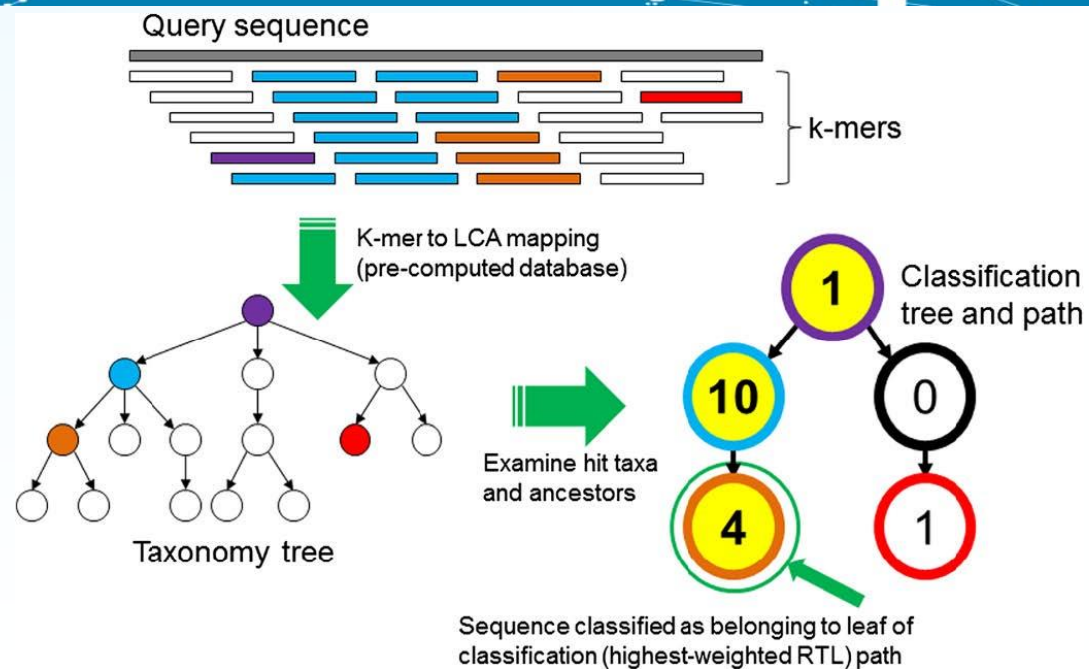




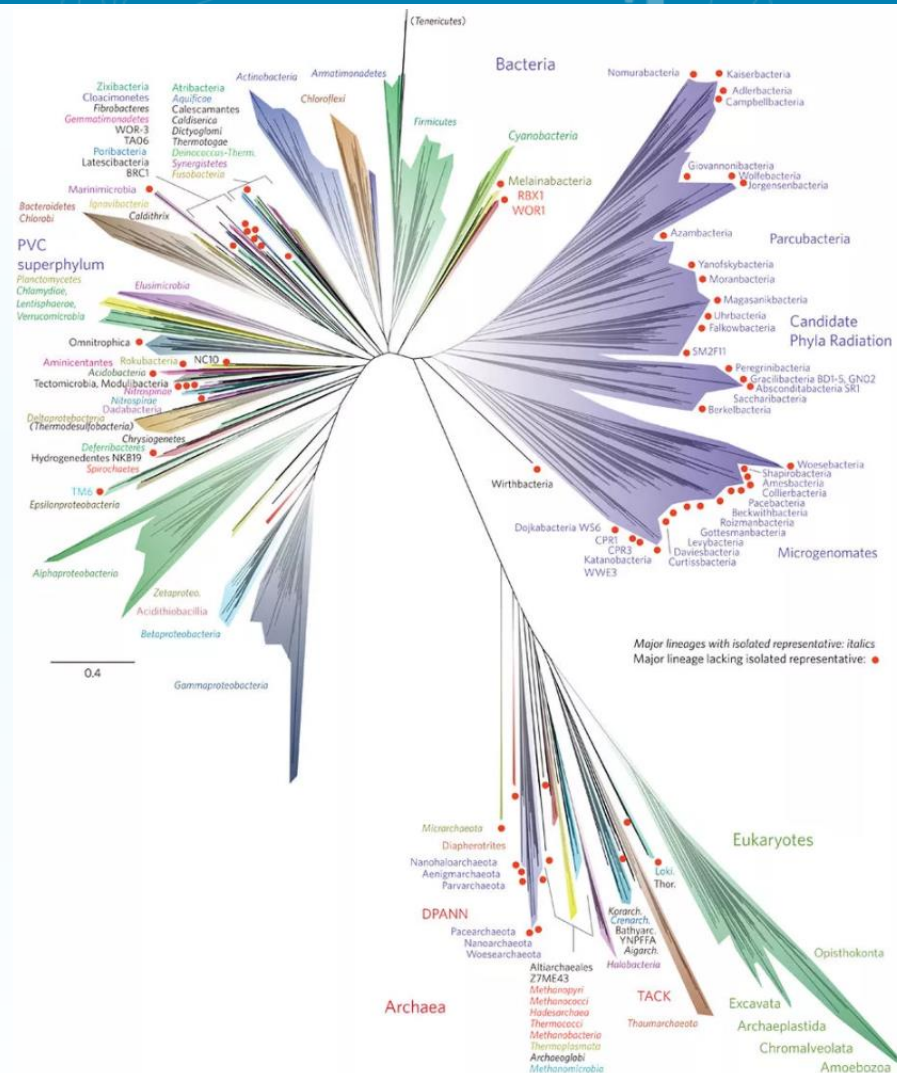
25 Kraken2物种注释

易生信
2023年11月25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

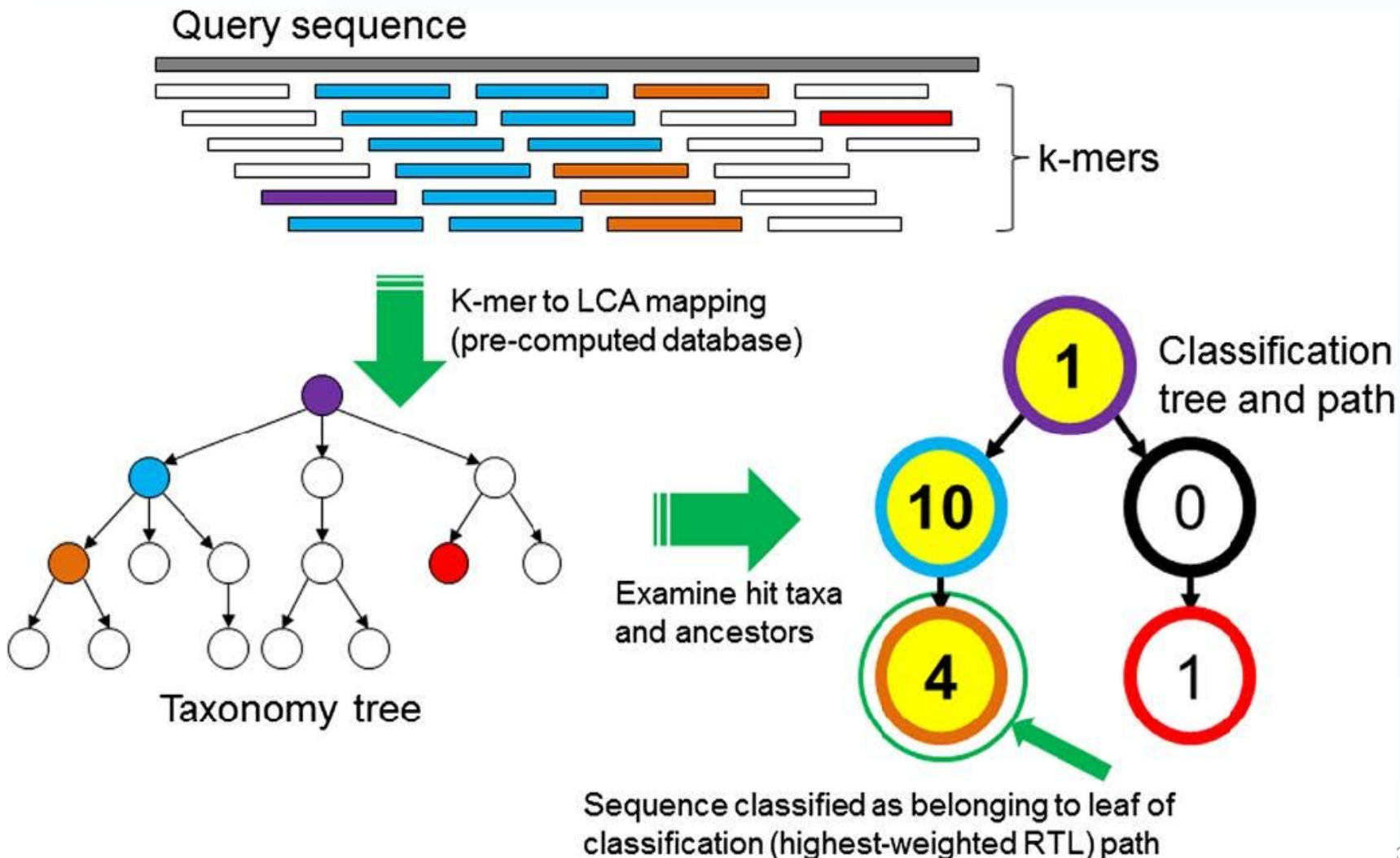


- NCBI——NR非冗余序列，NCBI发布的序列包含物种Taxonomy ID
- MetaPhlAn2——整理已发表基因组Marker基因数据库
- GTDB——基因组细菌120/古菌122单拷贝基因
- GreenGenes/RDP——原核生物核糖体(16S)数据库
- SILVA——原核、真核核糖体(16/18S)数据库



- 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



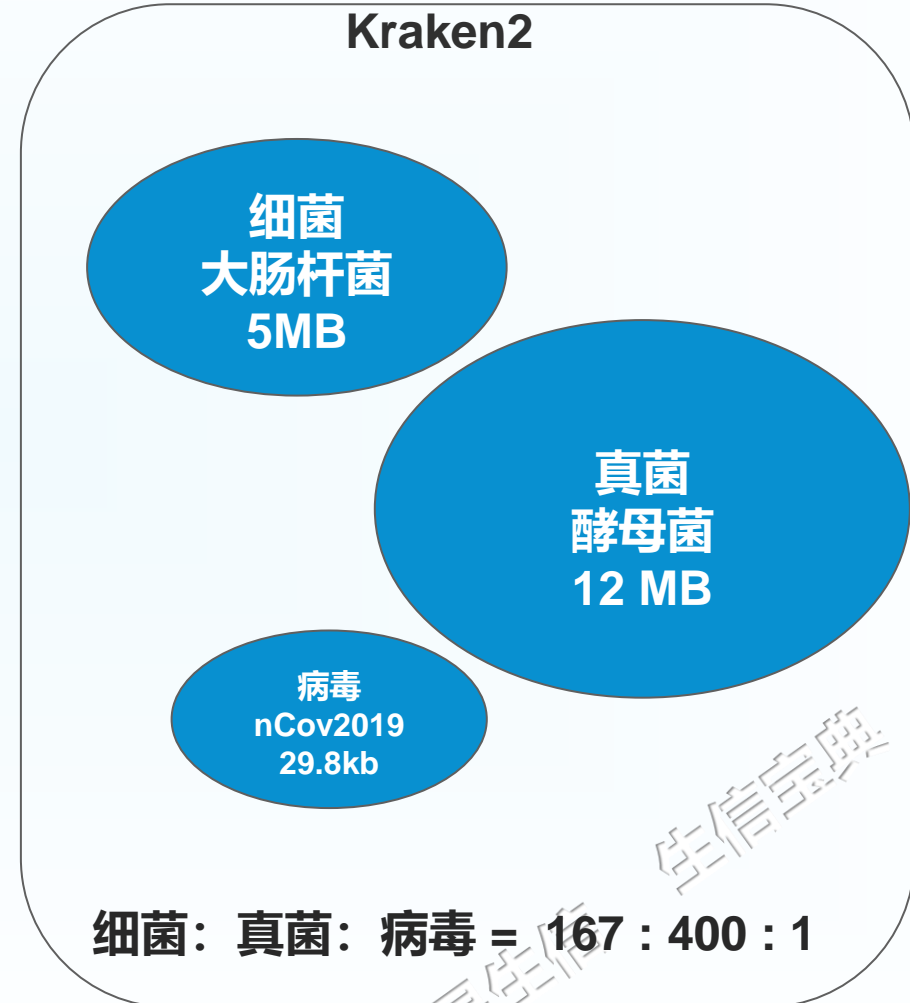
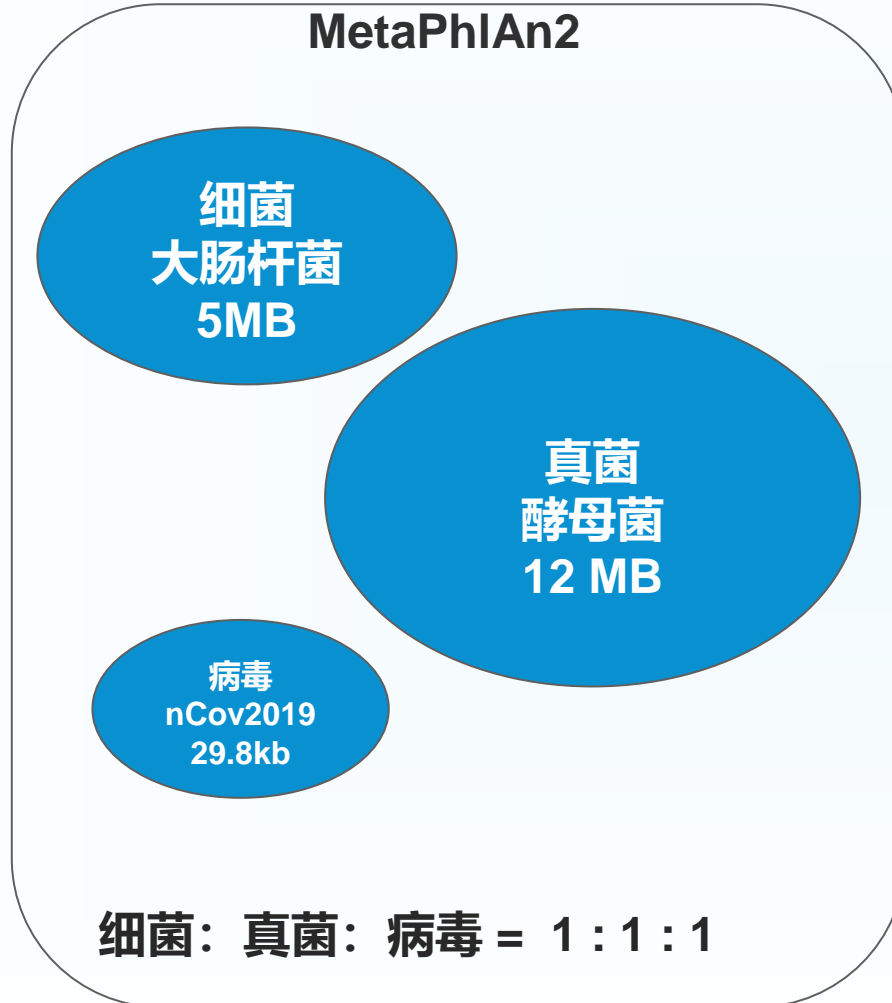
Lower Common Ancestor
最低共同祖先

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：刘洋或、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



- 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>

DerrickWood / **kraken2** Public

<> Code Issues 327 Pull requests 16 Actions Proj

master 5 branches 7 tags Go to file

BenLangmead Merge pull request #697 from ch4rr0/pyth... df20a8f

data	Add small viral testing set
docs	Prep for 2.1.2
scripts	Merge pull request #697 from ch4rr0/python
src	Merge pull request #675 from ch4rr0/maskin

Scholar

Kraken

About 34,300 results (0.06 sec)

[HTML] **Kraken**: ultrafast metagenomic sequence classification using exact alignments

DE Wood, SL Salzberg - Genome biology, 2014 - genomebiology.biomedcentral.com

... **Kraken**, along with a reduced memory version of **Kraken** (MiniKraken), quick execution versions of both (**Kraken**-Q and MiniKraken-Q), and **Kraken** ... genomes from GenBank (**Kraken**-GB).

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[HTML] Improved metagenomic analysis with **Kraken 2**

DE Wood, J Lu, B Langmead - Genome biology, 2019 - Springer

Although **Kraken**'s k-mer-based approach provides a fast taxonomic classification of metagenomic sequence data, its large memory requirements can be limiting for some applications. ...

☆ Save Cite Cited by 2078 Related articles All 14 versions

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>



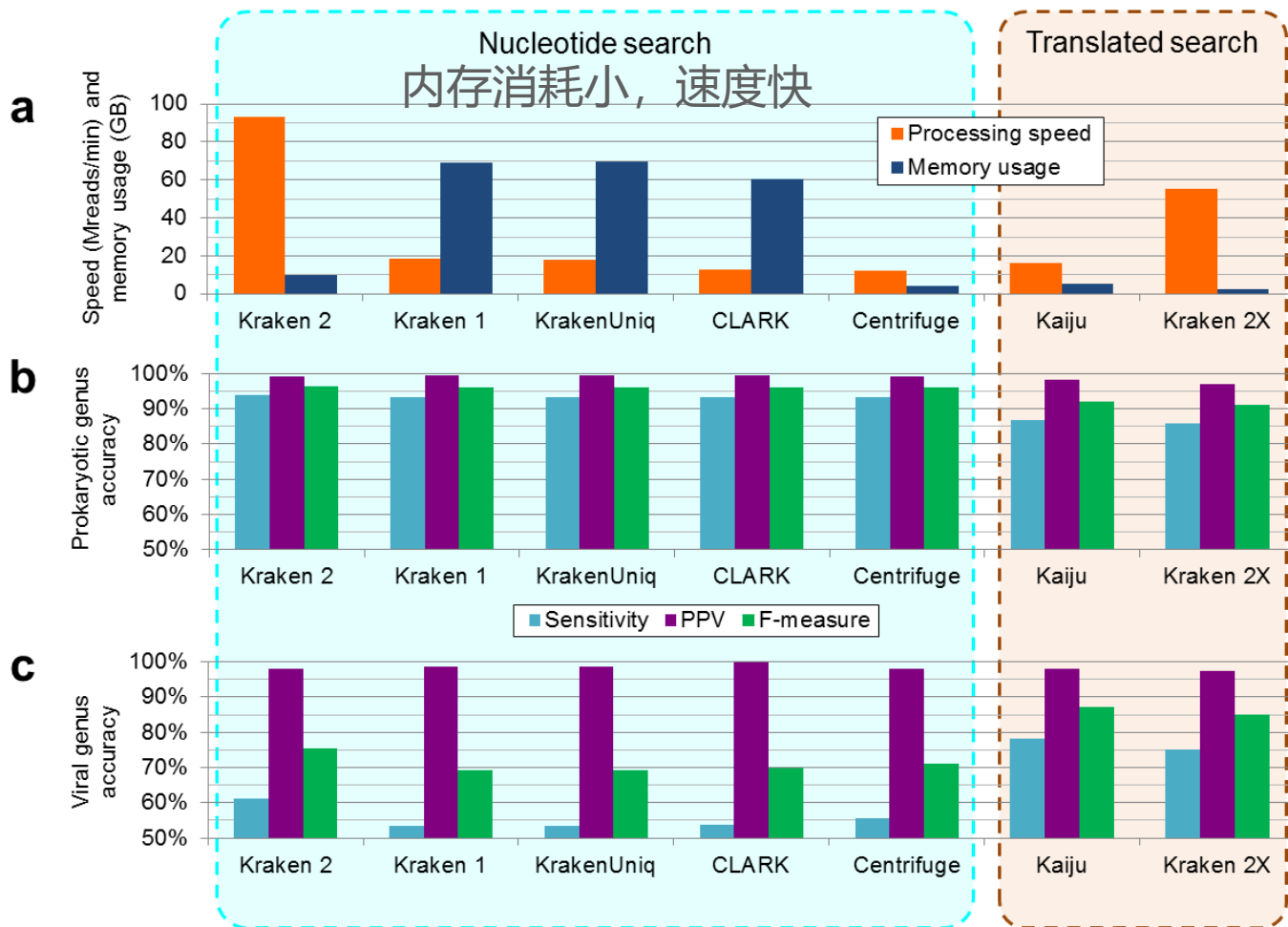
Kraken2与其它工具比较

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(PlusPFP-8/16) , 包括标准+原生动物+真菌+植物及Bracken2索引, 仅8G或16G, 可选完整版144G
- **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/plusfp8g --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



Kraken2实现kraken2结果的格式转换和筛选

○ 安装

```
conda install kraken2 -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
```

易生信
毕生缘
宏基因组



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	C4	C5	C6	N1	N2	N3	N4	N5	N6	
d__Bacteria	44672	21242	19694	42120	45493	48646	33573	22230	45868	50203	52489	45116	
d__Bacteria p__Actinobacteria			304	880	383	2330	107	561	1402	6151	5284	2732	139
d__Bacteria p__Actinobacteria c__Actinobacteria				54	871	380	2319	103	416	1118	6081	5279	272
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales					12	394	61	46	28	63	571	1393	457
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae							12	394	61	46	28	63	571
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces								9	388	57	25	27	49
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces gaoshouyii										1	0	0	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces hongkongensis										0	0	2	7
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces howellii										0	2	0	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces israelii										0	0	0	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces oris										0	319	29	2
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces pacaensis										8	0	3	13
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces radicidentis										0	2	2	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces slackii										0	3	1	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces sp. Chiba101										0	1	1	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces sp. dk561										0	0	0	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces sp. oral taxon 414											0	4	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces sp. oral taxon 897											0	1	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces sp. Z16										0	3	0	0
d__Bacteria p__Actinobacteria c__Actinobacteria o__Actinomycetales f__Actinomycetaceae g__Actinomyces s__Actinomyces viscosus										0	37	13	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



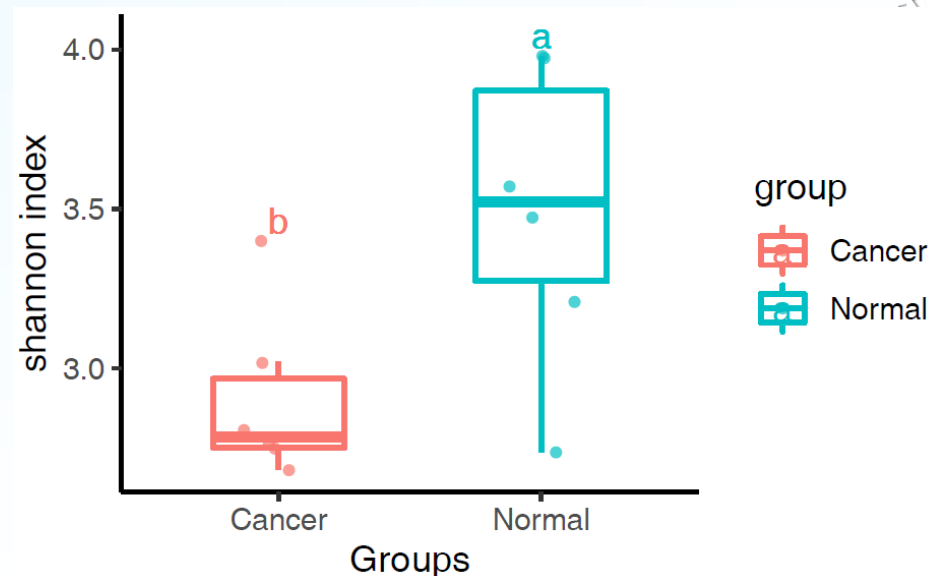
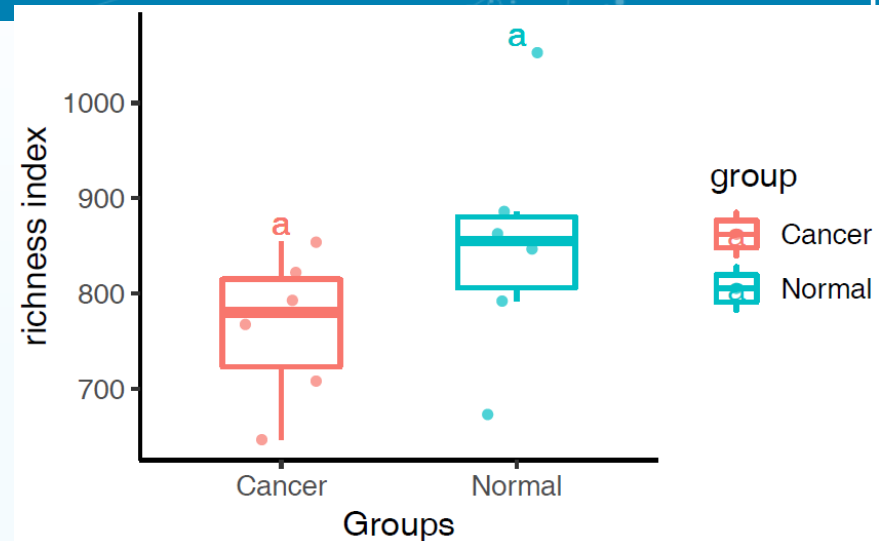
3StatPlot.sh - 物种Kraken2 - Alpha多样性

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
```

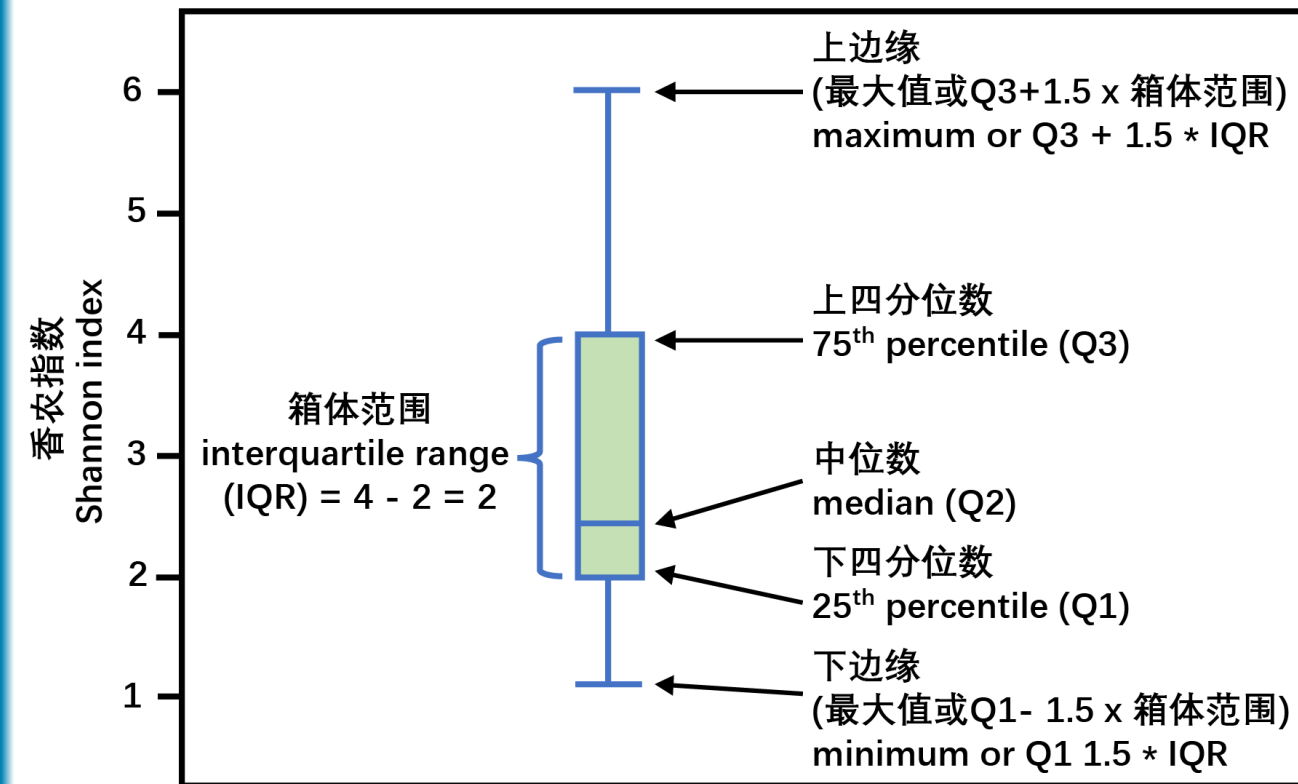
绘制箱线图,可选richness/chao1/shannon...

```
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
```

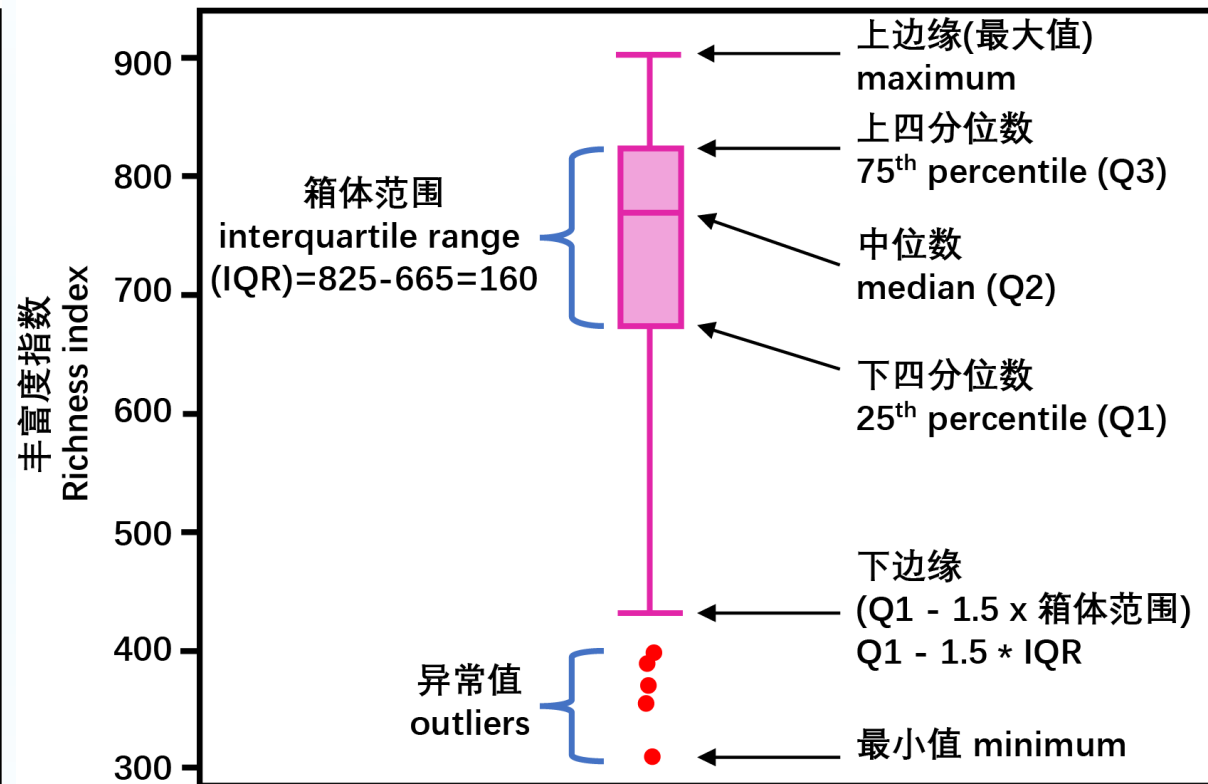


箱线图的基本知识

Alpha多样性香农指数箱线图(Boxplot of Shannon index)



Alpha多样性丰富度指数箱线图(Boxplot of richness index)



中文：百度百科箱形图：<https://baike.baidu.com/item/箱形图>

英文：斯坦福大学统计公开课<https://lagunita.stanford.edu/courses/Medicine/MedStats-SP/SelfPaced/about>

图注写法：[NBT封面：水稻NRT1.1B基因调控根系微生物组参与氮利用*](#)

3StatPlot.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

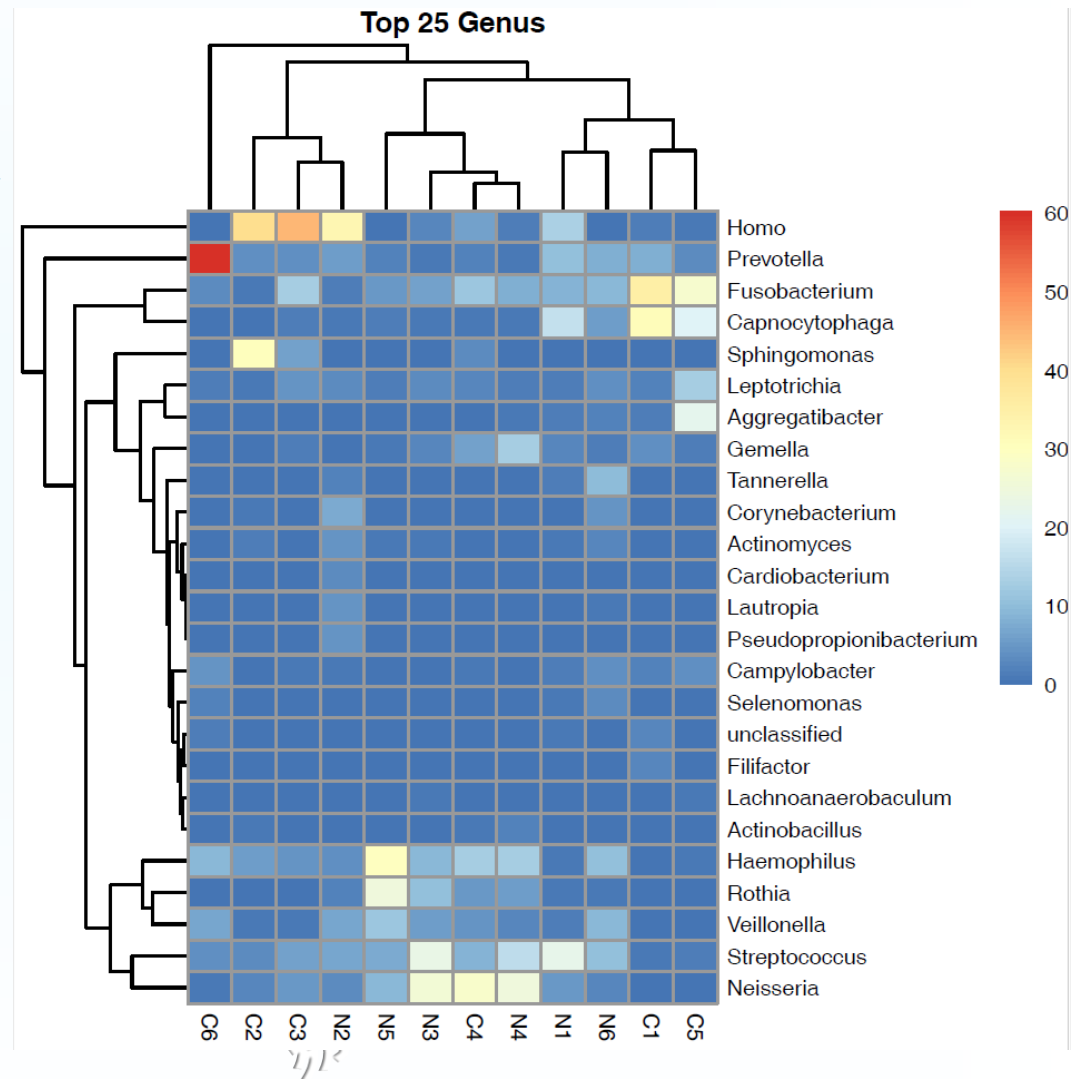
db/script/metaphlan_hclust_heatmap.R \

-i result/kraken2/tax_count.spf \

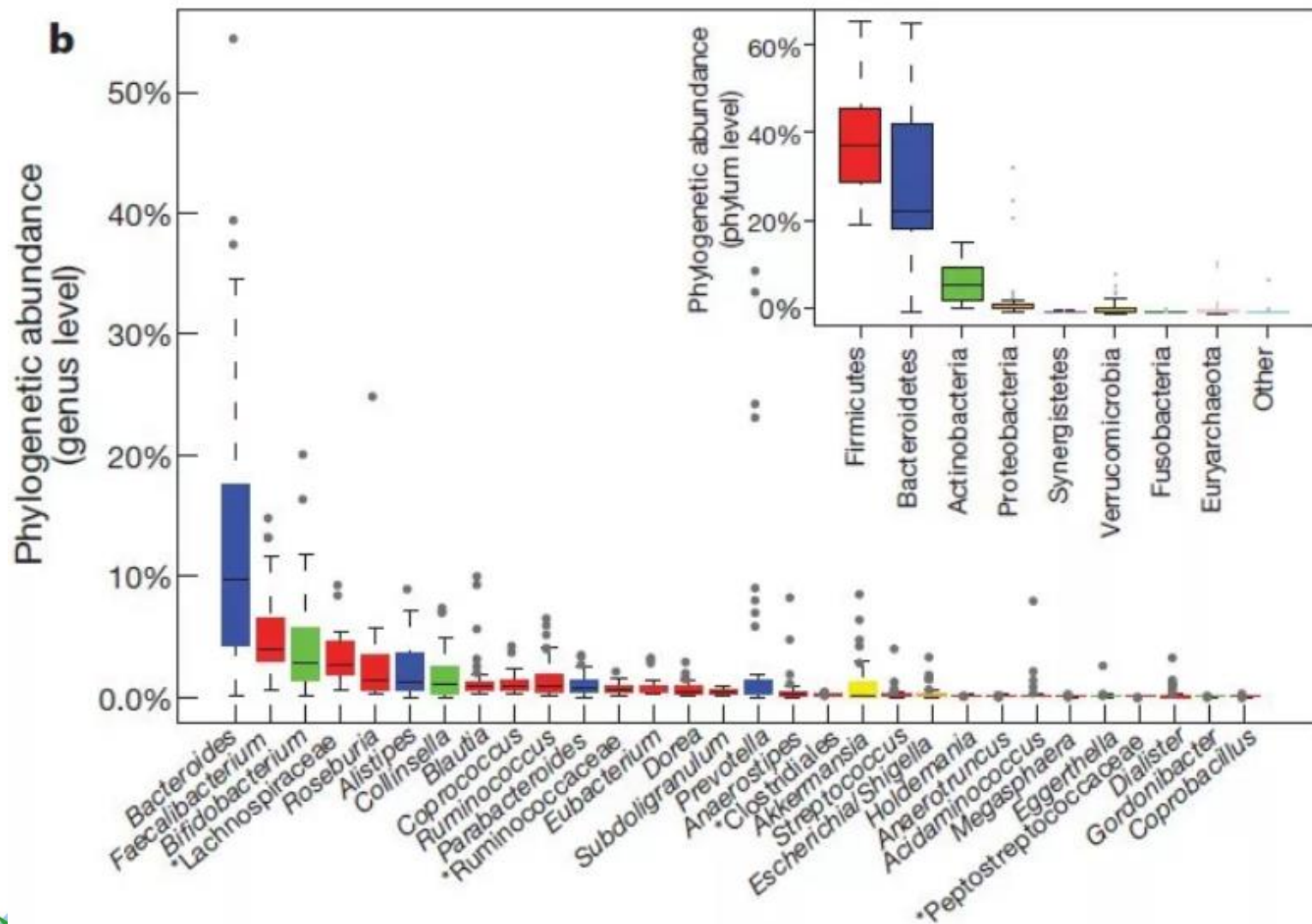
-t **Genus** \

-n **25** \

-o result/kraken2/heatmap_Genus



箱线图展示最高丰度的30个属和8个门

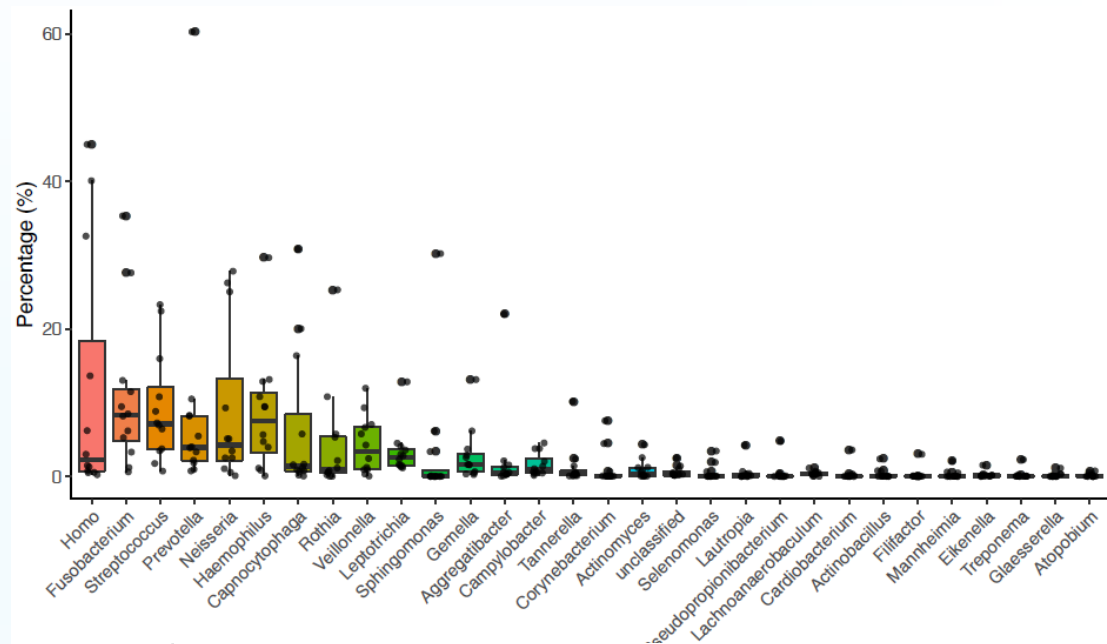


箱线图展示最高丰度的30个属。按门着色。同时角上有门水平箱线图。属和门水平丰度计算采用有参比对，85%相似度，65%覆盖度的阈值。未分类的属显示更高水平标注了星号。

3StatPlot.sh - 物种Kraken2 - 箱线图

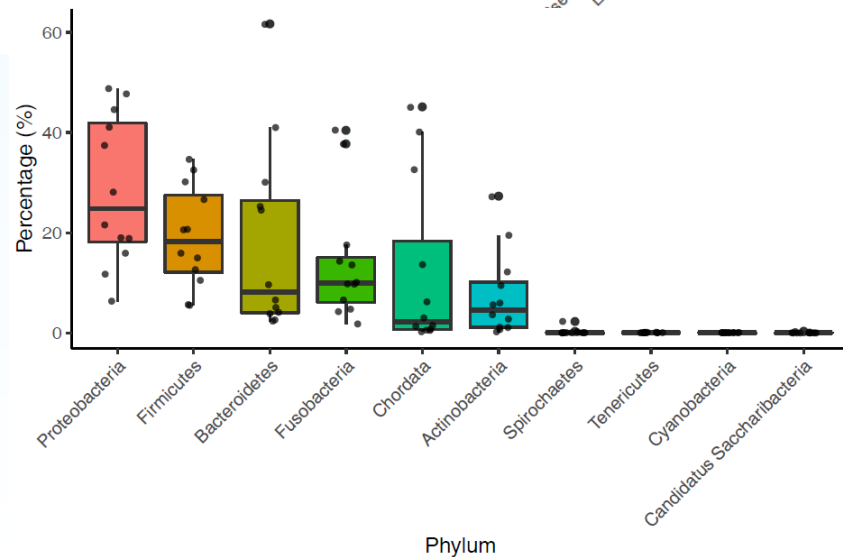
绘制属水平Top30箱线图

```
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

- 样本整合为表，同Kraken2类似
- Microbiome Helper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



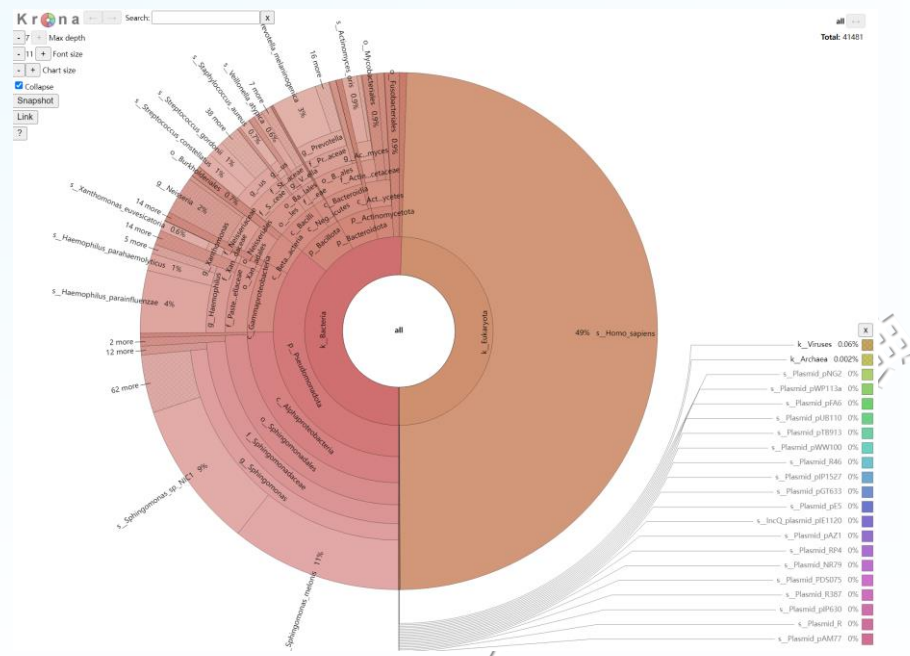
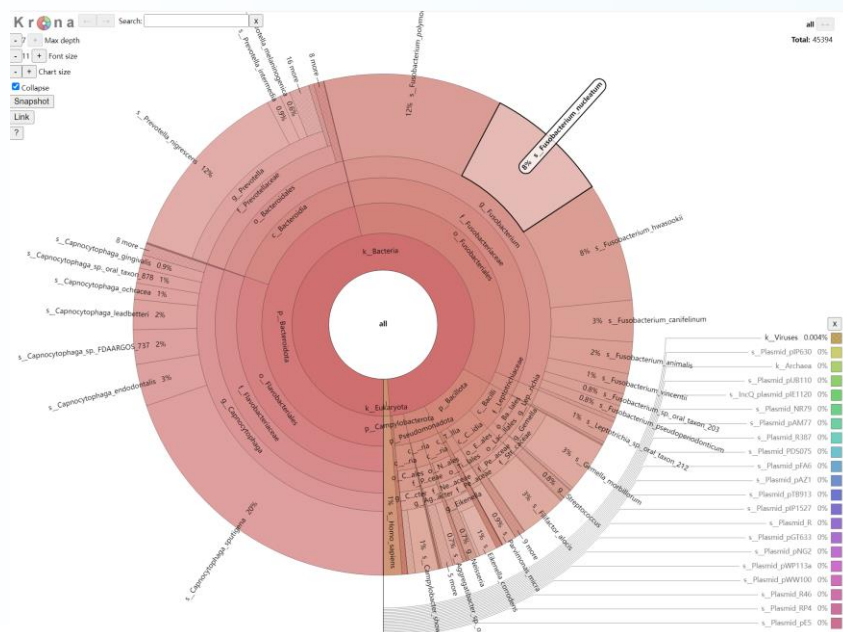
Beta多样性计算

- `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
```



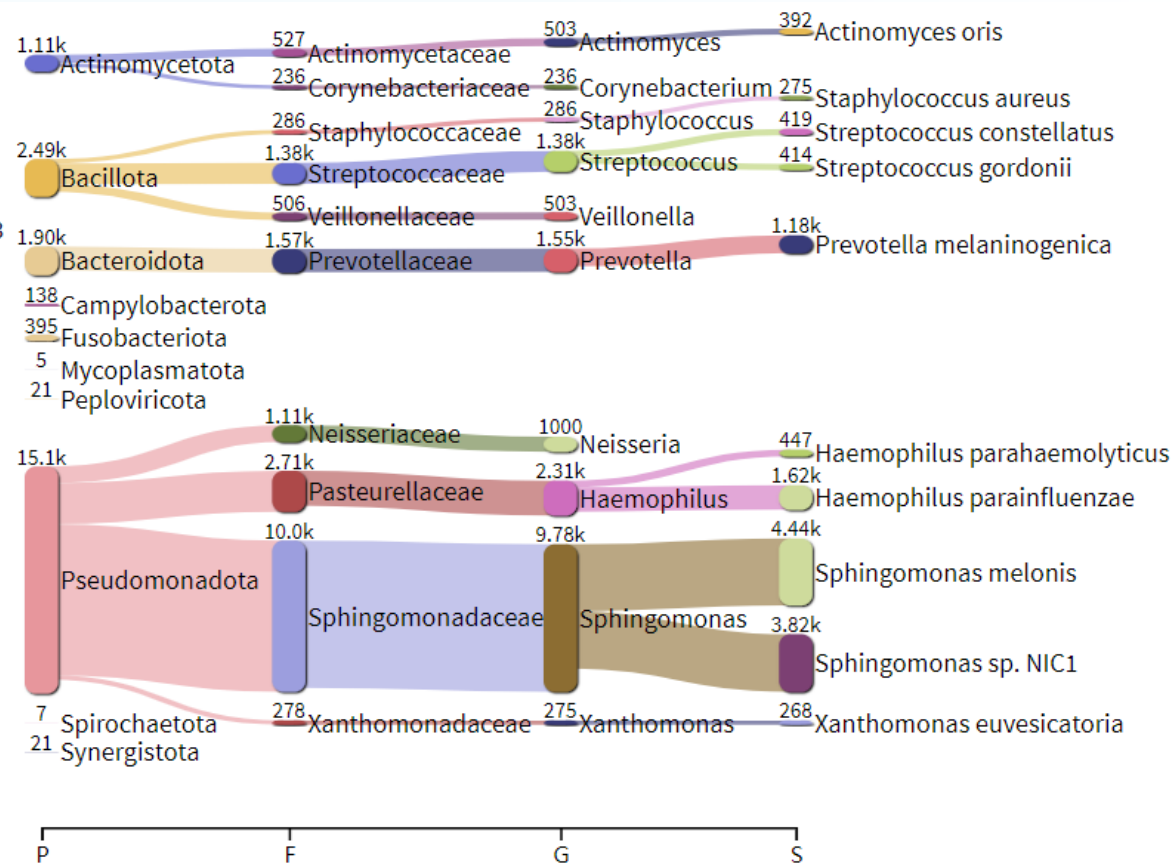
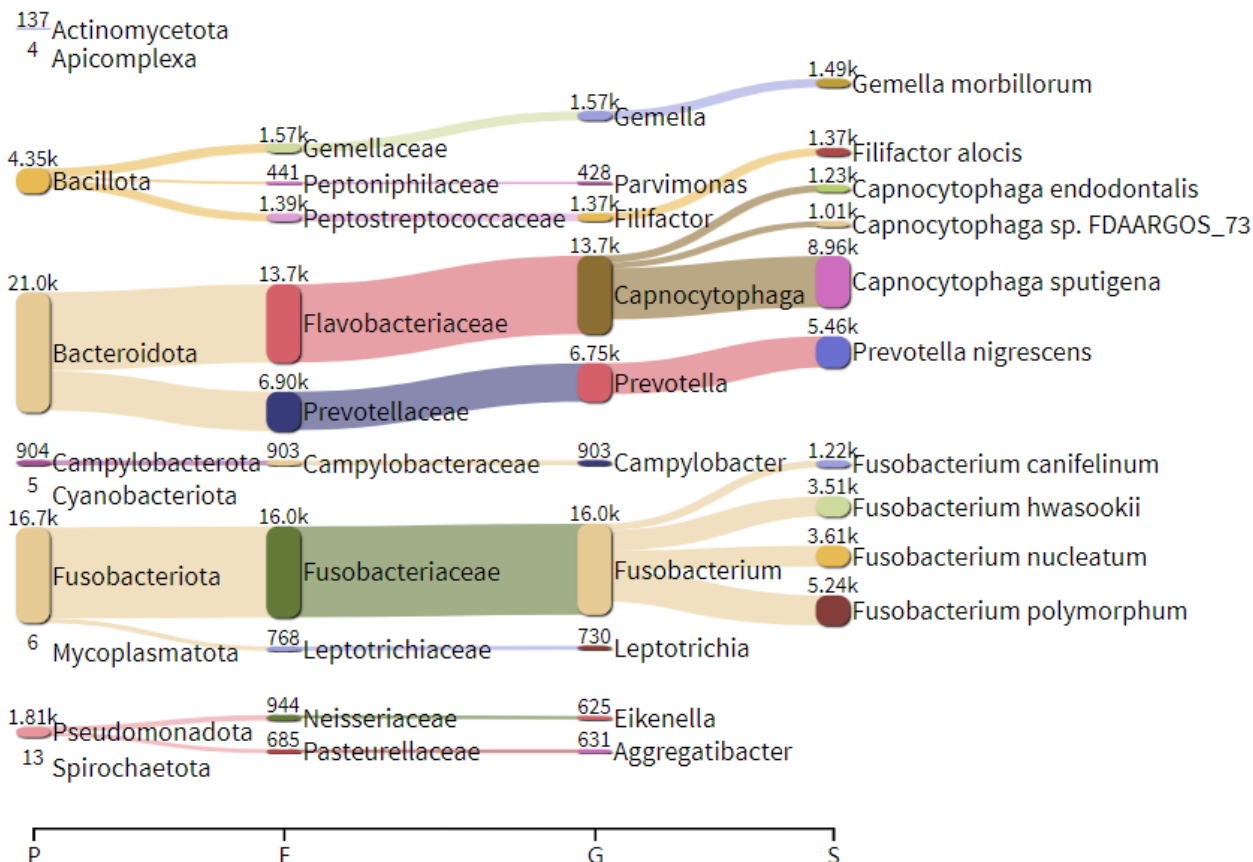

```
for i in `tail -n+2 result/metadata.txt|cut -f1`;do
    kreport2krona.py -r temp/bracken/${i}.report -o temp/bracken/${i}.krona --no-
    intermediate-ranks
    ktlImportText temp/bracken/${i}.krona -o result/kraken2/krona.${i}.html
done
```



宏基因组

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



3StatPlot.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/
```



3StatPlot.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:

beta_pcoa_stat.txt

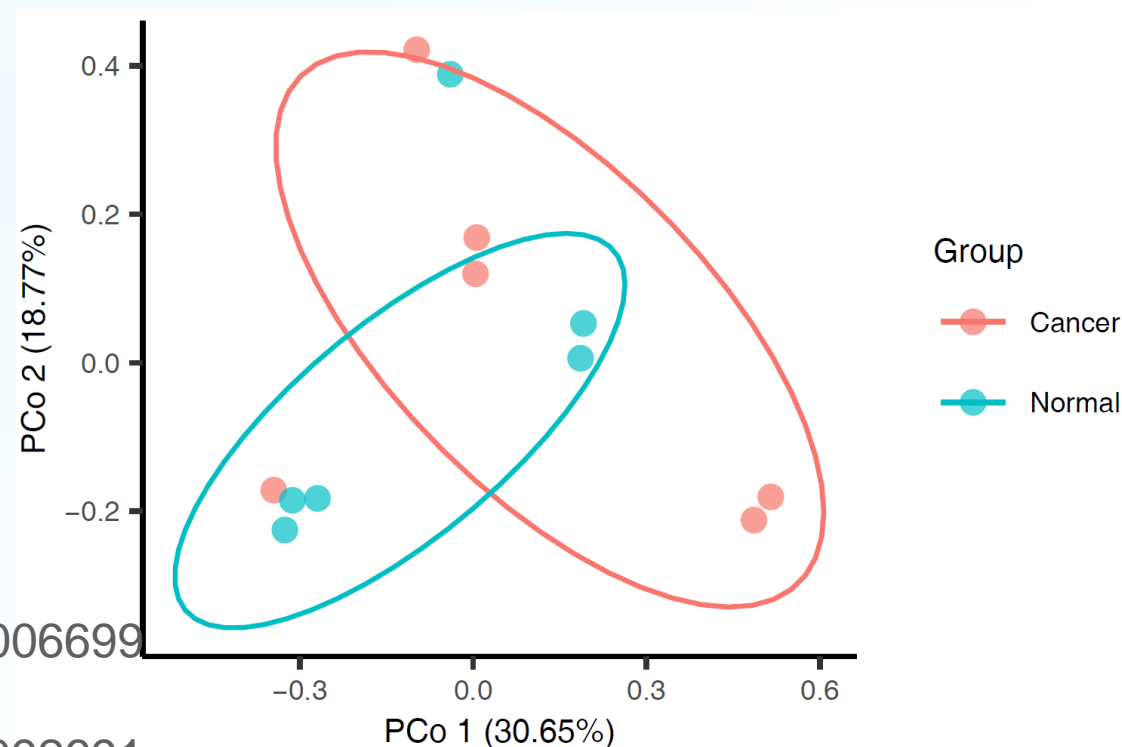
P值有波动但比较稳定

Sun Jan 03 16:19:07 2021

Cancer Normal 0.300669933006699

Sun Jan 03 17:55:04 2021

Cancer Normal 0.309269073092691



3StatPlot.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

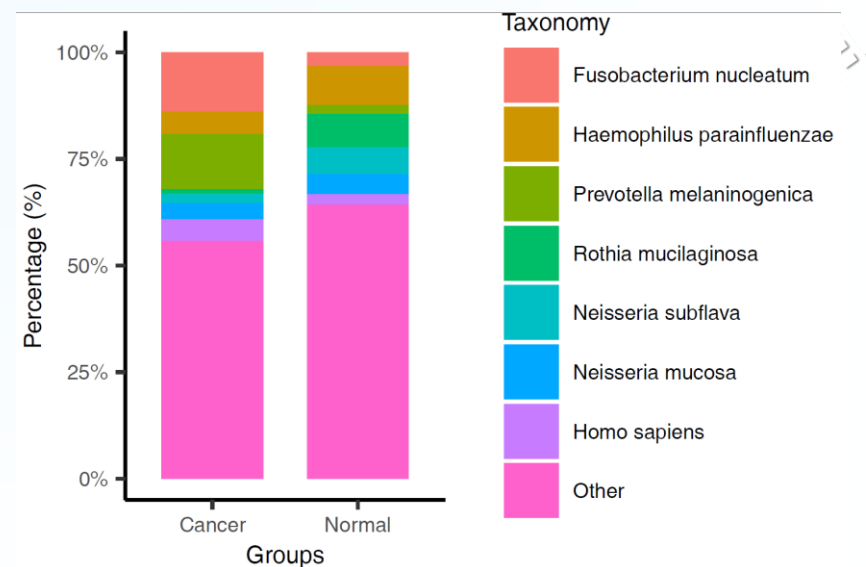
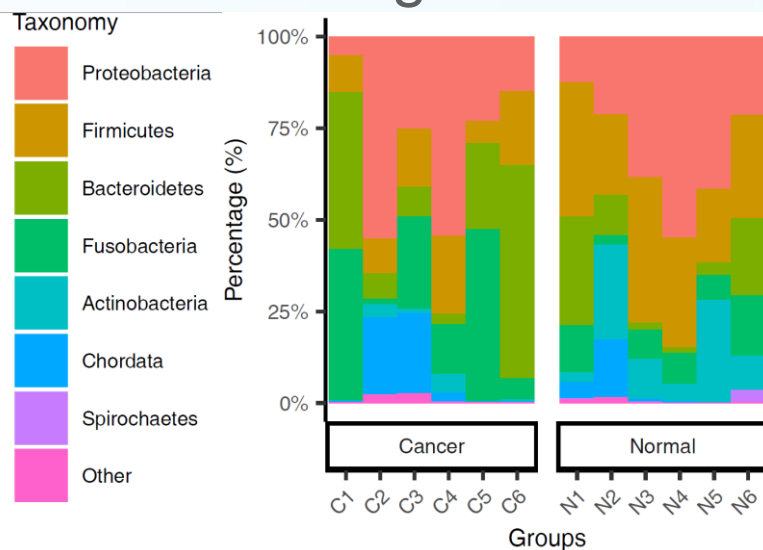
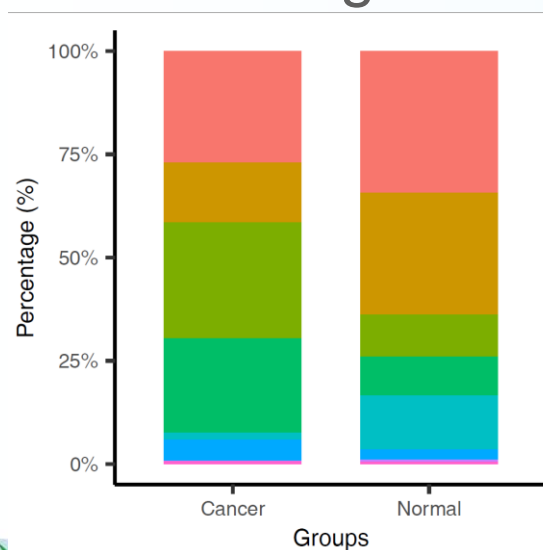
tax=S

```
Rscript ${sd}//tax_stackplot.R \
```

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

```
--group Group --output result/kraken2/bracken.${tax}.stackplot \
```

```
--legend 8 --width 89 --height 59
```



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"
```

易生信 毕生缘 基因组



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.

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

Jennifer Lu, Natalia Rincon, Derrick E. Wood, Florian P. Breitwieser, Christopher Pockrandt, Ben Langmead, Steven L. Salzberg, Martin Steinegger. 2022. Metagenome analysis using the Kraken software suite. **Nature Protocols** 17: 2815-2839. <https://doi.org/10.1038/s41596-022-00738-y>



- 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



- 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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