecules in all ribosomes. Prokaryotic ribosomes also contain one 120-base-long rRNA that sediments at 5S, whereas eukaryotic ribosomes have two small rRNAs: a 5S RNA molecule similar to the prokaryotic 5S and a 5.8S molecule 160 bases long.

Griffiths in sod. 2015. Introduction to genetic analysis. 11. izd.

Variable regions of 16S rRNA (16s rDNA)

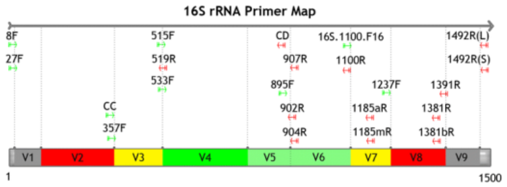
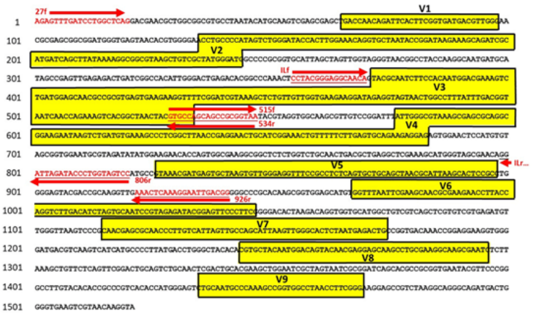


Illustration of different variable regions. Red regions (V2, V8) have a poor phylogenetic resolution at the phylum level. Green regions (V4, V5, V6) are associated with the shortest geodesic distance, which suggests that they may be the best choice for phylogeny-related analyses and the phylogenetic analysis of novel bacterial phyla. The figure refers to the primer map from Lutzonilab (http://lutzonilab.org/16s-ribosomal-dna/).

Bo Yang et al. 2017



Ribosomal RNA gene sequence of *Lactobacillus acidophilus* (EF533992.1) with putative primer binding sites and variable regions shown.

http://omegabioservices.com/index.php/16s-reference/

ITS region (internal transcribed spacer (slo: notranji prepisani vmesnik), located between 18S, 5.8S and 25S rRNA

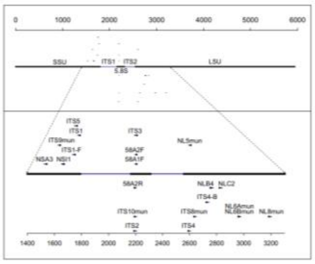


Figure 1

Diagram of primer locations in the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2, and LSU rDNA.
Primers are positioned above (forward primers) or below (reverse) their sequence positions. ITS1, ITS2, ITS3, and ITS4 from White et al. [5], primers ITS8mun, ITS9mun, ITS10mun, NL5mun, NL6Amun, NL6Bmun, NL8mun from Egger [16], primers ITS1-F, ITS4-B from Gardes and Bruns [6] and the remaining primers (NSA3, NSI1, 58A1F, 58A2F, 58A2R, NLB4, NLC2) from this study. Scale is in base pairs according to the extension of the Gargas and DePriest [23] nomenclature system described in this study.

Kendall in Rygiewicz, 2005

Reference databases

- GreenGenes. 16S rRNA.
http://greengenes.secondgenome.com/
- ITS2.
http://its2.bioapps.biozentrum.uni-wuerzburg.de/
- RDP. 16S in 28S rRNA
https://www.canr.msu.edu/cme/resources/
- PR2. 18S rRNA.
https://github.com/vaulot/pr2database
- Silva.
https://www.arb-silva.de/
- UNITE: ITS
https://unite.ut.ee/

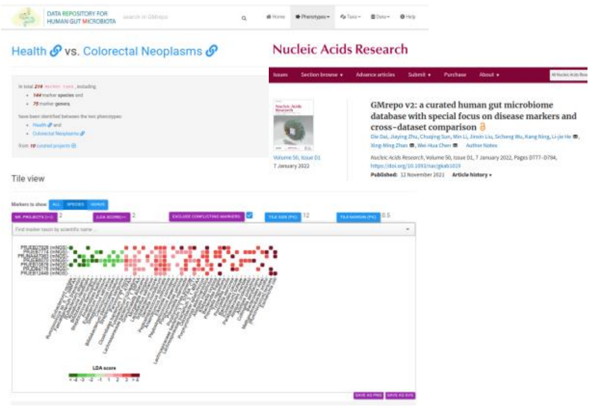
Several projects based on microorganism metabarcoding

Human Microbiome Project
http://commonfund.nih.gov/hmp

INTERNATIONAL CENSUS OF MARINE MICROBES
http://icomm.mbl.edu/

Earth Microbiome Project
http://www.earthmicrobiome.org/

Terragenome – International Soil Metagenome Sequencing Consortium
http://www.terragenome.org/about/



DATA REPOSITORY FOR HUMAN GUT MICROBIOTA

Health vs. Colorectal Neoplasms

Tile view

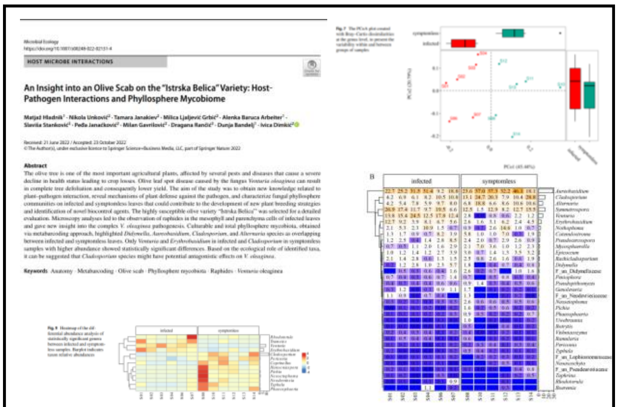
Nucleic Acids Research

GMrepo v2: a curated human gut microbiome database with special focus on disease markers and cross-dataset comparison

Die Dai, Jiaying Zhu, Chuqing Sun, Min Li, Jinxin Liu, Sicheng Wu, Kang Ning, Li-jie He, Xing-Ming Zhao, Wei-Hua Chen

Nucleic Acids Research, Volume 50, Issue D1, 7 January 2022, Pages D777–D794, https://doi.org/10.1093/nar/gkab1019

Published: 12 November 2021



Microbial Ecology
https://doi.org/10.1007/s00248-022-02131-4

HOST MICROBE INTERACTIONS

An Insight into an Olive Scab on the "Istrska Belica" Variety: Host-Pathogen Interactions and Phyllosphere Mycobiome

Matjaž Hladnik · Nikola Unković · Tamara Janakiev · Milica Ljaljević Grbić · Alenka Baruca Arbeiter · Slaviša Stanković · Peđa Janaćković · Milan Gavrilović · Dragana Rančić · Dunja Bandelj · Ivica Dimkić

Received: 21 June 2022 / Accepted: 23 October 2022

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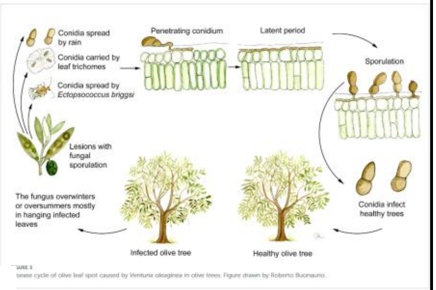
Abstract

The olive tree is one of the most important agricultural plants, affected by several pests and diseases that cause a severe decline in health status leading to crop losses. Olive leaf spot disease caused by the fungus *Venturia oleaginea* can result in complete tree defoliation and consequently lower yield. The aim of the study was to obtain new knowledge related to plant–pathogen interaction, reveal mechanisms of plant defense against the pathogen, and characterize fungal phyllosphere communities on infected and symptomless leaves that could contribute to the development of new plant breeding strategies and identification of novel biocontrol agents. The highly susceptible olive variety "Istrska Belica" was selected for a detailed evaluation. Microscopy analyses led to the observation of raphides in the mesophyll and parenchyma cells of infected leaves and gave new insight into the complex *V. oleaginea* pathogenesis. Culturable and total phyllosphere mycobiota, obtained via metabarcoding approach, highlighted *Didymella*, *Aureobasidium*, *Cladosporium*, and *Alternaria* species as overlapping between infected and symptomless leaves. Only *Venturia* and *Erythrobasidium* in infected and *Cladosporium* in symptomless samples with higher abundance showed statistically significant differences. Based on the ecological role of identified taxa, it can be suggested that *Cladosporium* species might have potential antagonistic effects on *V. oleaginea*.

Keywords Anatomy · Metabarcoding · Olive scab · Phyllosphere mycobiota · Raphides · *Venturia oleaginea*

Olive leaf spot disease
Venturia oleaginea (syn. *Spilocaea oleagina*)

Olive leaf with characteristic disease symptoms

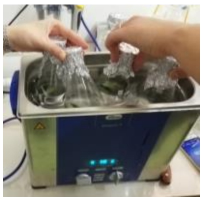


Disease cycle of olive leaf spot caused by *Venturia oleaginea* (Source: Roberto Buonaurio (Buonaurio et al., 2023))

Microbiota detection workflow

1) Sampling of infected and symptomless leaves.

3) Filtration of buffer with leaves using a sterile filter to collect all microorganisms.



2) Washing of leaves in 1X PBS buffer in ultrasonic bath.

4) Extraction of DNA, DNA barcodes amplification and sequencing with Ion S5 (Ion 530 chip).

Data analysis workflow

Mycobiota (ITS1)

- Amplification of ITS1 with (P1-)**ITS1f** and (A-barcode-)**ITS2**
- Extraction of ITS using ITSx tool
- QIIME2 analysis package
  - DADA2 (`denoise-pyro`)
  - `classify-sklearn` with a Naive Bayes classifier based on UNITE database
- ASVs blasted against GeneBank nucleotide database and Fungi RefSeq ITS database

`(blastn –task blastn –word_size 7)`

Bacteriobiota (V4 16S rRNA)

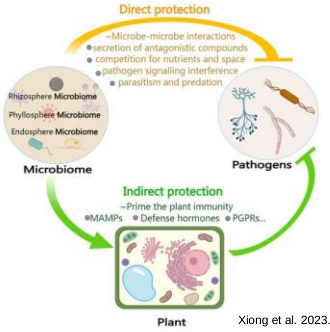
- Amplification of V4 16S rRNA with (A-barcode-)515F and (P1-)806R
- QIIME2 analysis package
  - `qiime cutadapt`

```
qiime cutadapt trim single \
 --p-adapter
^GTGYCAGCMGCCGCGGTAA...ATTAGAWACCCBNGTAGTCC$ \ --p-
discard untrimmed
```

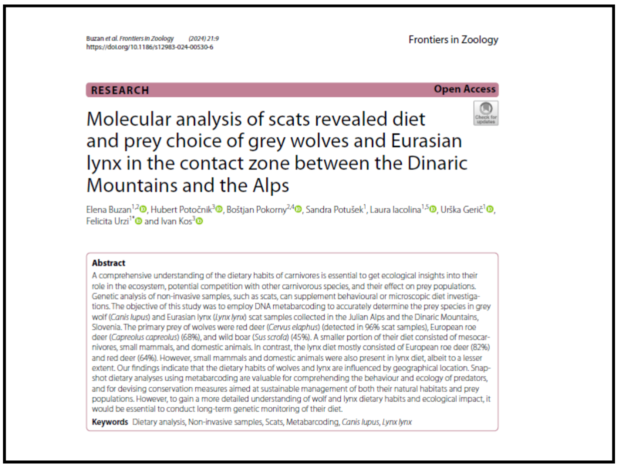
  - DADA2 (denoise-pyro)
  - `classify-sklearn` with a Naive Bayes classifier based on SILVA database (Silva 138 SSURef NR99 515F/806R)

M. Hladnik et al. 2022. An Insight into an Olive Scab on the “Istrska Belica” Variety: Host-Pathogen Interactions and Phyllosphere Mycobiome’, Microb. Ecol.

For statistical analysis methods implemented in R packages were used:
- Data wrangling: qiime2R, microviz, ggClusterNet, phyloseq
- Diversity analysis: phyloseq, vegan, MicrobiotaProcess
- Differential abundance analysis: MaASLin2, ANCOMBC, DESeq2



Xiong et al. 2023.



Buzan *et al. Frontiers in Zoology* (2024) 21:9
https://doi.org/10.1186/s12983-024-00530-6

Frontiers in Zoology

RESEARCH Open Access

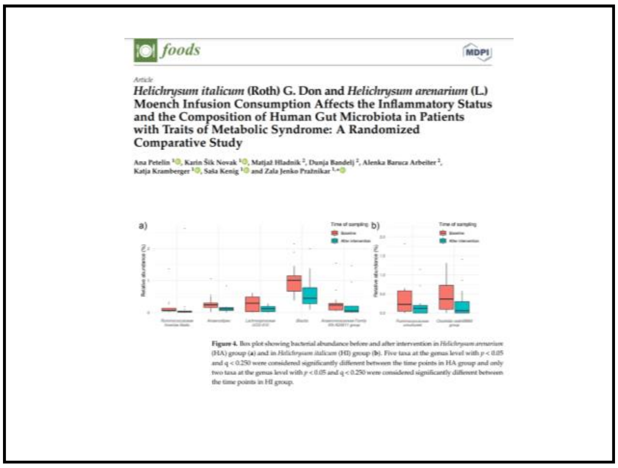
Molecular analysis of scats revealed diet and prey choice of grey wolves and Eurasian lynx in the contact zone between the Dinaric Mountains and the Alps

Elena Buzan[1,2], Hubert Potočnik[3], Boštjan Pokorny[2,4], Sandra Potušek[1], Laura Iacolina[1,5], Urška Gerič[1], Felicita Urzi[1*] and Ivan Kos[3]

Abstract

A comprehensive understanding of the dietary habits of carnivores is essential to get ecological insights into their role in the ecosystem, potential competition with other carnivorous species, and their effect on prey populations. Genetic analysis of non-invasive samples, such as scats, can supplement behavioural or microscopic diet investigations. The objective of this study was to employ DNA metabarcoding to accurately determine the prey species in grey wolf (*Canis lupus*) and Eurasian lynx (*Lynx lynx*) scat samples collected in the Julian Alps and the Dinaric Mountains, Slovenia. The primary prey of wolves were red deer (*Cervus elaphus*) (detected in 96% scat samples), European roe deer (*Capreolus capreolus*) (68%), and wild boar (*Sus scrofa*) (45%). A smaller portion of their diet consisted of mesocarnivores, small mammals, and domestic animals. In contrast, the lynx diet mostly consisted of European roe deer (82%) and red deer (64%). However, small mammals and domestic animals were also present in lynx diet, albeit to a lesser extent. Our findings indicate that the dietary habits of wolves and lynx are influenced by geographical location. Snapshot dietary analyses using metabarcoding are valuable for comprehending the behaviour and ecology of predators, and for devising conservation measures aimed at sustainable management of both their natural habitats and prey populations. However, to gain a more detailed understanding of wolf and lynx dietary habits and ecological impact, it would be essential to conduct long-term genetic monitoring of their diet.

Keywords Dietary analysis, Non-invasive samples, Scats, Metabarcoding, *Canis lupus*, *Lynx lynx*



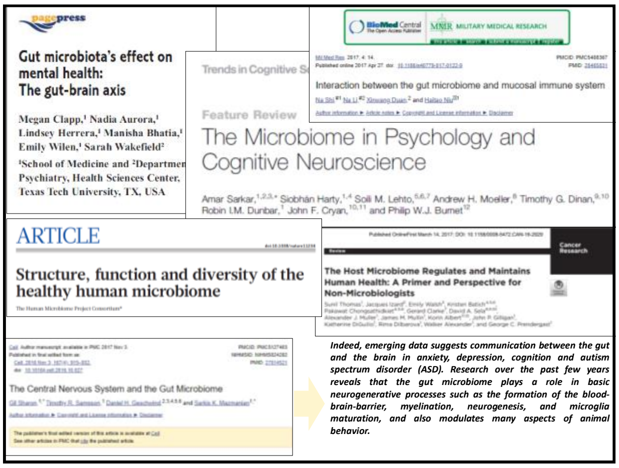
foods MDPI

Article

Helichrysum italicum **(Roth) G. Don and** ***Helichrysum arenarium*** **(L.) Moench Infusion Consumption Affects the Inflammatory Status and the Composition of Human Gut Microbiota in Patients with Traits of Metabolic Syndrome: A Randomized Comparative Study**

Ana Petelin [1], Karin Šik Novak [1], Matjaž Hladnik [2], Dunja Bandelj [2], Alenka Baruca Arbeiter [2], Katja Kramberger [1], Saša Kenig [1] and Zala Jenko Pražnikar [1,*]

Figure 4. Box plot showing bacterial abundance before and after intervention in *Helichrysum arenarium* (HA) group (**a**) and in *Helichrysum italicum* (HI) group (**b**). Five taxa at the genus level with $p < 0.05$ and $q < 0.250$ were considered significantly different between the time points in HA group and only two taxa at the genus level with $p < 0.05$ and $q < 0.250$ were considered significantly different between the time points in HI group.



pagepress

Gut microbiota's effect on mental health: The gut-brain axis

Megan Clapp,[1] Nadia Aurora,[1] Lindsey Herrera,[1] Manisha Bhatia,[1] Emily Wilen,[1] Sarah Wakefield[2]
[1]School of Medicine and [2]Departmen
Psychiatry, Health Sciences Center, Texas Tech University, TX, USA

Trends in Cognitive S

Feature Review

The Microbiome in Psychology and Cognitive Neuroscience

Amar Sarkar,[1,2,3,*] Siobhán Harty,[1,4] Soili M. Lehto,[5,6,7] Andrew H. Moeller,[8] Timothy G. Dinan,[9,10] Robin I.M. Dunbar,[1] John F. Cryan,[10,11] and Philip W.J. Burnet[12]

BioMed Central The Open Access Publisher MMR MILITARY MEDICAL RESEARCH

Mil Med Res. 2017; 4: 14.
Published online 2017 Apr 27. doi: 10.1186/s40779-017-0122-9
PMCID: PMC5408367
PMID: 28465831

Interaction between the gut microbiome and mucosal immune system

Na Shi,[#1] Na Li,[#2] Xinwang Duan,[2] and Haitao Niu[2†]

Author information ► Article notes ► Copyright and License information ► Disclaimer

ARTICLE

Structure, function and diversity of the healthy human microbiome

The Human Microbiome Project Consortium*

Cancer Research

Review

The Host Microbiome Regulates and Maintains Human Health: A Primer and Perspective for Non-Microbiologists

The Central Nervous System and the Gut Microbiome

Gil Sharon, Timothy R. Sampson, Daniel H. Geschwind and Sarkis K. Mazmanian

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See other articles in PMC that cite the published article.

Indeed, emerging data suggests communication between the gut and the brain in anxiety, depression, cognition and autism spectrum disorder (ASD). Research over the past few years reveals that the gut microbiome plays a role in basic neurogenerative processes such as the formation of the blood-brain-barrier, myelination, neurogenesis, and microglia maturation, and also modulates many aspects of animal behavior.

Clinical Psychology Review
Volume 83, February 2021, 101943

Review

The gut microbiota in anxiety and depression – A systematic review

Carra A. Simpson [a, b], [✉], Carmela Diaz-Arteche [b], Djamila Eliby [a, b], Orli S. Schwartz [c], Julian G. Simmons [a, b, 1], Caitlin S.M. Cowan [d, 1]

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https://doi.org/10.1016/j.cpr.2020.101943   Get rights and content

Abstract

Growing evidence indicates the community of microorganisms throughout the gastrointestinal tract, (i.e., gut microbiota), is associated with anxiety and depressive disorders. We present the first systematic review of the gut microbiota in anxiety disorders, along with an update in depression. Consideration of shared underlying features is essential due to the high rates of comorbidity. Systematic searches, following PRISMA guidelines, identified 26 studies (two case-control comparisons of the gut microbiota in generalised anxiety disorder, 18 in depression, one incorporating both anxiety/depression, and five including symptom-only measures). Alpha and beta diversity findings were inconsistent; however, differences in bacterial taxa indicated disorders may be characterised by a higher abundance of proinflammatory species (e.g., *Enterobacteriaceae* and *Desulfovibrio*), and lower short-chain fatty acid producing-bacteria (e.g., *Faecalibacterium*). Several taxa, and their mechanisms of action, may relate to anxiety and depression pathophysiology via communication of peripheral inflammation to the brain. Although the gut microbiota remains a promising target for prevention and therapy, future research should assess confounders, particularly diet and psychotropic medications, and should examine microorganism function.

THE FASEB JOURNAL
The Journal of the Federation of American Societies for Experimental Biology

Research Communication | Free Access

Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells

Christopher S. Reigstad, Charles E. Salmonson, John F. Rainey III, Joseph H. Szurszewski, David R. Linden, Justin L. Sonnenburg, Gianrico Farrugia, Purna C. Kashyap

First published: 30 December 2014 | https://doi.org/10.1096/fj.14-259598 | Citations: 30

This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.

Example of analysis with qiime2

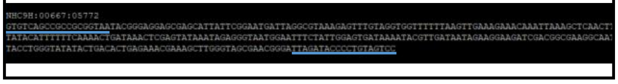
Example of primers 515F in 806R for amplification of V4 16S rRNA with Ion Torrent technology.

	P1 ADAPTER	806R PRIMER
P1-806R	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT	GGACTACNVGGGTWTCTAAT

Complementary sequence of 806R - `ATTAGAWACCCBNGTAGTCC`

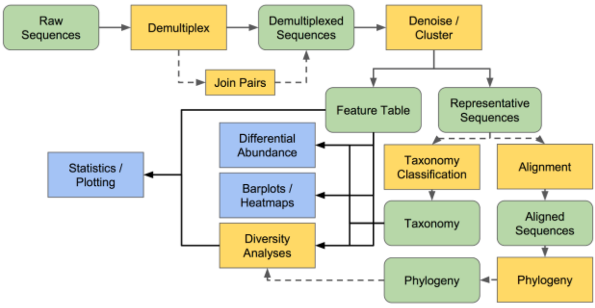
	A ADAPTER	BARKODA	LINKER	515F SEQUENCE
IonXpress_1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTAAGGTAAC	GAT	GTGYCAGCMGCCGCGGTAA

A shorter sequence added to the 5’ end of primer, so that when sequencing with several samples simultaneously is performed, distribution of reads per sample is possible.(This barcode has nothing to do with taxon identification!)



```
NHC9H:00667:05772
GTGTCAGCCGCCGCGGTAATACGGGAGGAGCGAGCATTATTCGGAATGATTAGGCGTAAAGAGTTTGTAGGTGGTTTTTTAAGTTGAAAGAAACAAATTAAAGCTCAACT
TATACATTTTTTCAAAACTGATAAACTCGAGTATAAATAGAGGGTAATGGAATTTCTATTGGAGTGATAAAATACGTTGATAATAGAAGGAAGATCGACGGCGAAGGCAA
TACCTGGGTATATACTGACACTGAGAAACGAAAGCTTGGGTAGCGAACGGGATTAGATACCCCTGTAGTCC
```

Conceptual overview of QIIME 2



Raw Sequences

Demultiplex

Demultiplexed Sequences

Denoise / Cluster

Join Pairs

Feature Table

Representative Sequences

Differential Abundance

Statistics / Plotting

Barplots / Heatmaps

Diversity Analyses

Taxonomy Classification

Alignment

Taxonomy

Aligned Sequences

Phylogeny

Phylogeny

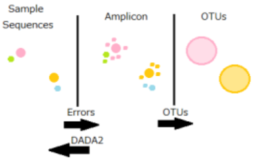
https://docs.qiime2.org/2024.2/tutorials/overview/

An example of data analysis with qiime2

Tutorial with qiime2:
https://docs.qiime2.org/2024.2/tutorials/

OTU: *angl. operational taxonomic unit (slo* operativne taksonomske enote)

- Clustering of sequences with 97 % similarity or 99 %
- New methods – DADA2, Deblur – identification of ASV, amplicon sequence variant (slo: različica pomnoženega zaporedja)

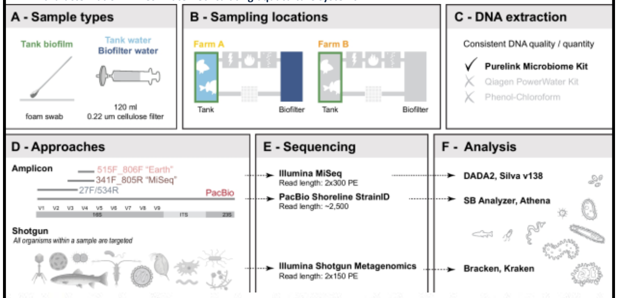


Vir: http://crusade1096.web.fc2.com/qiime2_renew.html

Bioinformatics analysis steps

- Demultiplexing
- Denoising / clustering
- Taxonomy classification
  - Alignment based methods
  - Machine-learning based classification methods (the multinomial Naive Bayes machine learning classifier in q2-feature classifier)
- Diversity analysis
  - alpha diversity
  - beta diversity

Metagenomics and metabarcoding experimental choices and their impact on microbial community characterization in freshwater recirculating aquaculture systems



Study design and experimental steps. **A** Three types of samples were taken. Tank biofilm was collected by rubbing a foam swab against the sidewall of a tank. Tank and biofilter water was collected by filtering 120 ml of water through a 0.22 um cellulose filter. **B** Samples were taken at two farms, A and B. In farm A, all three sample types were collected. In farm B, sampling focused on tank biofilm. **C** Three DNA extraction methods were compared. The Purelink Microbiome Kit outperformed other DNA extraction methods in quality, quantity, and consistent yield. **D** Two amplicon approaches (short and long reads) and an amplification-free shotgun approach were used. **E** Short amplicons were sequenced on an Illumina MiSeq with a v3 2 × 300PE kit. Long amplicons were sequenced with the PacBio Shoreline StrainID kit, producing an average read length of 2500 bp. Shotgun sequencing was performed with a 2 × 150PE Illumina kit. **F** Illumina MiSeq sequencing data were processed with the DADA2 pipeline, and ASVs were blasted against the SILVA v138 database for taxonomic assignment. PacBio long-read data was processed with the SBAnalyzer program and taxonomically assigned using Athena. Shotgun metagenomics sequencing data were processed with an in-house pipeline that uses the Kraken-Bracken method

https://environmentalmicrobiome.biomedcentral.com/articles/10.1186/s40793-023-00459-z

---

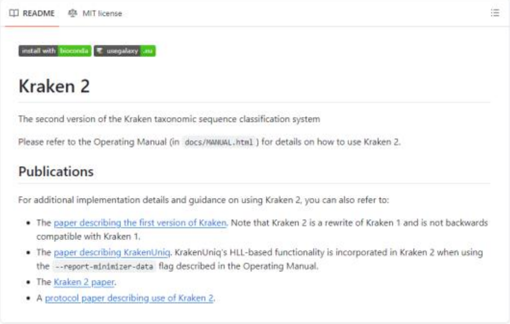
QIIME 2 2024.2 distributions

As of 2024.2, QIIME 2 releases now include the following QIIME 2 distributions that are available for install:

- `amplicon`
- `shotgun`
- `tiny`

---

https://github.com/DerrickWood/kraken2



Kraken 2

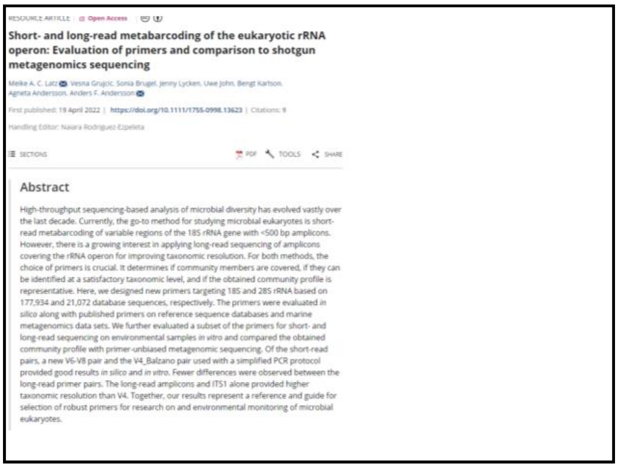
The second version of the Kraken taxonomic sequence classification system

Please refer to the Operating Manual (in `docs/MANUAL.html`) for details on how to use Kraken 2.

Publications

For additional implementation details and guidance on using Kraken 2, you can also refer to:

- The paper describing the first version of Kraken. Note that Kraken 2 is a rewrite of Kraken 1 and is not backwards compatible with Kraken 1.
- The paper describing KrakenUniq. KrakenUniq's HLL-based functionality is incorporated in Kraken 2 when using the `--report-minimizer-data` flag described in the Operating Manual.
- The Kraken 2 paper.
- A protocol paper describing use of Kraken 2.



RESOURCE ARTICLE | Open Access

Short- and long-read metabarcoding of the eukaryotic rRNA operon: Evaluation of primers and comparison to shotgun metagenomics sequencing

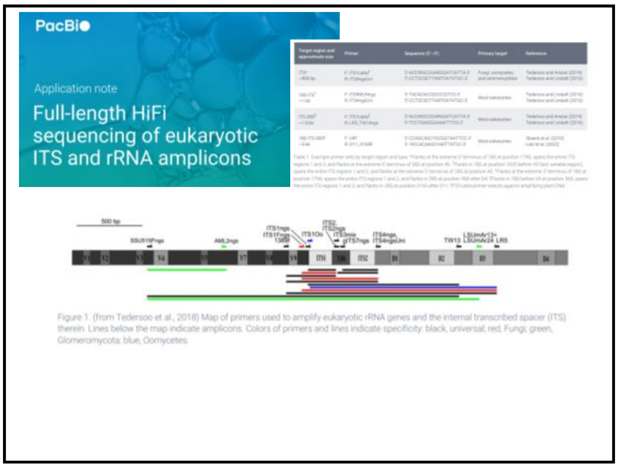
Meike A. C. Latz, Vesna Grujcic, Sonia Brugel, Jenny Lycken, Uwe John, Bengt Karlson, Agneta Andersson, Anders F. Andersson

First published: 19 April 2022 | https://doi.org/10.1111/1755-0998.13623 | Citations: 9

Handling Editor: Naiara Rodriguez-Ezpeleta

Abstract

High-throughput sequencing-based analysis of microbial diversity has evolved vastly over the last decade. Currently, the go-to method for studying microbial eukaryotes is short-read metabarcoding of variable regions of the 18S rRNA gene with <500 bp amplicons. However, there is a growing interest in applying long-read sequencing of amplicons covering the rRNA operon for improving taxonomic resolution. For both methods, the choice of primers is crucial. It determines if community members are covered, if they can be identified at a satisfactory taxonomic level, and if the obtained community profile is representative. Here, we designed new primers targeting 18S and 28S rRNA based on 177,934 and 21,072 database sequences, respectively. The primers were evaluated *in silico* along with published primers on reference sequence databases and marine metagenomics data sets. We further evaluated a subset of the primers for short- and long-read sequencing on environmental samples *in vitro* and compared the obtained community profile with primer-unbiased metagenomic sequencing. Of the short-read pairs, a new V6-V8 pair and the V4_Balzano pair used with a simplified PCR protocol provided good results *in silico* and *in vitro*. Fewer differences were observed between the long-read primer pairs. The long-read amplicons and ITS1 alone provided higher taxonomic resolution than V4. Together, our results represent a reference and guide for selection of robust primers for research on and environmental monitoring of microbial eukaryotes.



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Application note

Full-length HiFi sequencing of eukaryotic ITS and rRNA amplicons

Figure 1. (from Tedersoo et al., 2018) Map of primers used to amplify eukaryotic rRNA genes and the internal transcribed spacer (ITS) therein. Lines below the map indicate amplicons. Colors of primers and lines indicate specificity: black, universal; red, Fungi; green, Glomeromycota; blue, Oomycetes.

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Full-length 16S rRNA gene amplicon analysis of human gut microbiota using MinION™ nanopore sequencing confers species-level resolution

Yoshiyuki Matsuo, Shinnosuke Komiya, Yoshiaki Yasumizu, Yuki Yasuoka, Katsura Mizushima, Tomohisa Takagi, Kirill Kryukov, Aisaku Fukuda, Yoshiharu Morimoto, Yuji Naito, Hidetaka Okada, Hidemasa Bono, So Nakagawa & Kiichi Hirota

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Comparative analysis of full-length 16s ribosomal RNA genome sequencing in human fecal samples using primer sets with different degrees of degeneracy

Christian Waechter, Leon Fehse, Marius Welzel, Dominik Heider, Lek Babalija, Juan Cheko, Julian Mueller, Jochen Pöling, Thomas Braun, Sabine Pankuweit, Eberhard Weihe, Ralf Kinscherf, Bernhard Schieffer, Ulrich Luesebrink, Muhidien Soufi and Volker Ruppert

Amplification of bacterial full-length 16S gene with barcoded primers

Procedure & checklist

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This procedure describes the PCR amplification of bacterial full-length 16S rRNA genes (V1–V9 regions) using up to 192 dual indices. See Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 for a detailed library preparation procedure.

Overview	
Samples	1–192
Metagenomic DNA input for PCR	1-2 ng
16S rRNA degenerate forward primer sequence*	[illegible]
16S rRNA degenerate reverse primer sequence*	[illegible]

*Full sequence with barcode shown in the appendix

NATURE PROTOCOLS PROTOCOL

Rapid in situ identification of biological specimens via DNA amplicon sequencing using miniaturized laboratory equipment