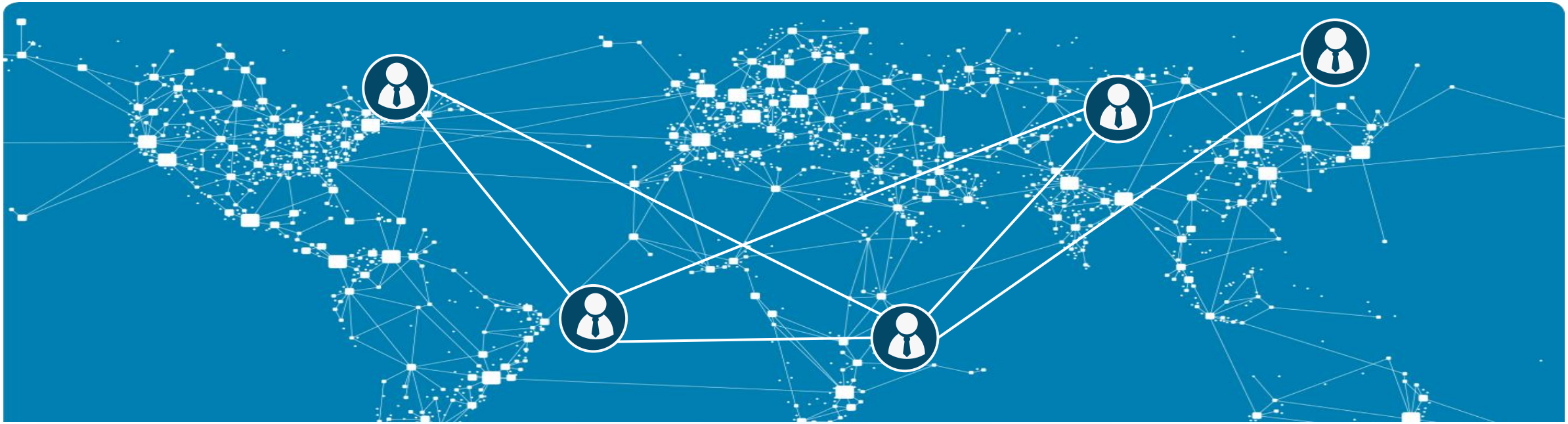
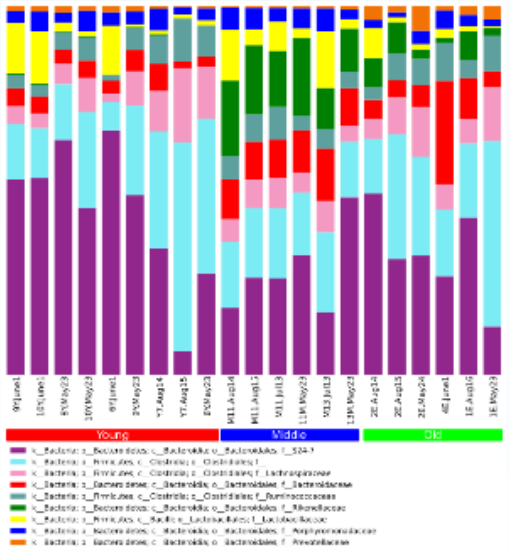
微生物组—宏基因组分析专题研讨会第19期

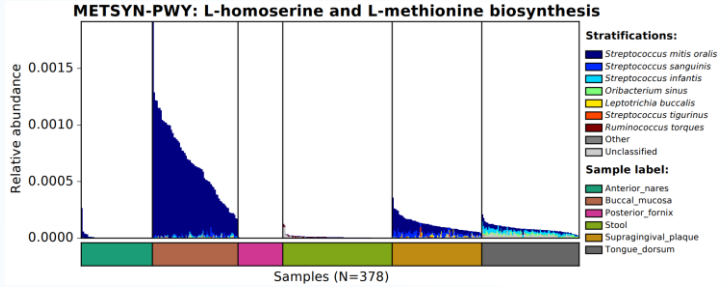


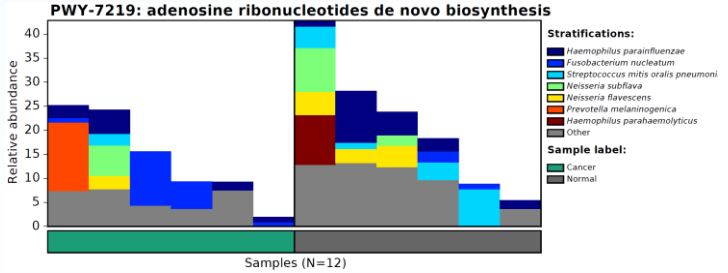
23humann物种和功能组成

易生信

2023年7月22日







宏基因组基于读长(Read-based)的分析流程

一. 软件安装和数据库部署

二. KneadData质控

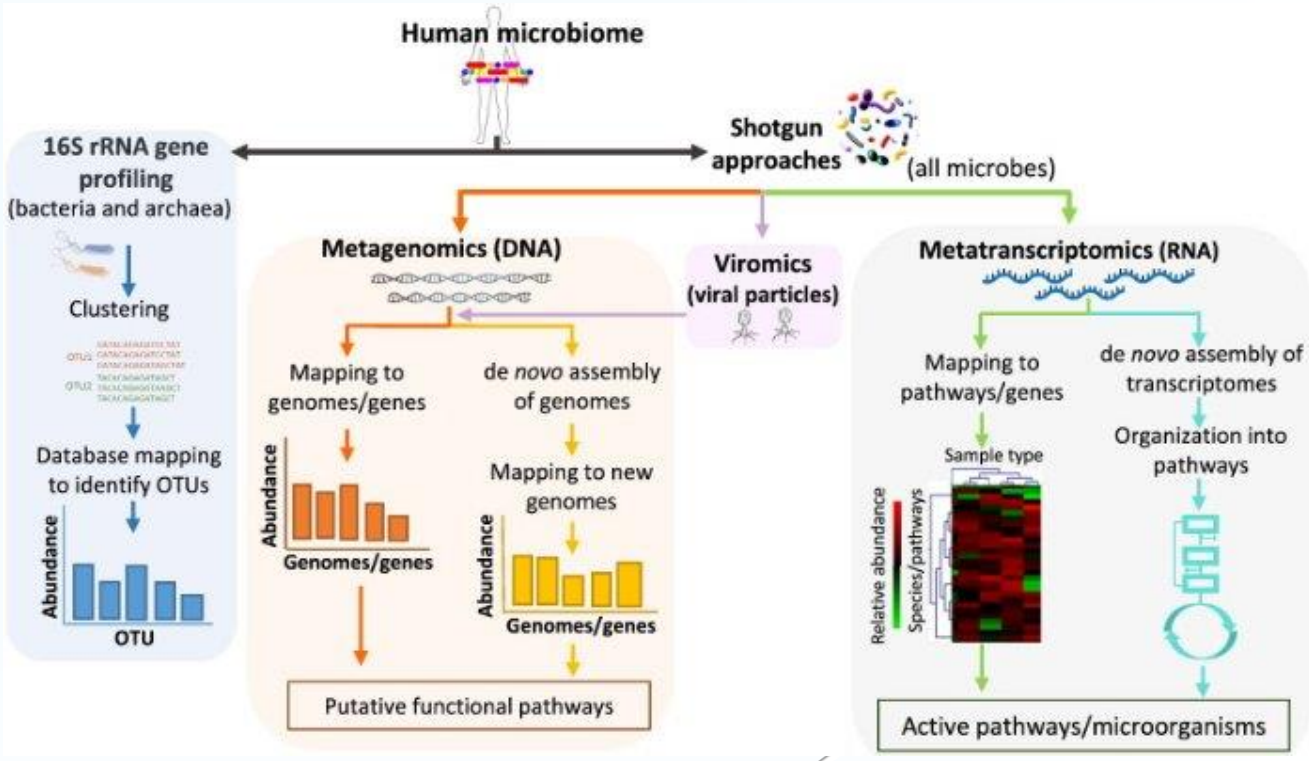
三. **MetaPhlAn4物种组成**

四. HUMAnN3功能组成

五. GraPhlAn可视化物种

六. LEfSe分析物种差异

七. STAMP功能组成分析

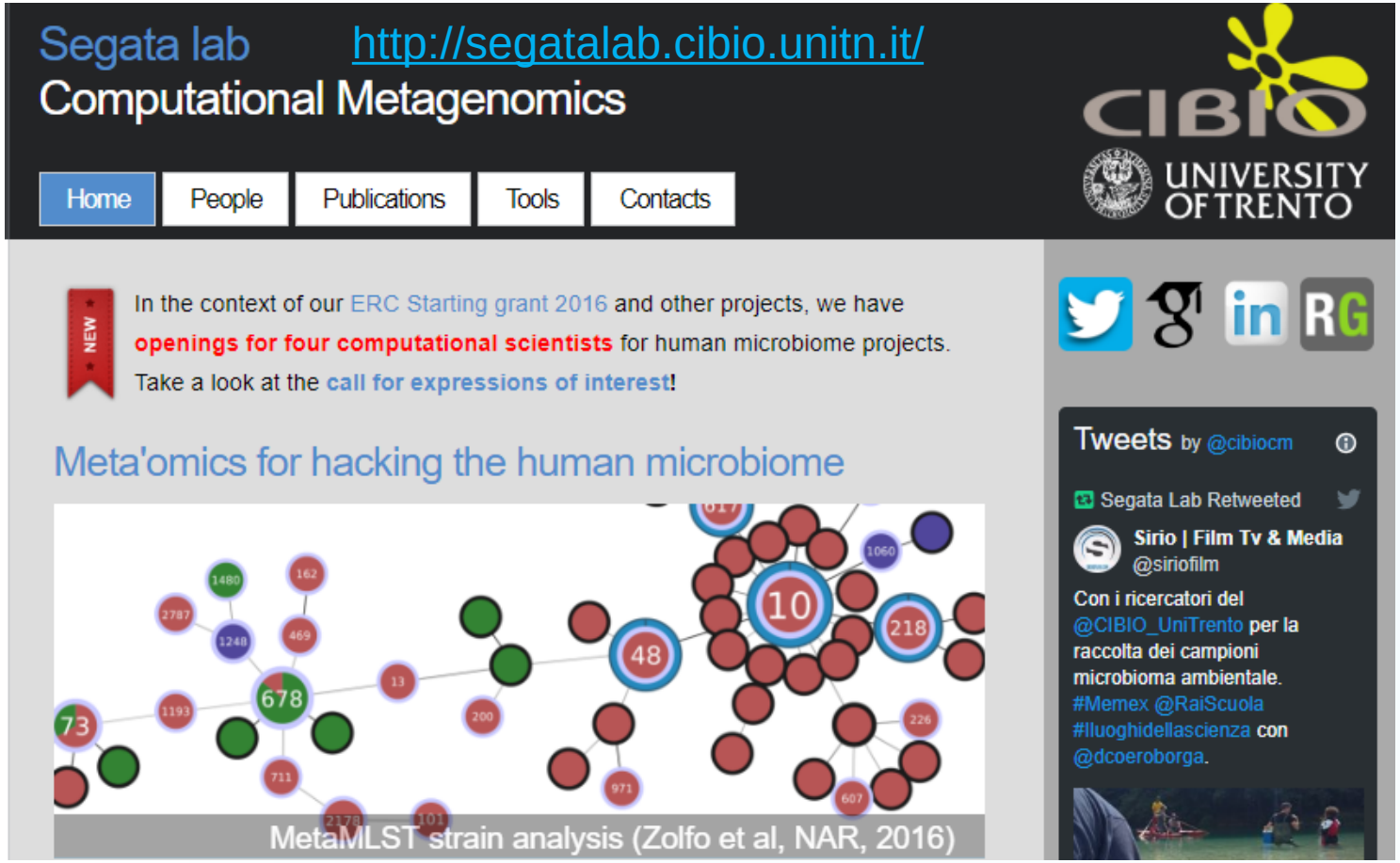


MetaPhlAn4 (metagenomic phylogenetic analysis，宏基因组系统发育分析，2012-2023)

- MetaPhlAn4是分析微生物群落(细菌、古菌、真核生物和病毒)组成的工具，只需一条完命令即可获得微生物的物种丰度信息。同时提供脚本可进一步统计和可视化。
- 主页：http://segatalab.cibio.unitn.it/tools/MetaPhlAn4/

- Nicola Segata, Levi Waldron, Annalisa Ballarini, Vagheesh Narasimhan, Olivier Jousson, Curtis Huttenhower. 2012. Metagenomic microbial community profiling using unique clade-specific marker genes. ***Nature Methods*** 9: 811. https://doi.org/10.1038/nmeth.2066 Cited by 1758
- Duy Tin Truong, Eric A. Franzosa, Timothy L. Tickle, Matthias Scholz, George Weingart, Edoardo Pasolli, Adrian Tett, Curtis Huttenhower, Nicola Segata. 2015. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. ***Nature Methods*** 12: 902-903. https://doi.org/10.1038/nmeth.3589 Cited by 1778
- Aitor Blanco-Míguez, et al. 2023. Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. ***Nature Biotechnology*** https://doi.org/10.1038/s41587-023-01688-w Cited by 38

意大利特伦托大学Nicola Segata组——宏基因组软件(10-12年哈佛公共卫生学院Huttenhower组博后)



Cell：宏基因组分箱15万人体微生物基因组

开发维护和参与的软件

- curatedMD (2017) 发表人类微生物组物种和功能组成整理数据库R包
- MetaMLST (2016)宏基因组多位点序列分型
- StrainPhlAN (2016)菌株水平群体基因组分析
- MetAML (2016)3000个宏基因组数据微生物与表型关联预测
- PanPhlAn (2016)预测菌株水平基因组成和转录活性
- MetaPhlAn2 (2015) 宏基因组物种组成
- GraPhlAn (2015) 物种或进化树圈图美化
- ShortBRED (2015)蛋白归类基因家族和宏基因组功能定量
- MicroPITA (2014)宏基因样本挑选
- MetaRef (2014)微生物类特异基因数据库
- PhyloPhlAn (2013)新微生物基因组分类和进化关系鉴定
- HUMAnN (2012) 宏基因组功能组成定量
- LEfSe (2011)生物标志物挖掘

Nicola Segata

Department CIBIO, University of Trento
Verified email at unitn.it - Homepage

FOLLOW

Human Microbiome Computational Biology Metagenomics Microbial Genomics Machine Learning

TITLE	CITED BY	YEAR
Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2 E Bolyen, JR Rideout, MR Dillon, NA Bokulich, CC Abnet, GA Al-Ghalith, ... Nature biotechnology 37 (8), 852-857	10550	2019
Metagenomic biomarker discovery and explanation N Segata, J Izard, L Waldron, D Gevers, L Miropolsky, WS Garrett, ... Genome biology 12 (6), R60	10250	2011
Structure, function and diversity of the healthy human microbiome nature 486 (7402), 207-214	9255	2012
A framework for human microbiome research nature 486 (7402), 215-221	2347	2012
MetaPhlAn2 for enhanced metagenomic taxonomic profiling DT Truong, EA Franzosa, TL Tickle, M Scholz, G Weingart, E Pasolli, ... Nature methods 12 (10), 902-903	1778	2015
Metagenomic microbial community profiling using unique clade-specific marker genes N Segata, L Waldron, A Ballarini, V Narasimhan, O Jousson, ... Nature methods 9 (8), 811-814	1758	2012
Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis JU Scher, A Sczesnak, RS Longman, N Segata, C Ubeda, C Bielski, ... elife 2, e01202	1685	2013
Microbial co-occurrence relationships in the human microbiome	1365	2012

QIIME 2

LEfSe

MetaPhlAn 2

MetaPhlAn 1

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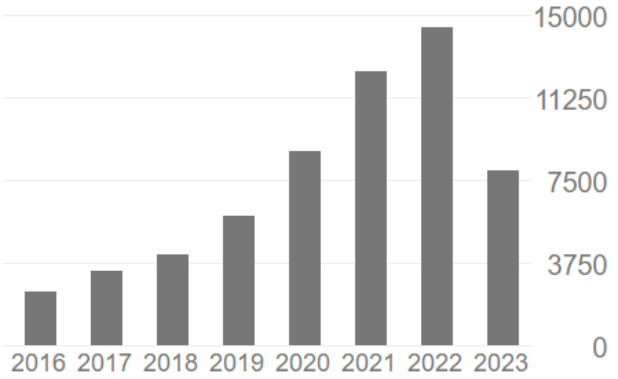

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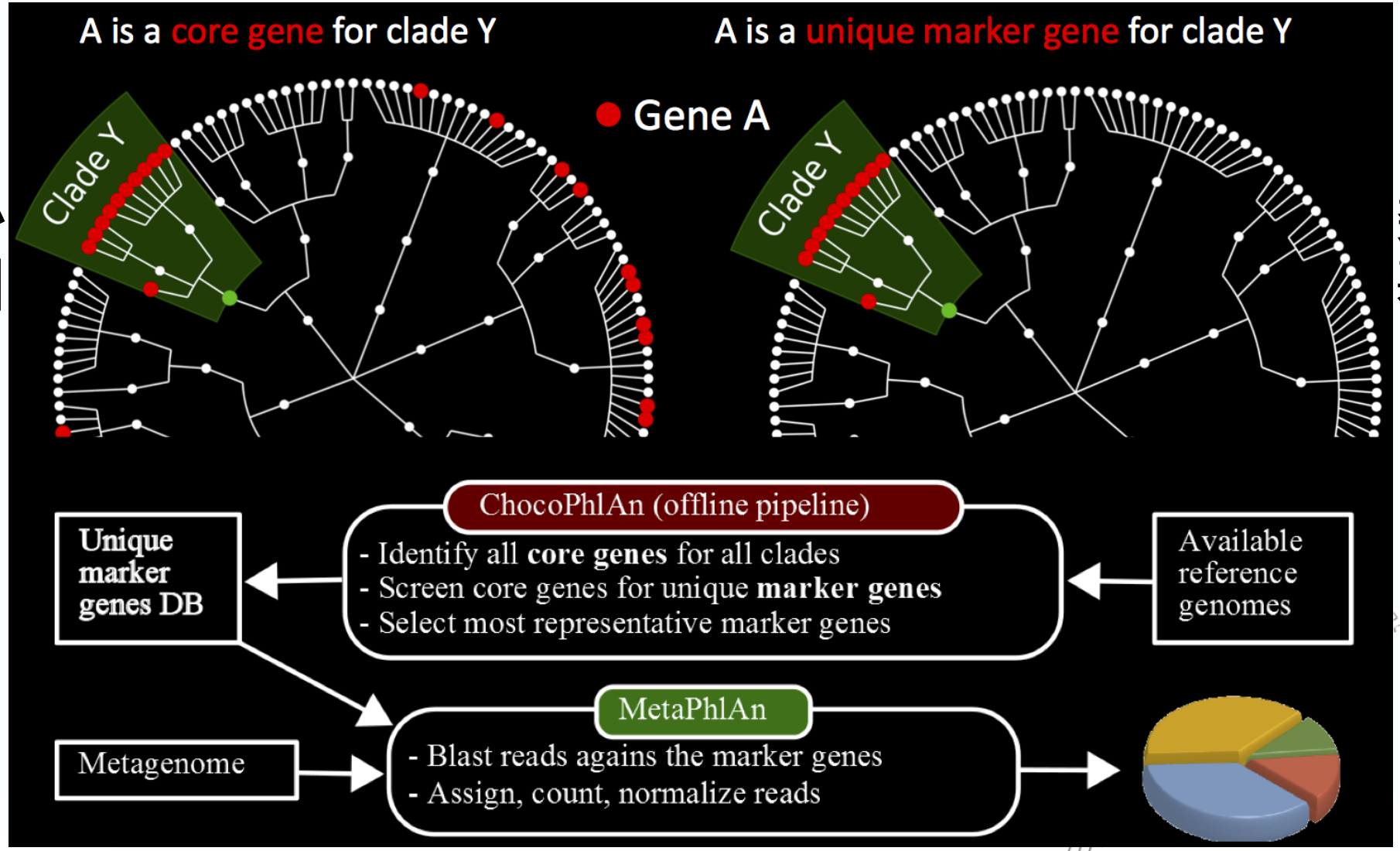
Curtis Huttenhower
Department of Biostatistics, Harv…
5

MetaPhlAn Marker的选择(核心算法)



类核心基因

类特异基因

泛基因组数据库

最新的MetaPhlAn4

- humann.6可基于MetaPhlAn4结果继续运行
- 整合了分离培养基因组+1百万宏基因组组装基因组(MAGs)
- 26,970种水平MAGs，其中4,992个未定义的种
- 默认移除低质量结果
- 安装 conda install -c bioconda metaphlan=4.0.6
- 使用 https://github.com/biobakery/biobakery/wiki/metaphlan4
- Aitor Blanco-Míguez, et al. 2023. Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. ***Nature Biotechnology*** https://doi.org/10.1038/s41587-023-01688-w Cited by 1

MetaPhlAn4的使用

```
# 设置数据库位置，再运行程序
DEFAULT_DB_FOLDER=~/db/metaphlan4
time metaphlan --input_type fastq temp/concat/C1.fq \
  temp/metaphlan4/C1.txt --nproc 4
```

输入fastq单个文件，输出物种组成表，可选bowtie2比对中间文件

- 软件安装代码见1soft_db.sh或2pipeline.sh
- 除非只关注物种组成，否则MetaPhlAn很少单独使用
- humann整合了MetaPhlAn4软件，可实现一条命令完成物种、功能、以及功能对应物种组成三个文件，多角度挖掘宏基因组数据。

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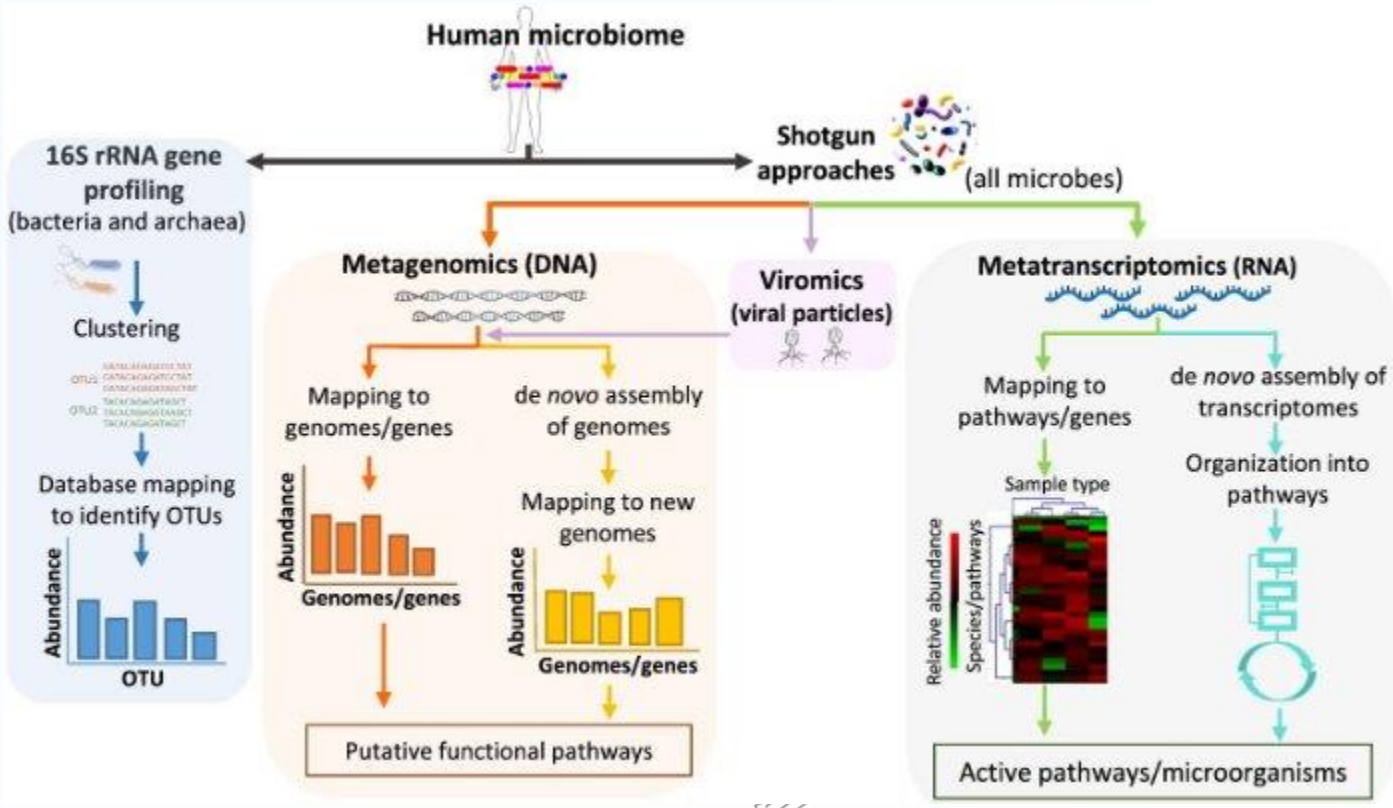
七. 功能组成统计分析



HUMAnN3 http://www.huttenhower.org/humann

- **HUMAnN3**: The HMP Unified Metabolic Analysis Network 3, HUMAnN是基于**宏基因组、宏转录组数据分析**微生物通路丰度的有效工具。这一过程称为功能谱，目的是描述群体成员的代谢潜能。可以**回答微生物群体成员可能干什么，或在干什么的问题。**

[HTML] Metabolic reconstruction for metagenomic data and its application to the human microbiome

S Abubucker, N Segata, J Goll… - PLoS computational …, 2012 - journals.plos.org

Microbial communities carry out the majority of the biochemical activity on the planet, an they play integral roles in processes including metabolism and immune homeostasis in t human microbiome. Shotgun sequencing of such communities' metagenomes provides information complementary to organismal abundances from taxonomic markers, but the resulting data typically comprise short reads from hundreds of different organisms and a best challenging to assemble comparably to single-organism genomes. Here, we descri

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Species-level functional profiling of metagenomes and metatranscriptomes

EA Franzosa, LJ McIver, G Rahnavard, LR Thompson… - Nature …, 2018 - nature.com

Functional profiles of microbial communities are typically generated using comprehensive metagenomic or metatranscriptomic sequence read searches, which are time-consuming, prone to spurious mapping, and often limited to community-level quantification. We developed HUMAnN2, a tiered search strategy that enables fast, accurate, and species-resolved functional profiling of host-associated and environmental communities. HUMAnN2 identifies a community's known species, aligns reads to their pangenomes, performs …

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Abubucker, S. *et al.* Metabolic Reconstruction for Metagenomic Data and Its Application to the Human Microbiome. *PLOS Computational Biology* **8**, e1002358, doi:10.1371/journal.pcbi.1002358 (2012).

Franzosa, E. A. *et al.* Species-level functional profiling of metagenomes and metatranscriptomes. *Nature Methods* **15**, 962-968, doi:10.1038/s41592-018-0176-y (2018).

Nature Methods：humann实现宏基因组和宏转录组种水平功能组成分析

HUMAnN2人类微生物组统一代谢网络分析2　　宏基因组有参流程 (HUMAnN2)

HUMAnN3的依赖关系

- MetaPhlAn4 (version >= 4.0.1)
- Bowtie2 (version >= 2.2) (automatically installed)
- Diamond (0.9.0 > version >= 0.8.22) (automatically installed)
- Python (version >= 2.7)
- MinPath (automatically installed)
- Usearch (version >= 7.0) (only required if using usearch for translated search)
- SAMtools (only required if bam input files are provided)
- Biom-format (only required if input or output files are in biom format)

HUMAnN3的特点

- 可对已知和未知生物分析群体功能谱
  MetaPhlAn4和ChocoPhlAn泛基因组数据库
- 可获得基因组、基因和通路层面的结果
  UniRef基因家族，MetaCyc基因通路，MinPath定义最小通路集
- 简单的使用界面(单行命令实现全部工作流程)
  用户只需提供质控后的宏基因组或宏转录组数据
- 加速序列比对
  采用Bowtie2加速核酸水平比对
  采用Diamond加速核酸翻译蛋白水平比对

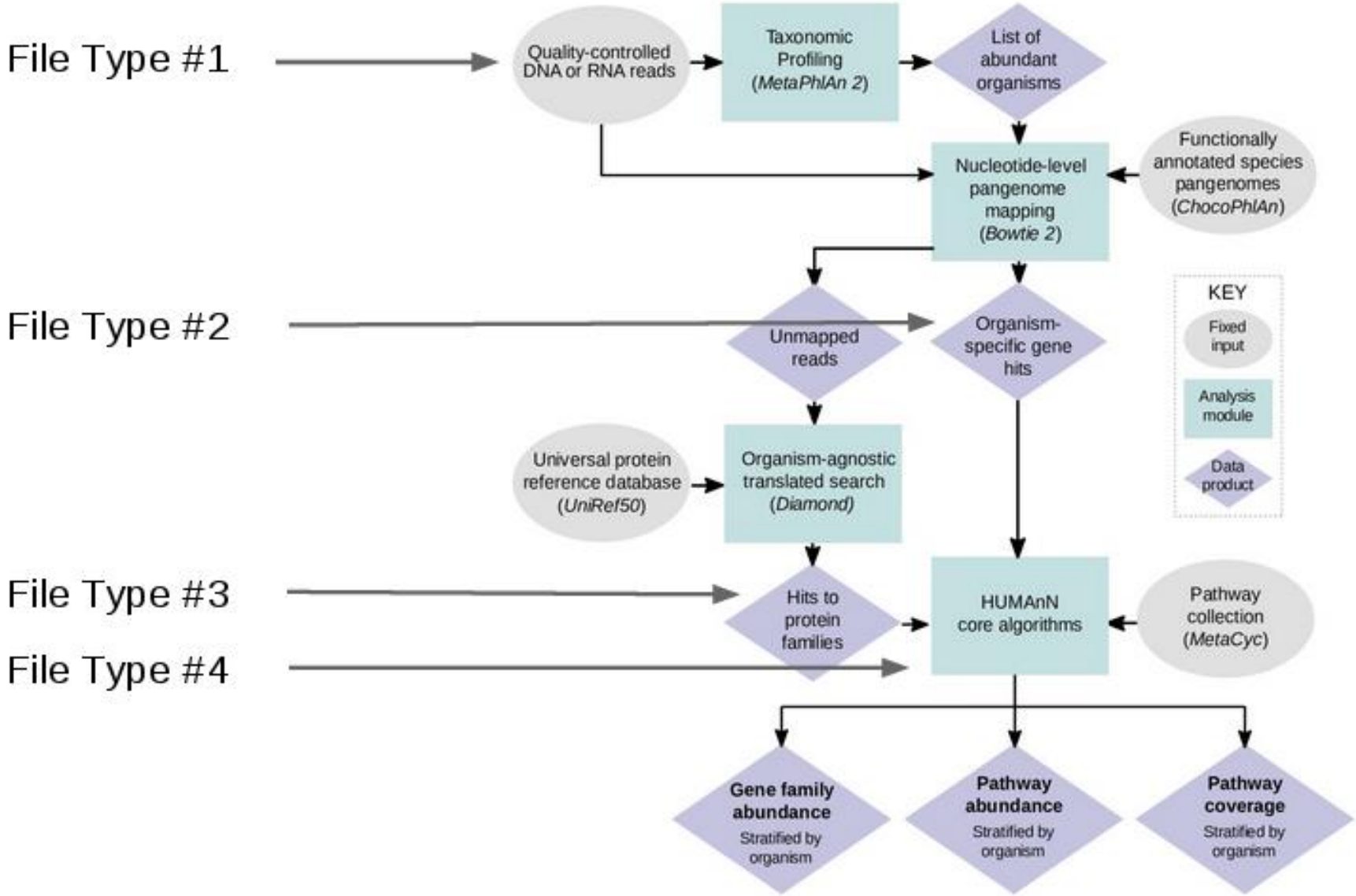
HUMAnN3工作原理

- File Type 1 (a quality-controlled metagenome or metatranscriptome)
  - fastq (fastq.gz)
  - fasta (fasta.gz)

- File Type 2 (alignment results type 1)
  - sam
  - Bam

- File Type 3 (alignment results type 2)
  - blast-like tsv

- File Type 4 (gene table)
  - tsv
  - biom



软件安装和数据库下载

- **humann有参宏基因组物种和功能定量流程**

  conda install humann

- 查看可用数据库并设置下载位置

  humann_databases # 显示可用数据库

- 输助比对数据库 2.6G

  humann_databases --download utility_mapping full ~/db/humann3

- 微生物物种核心基因 16G

  humann_databases --download chocophlan full ~/db/humann3

- 功能基因diamond索引 20G

  humann_databases --download uniref uniref90_diamond ~/db/humann3

http://huttenhower.sph.harvard.edu/humann_data/uniprot/uniref_annotated/

目前数据为2016版，2019版为humann的数据库，数据量增大一倍

配置数据库配置

- 查看参数和数据库位置是否正确

  humann_config --print

- 常用修改线程数、核酸、蛋白库和多种功能注释数据库位置

  humann_config --update run_modes threads 8

  humann_config --update database_folders utility_mapping

  ~/db/humann3/utility_mapping

  humann_config --update database_folders nucleotide ~/db/humann3/chocophlan

  humann_config --update database_folders protein ~/db/humann3/uniref

HUMAnN3物种和功能数据库

```
# humann3数据库无法自动下载，备用链接下载安装
wget -c ftp://download.nmdc.cn/tools/meta/humann3/full_chocophlan.v201901_v31.tar.gz
wget -c ftp://download.nmdc.cn/tools/meta/humann3/uniref90_annotated_v201901b_full.tar.gz
wget -c ftp://download.nmdc.cn/tools/meta/humann3/full_mapping_v201901b.tar.gz
# 安装、解压
mkdir -p ${db}/humann3/chocophlan
tar xvzf full_chocophlan.v201901_v31.tar.gz -C ${db}/humann3/chocophlan
mkdir -p ${db}/humann3/uniref
tar xvzf uniref90_annotated_v201901b_full.tar.gz -C ${db}/humann3/uniref
mkdir -p ${db}/humann3/utility_mapping
tar xvzf full_mapping_v201901b.tar.gz -C ${db}/humann3/utility_mapping
```

2.1 合并双端文件

- 有参宏基因组不考虑双端，将双端文件直接合并为一个文件

```
# 创建目录存放合并后的序列
mkdir -p temp/concat
# for循环调用cat合并每一个样品
for i in `tail -n+2 result/metadata.txt | cut -f 1`;do \
    cat temp/qc/${i}*_?.fastq > temp/concat/${i}.fq; done
# 查看样品数量和大小
ls -sh temp/concat/*.fq
```

大文件加速(只用单端和/或截取，牺牲精度换速度)

- 方法1. 仅链接左端为输入文件(提速50%，节省1倍文件空间)

```
for i in `tail -n+2 result/metadata.txt|cut -f1`;do
  ln -sf `pwd`/temp/qc/${i}_1.fastq temp/concat/${i}.fq
done
```

- 方法2. 控制标准样比对时间。测序数据量通常为6~50G，同一样本分析时间可达10h~100h，严重浪费时间而浪费硬盘空间。可用head对单端分析截取20M序列，即3G，则为80M行

```
for i in `tail -n+2 result/metadata.txt|cut -f1`;do
   head -n80000000 temp/qc/${i}_1.fastq  > temp/concat/${i}.fq
done
```

2.2 HUMAnN3计算物种和功能组成

```
mkdir -p temp/humann3
```

- 如果数据库位置正确，只需输入文件和输出目录，经rush管理批量任务队列

```
tail -n+2 result/metadata.txt|cut -f1|rush -j 2 \
  'humann --input temp/concat/{1}.fq  \
  --output temp/humann3/’
# 核心步骤，测序数据2 X 8 = 16线程，用时1h，真实数据可能要几小时至几天
```

2.3 物种组成分析

```
mkdir -p result/metaphlan4
```

- # 样品结果合并

```
merge_metaphlan_tables.py
temp/humann/*_humann_temp/*_metaphlan_bugs_list.tsv | \
  sed 's/_metaphlan_bugs_list//g' > result/metaphlan4/taxonomy.tsv
```

- # 转换为spf格式方便stamp分析

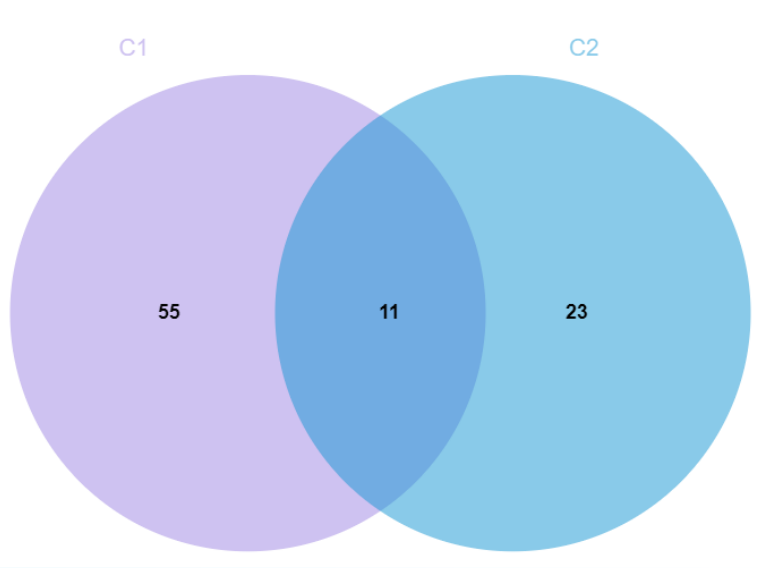
```
metaphlan_to_stamp.pl result/metaphlan4/taxonomy.tsv \
    > result/metaphlan4/taxonomy.spf
```

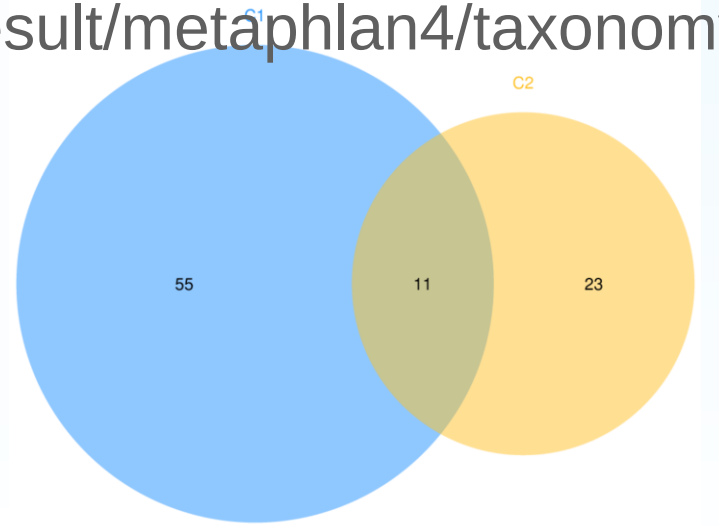
- metaphlan_to_stamp.pl脚本来自microbiome_helper项目，整合至EasyMicrobiome

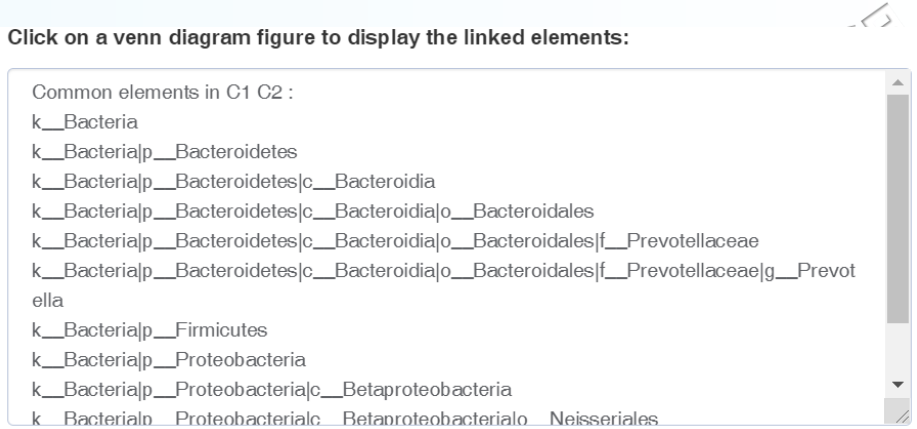
筛选样本中某一丰度的分类(2StatPlot.sh)

- # (可选)筛选>0.5%的分类，绘制维恩图

```
awk 'BEGIN{OFS=FS="\t"}{if(FNR==1)
{for(i=2;i<=NF;i++) a[i]=$i;} \
   else {for(i=2;i<=NF;i++) if($i>0.5) print $1, a[i];}}' \
   result/metaphlan4/taxonomy.tsv \
   > result/metaphlan4/taxonomy_high.tsv
wc -l result/metaphlan4/taxonomy_high.tsv
```







http://www.ehbio.com/test/venn/#/

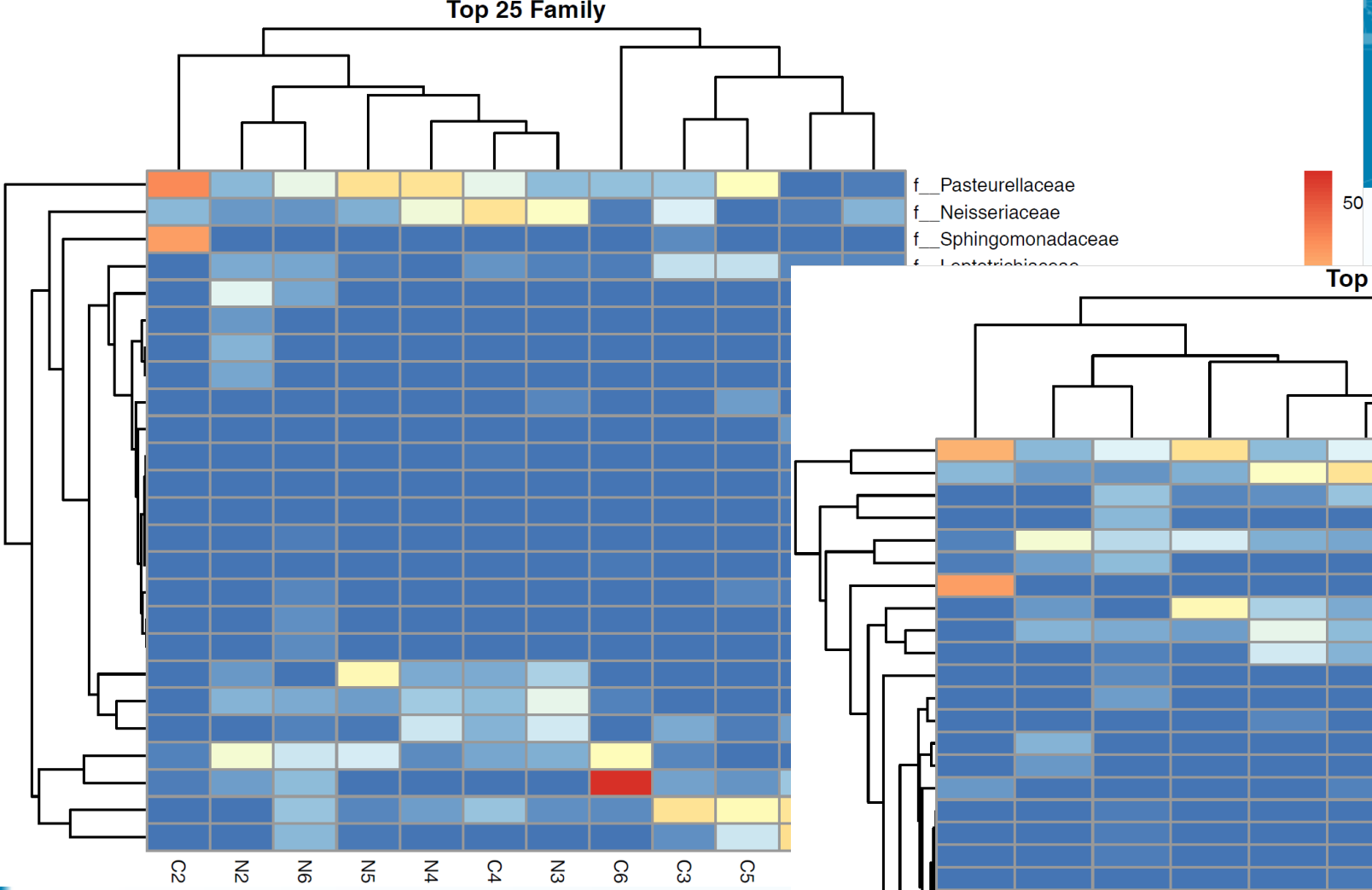
Tong Chen, Haiyan Zhang, Yu Liu, Yong-Xin Liu & Luqi Huang. (**2021**). EVenn: Easy to create repeatable and editable Venn diagrams and Venn networks online. ***Journal of Genetics and Genomics***, doi: 10.1016/j.jgg.2021.07.007

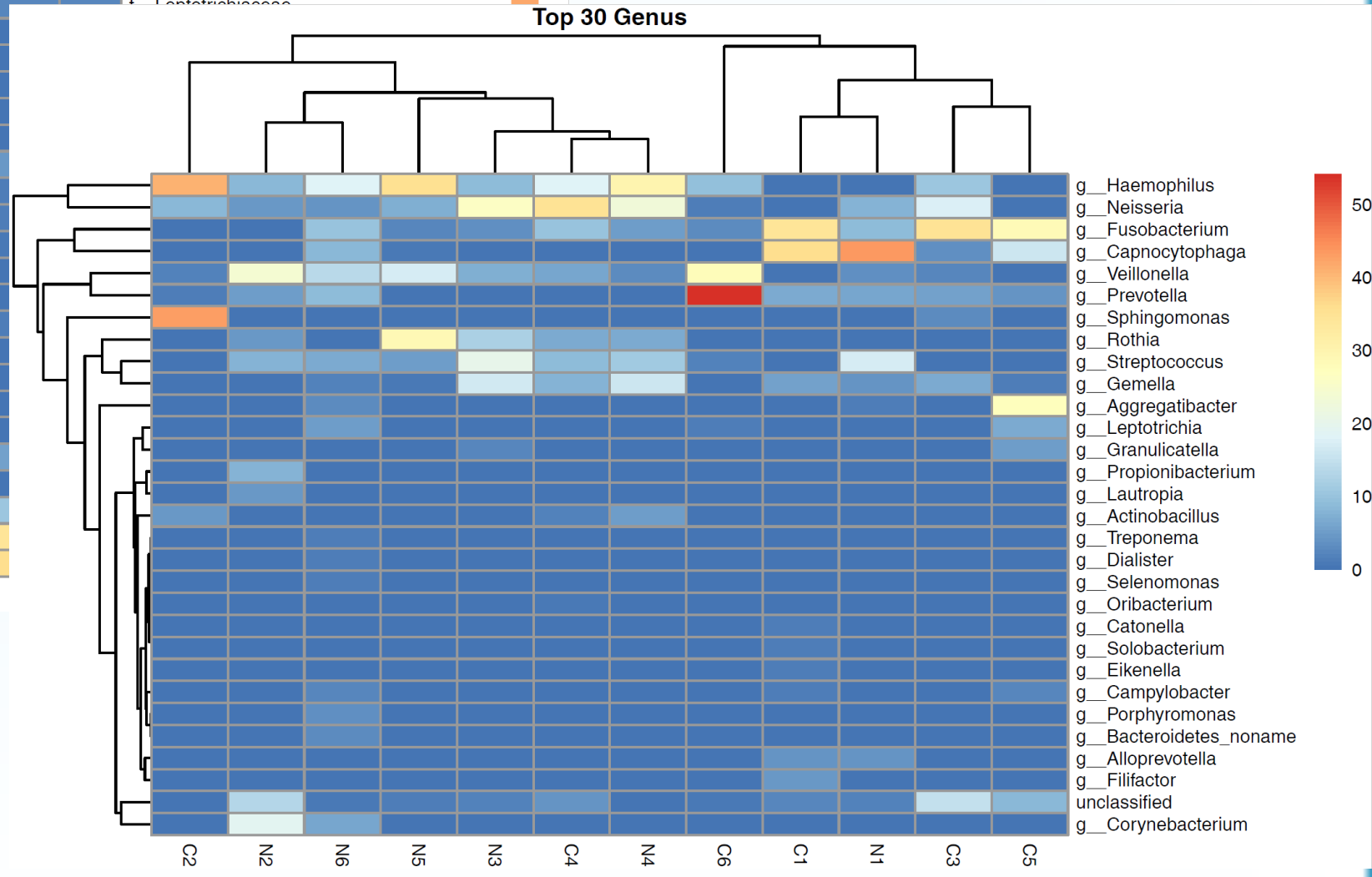
评估样本物种组成和分组聚类——更灵活绘制热图

- 方法1. metaphlan_hclust_heatmap.py 脚本服务器绘制热图，依赖关系和环境变量复杂
- 方法2. Excel筛选 metaphlan4/taxonomy.tsv 并在线绘制热图
- 方法3. R数据筛选taxonomy.spf并用pheatmap绘制热图，可指定分类级别、物种数量

```
Rscript $sd/metaphlan_hclust_heatmap.R \
    -i result/metaphlan4/taxonomy.spf \
    -t Order \
    -n 25 \
    -o result/metaphlan4/heatmap_Order
```

R脚本可以windows下运行，详见 3StatPlot.sh


Top 25 Family
f__Pasteurellaceae
f__Neisseriaceae
f__Sphingomonadaceae
50
C2
N2
N6
N5
N4
C4
N3
C6
C3
C5


Top 30 Genus
g__Haemophilus
g__Neisseria
g__Fusobacterium
g__Capnocytophaga
g__Veillonella
g__Prevotella
g__Sphingomonas
g__Rothia
g__Streptococcus
g__Gemella
g__Aggregatibacter
g__Leptotrichia
g__Granulicatella
g__Propionibacterium
g__Lautropia
g__Actinobacillus
g__Treponema
g__Dialister
g__Selenomonas
g__Oribacterium
g__Catonella
g__Solobacterium
g__Eikenella
g__Campylobacter
g__Porphyromonas
g__Bacteroidetes_noname
g__Alloprevotella
g__Filifactor
unclassified
g__Corynebacterium
50
40
30
20
10
0
C2
N2
N6
N5
N3
C4
N4
C6
C1
N1
C3
C5

2.4.1 功能组成合并、标准化和分层

- # 合并所有样品

```
humann_join_tables --input temp/humann --file_name pathabundance \
  --output result/humann3/pathabundance.tsv
sed -i 's/_Abundance//g' result/humann3/pathabundance.tsv
```

- # 标准化为相对丰度relab或百万分数cpm

```
humann_renorm_table --input result/humann3/pathabundance.tsv --units relab \
  --output result/humann3/pathabundance_relab.tsv
```

- # 分层结果

```
humann_split_stratified_table --input result/humann3/pathabundance_relab.tsv \
  --output result/humann3/
```

2.4.2 添加分组和差异比较

手动或用Shell有表头下面添加分组行

# Pathway	C1	C2	C3	C4	C5	C6	N1	N2	N3	N4	N5	N6
Group	Cancer	Cancer	Cancer	Cancer	Cancer	Cancer	Normal	Normal	Normal	Normal	Normal	Normal
ANAGLYCOLYSIS-PWY: glycolysis III (from glucose)				12.7763613320	3.3803273747	3.4161087641	15.4265476544	12.7441068874	8.6332745611	0	3.5400735715	18.6514
ANAGLYCOLYSIS-PWY: glycolysis III (from glucose)|g__Fusobacterium.s__Fusobacterium_nucleatum							5.0005085292	0	0	0	4.1457115522	0
ANAGLYCOLYSIS-PWY: glycolysis III (from glucose)|g__Haemophilus.s__Haemophilus_parainfluenzae							0	0	0	3.5541892578	0	2.0826450377

KW差异比较：输入input、分组focal、分组类型type、分组行结果、FDR和输出output；结果包括特征对应各组均值和统计值

```
humann_associate --input result/humann/pathabundance.pcl  \
        --focal-metadatum Group --focal-type categorical \
        --last-metadatum Group --fdr 0.05 \
        --output result/humann3/associate.txt
```

样本少无显著差异，改用HMP中每组各十个样本的数据集

官方教程 https://bitbucket.org/biobakery/biobakery/wiki/humann#rst-header-downstream-analyses

HMP数据——差异比较

使用HMP示例数据，样本名下面有分组信息，需要手动制作

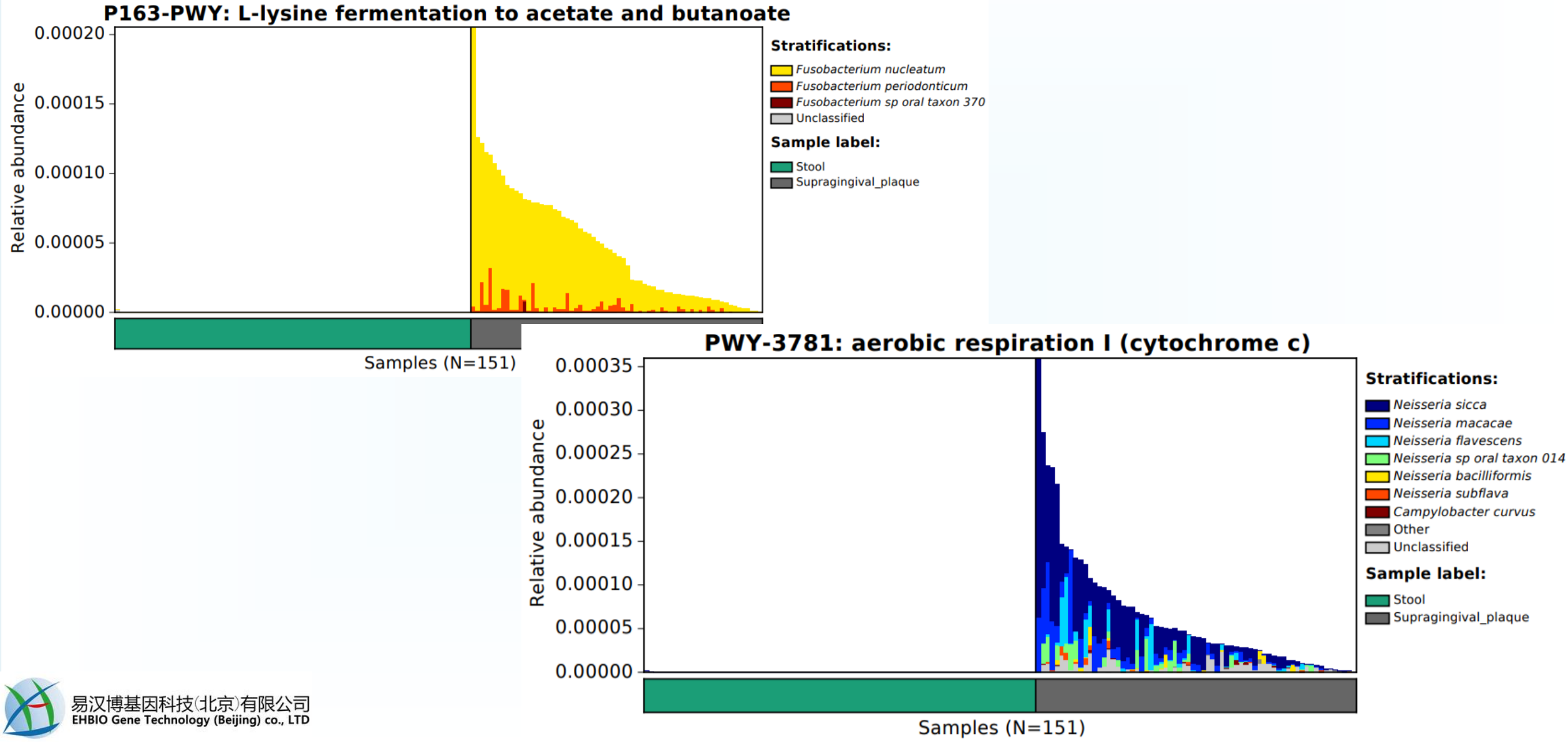
FEATURE \ SAMPLE	SRS011084	SRS011086	SRS011090
Group	Stool	Stool	Stool
1CMET2-PWY: N10-formyl-tetrahydrofolate biosynthesis	0.000498359	0.000628096	0.000304951
1CMET2-PWY: N10-formyl-tetrahydrofolate biosynthesis|g__Acidovorax.s__Acidovorax_ebreus	0	0	0

```
humann_associate --input hmp_pathabund.pcl --focal-metadatum Group --focal-type categorical --last-metadatum Group --fdr 0.05 --output associate.txt
```

# Feature	Level means (|ed)	P-value	Q-value
P163-PWY: L-lysine fermentation to acetate and butanoate	Stool:3.133e-08|Supragingival_plaque:5.385e-05	1.253e-30	4.261e-28
PWY-3781: aerobic respiration I (cytochrome c)	Stool:1.464e-07|Supragingival_plaque:0.0002634	4.076e-30	4.62e-28
PWY66-409: superpathway of purine nucleotide salvage	Stool:0|Supragingival_plaque:0.000106	2.986e-30	4.62e-28
PWY1F-823: leucopelargonidin and leucocyanidin biosynthesis	Stool:4.877e-09|Supragingival_plaque:4.695e-05	1.402e-29	1.192e-27

```
humann_barplot --input hmp_pathabund.pcl --focal-feature PWY-3781 --focal-metadata Group --last-metadata Group --output barplot_PWY-3781.pdf --sort sum metadata
```

--sort sum metadata先按分组，再按丰度



2.4.3 转换为KEGG注释

```
# 转换基因家族为KO(uniref90_ko)，可选eggNOG(uniref90_eggnog)或酶(uniref90_level4ec)
for i in `tail -n+2 result/metadata.txt|cut -f1`;do
  humann_regroup_table \
    -i temp/humann3/${i}_genefamilies.tsv \
    -g uniref90_ko \
    -o temp/humann3/${i}_ko.tsv
done
# 合并，并修正样本名
humann_join_tables \
  --input temp/humann3/ \
  --file_name ko \
  --output result/humann3/ko.tsv
sed -i '1s/_Abundance-RPKs//g' result/humann3/ko.tsv
```

# Gene Family	KO1	WT1
K00029	0	23.999
K00029|g__Pseudomonas.s__Pseudomon	0	23.999
K00031	8.81	7.018
K00031|g__Agrobacterium.s__Agrobacter	0	1.802
K00031|unclassified	8.81	5.215
K00032	0	8.48
K00032|g__Pseudomonas.s__Pseudomon	0	8.48
K00033	0	12.989
K00033|g__Bacillus.s__Bacillus_megateriu	0	3.454
K00033|g__Pseudomonas.s__Pseudomon	0	9.535
K00035	0	1.285
K00035|g__Agrobacterium.s__Agrobacter	0	1.285
K00036	0	46.161
K00036|g__Agrobacterium.s__Agrobacter	0	0.754
K00036|g__Bacillus.s__Bacillus_megateriu	0	5.084
K00036|g__Pseudomonas.s__Pseudomon	0	40.324

总结

- humann调用MetaPhlAn4基于bowtie2比对上万个物种的数据库，可快速、准确获得细菌、真菌、古菌、病毒、真核生物等的物种组成
- merge_metaphlan_tables.py合并、metaphlan_hclust_heatmap.py绘制丰度热图，metaphlan_to_stamp.pl生成STAMP输入文件
- humann采用diamond比对UniRef数据库获得功能组成；注意数据库位置设置；了解其依赖关系以便解决依赖关系错误的问题
- 结果包括功能通路丰度组成和**功能通路具体来源的物种**，提供join, norm, stratified等脚本实现合并、标准化和分层
- associate, barplot脚本实现组间KW检验和通路组成可视化
- humann_regroup_table脚本转换基因家族为KO/eggNOG/EC等注释

参考资源

- 宏基因组公众号文章目录　　生信宝典公众号文章目录
- 科学出版社《微生物组数据分析》——50+篇
- Bio-protocol《微生物组实验手册》——153篇
- Protein Cell：扩增子和宏基因组数据分析实用指南
- CMJ：人类微生物组研究设计、样本采集和生物信息分析指南
- 加拿大生信网 https://bioinformatics.ca/ 宏基因组课程中文版
- 美国高通量开源课程 https://github.com/ngs-docs
- Curtis Huttenhower http://huttenhower.sph.harvard.edu/
- Nicola Segata http://segatalab.cibio.unitn.it/

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