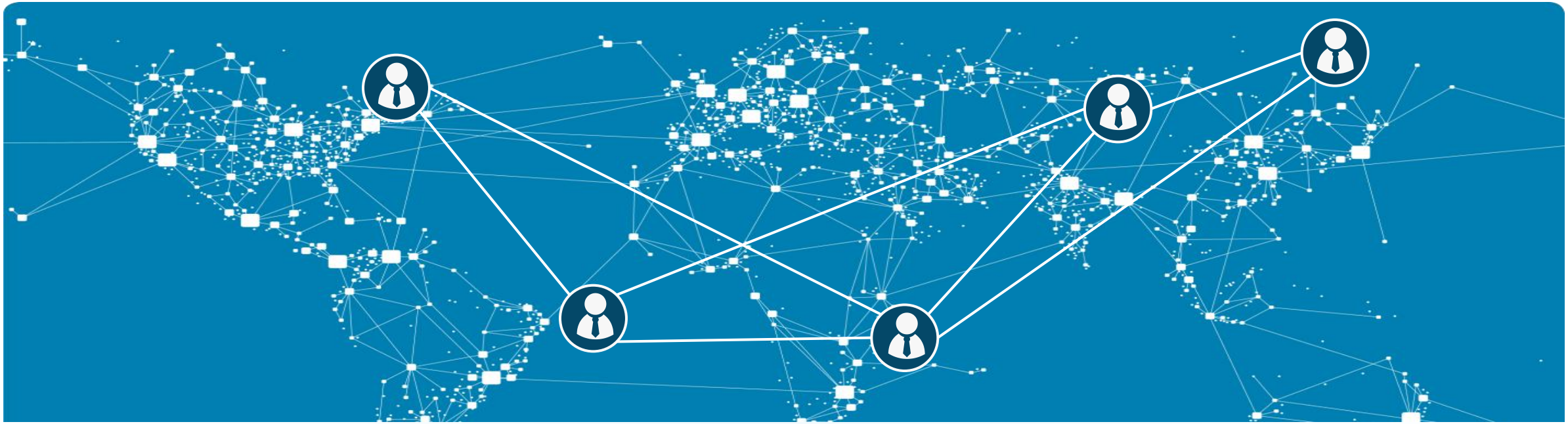
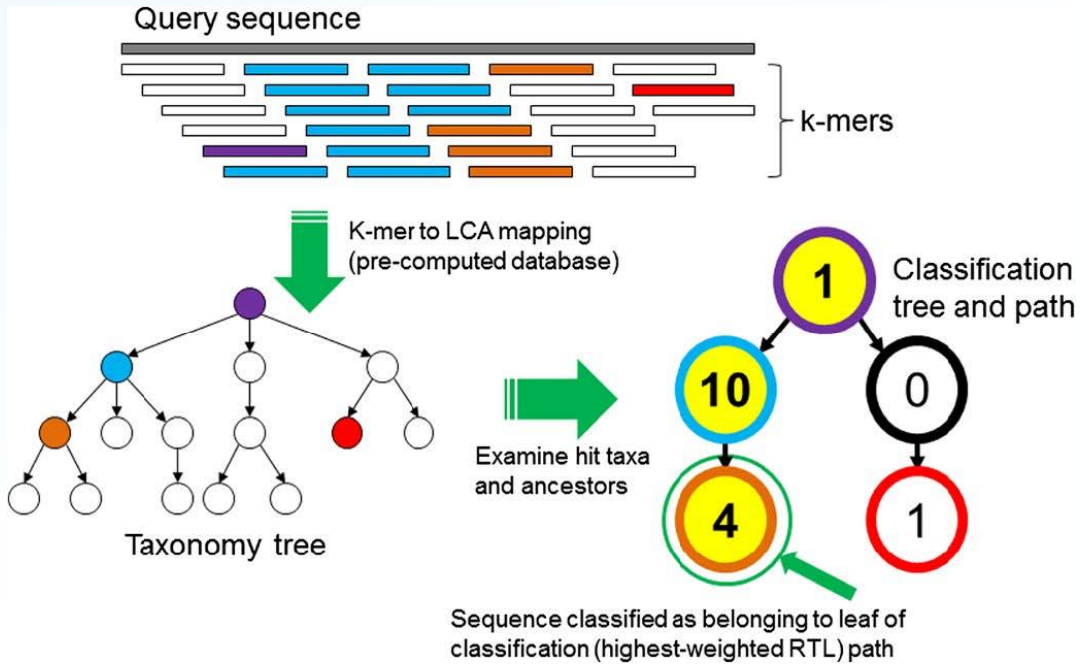
微生物组—宏基因组分析专题研讨会第24期



25 Kraken2物种注释

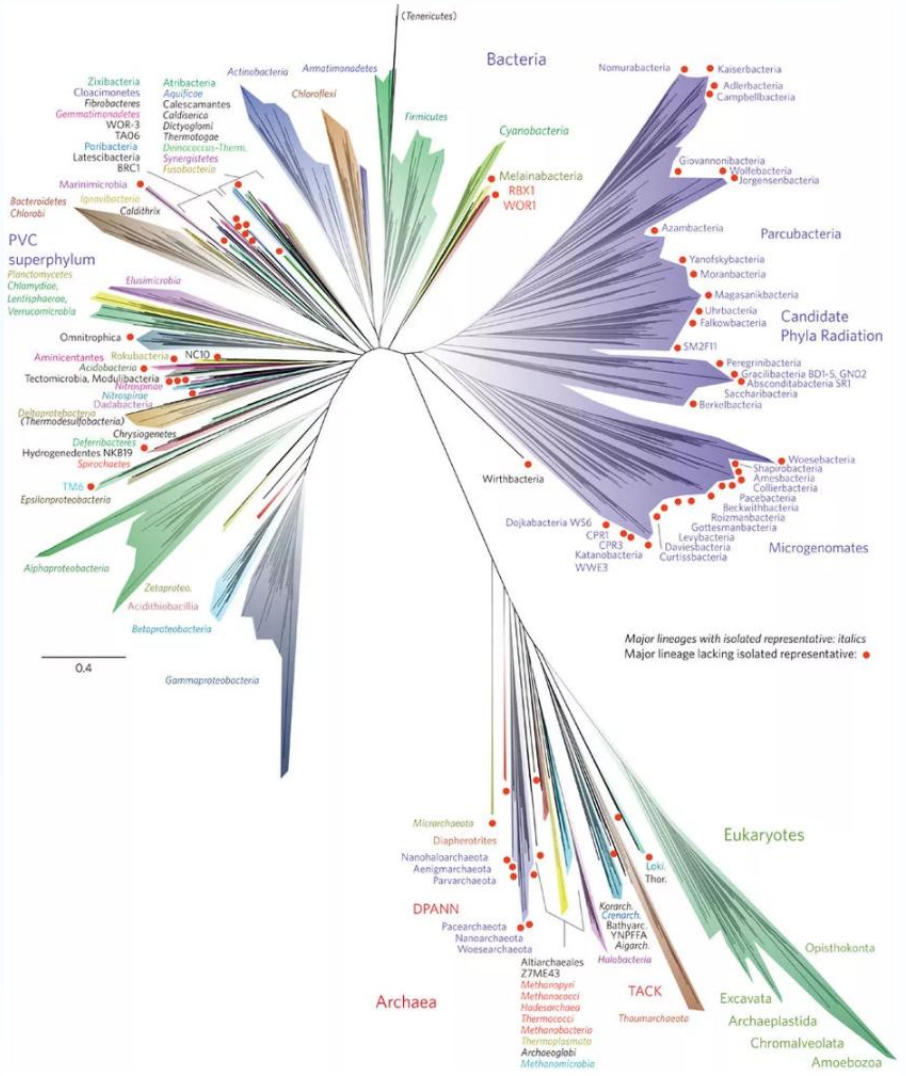
刘永鑫

2025年11月29日



物种分类学注释

- 分类学(taxonomy)：是一门研究生物类群间的异同以及异同程度，阐明生物间的亲缘关系、进化过程和发展规律的科学。
- 主要分为细菌、古菌和真核生物三大类；
- 常用七级分类法：界(Kingdom)、门(Phylum)、纲(Class)、目(Order)、科(Family)、属(Genus)、种(Species)



Laura A. Hug, …, Jillian F. Banfield. 2016. A new view of the tree of life. ***Nature Microbiology*** 1: 16048. https://doi.org/10.1038/nmicrobiol.2016.48

物种注释——相当于地址

- 界(Kingdom)、门(Phylum)、纲(Class)、目(Order)、科(Family)、属(Genus)、种(Species)
- 动物界、脊索动物门、哺乳纲、食肉目、熊科、大熊猫属、大熊猫
- 动物界、脊索动物门、哺乳纲、灵长目、人科、人属、智人种
- 国、省、市、县、镇、村、屯
- 中国、黑龙江省、哈尔滨市、五常县、冲河镇、三家子村、大排地屯
- 微生物进化快，属种不能保证与功能一致，常用株(Strain)关联功能
- 扩增子只测序部分16S序列，信息有限，仅能确定属水平

https://baike.baidu.com/item/%E7%95%8C%E9%97%A8%E7%BA%B2%E7%9B%AE%E7%A7%91%E5%B1%9E%E7%A7%8D

物种注释数据库

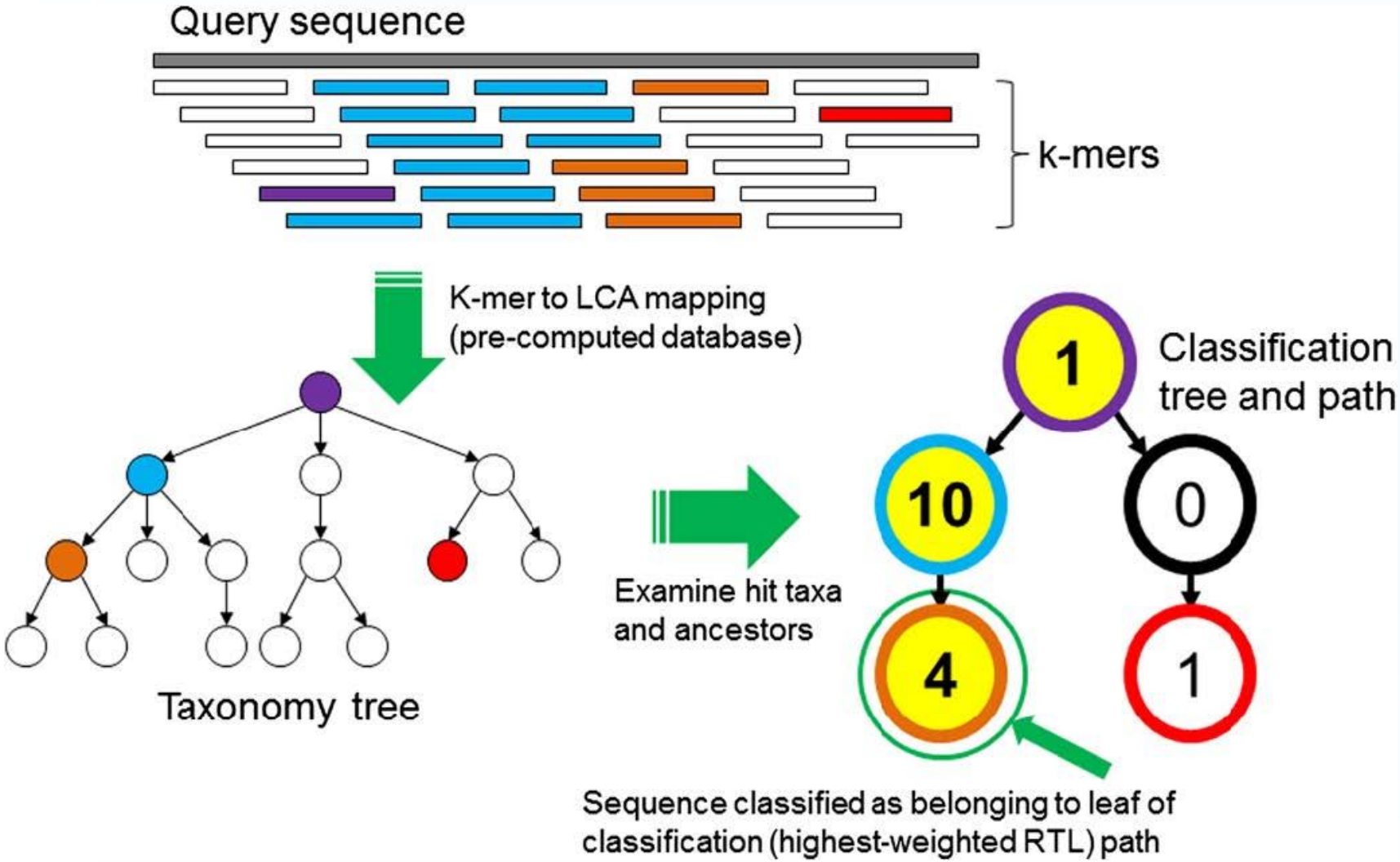
- NCBI——NR非冗余序列，NCBI发布的序列包含物种Taxonomy ID
- MetaPhlAn2——整理已发表基因组Marker基因数据库
- GTDB——基因组细菌120/古菌122单拷贝基因
- GreenGenes/RDP——原核生物核糖体(16S)数据库
- SILVA——原核、真核核糖体(16/18S)数据库

物种注释方法

- 比对方法：与有物种注释的序列数据库比对，通过相似度进行物种注释；这种方法受限于数据库，且比对结果不准确。常用blast、diamond等。

- LCA(Lower Common Ancestor最低共同祖先)：此类方法常基于K-mer进行分类注释；目前认为方法较准确，但是注释到的物种信息很少，常用软件有Kraken系列、RDP classifier、Sintax等。

Kraken序列分类算法：LCA



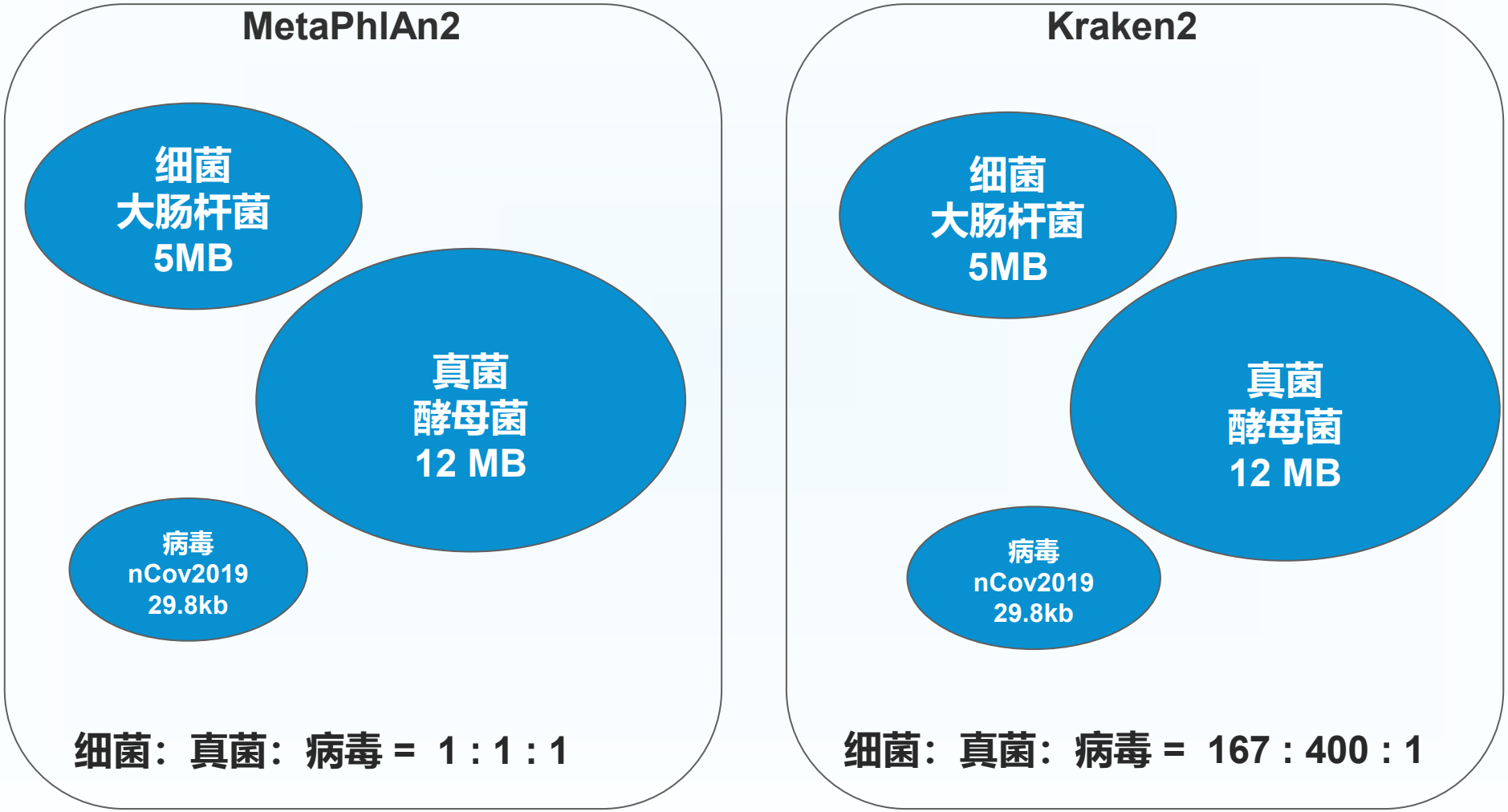
Lower Common Ancestor
最低共同祖先

软件默认认阈值为0，
存在过分类问题

[Kraken：使用精确比对的超快速宏基因组序列分类软件]

相对丰度：分类(taxonomic) vs 序列(sequence)

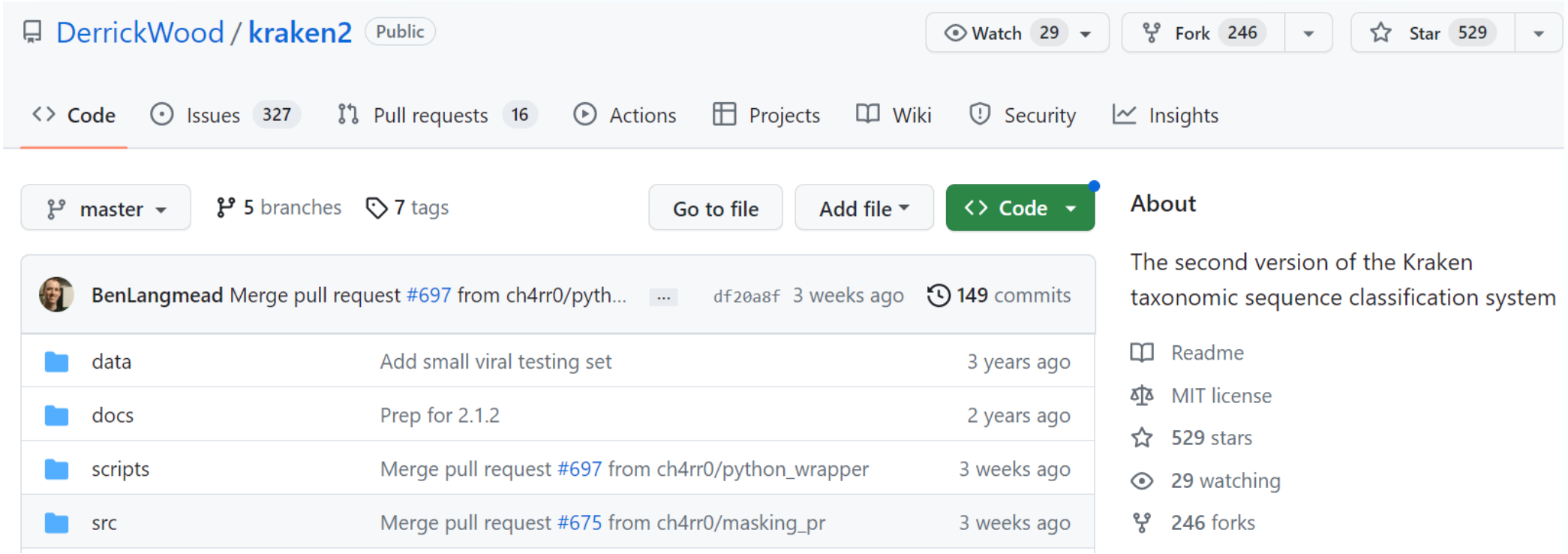
Nature子刊：刘洋彧、Rob Knight等评测不同宏基因组物种定量方法及其对结果的影响



Zheng Sun, Shi Huang, Meng Zhang, Qiyun Zhu, Niina Haiminen, Anna Paola Carrieri, Yoshiki Vázquez-Baeza, Laxmi Parida, Ho-Cheol Kim, Rob Knight & Yang-Yu Liu. (**2021**). Challenges in benchmarking metagenomic profilers. ***Nature Methods***, doi: https://doi.org/10.1038/s41592-021-01141-3

Kraken2

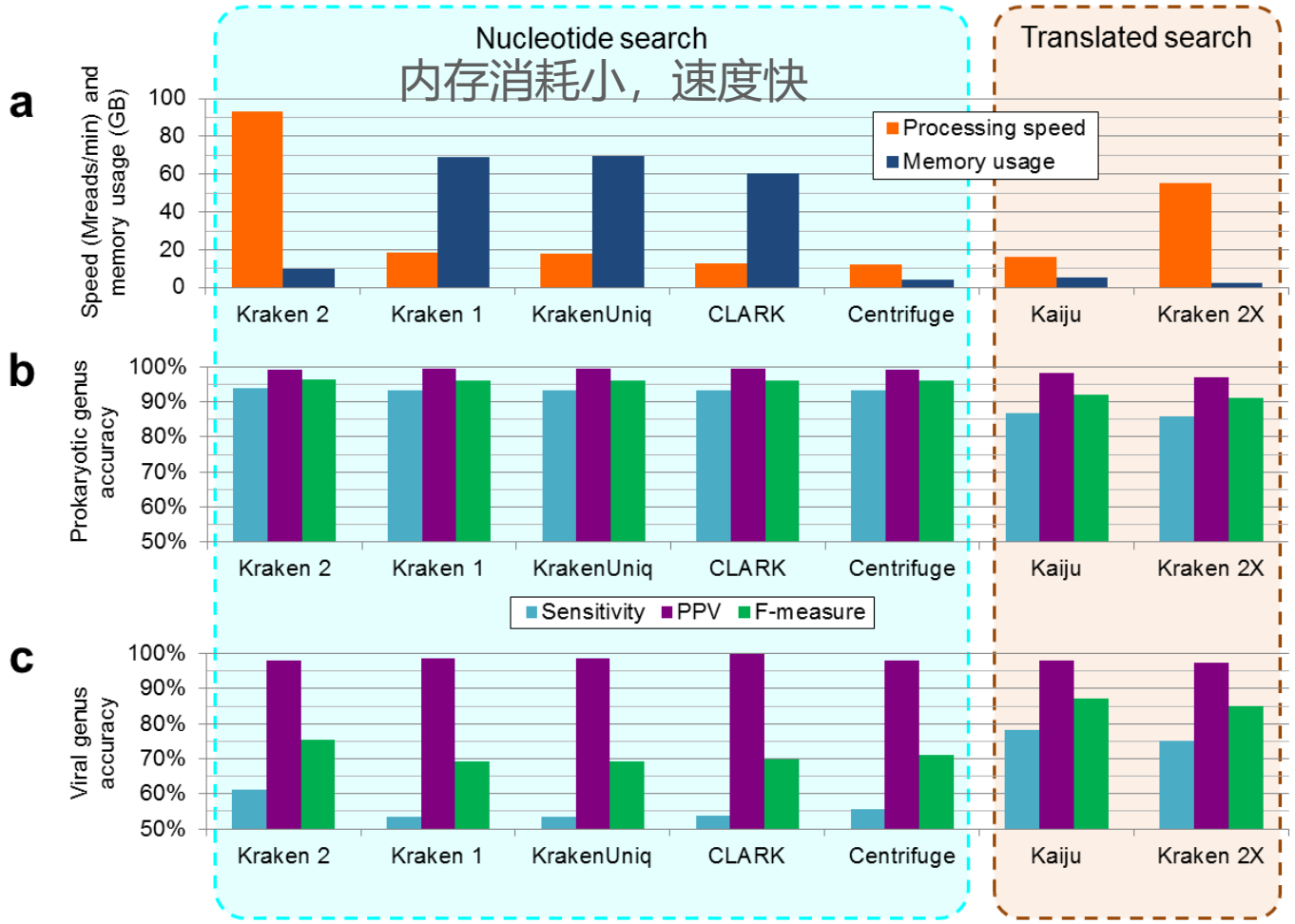
- Kraken有安装数据库过大，结果可读性差，需要二次转换等缺点。
- kraken2横空出世 https://github.com/DerrickWood/kraken2



Derrick E. Wood, Jennifer Lu, Ben Langmead. 2019. Improved metagenomic analysis with Kraken 2. ***Genome Biology*** 20: 257. https://doi.org/10.1186/s13059-019-1891-0

Kraken2与其它工具比较

图1. Kraken 2与其他工具之间的比较。（a）显示了每个分类器的处理速度（M）和内存使用情况（GB），对16线程下的5000万对配对模拟读长进行评估的。 显示了（b）40个原核基因组和（c）10个病毒基因组的准确性结果。 此处显示的是灵敏度Sensitive，阳性预测值（PPV）和F值的结果。 “ Kraken 2X”是针对蛋白质数据库进行翻译搜索的Kraken 2



Simon H. Ye, Katherine J. Siddle, Daniel J. Park, Pardis C. Sabeti. 2019. Benchmarking metagenomics tools for taxonomic classification. ***Cell*** 178: 779-794.
https://doi.org/10.1016/j.cell.2019.07.010

Cell：20种宏基因组学物种分类工具大比拼

Kraken2安装和数据库配置

- 安装基于LCA算法的物种注释软件Kraken2

conda install kraken2

- 下载数据库：https://benlangmead.github.io/aws-indexes/k2
- 小内存/演示使用迷你库(PlusPF-16），包括标准+原生动物+真菌及Bracken2索引，仅16G，可选标准100G或包括植物的214G
- **不是数据库时间、体积大小版对注释比例影响非常大**
- 自定义数据库，标准模式只下载5种数据库：古菌archaea、细菌bacteria、人类human、载体UniVec_Core、病毒viral

kraken2-build --standard --threads 24 --db ~/db/kraken2

基于NCBI数据库的Kraken2物种注释

```
### 多样本并行物种注释，推荐1个任务，最多3个，使用3倍内存
mkdir -p temp/kraken2
tail -n+2 result/metadata.txt|cut -f1|rush -j 1 \
 'kraken2 --db ${db}/kraken2/pluspf16g --paired temp/qc/{1}_?.fastq \
  --threads 3 --use-names --report-zero-counts \
  --report temp/kraken2/{1}.report \
  --output temp/kraken2/{1}.output'
# 屏幕会输出各样品注释比例，和运行时间 10 - 20 min
```

Krakentools实现kraken2结果的格式转换和筛选

- 安装

```
conda install krakentools -c bioconda
```

- 批量转换kraken2的report结果为mpa格式(metaphlan格式，可直接进行LEfSe分析)

```
for i in `tail -n+2 result/metadata.txt|cut -f1`;do
  kreport2mpa.py -r temp/kraken2/${i}.report \
    --display-header \
    -o temp/kraken2/${i}.mpa
done
```

Kraken2基于NCBI数据库注释reads层面

```
### 汇总样品物种组成表
mkdir -p result/kraken2
tail -n+2 result/metadata.txt|cut -f1|rush -j 1 \
 'tail -n+2 temp/kraken2/{1}.mpa | sort | cut -f 2 | sed "1 s/^/{1}\n/" > temp/kraken2/{1}_count '
header=`tail -n 1 result/metadata.txt | cut -f 1`
tail -n+2 temp/kraken2/${header}.mpa | sort | cut -f 1 | sed "1 s/^/Taxonomy\n/" > temp/kraken2/0header_count
paste temp/kraken2/*count > result/kraken2/tax_count.mpa
```

物种组成表

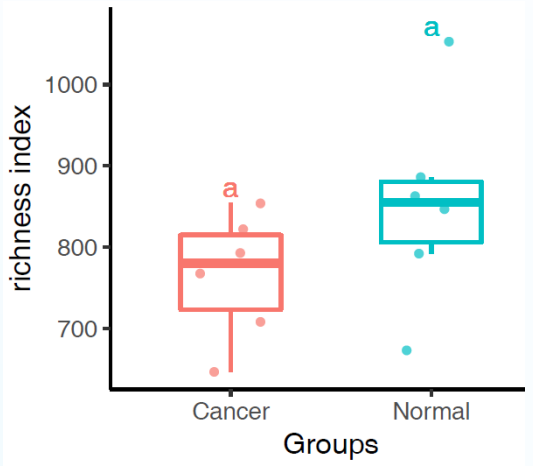
Taxonomy	C1	C2	C3	Y1	Y2	Y3
k__Bacillati	101803	83213	86297	759349	616349	655680
k__Bacillati|p__Actinomycetota	9058	4055	12490	40165	67168	233261
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia	38	29	43	5	10	4
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales	38	28	43	5	10	4
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Acidimicrobiaceae	3	0	3	0	0	0
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Acidimicrobiaceae|g__Acidimicrobium	3	0	3	0	0	
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Acidimicrobiaceae|g__Acidimicrobium|s__Acidimicrobium_ferrooxidans	3	0				
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Candidatus_Hopanoidivoransaceae	10	7	7	0	0	
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Candidatus_Hopanoidivoransaceae|g__Candidatus_Poriferisocius	10	7	7			
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Candidatus_Hopanoidivoransaceae|g__Candidatus_Poriferisocius|s__Candidatus_Poriferisocius_sp.						
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Candidatus_Poriferisodalaceae	11	3	1	1	6	
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Candidatus_Poriferisodalaceae|g__Candidatus_Poriferisodalis	11	3	1			
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__Candidatus_Poriferisodalaceae|g__Candidatus_Poriferisodalis|s__Candidatus_Poriferisodalis_sp.						
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__lamiaceae	11	12	18	4	3	0
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__lamiaceae|g__Actinomarinicola	6	1	4	0	2	
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__lamiaceae|g__Actinomarinicola|s__Actinomarinicola_tropica	6	1	4			
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__lamiaceae|g__Aquihabitans	2	4	13	4	1	
k__Bacillati|p__Actinomycetota|c__Acidimicrobiia|o__Acidimicrobiales|f__lamiaceae|g__Aquihabitans|s__Aquihabitans_daechungensis	0	0	0			

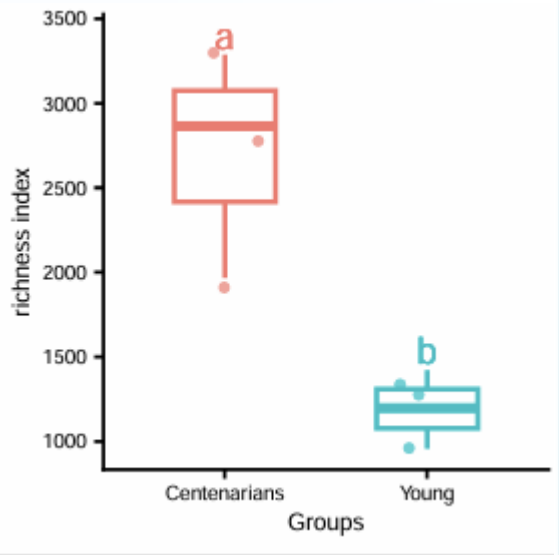
- 本地/在线使用LEfSe差异比较，GraPhlAn或microbiomeViz可视化
- R语言统计分析alpha, beta和物种组成和可视化
- 直接使用STAMP差异比较和可视化

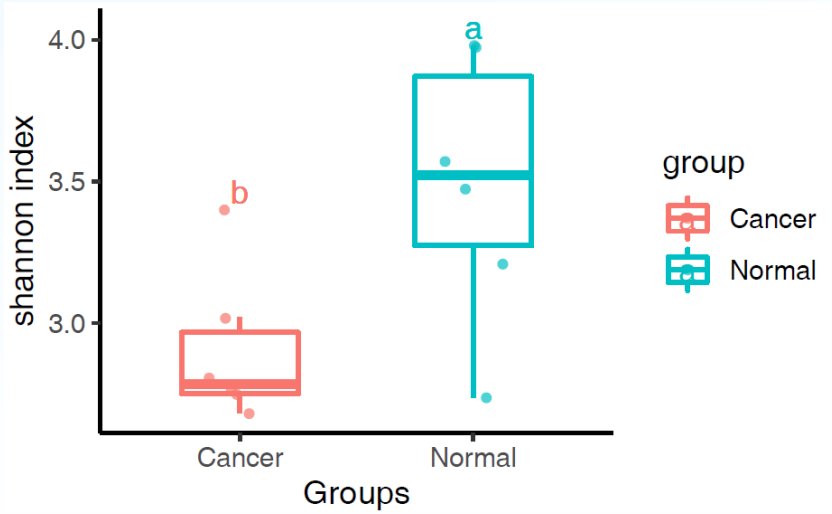
microbiomeViz：绘制lefse结果中Cladogram

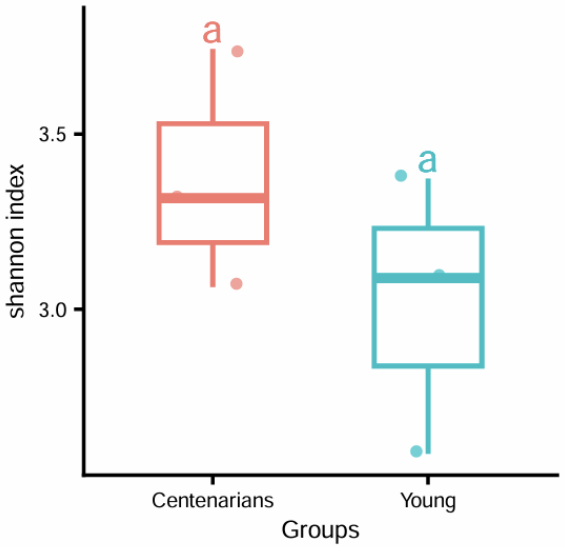
2StatPlot.sh - 物种Kraken2 - Alpha多样性

```
# 提取种级别、抽平、计算6种alpha多样性指数
Rscript $sd/kraken2alpha.R \
  --input result/kraken2/tax_count.mpa \
  --depth 0 \
  --species result/kraken2/tax_count.txt \
  --normalize result/kraken2/tax_count.norm \
  --output result/kraken2/tax_count.alpha
# 绘制箱线图,可选richness/chao1/shannon…
Rscript $sd/alpha_boxplot.R \
  -i result/kraken2/tax_count.alpha \
  -a shannon \
  -d result/metadata.txt \
  -n Group \
  -o result/kraken2/ \
  -w 89 -e 59
```

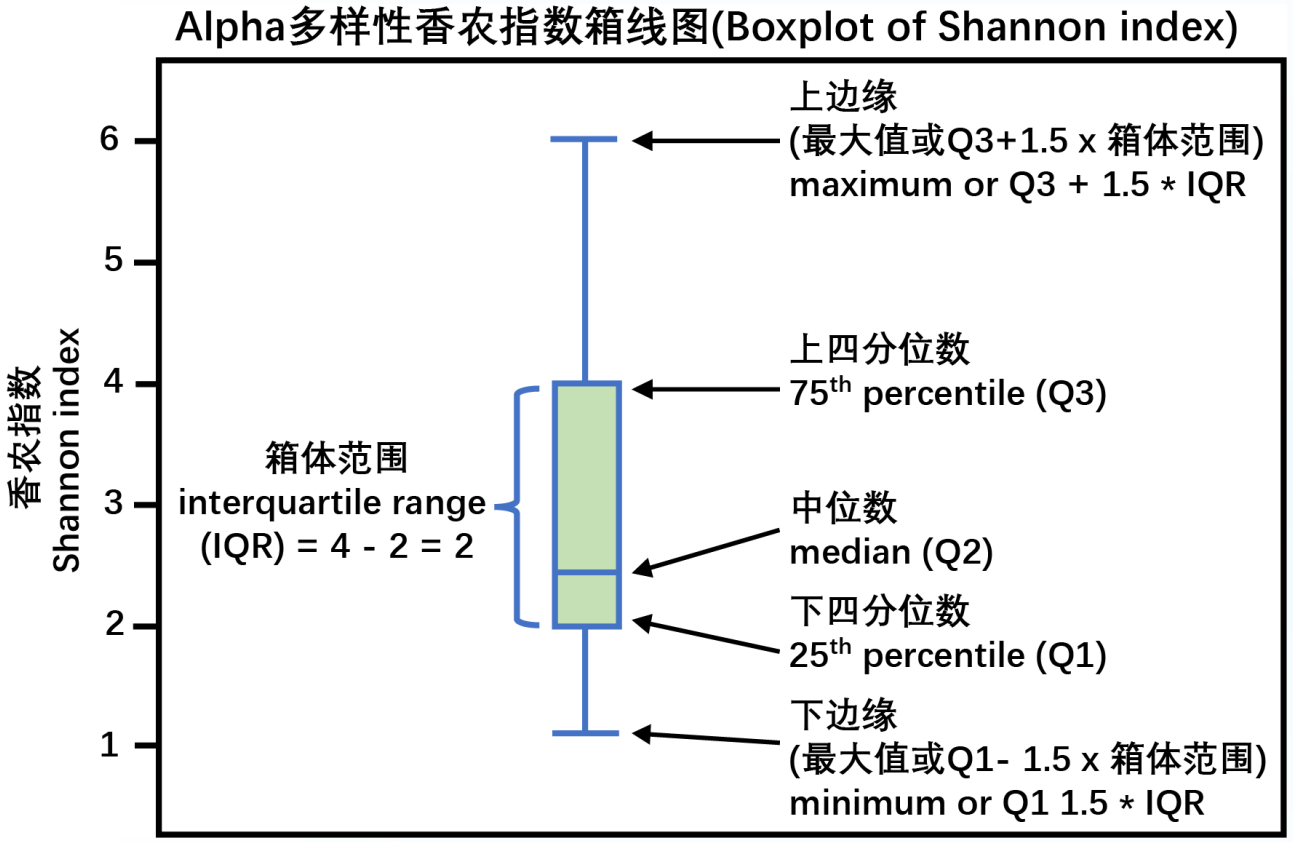


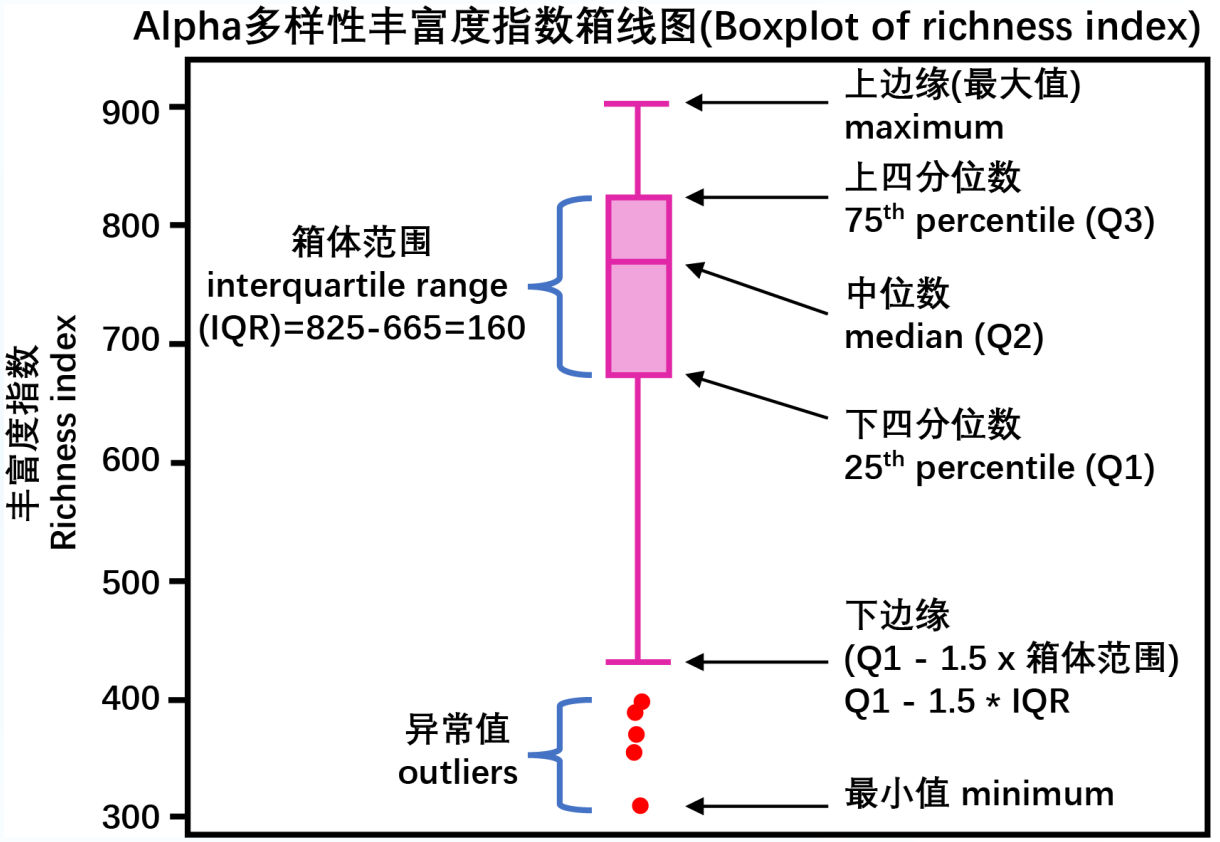






箱线图的基本知识





[iMeta | 刘永鑫组-开发微生物组数据分析与可视化平台MicrobiomeStatPlots]

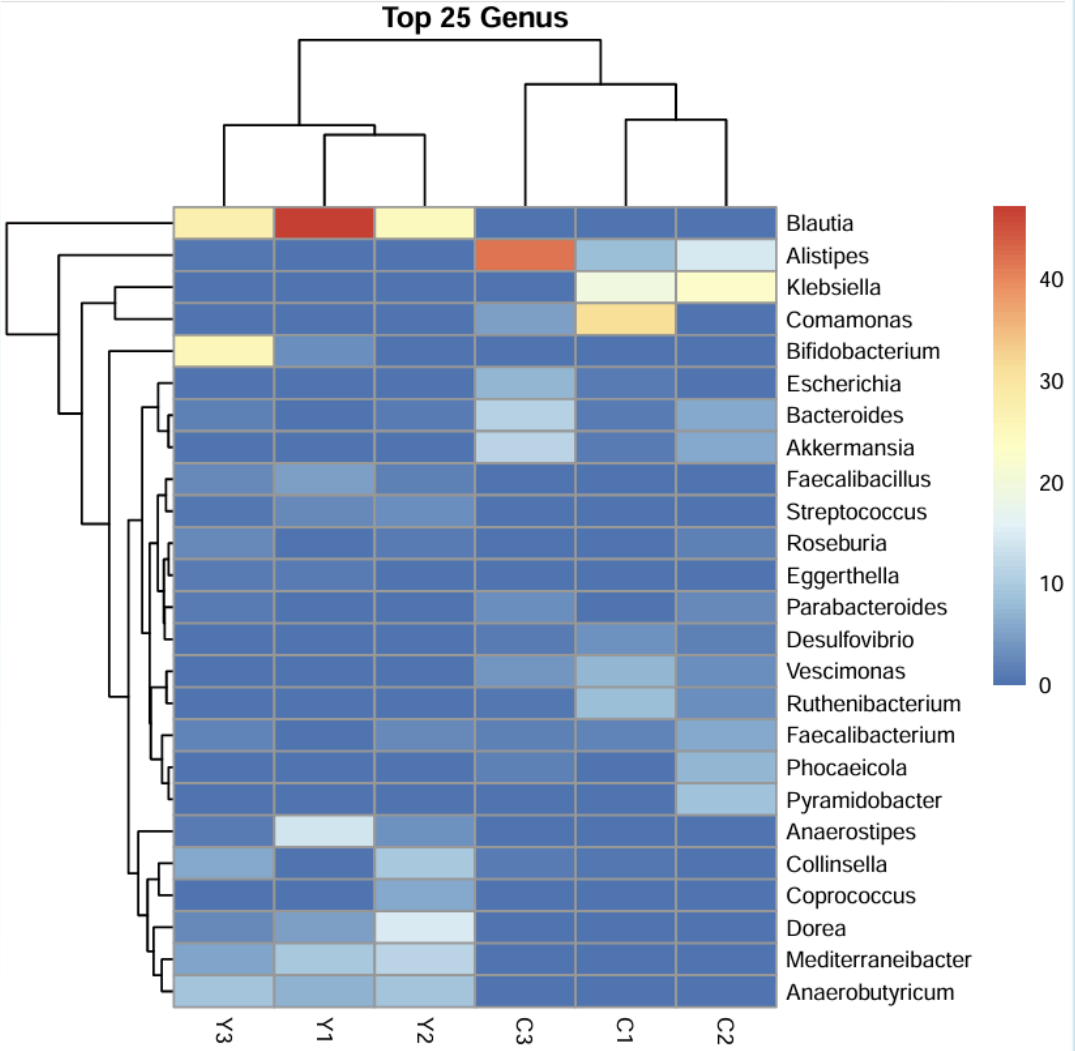
《微生物组数据分析与可视化实战》

2StatPlot.sh - 物种Kraken2 - 热图

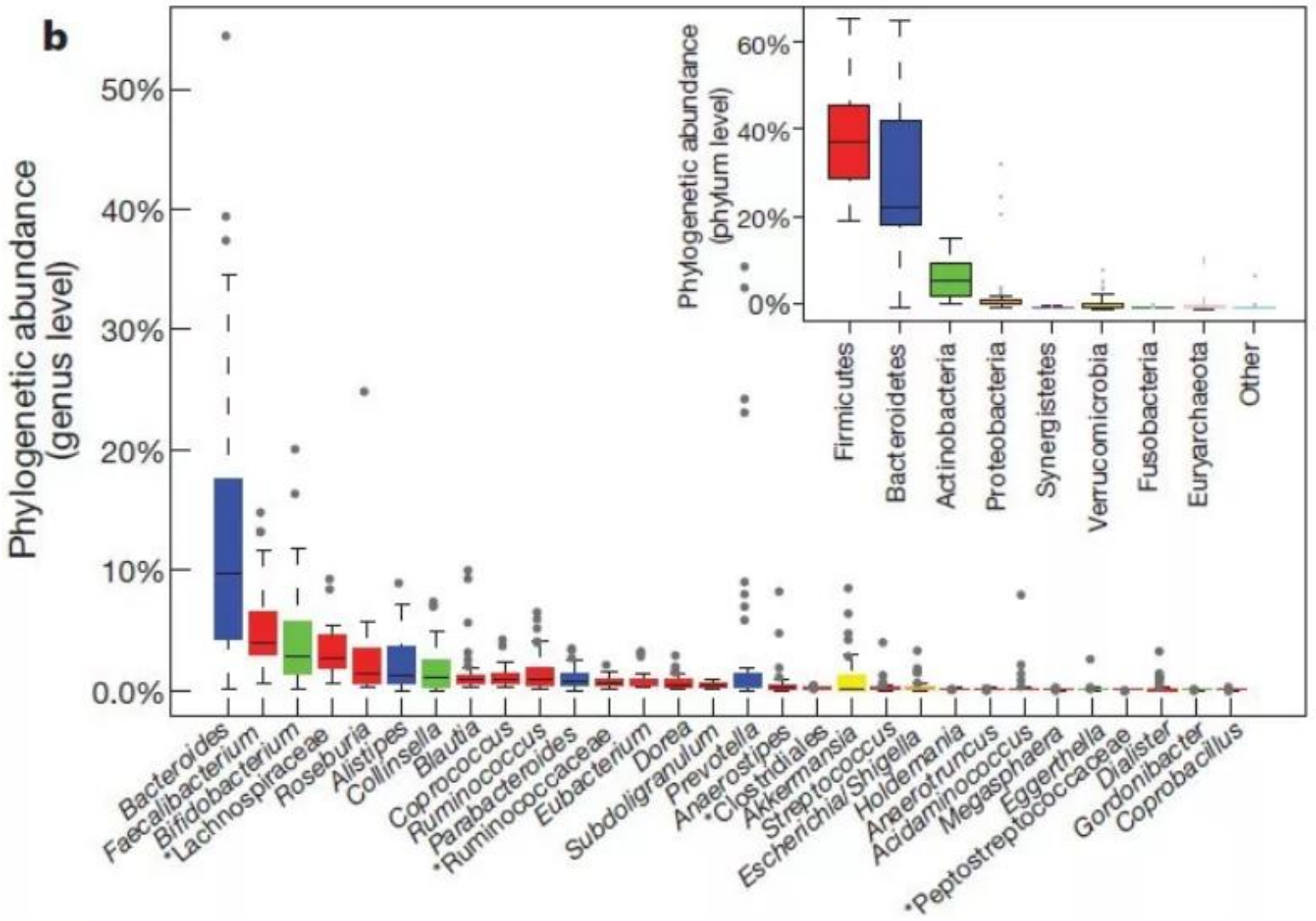
调整输入文件为spf文件，即物种丰度表格

可选分类级Kingdom / Phylum / Class / Order / Family / Genus / Species、分类显示数量

```
Rscript db/script/metaphlan_hclust_heatmap.R \
  -i result/kraken2/tax_count.spf \
  -t Genus \
  -n 25 \
  -o result/kraken2/heatmap_Genus
```



箱线图展示最高丰度的30个属和8个门



箱线图展示最高丰度的30个属。按门着色。同时角上有门水平箱线图。属和门水平丰度计算采用有参比对，85%相似度，65%覆盖度的阈值。未分类的属显示更高水平标注了星号。

Nature：人类肠道微生物组的肠型

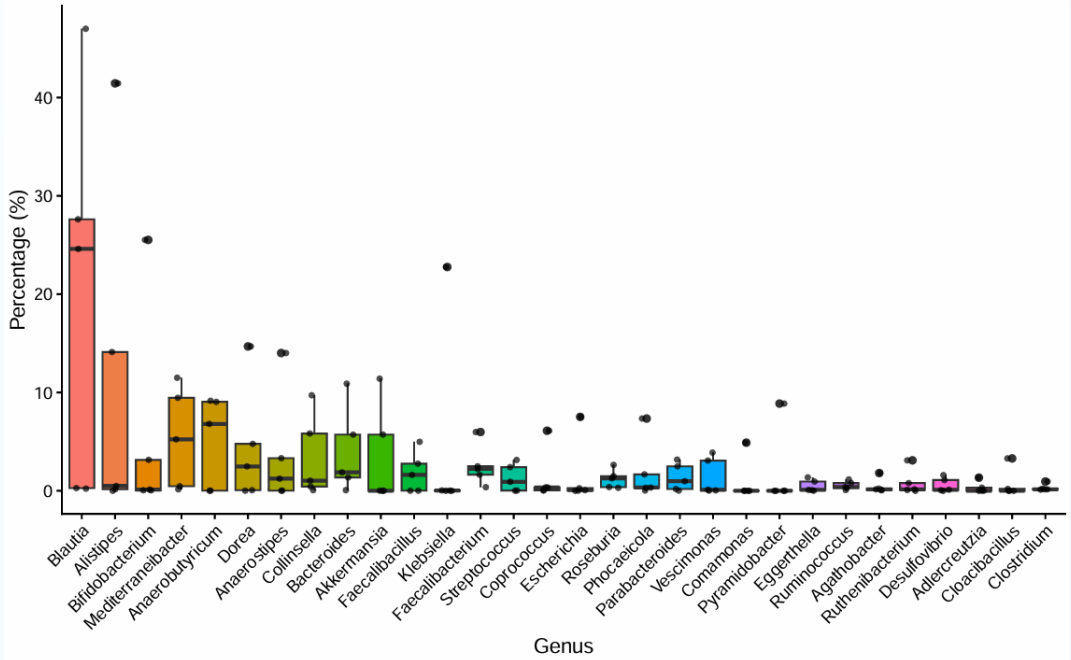
2StatPlot.sh - 物种Kraken2 - 箱线图

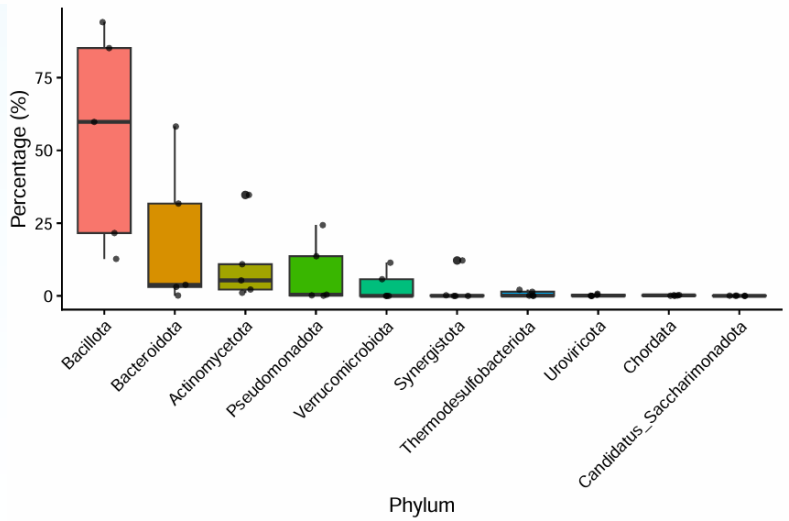
- # 绘制属水平Top30箱线图

```
Rscript ${db}/script/metaphlan_boxplot.R \
  -i result/kraken2/tax_count.spf \
  -t Genus \
  -n 30 \
  -o result/kraken2/boxplot_Genus
```

- # 绘制门水平Top10箱线图

```
Rscript ${db}/script/metaphlan_boxplot.R \
  -i result/kraken2/tax_count.spf \
  -t Phylum \
  -n 10 -w 4 -e 2.5 \
  -o result/kraken2/boxplot_Phylum
```





Bracken估计Kraken2结果丰度

- -d为数据库与kraken2一致，-i为kraken2报告文件
- r是读长，此处默认为100，通常为150
- l为分类级，本次种级别(S)丰度估计，可选域、门、纲、目、科、属、种：**D,P,C,O,F,G,S**，t是阈值，默认0，越大越可靠，但可用数据越少

```
tax=P
for i in `tail -n+2 result/metadata.txt|cut -f1`;do
bracken -d ${db}/kraken2/mini \
    -i temp/kraken2/${i}.report \
    -r 100 -l ${tax} -t 0 \
    -o temp/bracken/${i}
done
```

Bracken结果描述

- 结果描述：共7列，分别为物种名、ID、分类级、读长计数、补充读长计数、**总数、百分比**

name	Taxonomy id	Taxonomy lvl	Kraken assigned reads	Added reads	New est reads	Fraction Total reads
Phixviricota	2732412	P	0	0	**0**	0
Microsporidia	6029	P	0	0	**0**	0
Hofneiviricota	2732410	P	0	0	**0**	0
Proteobacteria	1224	P	1869	14	**1883**	0.05368
Peploviricota	2731361	P	0	0	**0**	0
Spirochaetes	203691	P	9	0	**9**	0.00026
Cercozoa	136419	P	0	0	**0**	0

Bracken结果整合和筛选

- 样本整合为表，同Kraken2类似
- Microbiome Helpler中filter_feature_table.R按出现频率筛选，如1%至少筛选掉全为0的行，默认为20%

```
Rscript ~/db/EasyMicrobiome/script/filter_feature_table.R \
  -i result/kraken2/bracken.${tax}.txt \
  -p 0.01 \
  -o result/kraken2/bracken.${tax}.0.01
```

- # 种水平去除人类P:Chordata,S:Homo sapiens

```
grep -v 'Homo sapiens' result/kraken2/bracken.S.0.01 \
  > result/kraken2/bracken.S.0.01-H
```

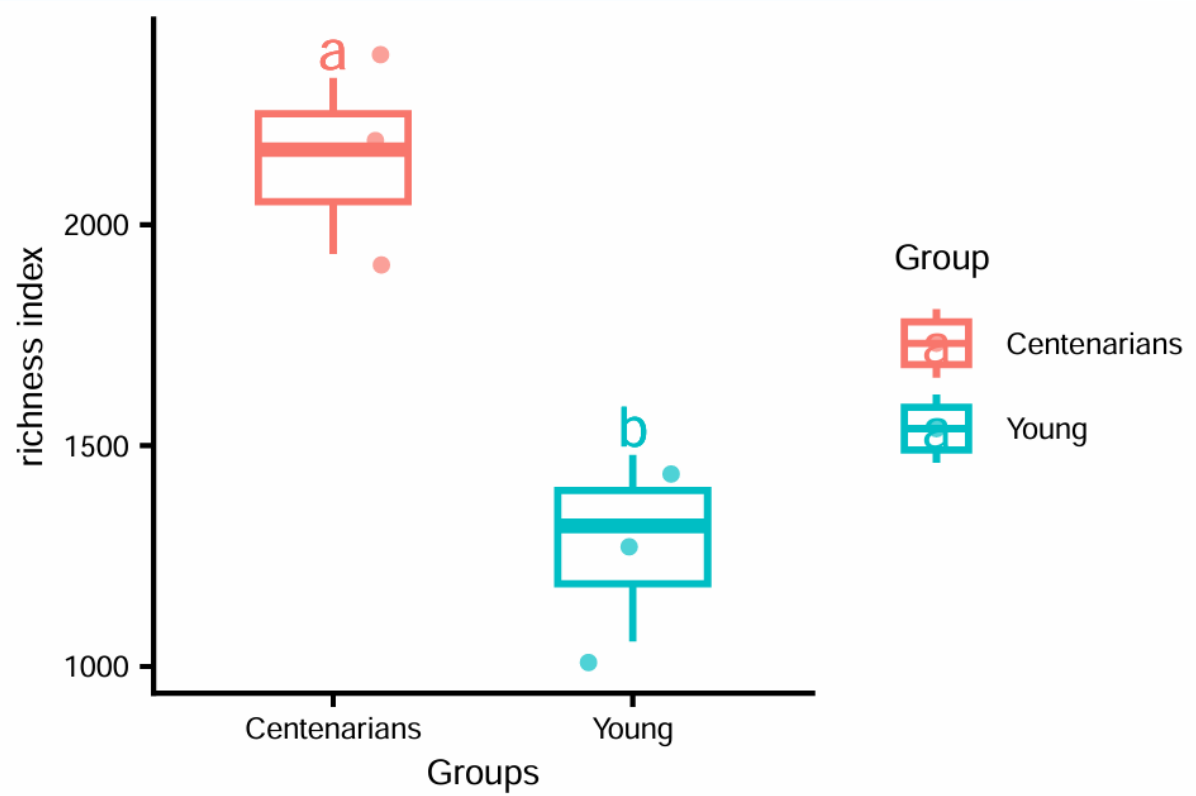
Alpha多样性计算

```
mkdir -p result/kraken2
echo -e "SampleID\tBerger Parker\tSimpson\tinverse Simpson\tShannon" >
result/kraken2/alpha.txt
for i in `tail -n+2 result/metadata.txt|cut -f1`;do
    echo -e -n "$i\t" >> result/kraken2/alpha.txt
    for a in BP Si ISi Sh;do
        alpha_diversity.py -f temp/bracken/${i}.brk -a $a | cut -f 2 -d ':' | tr '\n' '\t' >>
result/kraken2/alpha.txt
    done
    echo "" >> result/kraken2/alpha.txt
done
```

SampleID	Berger Parker	Simpson	inverse Simpson	Shannon
C1	0.197471031414	0.914538184071	11.701132127	3.14863934361
C2	0.494250379692	0.73255534872	3.73909141653	2.54229592925

2StatPlot.sh – ## Braken2 – ### Alpha diversity

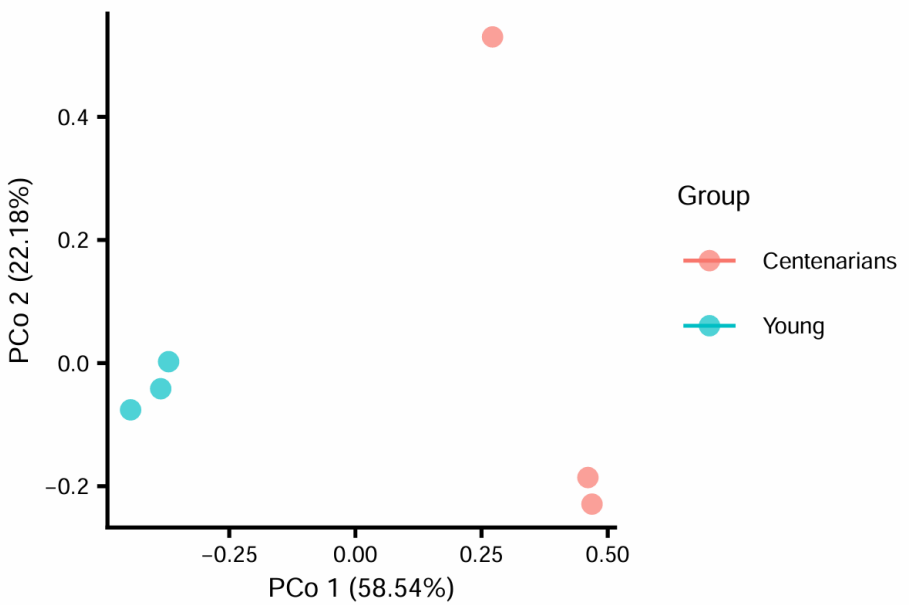
```
tax=S

Rscript $sd/alpha_boxplot.R \
  -i kraken2/bracken.${tax}.alpha \
  -a richness \
  -d metadata.txt \
  -n Group \
  -o kraken2/${tax} \
  -w 89 -e 59
```



Beta多样性计算

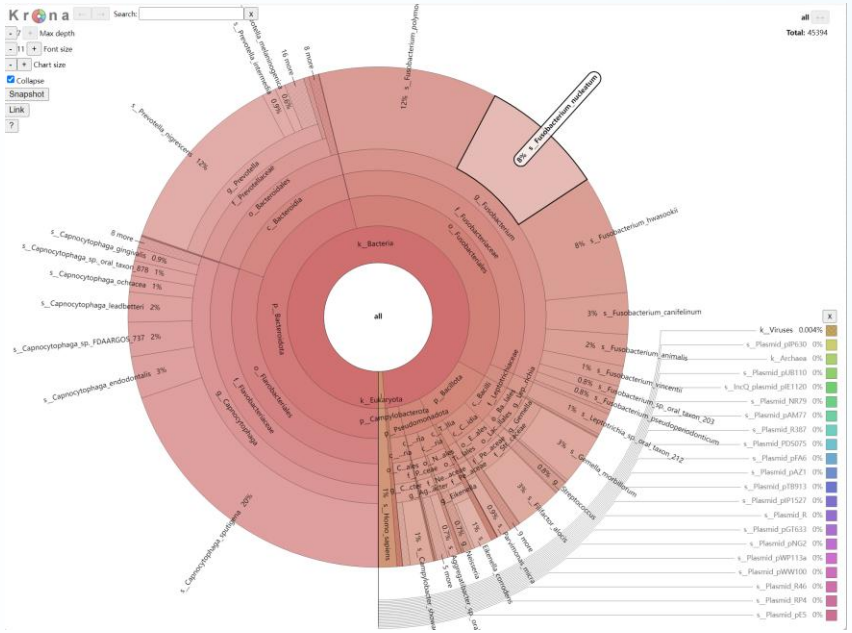
- beta_diversity.py -i temp/bracken/*.brk --type bracken \
- > result/kraken2/beta.txt
- cat result/kraken2/beta.txt

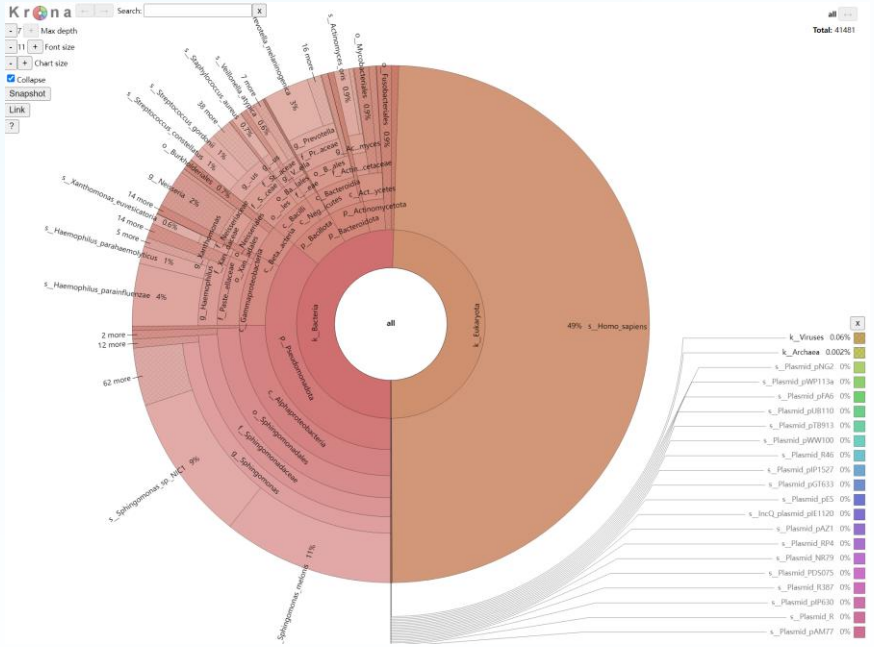
```
#0      temp/bracken/C1.brk (45394 reads)
#1      temp/bracken/C2.brk (41481 reads)
x       0       1
0       0.000   0.940
1       x.xxx   0.000
```



Krona图

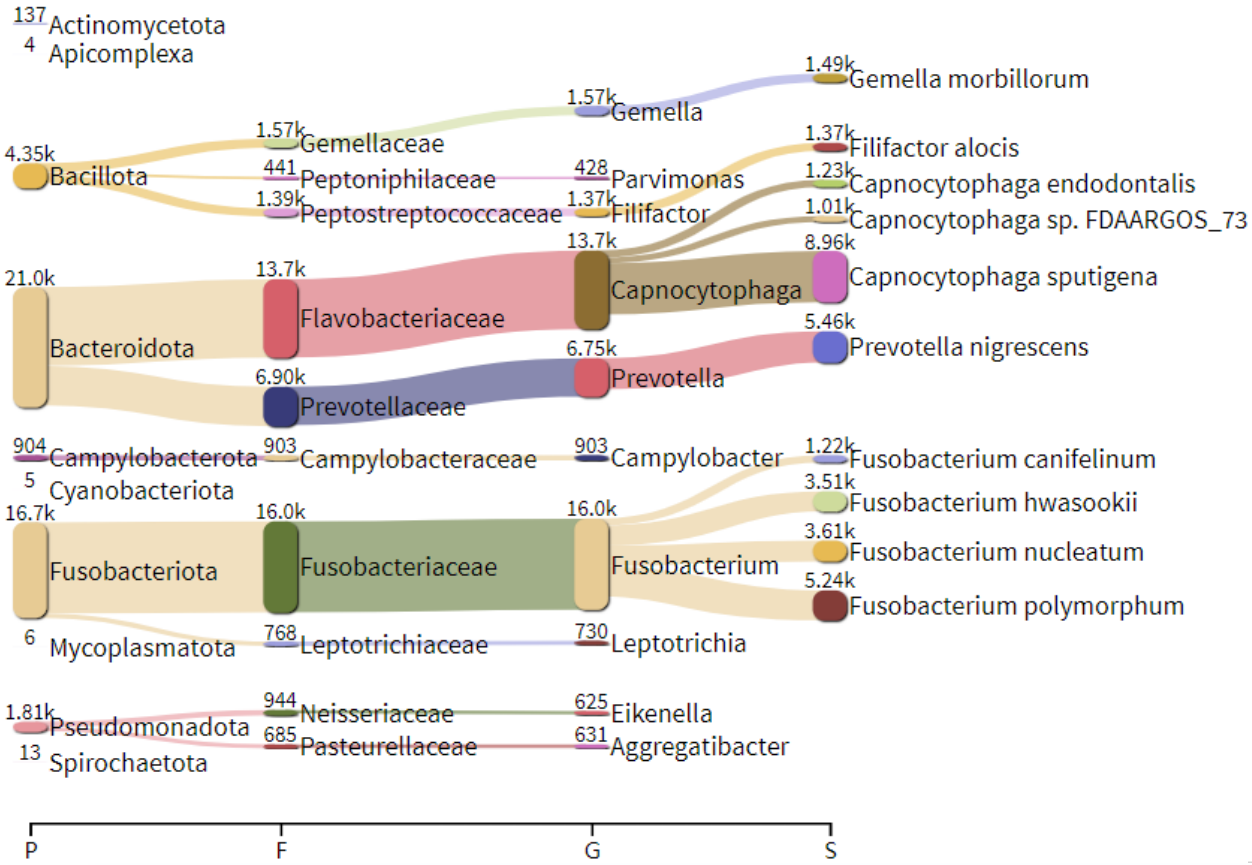
```
for i in `tail -n+2 result/metadata.txt|cut -f1`;do
    kreport2krona.py -r temp/bracken/${i}.report -o temp/bracken/${i}.krona --no-intermediate-ranks
    ktImportText temp/bracken/${i}.krona -o result/kraken2/krona.${i}.html
done
```

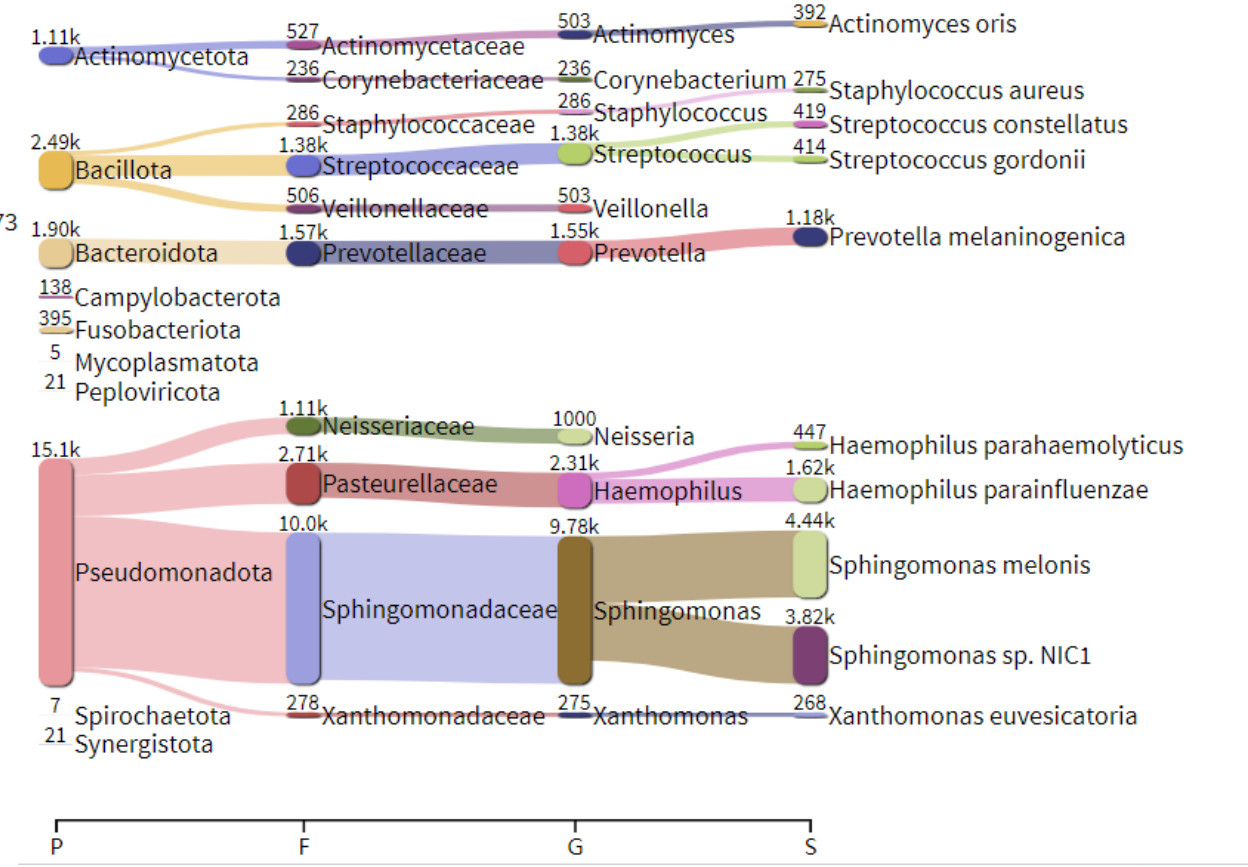




Pavian桑基图

Pavian桑基图：https://fbreitwieser.shinyapps.io/pavian/ 在线可视化:，左侧菜单，Upload sample set (temp/bracken/*.report)，支持多样本同时上传；Sample查看结果，Configure Sankey配置图样式，Save Network下载图网页





2StatPlot.sh - 物种Kraken2 – Alpha/Beta多样性

- 多样性计算需要抽平并计算alpha多样性，-d指定最小样本量，默认0为最小值，抽平文件bracken.S.norm，alpha多样性bracken.S.alpha

```
tax=S
Rscript $sd/otutab_rare.R \
  --input result/kraken2/bracken.${tax}.txt \
  --depth 0 --seed 1 \
  --normalize result/kraken2/bracken.${tax}.norm \
  --output result/kraken2/bracken.${tax}.alpha
```

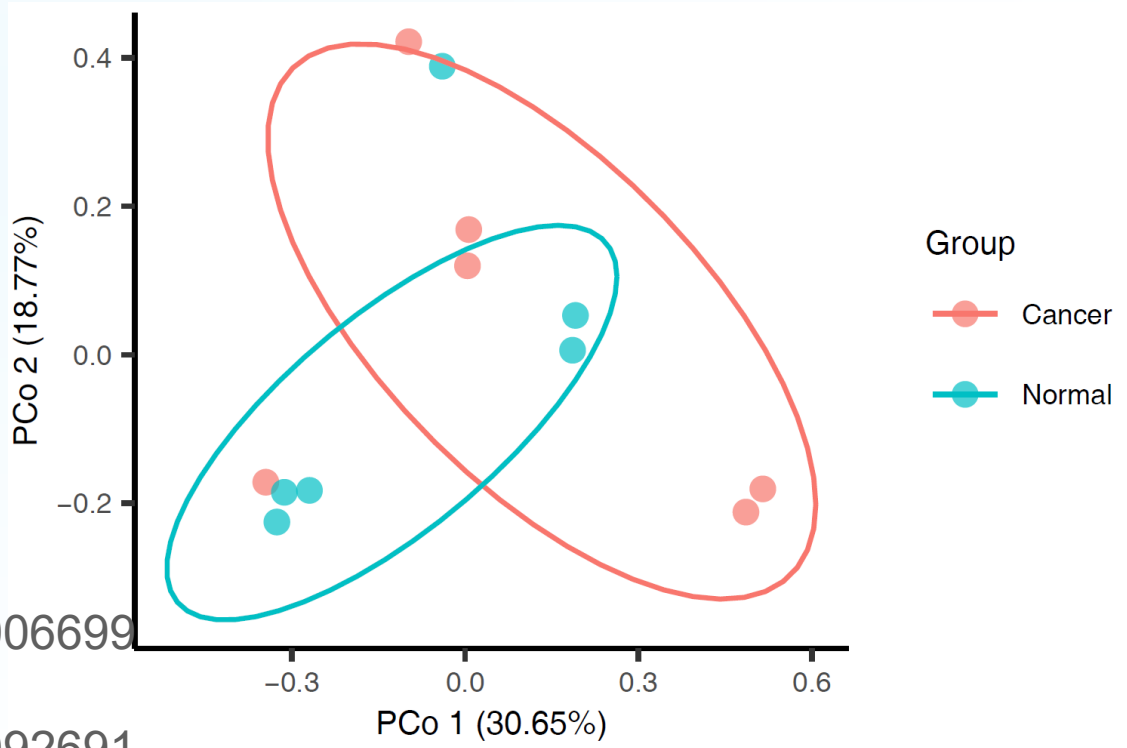
- Beta多样性距离矩阵计算

```
usearch -beta_div result/kraken2/bracken.${tax}.norm \
    -filename_prefix result/kraken2/beta/
```

2StatPlot.sh - 物种Kraken2 –Beta多样性

- Bracken的Reads更多，Alpha多样性丰富度大于Kraken2的结果
- Beta多样性可选距离有 bray_curtis, euclidean, jaccard, manhattan

```
dis=bray_curtis
Rscript $sd/beta_pcoa.R \
  --input result/kraken2/beta/${dis}.txt \
  --design result/metadata.txt \
  --group Group \
  --width 89 --height 59 \
  --output result/kraken2/pcoa.${dis}.pdf
```

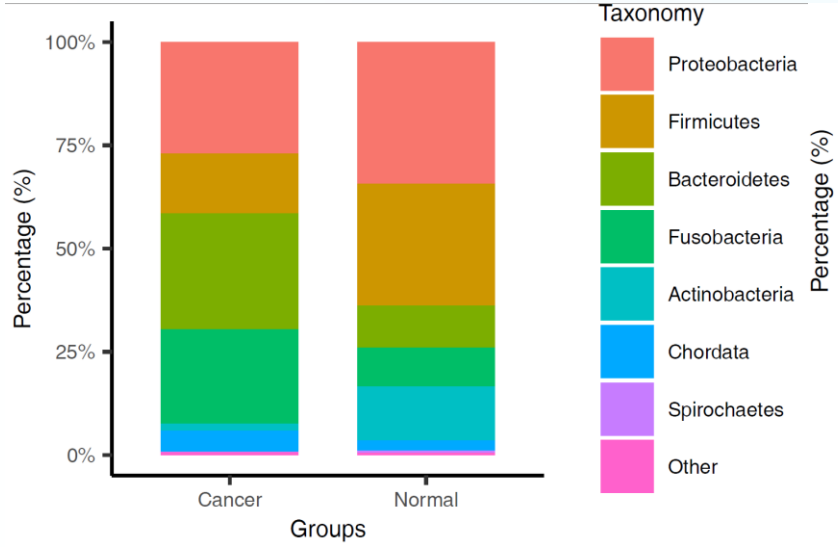


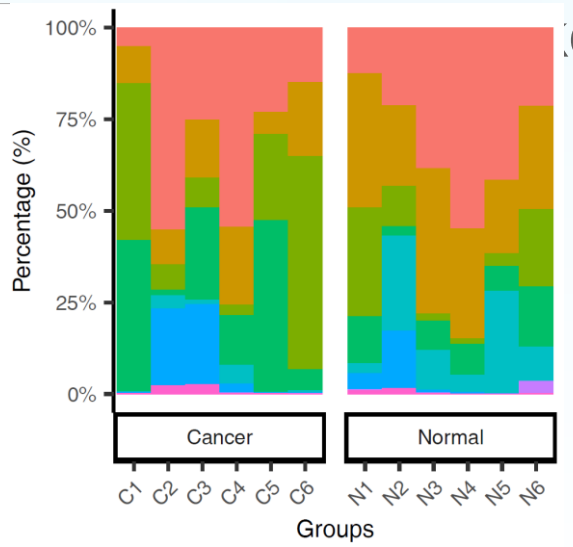

```
统计结果文件:          Sun Jan 03 16:19:07 2021
beta_pcoa_stat.txt     Cancer   Normal   0.300669933006699
P值有波动但比较稳定    Sun Jan 03 17:55:04 2021
                       Cancer   Normal   0.309269073092691
```

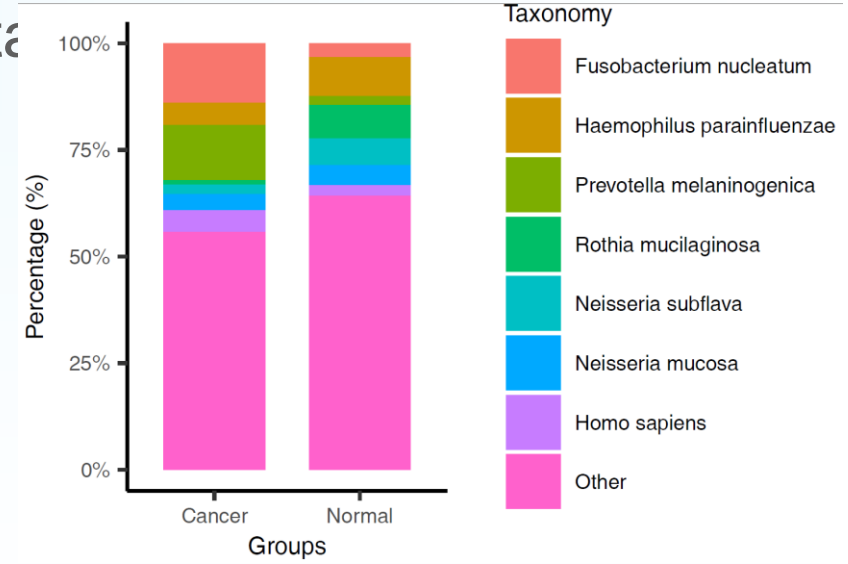
2StatPlot.sh - 物种Kraken2 – 堆叠柱状图

- 以门(P)/种(S)水平为例，结果包括output.sample/group.pdf两个文件

```
tax=S

Rscript ${sd}//tax_stackplot.R \

  --input result/kraken2/bracken.${tax}.txt --design result/metadata.txt \
```







extract_kraken_reads.py去宿主-提取非植物33090和动物(人)33208序列

```
tail -n+2 result/metadata.txt|cut -f1 |rush -j 3 \
    "extract_kraken_reads.py \
    -k temp/kraken2/{1}.output \
    -r temp/kraken2/{1}.report \
    -1 temp/qc/{1}_1_kneaddata_paired_1.fastq \
    -2 temp/qc/{1}_1_kneaddata_paired_2.fastq \
    -t 33090 33208 --include-children --exclude \
    --max 20000000 --fastq-output \
    -o temp/kraken2_qc/{1}_1.fq \
    -o2 temp/kraken2_qc/{1}_2.fq"
```

KrakenUniq: 基于唯一K-mer获得特异宏基因组分类

内存消耗很大，适合敏感的病原检测

- https://github.com/fbreitwieser/krakenuniq

KrakenUniq: confident and fast metagenomics classification using unique k-mer counts

False-positive identifications are a significant problem in metagenomics classification. KrakenUniq (formerly KrakenHLL) is a novel metagenomics classifier that combines the fast k-mer-based classification of Kraken with an efficient algorithm for assessing the coverage of unique k-mers found in each species in a dataset. On various test datasets, KrakenUniq gives better recall and precision than other methods and effectively classifies and distinguishes pathogens with low abundance from false positives in infectious disease samples. By using the probabilistic cardinality estimator HyperLogLog, KrakenUniq runs as fast as Kraken and requires little additional memory.

F. P. Breitwieser, D. N. Baker, S. L. Salzberg. 2018. KrakenUniq: confident and fast metagenomics classification using unique k-mer counts. ***Genome Biology*** 19: 198. https://doi.org/10.1186/s13059-018-1568-0

Jennifer Lu, Natalia Rincon, Derrick E. Wood, *et al.* 2022. Metagenome analysis using the Kraken software suite. ***Nature Protocols*** 17: 2815-2839. https://doi.org/10.1038/s41596-022-00738-y

总结

- 物种注释(界门纲目科属种)类似于地址，表明物种间关系远近，不同分类数据库结果差别较大，分类方法常用比对Best hit和精确匹配LCA；
- Kraken2运行速度快、数据库可大可小、结果为计数型counts格式，可抽平开展多样性分析，也可绘制各级热图和箱线图进行整体描述；
- 下游有Bracken2丰度重估计，KrakenTools格式转换和筛选；
- 物种组成表下游STAMP/LEfSe和扩增子课程R语言多样性分析；
- 常用的物种可视化工具有GranPhlAn(公认最美，使用复杂、输入文件准备复杂)、microbiomeViz(R中重复LEfSe结果)、Metacoder(非常有特色)和Krona(跨平台、交互式网页结果)等多种风格可选

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
- iMeta | 易宏基因组(EasyMetagenome): 用户友好且灵活的宏基因组测序数据分析流程
- iMeta | MicrobiomeStatPlot 微生物组数据分析——50+篇
- iMetaOmics | 易扩增子(EasyAmplicon)：用户友好的扩增子测序数据分析指南
- 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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